Invited Review

Retinal Photodamage by Endogenous and Xenobiotic Agents†

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ABSTRACT

The human eye is constantly exposed to sunlight and artificial lighting. Light transmission through the eye is fundamental to its unique biological functions of directing vision and circadian rhythm and therefore light absorbed by the eye must be benign. However, exposure to the very intense ambient radiation can pose a hazard particularly if the recipient is over 40 years of age. There are age-related changes in the endogenous (natural) chromophores (lipofuscin, A2E and all-trans-retinal derivatives) in the human retina that makes it more susceptible to visible light damage. Intense visible light sources that do not filter short blue visible light (400–440 nm) used for phototherapy of circadian damage. Intense visible light sources that do not filter short blue light in general and short blue visible light in particular dramatically raises the risk of photodamage to the retina. Moreover, many drugs, dietary supplements, nanoparticles and diagnostic dyes (xenobiotics) absorb ocular light and have the potential to induce photodamage to the retina, leading to transient or permanent blinding disorders. This article will review the underlying reasons why visible light in general and short blue visible light in particular dramatically raises the risk of photodamage to the human retina.

Abbreviations: A2E, N-retinylidene-N-retinylethanolamine; A2Fox, A2E oxidation products; A2PE, N-retinylidene-N-retinyl phosphatidylethanolamine; A2PE-H2, dihydro-N-retinylidene-N-retinyl phosphatidylethanolamine; ABCA4, photoreceptor-specific ATP-binding cassette transporter; AMD, age-related macular degeneration; APE, all-trans-retinal-PE Schiff base (N-retinylidene-phosphatidylethanolamine); atRal, all-trans-retinal; atRol, all-trans-retinol; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; DMSO, dimethyl sulfoxide; EPR, electron paramagnetic resonance; GA, geographic atrophy; GSH, glutathione reduced form; GSSG, oxidized form of glutathione; HLE, human lens epithelium; HNE, 4-hydroxynonenal; ICG, indocyanine green; IRBP, interphotoreceptor retinoid-binding protein; iso-A2E, 13cis isomer of A2E; LDH, lactate dehydrogenase; LDL, low-density lipoprotein; LF, lipofuscin; MS, mass spectrometry; MTS, a cell proliferation assay compound; oxidase; PCV, polypoidal choroidal vasculopathy; PDT, photodynamic therapy; PE, phosphatidylethanolamine; POS, photoreceptor outer segments; RA, retinoic acid; RDH, all-trans-retinol dehydrogenase; RGR, retinal G protein-coupled receptor; ROS, reactive oxygen species; RP, retinyl palmitate; RPE, retinal pigment epithelium; SAD, seasonal affective disorder; TBARS, thiobarbituric acid reactive substances; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; UV, ultraviolet radiation (100–400 nm); UV-A, ultraviolet B (315–400 nm); UV-B, ultraviolet B (280–315 nm); UV-C, ultraviolet C (100–280 nm); y.o., years old.

INTRODUCTION

Aside from the skin, the eye is the organ most susceptible to sunlight and artificial lighting induced damage. Solar radiation exposes the eye to UV-B (280–315 nm), UV-A (315–400 nm) and visible light (400–700 nm) (1). Light is transmitted through the eye and then signals the brain directing both sight and circadian rhythm. Therefore, light absorbed by the eye must be benign. However, under certain circumstances, the eye may be damaged by solar and artificial radiation.

Factors which determine light damage to the eye

The primary factors, which determine whether ambient radiation will injure the human eye are the intensity of the light, the wavelength emitted and received by ocular tissues, and the age of the recipient (2).

UV and visible light intensity. The greater the intensity of light, the more likely it is to damage the eye. Light that may not ordinarily be harmful can do acute damage if it is sufficiently intense. For example, it is well known that the eye can be damaged (temporarily or permanently) by exposure to reflective sunlight from snow (snow blindness), or from staring at the sun during an eclipse (3,4). There is an increase in UV radiation with a thinning of the protective ozone layer (5). Similarly, the eye can sustain damage from artificial light sources that emit UV-A or UV-B (6). Cumulative light damage results from less-intense exposure over a longer period of time and is often a result of an underlying age-related loss of antioxidant protection (7,8).
Absorbed by the lens. Ambient radiation, from the sun or from artificial light sources, contains varying amounts of UV-C (100–280 nm), UV-B, UV-A and visible light (1). The shorter the wavelength, the greater the energy and therefore the greater the potential for the radiation to do biological damage. However, although the longer wavelengths are less energetic, they more deeply penetrate ocular tissues (9).

For a photochemical reaction to occur, the emitted wavelengths of light from the source must be absorbed in a particular ocular tissue. The primate/human eye has unique filtering characteristics that determine in which area of the eye each wavelength of light will be absorbed (Fig. 1). All radiation below 295 nm is filtered by the human cornea (all UV-C and some UV-B) before they reach the human lens (10). The transmission characteristics of the human lens changes with age.

In adults, the lens absorb UV-B above 295 nm and all of UV-A. However, the very young human lens transmit a small window of UV-B light (320 nm) to the retina, whereas the elderly lens filters out much of the short blue visible light (400–500 nm) (11). Transmission characteristics through ocular tissues also differs with species; the lenses of mammals other than primates transmit ultraviolet light longer than 295 nm to the retina (12).

Antioxidant system efficacy. Most damage by ambient light to the young and adult eye is avoided because the eye is protected by a very efficient antioxidant system. In addition, there are protective pigments located in the young and adult retina and lens, which absorb ambient radiation and dissipate its energy without causing damage (13).

Ordinarily lutein and zeaxanthan in the macular (center) of the retina (14), vitamin E (15,16), glutathione (7) and other antioxidants (17) throughout the eye protects the retina against both inflammatory and photooxidative damage.

Unfortunately with age, these protective agents become depleted. After middle age (40-year old) there is a decrease in the production of ocular antioxidant enzymes and low-molecular weight antioxidants and the retina loses its protection against reactive oxygen species (ROS) toxicity (7,18,19). In addition, the content and function of cardiolipin which facilitates retention of cytochrome c in mitochondria, preventing the cells from apoptosis diminishes progressively in humans with age to the level constituting ca 60% of the value detected in children (20).

The RPE and choroid contains melanin, which absorbs UV and short-wavelength visible light and protects the retina against photic damage. However, with age ocular melanin is photobleached and this decreases its protective effectiveness against UV damage (21).

At the same time, the protective pigments are chemically modified and now these ocular pigments damage the lens and retina on exposure to ambient radiation (21–23).

Mechanism of light damage to the eye

The role of chromophores. Chronic exposure to less-intense radiation does damage to the eye through a photooxidation reaction. In such type of oxidation reactions, a chromophore in the eye absorbs UV or visible light, produces ROS such as singlet oxygen and superoxide and these damage ocular tissues. The chromophore may be endogenous (natural) or exogenous (drug, herbal medication or nanoparticle) that has accumulated in the eye (24).

Absorption of light excites the chromophore to a transient singlet state (Fig. 2). The excess of energy of the chromophore singlet state can be dissipated (13), emitted as fluorescence or used for intersystem crossing to the triplet state. The chromophore can return to its ground energy level by dissipation of the excess of energy or phosphorescence. The chromophore lifetime at triplet state is long enough to let its molecules transfer electrons via a Type I and form free radical or the excess of energy via a Type II to different molecules, for example oxygen and produce its singlet form (singlet oxygen). The reactive species generated during these processes can cause the eventual damage. Photooxidation can occur in the eye either by Type-I or -II mechanism or both concurrently.

Human retina. The retina in the mammalian eye is composed of the photoreceptor cells (rods and cones) that receive light and the neural portion (ganglion, amacrine, horizontal and bipolar cells) that transduces light signals through the retina to the optic nerve. Behind the photoreceptor cells are the retinal pigment epithelial (RPE) cells, which are separated from choroid with Bruch’s membrane.

RPE cells. The RPE cells form a single-cell layer that is the most distal layer of the retina. The cells transport ions, water and metabolic end products from the subretinal space to the blood (25), and deliver nutrients, such as glucose, retinol and fatty acids in the opposite direction—from the blood to the photoreceptors. Moreover, the RPE cells phagocytize light-damaged photoreceptor outer segments (POS) (26) in a circadian regulated process, and then shed, digest, recycle and transport the essential elements of their contents back to the photoreceptors (25,27). However, a part of undigested material accumulates with age and is stored in lysosomes as an age pigment called lipofuscin (28) (Fig. 3).

The RPE cells are permanently exposed to factors that potentially induce oxidative stress. Those conditions assure that RPE cells contain high amounts of antioxidant enzymes, such as superoxide dismutase and catalase (25). In addition, the presence of aldehyde dehydrogenase was affirmed in the RPE cells (29). This cytosolic enzyme participates in the oxidation of various aldehydes, including all-trans-retinal. It was shown by Ishibashi et al. (30) that the expression of

![Figure 1](image-url)
aldehyde dehydrogenase 6 was reduced in the retinal pigment epithelial cells isolated from older human eyes.

UV damage and “blue light hazard”. The young retina is at particular risk for damage from UV exposure because the young lens has not as yet synthesized the yellow chromophore (3-hydroxykynurenine and its α-glucoside) that prevents UV transmission to the retina (31). UV damage to the eye is cumulative and may increase the possibility of developing eye disorders (ocular melanoma and macular degeneration) later in life.

In addition to UV damage to the young retina, short blue visible light (430 nm) damages the retina in persons over 50 years of age through photooxidation reactions (2,32–34). The human retinal pigment epithelium (RPE) and the anterior layers of the retina in adults constantly receive intense visible light (>400 nm) (35,36) (Fig. 1). Studies performed on animals including primates have shown that short-term light exposures (<12 h) at relatively high intensity can induce damage in photoreceptors and the RPE cells (33,37,38). The most phototoxic effect was produced by light at wavelengths around 430 nm, then referred as the “blue light hazard” (39,40). Agarwal et al. (41) observed that thickness of the retinal outer nuclear layer, photoreceptor segment length and RPE thickness were 60–70% reduced when rat retinas were evaluated 4 weeks after blue light exposure. Light damage observed in rats resulted in a retinal degeneration exhibiting features of atrophic age-related macular degeneration (AMD), including photoreceptor and RPE degeneration and choriocapillaris atrophy (38). The most recent experiments demonstrated that 6-h blue light (450 nm) exposure of rats caused significant shortening of the photoreceptor inner and outer segment lengths, when retinas were evaluated immediately after irradiation (33). In addition, 40–50% of the photoreceptor nuclei were pyknotic with condensation of chromatin and dark staining nuclei.

The photodamage observed in the RPE cells is oxygen-dependent (42,43) and is diminished by antioxidants (15,31,44,45), which suggests that the blue light effect is associated with oxidative stress. The dramatic changes observed in the photoreceptors and the RPE after light
exposure made investigators to find potential chromophores that would be able to absorb light in the blue region and convert excessive energy to production of reactive intermediates. Photoreceptor damage may be mediated through rhodopsin, as blue light (400 ± 10 nm) causes its photoreversal bleaching and increased generation of toxic rhodopsin intermediates (46,47). There are several endogenous RPE chromophores, including melanin, the mitochondrial enzyme cytochrome c oxidase and riboflavin that can act as potential photosensitizers and may be responsible for visible light short-wavelength damage to the RPE (48,49). The reduced form of cytochrome c oxidase absorbs mainly blue light with a maximum at 440 nm (50). Blue light (425 ± 20 nm) can induce generation of ROS by mitochondrial cytochromes (51), inhibit cytochrome c oxidase leading to ATP depletion (52,53), and lead to calcium accumulation (54) and apoptosis (55).

Nevertheless, photoreactivity of melanin, cytochrome c oxidase or riboflavin present in the RPE seems to be negligible compared with a visual cycle retinoid—all-trans-retinal (56–58) or chromophores present in the age pigment—lipofuscin accumulated in age in the RPE cells (59–62).

Macular degeneration. The relatively high concentration of oxygen in the retina, exposure to light, age-related changes in the level of antioxidants and the presence of chromophores formed during lifetime in situ or xenobiotics create favorable conditions for oxidative stress (36), which can be a major factor of retinal diseases including macular degeneration.

AMD is the major cause of visual impairment and blindness (63,64) among the elderly in the United States and in European and Australian (Oceanic, Pacific) nations (65–70). It is estimated that more than 1.5% of Caucasians at age more than 70 years old suffer advanced form of AMD and another 10% have symptoms of early macular degeneration (64,70). AMD is the disease involving photoreceptors, RPE and the choroid in the elderly eyes. It is initially characterized by thickening of Bruch’s membrane and accumulation of lipid material (drusen) on the inner collagenous layer of the membrane (70) (Fig. 3B). The early stages of AMD can evolve into “wet” form, where blood vessel proliferation is observed or to geographic atrophy (GA) associated with the RPE cell death.

Little is known about AMD etiology. However, several risk factors, namely: aging, tobacco smoking, female gender, hypertension, high-body mass index, a high fat diet and some genetic predispositions are considered as those that can contribute to development of the disease (70–73). Sunlight is an environmental factor that has been identified (39,74), but epidemiologic studies assessing the link between light exposure and AMD have provided inconsistent results. Three cohort based studies (34,75–78) showed an association; case control studies (79,80) failed to confirm this relationship, but the age-related eye disease study (AREDS) (81) did suggest a connection between increased light exposure and AMD. An improved study (AREDS2) is presently being conducted (82). In epidemiological studies performed on 4000+ participants, Fletcher et al. (83) found associations between blue light exposure and development of early stages of AMD as well as its neovascular form in persons who had low levels of antioxidants (vitamin C and E, zeaxanthin) in their blood and low uptake of dietary zinc. Indeed, the role of antioxidants seems to be considerable in AMD. Epidemiological studies have shown that, although there is no evidence that supplementation of antioxidants important in the retina metabolism (lutein, zeaxanthin, vitamin C and E and zinc) (14) prevents early AMD development, but it slows down progression to advanced AMD (81,84). Therefore, oxidative stress seems to be the major mechanism involved in development of the disease (85,86).

ENDOGENOUS CHROMOPHORES

Lipofuscin

Increased accumulation of fluorescent age pigment (lipofuscin) is observed in the RPE cells, especially in the rim of the geographic atrophy area. The role of lipofuscin and its photosensitizing chromophores in AMD development is still under debate. The accelerated accumulation of lipofuscin is also observed in a juvenile form of macular degeneration called Stargardt’s disease (87). The toxic effect of lipofuscin components in this disease is particularly visible.

Lipofuscin (LF) is a heterogenic material (88,89), which accumulates with age in lysosomal storage bodies generally in postmitotic cells (90–92), such as neurons, cardiac myocytes, striated muscle fibers, Sertoli cells in testes and RPE (88,93,94). Although morphology of the granules varies from one tissue to another (93), they are formed mostly of non completely digested biomolecules, such as proteins and lipids (88). Interestingly, the pigment exposed to UV irradiance emits fluorescence (93). The mechanism of LF formation and accumulation still remains obscure. It is suggested that oxidative stress, diminished activity of lysosomal proteolytic enzymes, not efficient phagocytosed and/or autophagocytosed material degradation and elimination from the cells are the main factors of lipofuscinogenesis (88,94,95).

LF amount and distribution

Lipofuscin in RPE cells. Lipofuscin in the retinal pigment epithelial cells appears as yellow–brown granules, which are brighter than melanosomes (96) with roughly spherical shape at diameter of 0.5–1 μm (89,93,96–99) and are made up of smaller aggregates at size ca 50 nm (99–101).

The cells that form the RPE are postmitotic cells. That fact has a fundamental significance in lipofuscin accumulation in the aging human eye (62,86,93,102,103) (Fig. 3). The electron microscopy images revealed that the RPE cells in infants contain no recognizable amount of LF (93). At 40 years of age LF granules are present in the subapical cytoplasm. In sixth decade of life the number of the age pigment granules equals or is even higher than the number of melanosomes and progressively increases in age. At the age of 70–80 y.o., LF granules can occupy 19–33% of cytoplasmic volume (60,103). Interestingly, the age pigment granules accumulate in young persons mostly in the basal cytoplasm and LF concentration is ca 1.4 times higher than in the apical part and is reversibly correlated with melanin distribution (104). In older persons, however, the granule distributions of both pigments become more uniform (103). In addition, a conglomerate of melanolipofuscin is accumulated (60,105) with melanin core and LF forming the outer layer (96,106) (Fig. 3B).
**Distribution in the eye fundus.** Spectroscopic measurements performed on human retinas by Delori et al. (103) showed that LF distribution in the eye fundus is not homogenous (Fig. 4A). Autofluorescence detected in the fovea was ca. 40% lower than in the surrounding region (7° eccentricity) and was asymmetrically distributed around the fovea with maxima at ca. 7° nasally, 13° superiorly, 11° temporally and 9° inferiorly (103). Interestingly, the intensity of LF autofluorescence was less along inferior meridian than along any other (103). In addition, studies performed on rhesus monkeys showed that lipofuscin autofluorescence is reversibly associated with drusen distribution (107). Autofluorescence detected in the RPE cells over “the dome” of a druse was considerably weaker that from the cells adjacent to the druse. However, a close relationship between drusen formation in the Bruch’s membrane and slower lipofuscin accumulation has not as yet been definitively proven. Moreover, there has been no evidence so far of material characteristic for proteins specific for POS or lysosomal enzymes in Bruch’s membrane drusen (108,109).

**Association with eye diseases.** Enhanced accumulation of LF in the RPE layer is associated with a several retina diseases, such as AMD, Stargardt’s disease, Best’s macular degeneration and Batten’s disease (98,103). Increased LF autofluorescence is clinically observed in the band of the human retina surrounding the region of geographic atrophy (110,111). It could suggest the association between the excessive LF accumulation and RPE cells and their death in the late stage of dry AMD.

**Lipofuscin granule composition**

Material that forms lipofuscin in the RPE cells originates mainly from the photoreceptor outer segment disks, which are shed on the apical surface of the RPE cells then phagocytozed and exposed to enzymatic digestion in the phagolysosomal system (92,112,113). However, a part of the phagocytosed material originated mostly from peroxidized POS is not entirely digested and accumulates inside the RPE cells forming the age pigment (94,108,113). The impairment of lysosomal degradation in autophagy of used up intracellular organelle, such as mitochondria, Golgi apparatus or endoplasmic reticulum can also contribute in lipofuscin accumulation in the RPE cells (62).

**Oxygen effect.** Oxygen concentration seems to have considerable significance in LF formation in RPE cells, which are located in the region of high O2 tension (ca. 70 mmHg) (48,62). In vitro studies have shown that increased oxygen partial pressure caused enhanced LF accumulation in RPE cells fed with native POS (114). In addition, high oxygenation facilitates peroxidation processes inside the RPE cells and in their close neighborhood. The RPE cells, which phagocytosed POS peroxidized or modified by lipid peroxidation products, accumulated much more LF than cells treated with native POS (94,115).

**Role of antioxidants and vitamins.** Antioxidant and vitamin level in cell culture medium or animal diet is another factor in the rate of LF accumulation in the RPE cells. Carotenoids (zeaxanthin and lutein) and α-tocopherol significantly reduced LF formation in the RPE cells in vitro (114). A number of studies performed on animals showed that deficiency of antioxidant nutrients, particularly vitamin E in diet caused dramatic accumulation of lipofuscin-like autofluorescent pigments in the RPE (113). It could be an important factor of lipofuscinogenesis in vivo since age-related decline in vitamin E levels in the macular RPE in persons older than 70 (48). On the other hand, the content of vitamin A in the diet is reversibly correlated with LF accumulation in the RPE of tested animals. Vitamin A shortage in the diet significantly reduced formation of the age pigment (113), but the diet supplemented with the retinoid enhanced LF build-up (116).

**Proteins in lipofuscin.** The age pigment granules in the RPE cells are composed of lipids (20–50%) and proteins (30–70%) (99,117–120). The usage of proteinase K for digestion of the extra-granular material followed by proteomic analyses revealed that lipofuscin contains up 2% proteins, whereas...
Lipids in lipofuscin. Biesemeier et al. (96) studies have shown relatively high phosphorus content in LF granules in the RPE cells, which implies that phospholipids make a significant fraction of all lipids in the age pigment. These suggestions are consistent with results obtained earlier by Haralampus-Grynaviski et al. (101) mass spectroscopy studies of LF extracts, where phosphatidyleholines and phosphatidylethanolamines of palmitic, stearic, oleic and arachidonic acid were identified with no evidence of docosahexaenoic acid, which is present at high abundance in POS. Sparrow and Boulton (123) suggest that fatty acid composition of POS is different than lipofuscin, the phospholipids extracted from LF granules originate from the phospholipid bilayer surrounding LF body than from phagocytosed POS.

Retinoids in lipofuscin. Phagocytosis of POS disks by RPE cells indicated that lipofuscin cells have specific components compared to the age pigment in different tissues. Beside proteins engaged in the visual cycle, the age pigment granules contain retinoid chromophores (92,105,112,124).

Fluorescence

Lipofuscin in the human RPE cells emits characteristic golden-yellow fluorescence. However, the fluorescence emission spectrum depends on the wavelength of excitation (101,124,125). RPE lipofuscin granules excited at 364 nm exhibit a strong broad band emission spectrum with a peak at 600 nm (91).

Spectrum characteristics. Detection of bulk LF fluorescence revealed that with increasing excitation wavelength from 400 to 532 nm, emission spectrum narrowed, its maximum shifted to the longer wavelengths, but the red arm of the spectrum stood still (101) (Table 1). It suggests that LF is a mixture of numerous fluorophores. Human retinal fundus autofluorescence detected in almost 150 persons when excited at 430 nm showed emission spectrum with maximal intensity at 608 ± 1 nm. Shifting of excitation wavelength to 545–550 nm caused the peak of emission spectrum to move to 630–640 nm (103,124). In reverse, when emission of LF from RPE cells was set up 610 nm, the excitation spectrum was characterized by two peaks—the main at ca 480 nm and a secondary at 405 nm (120).

Changes with age. Spectroscopic studies showed that optical density and fluorescence intensity of LF granules increases with age (39,126). The spectrum of LF fluorescence is slightly different in young and elderly persons. The blue component of the fluorescence spectrum is observed only in the granules extracted from young persons (126,127). However, the blue band is absent in LF from old persons and the intensity of yellow and orange bands is significantly enhanced. The diversity in fluorescence spectra between LF granules was confirmed using near-field scanning optical (NFSO) microscopy (98). The LF isolated from 60 to 90 y.o. persons excited at 458 nm showed varied fluorescence emission spectra between individual granules with maxima in range 550–645 nm. The images taken with NFSO and atomic force microscopy showed nonhomogenous distribution of orange fluorophores in LF granules, whose surface can be partially even non emissive (97,100). The effect on RPE cell metabolism

The age pigment accumulated in the RPE cells seems to have a negative effect on metabolism of the cells. Studies performed in vitro on RPE cells isolated from newborn pigmented rabbits showed that overloading of the cells with peroxidized POS (artificial lipofuscin) caused reduced phagocytotic capacity (88,114). Similar effect was observed in human fibroblasts. The cells first stimulated for accumulation of ceroid/lipofuscin then exposed to oxidative stress showed increased susceptibility to oxidative damage (128). However, diminished autophagocytosis and decreased survival time was observed in the cells preloaded with ceroid/lipofuscin and then starved with essential amino acids (128).

Photoreactivity

The age pigment present in the human RPE cells shows broad reactivity when exposed to UV or short-wavelength visible light. The RPE cells isolated from human donors exposed to blue light (408–495 nm) in vitro showed substantial oxygen consumption, which rate correlated with the age of donors suggesting a major role of LF in age-related photodamage of the retina (59).

Transient species. Indeed, photoexcitation of the extracts of human LF in organic solvents led to formation of transient species: LF triplet state observed at 430–440 nm with lifetime 7 and 10.3 μs for LF fractions soluble in chloroform and then dissolved in methanol (129) and LF fraction soluble in hexane and benzene (60), respectively. Another transient species postulated as a free radical insensitive to oxygen were detected and their spectrum had maximum at 580 nm (129).

Oxygen photoconsumption. The transient species photogenerated in LF extracts can interact with oxygen to produce its reactive forms. The electron spin resonance oximetry, in which photoinduced changes of oxygen concentration is measured revealed that action spectrum of O2 uptake in LF granules, its organic solvent extracts and LF insoluble material monotonically decreases with excitation wavelength and are similar to LF absorbance spectrum (61).
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<td>600 610 630–640</td>
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Abs, absorbance; Af, autofluorescence; DCM, dichloromethane; Fl, fluorescence; Lf, lipofuscin; MeCN, acetonitrile; MeOH, methanol; OD, ocular distribution; OS, outer segments; POS, photoreceptor outer segments; TrX-100, Triton X-100; wr, wavelength range; (?), the presence has not been absolutely proven.
**ROS photogeneration.** Oxygen photoconsumed by LF or its extract is at least partially converted into ROS. The electron paramagnetic resonance (EPR) spin trapping studies revealed that components of human LF extracted by chloroform and irradiated at \( \lambda > 300 \) nm produce superoxide anion, carbon-centered radicals and can abstract hydrogen or electron from methanol used as a solvent to yield hydroxymethyl radical, methoxyl radical cation and hydroxymethyl peroxyl radical (130) (Table 2). Photophysical studies performed on purified age pigment granules isolated from human RPE cells showed that visible light (400–730 nm) illumination induces LF to generate superoxide anion, hydrogen peroxide and singlet oxygen (59,60,105,131). The ROS were photogenerated by LF most efficiently in the blue region (400–520 nm) of the light spectrum (131). However, *in vitro* studies showed that in contrast to RPE melanosomes, no more than 3% of oxygen photoconsumed in the presence LF was converted into \( \text{H}_2\text{O}_2 \) (105).

**Singlet oxygen photoproduction.** Photogeneration of \( ^1\text{O}_2 \) by LF extract in benzene as well as chloroform-insoluble fraction is wavelength dependent (60,132,133) (Table 2). Avalle *et al.* (133) showed that LF fraction extracted with mixture of \( \text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O} \) (1:1:1), reconstituted in \( \text{CDCl}_3 \) and exposed to UV–VIS light (300–450 nm) produced singlet oxygen, with an action spectrum characterized by a single band in the range of 300–450 nm with a maximum at 380 nm. However, Różanowska *et al.* (60) observed that efficiency of \( ^1\text{O}_2 \) photogeneration of LF extracts in hexane, benzene or the Folch mixture decreases monotonically with increasing excitation wavelength in range 420–550 nm and is 10 times higher at 420 nm compared with 520 nm. The quantum yields for \( ^1\text{O}_2 \) photogeneration in air-saturated benzene is 0.08 ± 0.03 at 420 nm compared with 520 nm. The quantum yield of \( ^1\text{O}_2 \) photogeneration of LF extracts in hexane, benzene or the Folch mixture decreases monotonically with increasing excitation wavelength in range 420–550 nm and is 10 times higher at 420 nm compared with 520 nm. The quantum yield for \( ^1\text{O}_2 \) photogeneration in air-saturated benzene is 0.08 ± 0.03 at 355 nm and 0.05 ± 0.01 in blue light (420–440 nm). Singlet oxygen photogeneration by LF is oxygen-dependent—the quantum yield is almost twice higher in benzene solution saturated with oxygen. The authors of the studies suggest that this effect can indicate the possible presence of several photosensitizers, with different lifetime of their triplet states that might transfer energy to molecular oxygen (60). The quantum yield of \( ^3\text{O}_2 \) photoformation by LF extract is comparable to all-trans-retinal in methanol (129), whereas in benzene it is two to three and three to six times lower when excited at 355 nm and blue light (420–440 nm), respectively (60) (Table 2). On the other hand, still measureable amounts of singlet oxygen can be photoproduced in LF extract solution at 470 nm, whereas in all-trans-retinal that wavelength is out of range for \( ^1\text{O}_2 \) generation.

**Lipid and protein peroxidation.** In addition, lipofuscin-related photoproduction of lipid hydroperoxides and TBA reactive species indicate that the age pigment exposed to blue light (408–495 nm) enhances lipid peroxidation in the human RPE cells (59). Human LF granules exposed to visible light (408–495 and 515 nm) caused lipid peroxidation *in vitro* (105,134) in the human RPE cell culture and in the POS isolated from bovine eyes (135). Addition of specific antioxidants proved that LF-induced lipid photoperoxidation can be initiated by singlet oxygen (135). Moreover, LF photoinduced *in vitro* peroxidation of bovine serum albumin at pH 7.0 (105,135).

**The effect on enzyme activity.** ROS photoproduced in human RPE cells by LF caused dramatic drop of antioxidant potential expressed in significant inactivation of catalase (135,136) and superoxide dismutase and decline of intracellular glutathione level (136). The age pigment reduces activity of acid phosphatase both in the dark and when exposed to light (400–1100 nm) (135) and causes significant activity decrease of other lysosome enzymes (136). In addition, LF can stimulate photoreduction of cytochrome c under aerobic conditions (131).

<table>
<thead>
<tr>
<th>Chromophore</th>
<th>Form</th>
<th>Irradiation wavelength (nm)</th>
<th>Quantum yield (Φ)</th>
<th>Solvent</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipofuscin</td>
<td>( ^1\text{O}_2 ), CCR</td>
<td>&gt;300</td>
<td>-</td>
<td>Deuterated chloroform</td>
<td>(130)</td>
</tr>
<tr>
<td>Lipo</td>
<td>( ^1\text{O}_2 ), ( ^2\text{O}_2 ), ( ^1\text{O}_2 )</td>
<td>400–730</td>
<td>-</td>
<td>Benzene (air)</td>
<td>(60)</td>
</tr>
<tr>
<td>Lipofuscin</td>
<td>( ^1\text{O}_2 )</td>
<td>300–450</td>
<td>0.08 ± 0.03</td>
<td>Benzene (air)</td>
<td>(60)</td>
</tr>
<tr>
<td>Lipofuscin</td>
<td>( ^1\text{O}_2 )</td>
<td>355</td>
<td>0.05 ± 0.01</td>
<td>Benzene (air)</td>
<td>(60)</td>
</tr>
<tr>
<td>Lipofuscin</td>
<td>( ^1\text{O}_2 )</td>
<td>420–440</td>
<td>-</td>
<td>Benzene, hexane</td>
<td>(60)</td>
</tr>
<tr>
<td>Lipofuscin</td>
<td>( ^1\text{O}_2 )</td>
<td>420–550</td>
<td>-</td>
<td>Benzene, hexane</td>
<td>(60)</td>
</tr>
<tr>
<td>All-trans-retinal</td>
<td>( ^1\text{O}_2 ), CCR</td>
<td>&gt;300</td>
<td>-</td>
<td>DMSO/benzene mix</td>
<td>(57)</td>
</tr>
<tr>
<td>Lipofuscin</td>
<td>( ^1\text{O}_2 )</td>
<td>407</td>
<td>0.05</td>
<td>Methanol</td>
<td>(159)</td>
</tr>
<tr>
<td>Lipofuscin</td>
<td>( ^1\text{O}_2 )</td>
<td>250</td>
<td>0.25</td>
<td>Carbon tetrachloride</td>
<td>(159)</td>
</tr>
<tr>
<td>Lipofuscin</td>
<td>( ^1\text{O}_2 )</td>
<td>355</td>
<td>0.16–0.24</td>
<td>Benzene</td>
<td>(60)</td>
</tr>
<tr>
<td>Lipofuscin</td>
<td>( ^1\text{O}_2 )</td>
<td>420–440</td>
<td>0.10–0.30</td>
<td>Benzene</td>
<td>(60)</td>
</tr>
<tr>
<td>Lipofuscin</td>
<td>( ^1\text{O}_2 )</td>
<td>407</td>
<td>0.0003</td>
<td>DMSO/benzene mix</td>
<td>(61)</td>
</tr>
<tr>
<td>Lipofuscin</td>
<td>( ^1\text{O}_2 )</td>
<td>420</td>
<td>0.02</td>
<td>Acetonitrile</td>
<td>(190)</td>
</tr>
<tr>
<td>Lipofuscin</td>
<td>( ^1\text{O}_2 )</td>
<td>355</td>
<td>0.013</td>
<td>( \text{C}<em>6\text{D}</em>{12}/\text{CD}_3\text{CO} )</td>
<td>(195)</td>
</tr>
<tr>
<td>Lipofuscin</td>
<td>( ^1\text{O}_2 )</td>
<td>420</td>
<td>0.004</td>
<td>Hexafluorobenzene</td>
<td>(195)</td>
</tr>
<tr>
<td>Lipofuscin</td>
<td>( ^1\text{O}_2 )</td>
<td>435</td>
<td>0.004</td>
<td>Carbon tetrachloride</td>
<td>(174)</td>
</tr>
</tbody>
</table>

CCR, carbon-centered radical; \( \text{C}_6\text{D}_{12}/\text{CD}_3\text{CO} \), deuterated cyclohexane and deuterated acetone mixture; deox., deoxygenated; \( \lambda_{irrad.} \), irradiation wavelength used for reactive form generation.

*The rates for \(^1\text{O}_2 \) quenching have been determined as \( \text{ca} \ 3 \times 10^7 \text{ M}^{-1} \text{s}^{-1} \) and \( 2 \times 10^8 \text{ M}^{-1} \text{s}^{-1} \) in \( \text{CCl}_4 \) (198) and acetonitrile (174), respectively.
Blue light hazard. Overall, the studies of spectral dependence within visible spectrum of RPE lipofuscin phototoxicity showed that the age pigment granules or material extracted from LF produce ROS most efficiently when they are exposed to blue light (120). It could explain “blue light hazard” to the retina (39,120). The RPE cells loaded in vitro with lipofuscin and exposed to blue-green light (400–550 nm) exhibited significant changes in morphology associated with lipid peroxidation, protein oxidation, loss of lysosomal integrity (94,114,123), increased cytoplasmic vacuolization, cell membrane blebbing, which led to significant decrease of cell viability (114,136) and even death (137). However, the “amber” light (550–800 nm) showed no negative effect on the cells, suggesting that lipofuscin phototoxicity is light-wavelength-dependent at least in vitro (137).

Age effect on LF photoreactivity. Lipofuscin photoreactivity is age-related (105). The age pigment granules isolated from RPE cells of older persons photoinduced oxygen uptake and formation of superoxide-derived spin adducts much faster than those from young individuals.

ALL-TRANS-RETINAL

Visual cycle and lipofuscin formation

Retinoids—retinol, retinaldehyde (retinal) and retinyl esters—are vitamin A metabolites that are major constituents in the vertebrate visual cycle (138–141). 11-cis-retinal is reversibly bound to opsin—a protein present in the outer segments of rods and cones in the vertebrate eyes. Both the retinoid and the protein form a visual chromophore—rhodopsin. Light that reaches the retina induces photosomerization of 11-cis-retinal to all-trans-retinal (atRal) (48,138,141–143). Most of atRal is released from rhodopsin into the disk lumen (142). The retinoid is transported from the inside of disk membranes into the cytoplasm (142,144) by the photoreceptor-specific ATP-binding cassette transporter (ABCA4/ABCR) (142,145,146) localized in the rim of the photoreceptor disk (147). There, all-trans-retinal is reduced to the less reactive all-trans-retinol (atRol) by NADPH-dependent all-trans-retinol dehydrogenases (RDHs), such as RDH8, RDH11 and RDH12 (48,139–142,144,148). Photoreceptors are unable to convert all-trans-retinal or all-trans-retinol back to 11-cis-retinal (25). Thus, all-trans-retinal is transported to the retinal pigment epithelial cells, esterified by lecithin-retinol acyltransferase, recycled to 11-cis-retinal and translocated back to the photoreceptors (58,149).

Not all atRal molecules formed in the POS are reduced to all-trans-retinal. All-trans-retinal in its free form is a highly reactive molecule. When it is released from photoactivated rhodopsin it can react with phosphatidylethanolamine (PE), a primary amine that is present in membranes of the POS at relatively high abundance, accumulate there and undergo condensation processes before the product reaches RPE cells during phagocytosis. It was shown that the concentration of all-trans-retinal released from opsin in vivo is relatively high and can reach the level of 3 mM (48,142). In addition, it was postulated that all-trans-retinal can be present in phagocytosed outer segment disks in the RPE cells after intensive illumination of the retina, when substantial amounts of atRal are rapidly generated (35,150) (Table 1). On the basis of studies performed on Rdh8−/−Abca4−/− mice, Maeda et al. (142) suggested that loss of all-trans-RDHs, RDH8 and RDH12 enzymes, which facilitate conversion of all-trans-retinal to all-trans-retinol, can cause excessive atRal accumulation in the photoreceptors and its diffusion into the RPE cells. Yang and Fong (151) implied that all-trans-retinal can be synthesized directly inside the RPE cells. The oxidative cleavage of β,β-carotene catalyzed by β,β-carotene-15,15′-dioxygenase leads to atRal formation under dark or photic conditions directly in the RPE (151). Strauss (25) suggested that atRal synthesis is possible from all-trans-retinol of the retinylster pool in the RPE cells. It was shown that all-trans-retinol is oxidized efficiently by a specific retinol dehydrogenase and significant amounts of all-trans-retinal accumulate in ARPE-hRGR and bovine RPE cells in the absence of light (151). All-trans-retinal present in the RPE cells can be a substrate of retinal G protein-coupled receptor (RGR), a microsomal opsin detected in the cells (151).

Retinal plays an important role in lipofuscin formation in the RPE cells. The deficiency of all-trans-retinal and 11-cis isomer in Rpe65 knockout mice correlate with reduced accumulation of lipofuscin in the animal retina (152). Although Tolleson et al. (153) postulated ocular lipofuscin granules contain all-trans-retinal, but it is mostly stored in a dimer form (atRal dimer) and other condensation product such as A2E (142).

Dark cytotoxicity

RPE cells in vitro. All-trans-retinal causes distinct cytotoxicity in RPE cells in vitro (154). It stalls human RPE cell metabolism and makes cell membrane almost totally permeable after 24-h of incubation with 40 μM atRal or as soon as 3 h at 60 μM concentration. Similar cytotoxic effects of all-trans-retinal on cell metabolism were observed when ARPE-19 cells were incubated in the dark with atRal in its free form (49,58).

Mitochondria. Another study performed on isolated mitochondria determined that retinal as a product of β-carotene cleavage inhibited oxidative phosphorylation by 12% in isolated mitochondria at concentrations as low as 1 μM (155). In addition, all-trans-retinal inhibited mitochondrial oxidation and uncoupled oxidative phosphorylation at concentrations > 50 μM in mitochondria isolated from rat hearts (58).

Cell membrane. All-trans-retinal in its free form has a high affinity with biological membranes, including cytoplasmic membrane, and may alter their stability (58). It can react via a Schiff base linkage with primary amines (49) present in membrane phospholipids and proteins. Binding of all-trans-retinal to the amine residues in membrane proteins can modify the structure of the cellular membrane and make it more permeable.

Lipid peroxidation. Modification of cell membrane structure can be associated with lipid peroxidation. We observed increased level of lipid hydroperoxides in the human RPE cells treated in the dark with 50 μM atRal (154). The formation of malonic dialdehyde, a product of lipid peroxidation, was
also detected in mitochondria incubated with \( \beta \)-carotene cleavage products containing retinal (155). The thiobarbituric acid reactive substances (TBARS) assay showed a direct effect of all-trans-retinal on lipid peroxidation in ARPE-19 cells (147).

**Antioxidants.** All-trans-retinal can also affect antioxidant system in the RPE cells. Interaction of atRal with antioxidant enzymes can cause their deactivation, which could be associated with enhanced superoxide formation, increase level of hydrogen peroxide and induction of lipid peroxidation. The effect of all-trans-retinal on \( \text{H}_2\text{O}_2 \) formation in the dark was indirectly proven by noting the reduction in dark toxicity in the presence of catalase added to RPE cells preincubated with atRal (156). In our studies (154), we have shown that atRal modifies glutathione level in the RPE cells in vitro. The retinal at concentration of 25 \( \mu \text{M} \) slightly decreases intracellular level of glutathione reduced form (GSH) and enhances oxidation of the tripeptide after 5 h incubation. In addition, atRal induces parallel changes of cell membrane structure, which lead to significant glutathione leaking from the cells (154). Similar effects were observed in other studies, where \( \beta \)-carotene cleavage products containing retinal induced depletion of glutathione and protein-SH in isolated mitochondria (155). The level of GSH dramatically decreased in mitochondria in the presence of retinal and other cleavage products and a parallel increase in oxidized form of glutathione (GSSG) content was observed.

**Photochemistry**

Photochemical reactions of retinoids proceed through several possible routes—photoisomerization, photodegradation, photooxidation and photopolymerization (153,157).

**Photoisomerization.** Retinoids including all-trans-retinol and all-trans-retinal undergo isomerization into a mixture of trans- and cis-isomers (153) (Table 3). 13-cis and 9-cis-retinal were identified as the major photoproducts of atRal illumination with UV laser radiation in deoxygenated acetonitrile (157,158).

**Photodegradation.** The all-trans-retinal absorption spectrum consists of a broad band in the range 280–480 nm with a maximal absorbance at 382 nm (Table 1). However, continuous irradiation of atRal in argon-saturated methanol caused almost total bleaching of the chromophore and formation of 9,10-dihydroretinal (Table 3), which has an absorption spectrum shifted to below 300 nm (56,158). Although fluorescence emission intensity of free atRal (\( \lambda_{\text{max}} = 467 \text{ nm} \)) is relatively low, it is getting diminished during long UV irradiation (158) (Table 1).

### Table 3. Photoisomerization, photodegradation and oxidation products of ocular retinoids.

<table>
<thead>
<tr>
<th>Chromophore</th>
<th>Photoisomerization</th>
<th>Photodegradation</th>
<th>Product</th>
<th>Illumination wavelength</th>
<th>Solvent</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>all-trans-retinal</td>
<td>13-cis-retinal</td>
<td>9,10-dihydroretinal</td>
<td>all ( \text{trans},5,8)-peroxy-retinal</td>
<td>MeCN</td>
<td>(157,158)</td>
<td></td>
</tr>
<tr>
<td>9-cis-retinal</td>
<td>(UV, deox. MeCN)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2E</td>
<td>9-cis-A2E</td>
<td>9-cis-A2E</td>
<td>A2E polypeoxides</td>
<td>430 nm</td>
<td>MeCN</td>
<td>(157)</td>
</tr>
<tr>
<td>13-cis-A2E</td>
<td>(room light)</td>
<td>Bleaches in deox</td>
<td>A2E nonaioxiranes</td>
<td>430 nm</td>
<td>MeCN</td>
<td>(199)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A2E monoperoxides</td>
<td>&gt; 400 nm</td>
<td>PBS</td>
<td>(198)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A2E epoxides</td>
<td></td>
<td>MeOH</td>
<td>(198)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A2E ( 5,8)-furanoid oxide</td>
<td>430 nm</td>
<td>MeCN</td>
<td>(201)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A2E ( 5,8,5'8'-)-bis-furanoid oxide</td>
<td>430 nm</td>
<td>MeCN</td>
<td>(201)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Monoperoxy-A2E</td>
<td>430 nm</td>
<td>MeCN</td>
<td>(202)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bisperoxy-A2E</td>
<td>430 nm</td>
<td>MeCN</td>
<td>(202)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Aldehydes and ketones formed after oxidized A2E cleavage</td>
<td>430 nm</td>
<td>MeCN</td>
<td>(216,217)</td>
</tr>
<tr>
<td>HIDD</td>
<td>Bleaches in deox</td>
<td>MeCN and MeOH</td>
<td>Pyridinum terpenoid</td>
<td>425 nm</td>
<td>MeOH</td>
<td>(206)</td>
</tr>
<tr>
<td>HIDD-H(^{+})</td>
<td>Bleaches in deox</td>
<td>MeCN and MeOH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1E</td>
<td>Bleaches in deox</td>
<td>MeCN and MeOH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>atRal dimer (AD)</td>
<td></td>
<td></td>
<td>Pyridinum terpenoid</td>
<td>425 nm</td>
<td>MeOH</td>
<td>(207)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>( \text{cis} ) 5,8-peroxide</td>
<td>425 nm</td>
<td>CDCl(_3)</td>
<td>(207)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Oxygen adduct to AD double bond</td>
<td>430 nm</td>
<td>H(_2)O, CHCl(_3)</td>
<td>(209)</td>
</tr>
<tr>
<td>AD-PE</td>
<td></td>
<td></td>
<td>Oxygen adduct to AD-PE double bond</td>
<td>430 nm</td>
<td>H(_2)O, CHCl(_3)</td>
<td>(209–211)</td>
</tr>
</tbody>
</table>

CDCl\(_3\), deuterated chloroform; CHCl\(_3\), chloroform; deox., deoxygenated; MeCN, acetonitrile; MeOH, methanol.
**Photooxidation.** When retinoids are exposed to UV light in air-saturated solutions they oxidize, giving products, such as retinol, 5,6-epoxide- and 4-keto-retinol and 5,6-endoperoxide (153). All-trans-retinal is photochemically less stable than ocular lipofuscin extract. In benzene, atRal bleaches much faster than ocular age pigment when exposed to UV light (60). All-trans-5,8-peroxyretinal was detected in irradiated atRal solution in acetonitrile (157) (Table 3).

**Free radical photoformation.** In vitro studies showed that all-trans-retinal exposed to UV or visible light (300–407 nm) produced free radicals in the presence of hydrogen atom or electron donors (56,57,153,159,160). EPR spin trapping experiments revealed that all-trans-retinal in deoxygenated methanol irradiated with UV and visible light (>300 nm) was able to abstract hydrogen atom from the solvent and generate its carbon-centered radicals (159) (Table 2). However, the retinyl spin adduct with 5,5-dimethyl-1-pyrroline N-oxide (DMPO) was not detected. Similar effects of hydrogen transfer were observed when other retinoids were tested—retinol in methanol and retinyl palmitate (RP) in dimethylformamide (159), but free radicals were not detected in retinoic acid (RA) solution in methanol (159). However, Tolleson et al. (153) have shown that retinol in 70% ethanol exposed to UV produces carbon-centered radicals. Pulse radiolysis experiments performed by Rożanowska et al. (160) revealed that atRal cation radical can be formed as a result of interaction with hydroxyl radical.

**UV and light-induced production of superoxide anion.** All-trans-retinal is an efficient photosensitizer and generates ROS in aerated solutions (57,153,159,160). Exposure to visible light (<418 nm) atRal Schiff base is able to transfer an electron from an electron donor such as reduced form of nicotinamide adenine dinucleotide to an oxygen molecule and form superoxide anion (159). Pawlak et al. (57) observed characteristic superoxide anion spin adducts to DMPO in dimethyl sulfoxide (DMSO)-benzene mixture (9:1) during exposure to visible light (407 nm) (Table 2). The reactive oxygen form can be also generated after atRal radical anion with molecular oxygen (160). However, atRal at its non excited form does not interact with superoxide anion (160).

**Singlet oxygen photoproduction.** Compared with other retinoids—atRol, retinoic acid and retinyl palmitate, all-trans-retinal most efficiently forms an excited triplet state (56) with lifetime at 9.3 μs (60). All-trans-retinal is the most effective visual cycle component that photogenerates singlet oxygen in vitro (57,60,159) (Table 2). The quantum yields of 1O2 photogeneration by atRal in methanol was determined as 0.05 and it was three and at least five times higher than in atRol and RP methanol solutions, respectively (159). Measurements performed in carbon tetrachloride revealed that the 1O2 quantum yield for atRal can reach the level of 0.25 (159). The efficiency of atRal in 1O2 photoformation is comparable to the human ocular lipofuscin organic solvent extract when solubilized in methanol (129). However, it was determined that the quantum yield in benzene for atRal is two to three times higher than for LF extract when irradiated with UV (355 nm) and even two to six higher during blue light (420–440 nm) exposure (60). Contrary to LF extracts, the quantum yield of singlet oxygen photoinduced formation by atRal remains generally stable for 355 and 420 nm of excitation wavelength and is oxygen concentration independent in the range of 1.9–9.0 mm O2 (60). In vitro studies performed by Pawlak et al. (57) have shown that oxygen uptake in all-trans-retinal samples was used mostly for 1O2 photogeneration, which was confirmed by the formation of 5a-hydroperoxysterol, a specific indicator of Type-II photosensitized oxidation of cholesterol.

**Photobiology**

All-trans-retinal-mediated photodamage to lipids and proteins is oxygen-dependent (147,161,162). The semioxidized retinoid can further interact with tyrosine and cysteine, leading to protein oxidation (160).

**Light-induced photoreceptor damage.** All-trans-retinal or other retinoid chromophores can play an important role in light-induced damage of photoreceptors and RPE cells (147), whereas atRol and RP were shown to be efficient antioxidants protecting POS lipids in vitro from peroxidation (16,86). Other in vitro studies have shown that all-trans-retinal exposed to visible light impairs the ABCA4 transporter present in POS (161). In addition, all-trans-retinal can cause photodamage to interphotoreceptor retinoid-binding protein (IRBP), which sustains photoreceptors and is responsible for retinoid transport between them and the RPE cells (163). All-trans-retinal bound to IRBP and exposed to visible light caused tryptophan fluorescence decay and thiol group oxidation in the protein structure as well as reduction of retinol binding activity. All-trans-retinal produced from 9-cis-retinal in 661 W photoreceptor cells induced their apoptosis after illumination with visible light (453 nm) (164). Interestingly, the same in vitro study has shown that all-trans-retinol is not toxic to the photoreceptor cells under the same experimental conditions (164). Lipids in ARPE-19 cells preloaded with atRal underwent faster peroxidation when the cells were exposed to UVA and TBARS concentration rose during irradiation (147). In vivo, atRal can induce light-dependent degeneration in the retina of rpe65−/− mice (147). The absence of 11-cis-retinal and all-trans-retinal in the photoreceptors made the mouse retina resistant to light toxicity. Maeda et al. (142) in their studies observed acute form of retinopathy in light exposed mice that lack two enzymes—ABCA4 and RDH8, which are responsible for atRal clearing. The toxic effects were characterized by increased cell membrane permeability and mitochondria-derived apoptosis. Interestingly, the authors emphasized that excess accumulation of atRal, but not A2E or other product of retinoid condensation was the main reason of the observed light-induced retinopathy.

**Phototoxicity in the RPE.** Our studies have shown that human RPE cells partially impaired by the dark cytotoxic effect of all-trans-retinal (<30 μm) and then irradiated with visible light (>400 nm) were exposed to additional stress in the form of reactive intermediates of retinal and oxygen (154). Short light exposure caused further decline in cell metabolism and enhanced cell membrane permeability. A phototoxic effect of all-trans-retinal was also observed when ARPE-19 cells were preincubated with a higher (0.1 mm) retinal concentration in phosphatidylcholine liposomes and exposed to visible light.
In addition, irradiation with blue light (440 nm) caused photodamage in the same type of RPE cells and also in HEK293 cells derived from human kidneys as well at much lower concentrations of free all-trans-retinal (58). However, light effect of RPE cells pretreated with atRal is not unambiguous. In our studies, light (>400 nm) exposure of RPE cells fed with atRal (<50 μM) modified the level of intra- and extracellular glutathione and affected lipid peroxidation and formation of other hydroperoxides, but these effects depended on atRal concentration and time of light exposure (154). Prolonged irradiation induces bleaching, isomerization and autoxidation of the retinal, which in effect lead to decrease in formation of retinal-derived photoreactive products and ROS. The presence of antioxidants and phospholipids containing amine groups in the cell membrane counteract the negative effects of oxidative stress caused by enhanced level of atRal in its free form. Różanowski et al. (156) showed in their in vitro studies that phototoxicity of all-trans-retinal toward RPE cells is significantly reduced by γ-tocopherol and zeaxanthin. In addition, atRal toxicity was significantly inhibited in the ARPE-19 cells that were treated with liposomes containing the retinoid and phosphatidylethanolamine (156). Moreover, the semioxidized atRal-PE adducts are less damaging to proteins than semioxidized atRal (160).

A2E

The lipofuscin chromophore

Spectroscopic analyses have revealed that the lipofuscin present in the human RPE contains numerous chromophores (92,120,126,129). At least a dozen of them have been isolated and characterized.

A2E (chemical name: 2-[2,6-dimethyl-8-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E,7E-octatetraenyl]-1-(2-hydroxyethyl)-4-[4-methyl-6-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E-hexatrienyl]-pyridinium) (165), also called N-retinylidene-N-retinylethanolamine (166) is a major and the first isolated fluorophore from RPE lipofuscin (102,167). It occurs in the RPE cells mostly in 2 isomeric forms all-trans-A2E and 13-cis isoform (iso-A2E) (168,169). It is estimated that iso-A2E constitutes 15–20% of entire pool of A2E in the human ocular lipofuscin (61,169).

A2E biosynthesis and accumulation in age

Biosynthesis. All-trans-retinal in its free form is a highly reactive molecule. After release from photoactivated rhodopsin in POS, all-trans-retinal can bind to phosphatidylethanolamine (92,102,146,166,170), a primary amine that is present in POS membranes of at relatively high abundance (ca 40% of total phospholipids) (146,170). A fraction of the all-trans-retinal-PE Schiff base (N-retinylidene-PE; APE) can undergo further condensation (166,170,171). Another atRal molecule can bind to APE to produce dihydro-N-retinylidene-N-retinyl phosphatidylethanolamine (A2PE-H2), which is transported during POS phagocytosis to the RPE cells (166). After oxidation and cyclization, A2PE-H2 is transformed to N-retinylidene-N-retinyl phosphatidylethanolamine (A2PE) (166). The phosphate ester is hydrolyzed in phagolysosomes (166) more likely via enzymatic reaction (170–172) to yield A2E chromophore (103,166,170), inside the retinal pigment epithelial cells (20,166,170,173–175). However, Ben-Shabat et al. (172) observed that A2PE can be at least partially synthesized inside POS before internalization by RPE cells. In addition, they suggested that some amounts of A2PE could be converted into A2E inside non phagocytosed POS (172).

Accumulation in the RPE. The bis-retinoid together with other fluorescent chromophores accumulates with age in lipofuscin granules (102). Studies performed by Delori et al. (103) have shown that lipofuscin fluorescence increases linearly with age until 70 years. It implies that A2E could accumulate in the age pigment granules at the same rate. Indeed, the measurements of A2E level conducted by Bhosale et al. (176), on the human eyes from donors at age 20–88 y.o. have shown that A2E builds up in the RPE mostly in the linear manner. The constant accumulation of A2E in RPE cells in vitro and the saturation of that process observed by Schutt et al. (175) suggest that RPE cells do not possess a mechanism that could lead to A2E degradation. However, the recent studies of Wu et al. (177) have shown that A2E loaded in vitro to the RPE cells can be cleaved by horseradish peroxidase.

The A2E level and spatial distribution in the retina

A2E level in the human eye. The A2E amount in the human RPE cells in vivo can reach level of 60–130 ng 10⁻⁵ cells (169) or 830 pmol per eye (168), which is comparable to the bis-retinoid level in the RPE cells incubated in vitro for several hours with 15–30 μM A2E (169,174,178). Davies et al. (137) estimated that the age pigment isolated from the human eyes contains both isomeric forms of A2E on average: 7.8 10⁻²⁰ mole/LF granule or 2.4 10⁻¹⁷ mole/RPE cell. Most recently, Bhosale et al. (176) determined A2E and iso-A2E amount in the human RPE/choroid in two age groups. An average level of A2E and its isomeric form in donors younger than 50 y.o. was 6.4 and 0.6 ng per eye, respectively. Persons older than 50 y.o. had significantly higher level of both A2E forms—11.8 and 2.2 ng per eye, respectively.

A2E levels in Stargardt’s disease. Increased accumulation of A2E in young age is observed in persons suffering for Stargardt’s macular degeneration—genetically caused malfunction of the rod photoreceptor-specific ATP-binding cassette transporter responsible for flipping atRal-PE Schiff base adduct from the intradiscal to the cytosolic face of POS disk membrane what facilitates reduction of atRal by atRol dehydrogenase and constrains accumulation of A2E (146,166,170,171,179).

A2E in animals’ eyes. A2E accumulates also in other mammals, such as monkeys, cow, pigs, rats and mice (20,33,102,176). For example, Sutter et al. (20) determined 4 and 42 pmol A2E per eye in rats weighing 200 and 350 g, respectively. However, A2E in albino Sprague–Dawley rats (3-month old, male) was detected in the retina at the level of 6 pmol μmol⁻¹ fatty acids (33).

Spatial distribution. Similarly to lipofuscin autofluorescence, A2E distribution in the human RPE is not uniform (103). The level of A2E in the macula is on average ca four times lower
compared to the periphery of the retina in persons younger than 50 y.o. (176). With age, the bis-retinoid amount increases faster in the macula than in the periphery. The macula/periiphery ratio of A2E level in the group of donors older than 50 y.o. drops to 3.4. Similarly to the human eye, A2E shows diversified spatial distribution in the mouse eyes (Fig. 4B). Grey et al. (180) have recently reported that A2E was detected mostly in the central RPE region of the eyes in 2-month old mice. However, with age A2E accumulated also across the tissue. A good correlation between A2E and lipofuscin distribution has been also observed.

Interestingly, A2E distribution in the human RPE/choroid seems to be inversely correlated with the concentration diversity of the retinal carotenoids. The level of carotenoids in the macula is 3.5–4.0 times higher than in peripherial region of the human retina (176).

**A2E distribution inside RPE cells.** It has been shown that A2E accumulates in the RPE cells in vitro in the perinuclear region (169,181) being delivered mostly (ca 90%) to lysosomes (102,169,175,182). In addition, the bis-retinoid can accumulate to a lesser extent in mitochondria, Golgi apparatus and the cytoplasm membrane (175).

**Illumination effect.** Light exposure affects A2E biosynthesis. Studies using the Abca4-/- transgenic mouse model for Stargardt’s macular degeneration have shown that the biosynthesis of A2E greatly accelerated when the animals were housed in a cyclic light/dark mode when compared with those stored in complete dark (166,183). Different intensity of ambient light seems not to affect A2E level in RPE cells of the transgenic mice, both albino and pigmented (184).

**Dark cytotoxicity**

**A2E structure and chemical properties.** A2E is generally less reactive than its precursor all-trans-retinal. The engagement of a Ral formyl group in reaction with the amine group of phosphatidylamine during condensation reaction makes A2E less reactive than its precursor toward nucleophilic groups, such as amines in proteins. However, A2E is a quaternary amine with molecules of pyramidal shape (178) with two hydrophobic retinoid chains. Its structure predestinates A2E to act as an amphiphilic detergent, which is able to modify phospholipid membrane arrangement (102,169,178).

**Cell metabolism and membrane integration.** A2E presence in the RPE cells can affect their metabolism through several mechanisms. The fluorophore inhibits the growth of human RPE cells in vitro at concentration as low as 1 μM (185). A2E at concentration of 25 μM that is comparable to its level in human ocular lipofuscin does not impair human RPE cell metabolism tested with MTS assay (154). The intracellular level of glutathione was not affected in the cells treated with 12.5 μM A2E. However, twice higher concentration of A2E caused the intracellular level of oxidized glutathione form to be raised three-fold, but the total concentration of both tripeptide forms was unchanged (154). In addition, 25 μM A2E did not increase lipid hydroperoxide level in the RPE cells, did not enhance cytoplasm membrane permeability detected as lactate dehydrogenase (LDH) release from the RPE cells in vitro and did not induce significant glutathione efflux from the cells (154). However, Sparrow et al. (169) studies have shown that although A2E was not detected in the cytoplasm membrane, the elevated level of LDH release was observed in the RPE cells exposed to much higher concentrations (50–100 μM) of the bis-retinoid. In addition, A2E at the level of 50 μM impaired phagocytotic properties of the RPE cells in vitro (186).

**Mitochondria.** A2E toxicity toward RPE mitochondria seems to be ambiguous. Several studies have shown that the bis-retinoid at < 5 μM impaired function of isolated cytochrome oxidase (187), and at 30–45 μM A2E inhibited activity of the enzyme in mitochondria isolated from rat liver (20). A2E at concentration of 50 μM diminished mitochondrial ATP synthesis in human RPE-J cells (186). However, the lipofuscin fluorophore did not affect the respiratory chain activity upstream of cytochrome c (20). On the other hand, studies performed by Maeda et al. (58) revealed that while all-trans-retinal inhibited mitochondrial oxidation and uncoupled oxidative phosphorylation at concentrations >50 μM in mitochondria isolated from rat hearts, A2E did not affect these metabolic functions even at a concentration of 600 μM.

**Lysosomes.** The inhibition of the ATP-driven proton pump of lysosomes was observed in the RPE cells incubated with only 2 μM A2E (188). Although Holz et al. (182) initially suggested that A2E inhibited lysosomal digestion of proteins, Finнemann et al. (189) showed that lysosomal proteolysis is unaffected directly by A2E. In the same study, they presented significant inhibition of phagolysosomal degradation of photoreceptor phospholipids in the cells treated with 100 μM A2E (189). Further experiments of that group (178) revealed more details on A2E effect on lysosome activity and cholesterol metabolism in the RPE cells. A2E as a quaternary amine, in contrast to classical lysosomotropic amines, does not increase pHi in late endosomes and lysosomes and does not inhibit directly the activity of several lysosomal enzymes in the RPE cells (treated with 15 μM A2E for 6 h) in vitro. However, as it was demonstrated using liposomes as a model of a phospholipid bilayer, the bis-retinoid competes with cholesterol for a place in the membrane. Thus, during the digestion process of POS phagocytosed by RPE cells A2E present in the photoreceptor material accumulates in the endosome membrane and restricts cholesterol efflux from the late endosomes. Increased level of cholesterol in late endosomes and lysosomes induces inhibition of acid lipase and leads to buildup of cholesteryl esters and oxidized cholesterol in the cells.

**Apoptosis.** A2E can induce cell apoptosis in the dark. Suter et al. (20) have shown that 25–50 μM A2E treatment of the porcine RPE cells for 24 h induced internucleosomal fragmentation of genomic DNA and positive TUNEL staining. However, apoptosis can be induced by the bis-retinoid in neurons at lower concentrations. A2E at concentration of 20 μM triggered detachment of cytochrome c from the mitochondria and release of apoptosis-inducing factor after 20 h treatment of cerebellar granule cells isolated from 8-day-old BALB/c mice and then cultured in vitro (20).
Cytoxicity of A2E and its precursors. Despite the fact that A2E shows limited reactivity, a comparison of the cytotoxicity of A2E and all-trans-retinal leads to the general conclusion that condensation of atRal into A2E protects RPE cells from cytotoxic effect of increased levels of the retinal in phagocytosed POS (154,174). In vitro studies performed on ARPE-19 cells showed that atRal toxicity was significantly inhibited when phosphatidylethanolamine was present in liposomes containing the chromophore (156). The semioxidized atRal-PE adducts are also less damaging to proteins than semioxidized atRal (160).

Thus, it seems that the buildup of undigested material in the RPE and/or the destruction of the postmitotic cells could contribute to the development of AMD and progression to its severe atrophic form.

Spectrophotometric properties

**Light absorption.** The absorption spectrum of all-trans-retinal, an A2E precursor is located mostly in UV, characterized by one band with a maximum at 380 nm. The retinal absorbs only slightly in the visible light range up to 450 nm (56) (Table 1). However, the condensation of 2 atRal molecules into A2E form causes that the bis-retinoid, contrary to its precursor absorbs efficiently the short-wavelength light of the visible range. A2E absorption spectrum is characterized by two maxima at 336 and 430–439 nm (168,173,190) and at 550 nm its absorbance decreases to 5–10% of the long-wavelength peak (103).

**Fluorescence.** The fluorescence of lipofuscin in the RPE cells was of interest to scientists investigating photobiological properties of the age pigment. The characterization attempt of the major chromophore responsible for the LF fluorescence led to extraction and isolation of A2E (102). Intensity and the maximum of fluorescence emission spectrum depend on the environment in which A2E is present and the excitation wavelength (Table 1). In organic solvents, the fluorescence emission was detected at 585–610 nm (169) and 510–570 nm, respectively. The maximal intensity of fluorescence emission was detected at 585–610 nm (169) and 510–570 nm (173), when A2E samples were irradiated with 380 and 430 nm, respectively. The maximal intensity of fluorescence emission of A2E in Triton X-100 micelles illuminated at 430 nm was registered at 615 nm (173). The emission maxima of A2E incorporated in liposomes or RPE cells have been observed in the range of 550–590 nm (191) and 565–570 nm (169) when excited at 400 and 380 nm, respectively.

In vitro studies performed on synthetic A2E in ethanol showed that most of its photoexcited molecules return to the ground state via non radioactive emission (57,190). Although the quantum yield of A2E fluorescence detected in the micelles (173) or in the RPE cells in vitro (185) was 1.5–5 times higher than in pure solutions, the energy of light absorbed by the bis-retinoid is only partially (ca 1%) emitted back as fluorescence (101,173,190). More detailed studies have revealed that the presence of relatively intensive autofluorescence observed in the human retina when excited at 550 nm (103) suggests that A2E is not the main fluorophore responsible for long-wavelength fluorescence emission from LF granules. Nevertheless, A2E can serve as an energy acceptor from other blue light absorbing molecules present in lipofuscin (101,173,192).

Photoreactivity

Absorption of light mostly in the blue light range and golden fluorescence similar to lipofuscin (101) implied that A2E could be a good candidate for the most potent photosensitizer of the ocular lipofuscin (49).

**Photoisomerization.** A part of the energy of light absorbed by A2E can be used for conversion of A2E molecule into its 13-cis isomer (iso-A2E) (57,168,173) (Table 3). It is likely that the photoequilibrium between these two forms of A2E occurs also in vivo (169). Other isomeric forms of A2E (9-cis, 11-cis) have been also detected as products of A2E photoisomerization in room light (172).

**Photogeneration of the transient intermediates.** Reszka et al. (130) showed that irradiation of the products of reaction typically used for A2E formation in vitro between atRal and ethanolamine (2:1) then fractionated with HPLC led to generation of transient species (the triplet state and a free radical insensitive to oxygen), which characteristics were similar to LF extracted from human RPE cells. However, the carbon-centered radicals detected during the studies were slightly different than those obtained in extracts from natural human lipofuscin.

Only a small portion of the energy absorbed by A2E is used to produce its transient reactive forms. The bis-retinoid is characterized by inefficient intersystem crossing (57,173,190,192). In vitro research revealed that pure A2E does not efficiently produce a long-lived triplet excited state, an intermediate that is essential for generation of singlet oxygen or other ROS (173,190,192). The quantum yield of that process was estimated to be below 0.01 after A2E excitation at 355 nm in methanol and benzene (192) and at most 0.03 in methanol solution of the bis-retinoid illuminated at 430 nm (190).

A2E and other blue-light photosensitizers. The low quantum yield of transient species formation of A2E and its photophysical properties in association with its concentration in lipofuscin granules strongly suggest that this fluorophore is not responsible for LF phototoxicity (57,61,137,190,192). It was estimated that A2E-derived phototoxicity in cells loaded with the ocular age pigment was responsible for maximum 1% of total lipofuscin photodamaging potential (137). Generally, A2E possesses far less-photosensitizing properties than lipofuscin (60,61), all-trans-retinal (57,58,154), protoporphyrin (193), or other endogenous blue light chromophores in the RPE (194). For example, Pawlak et al. (57) using EPR oximetry determined that liposomes containing 150 μM A2E illuminated with light at wavelength of 407 nm consumed oxygen 20.6-fold slower than did those containing 390 μM all-trans-retinal. The comparison of the rates of oxygen photouptake by A2E and ocular lipofuscin suspended in liposomes determined that the bis-retinoid consumed oxygen ca 100 times slower than lipofuscin, when normalized to A2E content in the human ocular age pigment (61).

**Superoxide anion photoproduction.** In vitro studies proved that A2E shows insignificant photoreactivity and is generally much less efficient at producing ROS compared with other blue light chromophores in the aged retina (57,61,173,174,195) (Table 2). EPR spin trapping performed by Reszka et al. (130) have shown that the products of A2E synthesis can photogenerate
superoxide anion. The results of further experiments carried out in vitro using EPR spin trapping (57,61,196) and pulse radiolysis (197) proved that A2E is able to produce superoxide anion. Pawlak et al. (57) have shown that A2E illuminated with 407 nm light in DMSO/benzene (9:1) mixture produced superoxide anion 3.2 times slower than lipofuscin extract, but ca four times faster than all-trans-retinal, all normalized to the same absorbance at 403 nm. The quantum efficiency for O$_2^-$ generation by A2E determined in that study was very low (0.0003), when riboflavin (RF) was used as the model of superoxide anion generator ($\Phi_{RF} = 0.01$).

Light-induced singlet oxygen formation. A2E can also photo-generate singlet oxygen, but the quantum yield of that process is not higher than 2% (57,173,192) (Table 2). When detection of $^1$O$_2$ characteristic phosphorescence at 1270 nm was employed, $^1$O$_2$ quantum yield has been estimated as 0.02 in acetonitrile (190) and 0.013 in deuterated cyclohexane/deuterated acetone (4:1) mixture (195) when exposed to UV (355 nm). Interestingly, it was determined at much lower level when A2E solutions were illuminated with blue light—0.004 in CCl$_4$ at 435 nm (174) or hexafluorobenzene at 420 nm and even less in perdeuterated solvents (195). It is at least one order of magnitude lower than for all-trans-retinal (\( \Phi = 0.24\)-0.25 in CCl$_4$)—the precursor in A2E biosynthesis (159,174). Pawlak et al. (57) and Kanofsky et al. (195) showed even larger differences in quantum yield of $^1$O$_2$ photoproduction by A2E and its precursor when measured specific products of cholesterol peroxidation. A vestigial concentration of 5x-cholesterol hydroperoxide—the specific $^1$O$_2$-derived peroxidation product detected in A2E samples indicated that the rate of singlet oxygen formation during illumination of A2E at 412–500 nm is ca 70 lower than in samples containing atRAL, when normalized to the same absorbance of both chromatophores at 403 nm (57). Although irradiation of A2E also releases ROS, the quantum yield is insufficient to account for the amount of ROS produced by lipofuscin.

Antioxidant properties. A2E is a relatively efficient quencher of singlet oxygen, with a quenching rate of ca 3 $\times$ 10$^4$ M$^{-1}$ s$^{-1}$ in CCl$_4$ (198) and 2 $\times$ 10$^4$ M$^{-1}$ s$^{-1}$ in acetonitrile (174), which is comparable to vitamin E or ascorbic acid (174).

A2E photooxidation. A2E is a reactive molecule can be easily oxidized, particularly when irradiated (184,194,198–202) (Table 3). Illumination of A2E in vitro in air-saturated solvents leads to the formation of A2E oxidation products (184,194,198–200). Gaillard et al. (196) have shown that A2E photoactins in air-saturated solvents run faster in water versus chloroform and in hydrogenated versus perdeuterated methanol. It was implied that the solvent effect and the phenomenon that products of A2E photooxidation are characterized by sequential oxygen addition primarily through a free radical mechanism (196). Moreover, photooxidation mediated by $^1$O$_2$ would lead to the addition of two oxygen atoms to A2E molecule (196). A2E is not a sole photosensitizer present in the RPE lipofuscin, which absorb blue light (61). There are other chromophores that are sufficiently phototoxic (59,60) that could oxidize A2E both via free radical and singlet oxygen mechanism.

The experiments performed on laboratory animals have proven that A2E photooxidation can take place in vitro. The studies on the $Abca4^{−/−}$ transgenic mouse have revealed that A2E conversion to oxidized form(s) is greatly accelerated by light (166,184). Intensive blue light illumination (450 nm) of Sprague–Dawley rats caused the total amount of A2E and its isomer (iso-A2E) in the retina (normalized to fatty acid content) to decrease six- and four-fold, respectively in comparison to animals housed in the dark (33). Blue-light (450 nm) exposure promoted oxidation of A2E and iso-A2E and accumulation of A2E oxidative products (33,184) that could be toxic to retinal tissue.

Photobiology
A2E is the major fluorescent components of lipofuscin. The question has been raised whether A2E is the primary photooxidizing agent for blue light damage to the retina. Numerous studies have shown that A2E can be at least partially responsible for lipofuscin phototoxicity in the RPE cells.

Photobiological activity. A2E present in lysosomes of the RPE cells treated in vitro with bis-retinoid-LDL complex and exposed to blue light (390–550 nm) caused disruption of lysosomal membranes (203). Cytochrome oxidase inhibition was observed in mitochondria isolated from rat liver, pretreated with 15 $\mu$M A2E and illuminated with a tungsten lamp (20). It has been reported that A2E (at the concentration of 50–100 $\mu$M) is the source of blue light-mediated (480 nm) ROS that can lead to RPE cell apoptosis (204,205).

On the other hand, A2E at the physiological level (< 25 $\mu$M) did not affect metabolism (determined with MTS assay) and did not enhance cytoplasmic membrane permeability (measured as LDH release from the cells) in the human RPE exposed in vitro to visible light (\( \lambda > 400 \) nm) (154). Moreover, Roberts et al. (174) have shown that A2E protected the undifferentiated human RPE cells in vitro from UV-induced (> 300 nm, 80 mJ cm$^{-2}$) photodamage and breaks in DNA at the concentration of the fluorophore of 5 and 1–5 $\mu$M, respectively. However, the A2E protecting properties were not observed when the cells were illuminated with visible light (< 400 nm) or the fluorophore concentration reached 10 $\mu$M.

Photobiological role of A2E in ocular lipofuscin. Photophysical and photochemical properties of A2E markedly differ from those of either LF granules or LF extracts (60) and from its biosynthesis precursor—all-trans-retinal (57,154,156,160). Photoreactivity of A2E is at least two orders of magnitude lower than lipofuscin when compared to the endogenous equivalent of the retinoid derivative in LF granules (120). Although A2E may be harmful at high concentrations, its cytotoxic and phototoxic effects even at the highest physiological level (20–25 $\mu$M) are much lower compared to the free form of its precursor all-trans-retinal at the same concentration or other LF components.

RETINAL AND A2E DERIVATIVES
Since the time when A2E was described as the major fluorophore of ocular lipofuscin, many derivatives of the bis-retinoid have been identified in the retinas or theoretically proposed (102,166). One group is formed by the intermediates
of A2E synthesis, the other is made up of additional all-trans-retinal condensation forms, whereas the products of A2E oxidation can be rated among the compounds forming the third group.

### A2E synthesis intermediates

**atRal-PE Schiff base.** APE is an imine form of all-trans-retinal and phosphatidylethanolamine and is one the precursors of A2E (170). APE absorbs in the range of visible light similar to A2E, but is characterized by only single band with maximal absorbance in acetonitrile solution at 456 nm (170) (Table 1). **HIDD.** Synthetic APE analogues—(E,E,E,E)-2-[9-(2-hydroxyethyl)iminoo-3,7-dimethyl-1,3,5,7-decatrien-1-yl]-1,3,3-tri methylcyclohexene (HIDD) and its protonated form HIDD-H⁺, which are the products of atRal reaction with ethanolamine (EA) have been described by Harper and Gaillard (206). HIDD absorbs mostly in UV with maximum at 360–370 nm in acetonitrile, whereas the main HIDD-H⁺ absorption is much redshifted with its peak at ca 450 nm (Table 1). Both HIDD forms are weakly fluorescent. The intensity of HIDD emission is stronger than HIDD-H⁺, with λmax in dichloromethane at ca 520 and 610 nm, respectively. Direct excitation can induce generation of HIDD triplet state; whereas the intermediate form of HIDD-H⁺ can be produced only via energy transfer from the triplet state of other photosensitizer. In addition, HIDD bleaches faster than HIDD-H⁺ both in deaerated acetonitrile and methanol, when exposed to irradiation at the wavelengths at their respective absorption maximum (Table 3).

**A1E.** A1E is another product of reaction between atRal and EA, which has a structure like A2E with only one retinoid arm (207). It seems that this product because of its molecular structure is not physiologically relevant. Its absorption spectrum is characterized by one intensive band with maximum at 417 nm in methanol (Table 1). It shows very week fluorescence with λmax at 560 nm. Illumination of A1E solution with blue light (425 nm) in air-saturated methanol and perdeuterated chloroform leads to formation of two different photoproducts—a pyridine terpenoid (a product of cyclization; λabs max = 294 nm) and a cyclic 5,8-peroxide (λabs max = 363 nm), respectively (Table 3). Both photoproducts were detected when CHCl₃ was used a solvent. The peroxide was also produced during A1E peroxidation with O₂ generated by thermal decomposition of aromatic endoperoxide.

**A2PE-H₂.** Condensation of APE with another atRal molecule leads to formation of dihydro-N-retinylidene-N-retinyl phosphatidylethanolamine (A2PE-H₂). It was first observed in extracts from POS and RPE of Abca4⁻/⁻ mice by Mata et al. (166). Significant accumulation of the bis-retinoid with age was observed in that type of mice, as well as in the human retina and RPE of persons with macular disorders (166,183). The absorption spectrum of the chromophore is characterized by two bands—one at 205 nm and the second band is considerably redshifted in comparison to the long-wavelength band of A2E, with a maximum at 500 nm (166) (Table 1).

**A2PE.** It has been demonstrated by Mata et al. (166) that A2PE-H₂ in acidic environment can be oxidized to form N-retinylidene-N-retinyl phosphatidylethanolamine (A2PE)—the next intermediate in A2E biosynthesis. They detected A2PE, contrary to its precursors only in the RPE cells of mice (166,183). However, Liu et al. (170) observed A2PE both in the retina and the RPE isolated from rats. Fishkin et al. (208) identified a novel fluorophore—A2-Rh during incubation of excessive amount of atRal with isolated bovine rod outer segments (Table 1). A2-Rh is formed with 3 bis-retinoids (A2) bound to lysines in a rhodopsin molecule. Depending upon where exactly A2PE is generated, the pipyryl ring formation in the A2PE structure caused modification of the two-band absorption spectrum (Table 1). The maxima of A2PE extracted from 6 months Abca4⁻/⁻ mouse RPE were detected at 330 and 430 nm (166). However, A2PE in acetonitrile solution prepared by Liu et al. (170) had maxima at 337 and 456 nm. Ben-Shabat et al. (171,172) demonstrated that A2PE emits orange fluorescence.

### Additional products of all-trans-retinal condensation

**All-trans-retinal dimer.** Fishkin et al. (209) have demonstrated that two molecules of all-trans-retinal can form a dimer before reaction with PE and together produce another conjugate that could be present in the POS. The atRal dimer was found in bleached rod outer segments incubated with atRal. Absorption spectra of atRal dimer and its product of conjugation with PE (AD-PE) are characterized by two bands with maxima at 295 and 432 nm and 285 and 506 nm, respectively (Table 1). The dimer effectively oxidizes when exposed in air-saturated solution to blue light (430 nm), but the rate of photooxidation is higher in water than in chloroform (209) (Table 3). It has been suggested that the photooxidation products of atRal dimer are formed by addition of oxygen to double bonds and an aromatic demethylation can occur. Sparrow’s group has shown that atRal dimer (210,211), its oxidative products (212) and atRal dimer conjugates with PE (209–211) and ethanolamine (210) have been detected in Abca4⁻/⁻ mouse eyes, bovine RPE and neutral retina and in human RPE/choroid. However, a study performed by Bui et al. (213) did not support the atRal dimer presence in Abca4⁻/⁻ mouse eyes. Most recently, it has been demonstrated that atRal dimer can accumulate in relatively small amounts in the retina of Royal College of Surgeons (RCS) rats that possess impaired photoreceptors (214).

**A2-DHP-PE.** A2-dihydropyridine-phosphatidylethanolamine (A2-DHP-PE) is another atRal-related chromophore, similar to AD-PE and reported by Wu et al. (211). It is a fluorescent compound with two bands in absorption spectrum and maxima at 330 and ca 490 nm (Table 1). It has been detected in bovine and human RPE and in Abca4⁻/⁻ mouse eyes, where age-related accumulation was observed.

**A2-GPE.** Most recently, a new fluorescent bis-retinoid compound has been identified (215). It is a product of the reaction between 2 atRal molecules and glycerophospho-ethanolamine (A2-GPE). Similarly to the previously described atRal condensation products, its absorption spectrum possesses two
observed specific MS peaks at m/z treated three times with 10 μM A2E as the only one generated in calf’s primary RPE cells illuminated (200) observed again the mono-oxygenated product of et al. chloroform⁄methanol extracts from A2E-loaded RPE cells illuminated for 3 h revealed that they absorb in UV and the visible region below 550 nm (Table 1). They have two main bands in their absorption spectra with UV band maximum at 295–340 nm and the more intensive longer-wavelength one at 400–440 nm. Fluorescence measurements of A2E oxidation products showed that emission intensity of the oxidized forms is higher than the native bis-retinoid (212).

Cytotoxicity. Sparrow et al. (194) performed studies, which have revealed that the A2E photooxidation products considered by them as A2E epoxides cause lesions in nuclear DNA of RPE cells in vitro. Photooxidation of A2E in human ARPE-19 cells (treated with 10 μM A2E) with blue light (430 nm) resulted in formation of peroxy-A2E and furano-A2E, which induced complement component C3 activation, C3a formation (218) and also deposition of C3, factor B, H and membrane attack complex (MAC) (109). Antioxidants—vitamin E and C reduce in vitro DNA damage (194) and together with anthocyanins protect the RPE cells from death (194,202) induced by the A2E oxidation products generated during blue light (430 nm) exposure.

Oxidized A2E precursors. The intermediates of A2E biosynthesis can also be a target for oxidation. Kim et al. (219) have shown that A2PE illumination with blue light (430 nm) in PBS leads to oxidation of the A2E precursor and generation of oxidative products similar to those obtained during A2E photooxidation.

Nevertheless, A2E present in the lipofuscin granules may not be photooxidize itself. However, there are sufficient endogenous blue- or visible-light photoactive components in the ocular lipofuscin that may easily photooxidize A2E to its multioxidized products also in vivo (59,60,105,130,134,137,220–222). High concentrations of A2E may be cytotoxic to the retina and A2E itself has a low-phototoxic efficiency. However, the oxidation products of A2E that are formed in the dark or when exposed to visible, mostly blue light seem to be more toxic than A2E itself (194,217,223). They could not only accelerate further A2E oxidation process in lipofuscin granules but also affect biomolecules in the RPE cells and contribute to observed retinal pigment epithelial photodamage and maculopathy.
XENOBIOtIC AGENTS

“Xenobiotic” light sources

In addition to sunlight, some artificial light sources, particularly those that are used for phototherapy of seasonal affective disorder (SAD) (224–227) or circadian imbalance (228,229), may enhance normal age-related light damage to the eye. A light box adjusted to eye level is often used to administer between 2500 and 10 000 lux from the light source. These “full spectrum” light sources if not properly filtered may emit UV radiation and/or intense light in the 430 nm +/− regions (6). Phototherapeutic modification of the human circadian response can be of value (230), but only the action spectra (wavelength efficiency) 480 ± 20 nm is necessary to trigger an alert circadian response. Wavelengths lower than this are of no therapeutic value and put the patients over 40 years of age at risk for impaired vision and future retinal and macular degeneration (2).

It is also essential to remove very short blue visible light (400–440 nm) (2) from all therapeutic light sources (229), as these wavelengths of light present a risk of retinal damage to patients above 40 years old (2).

“Xenobiotic” drugs, nanoparticles and herbal supplements

The presence of a pharmaceutical or nutraceutical agent or nanoparticle, which crosses blood–retinal barrier (231) and absorbs light can severely damage the retina. The degree to which a particular exogenous photosensitizing substance is capable of producing phototoxic and toxic side effects in the retina depends on its chemical and photobiophysical parameters including: (l) the chemical structure; (2) the absorption spectrum of the drug; (3) the ability to cross blood–retinal barrier (amphiphilic or lipophilic drugs); and (4) binding of the drug to retinal tissue (proteins, melanin, DNA; Table 4). For instance, compounds that have either a tricyclic, heterocyclic or porphyrin ring structure, have absorption spectra above 400 nm and are incorporated into retinal tissues are potentially phototoxic agents to the retina (24).

PDT therapeutic drugs

Photodynamic therapy (PDT) is based on inducing a photooxidation in a tumor or cancer cell by systemically injecting a highly phototoxic dye, which will be (hopefully) taken up selectively by a tumor. Subsequent laser irradiation of the dye infused tumor with the radiation at the absorbance spectra of the drug will induce a strong photooxidation in the tumor cells. PDT has been used to treat ocular melanoma (232), but has been associated with side effects including neovascular glaucoma (233). Encapsulating PDT dyes in heat-sensitive liposomes that are released by the laser increases selectivity (234) and reduces potential side effects. The combination of PDT therapy of ocular melanoma with concurrent use of photoradiotherapy (235) has been found to increase effectiveness of treatment and decrease recurrence of metastasis.

PDT is broadly used in current clinical setting in the treatment of several retinal degenerative diseases, including wet macular degeneration, choroidal neovascularization (236) and polypoidal choroidal vasculopathy (PCV) (237). In that case, the dye is injected directly into the retina, and the subsequent photooxidation reaction directs a laser to destroy the excessive blood vessels (angiogenesis) that are the cause of the damage to the retina caused by wet macular degeneration (238). Combination therapy using anti-VEGF drugs concurrent with PDT therapy (239) has been effective at delaying the necessity for repeat treatment.

Nanoparticles

Nanoparticles have been recently studied as drug carriers to first bypass blood ocular barriers and then to release encapsulated drug into various compartments of the eye. However, there are few studies that examine the potential ocular cytotoxicity and phototoxicity of these newly engineered compounds.

For instance, the water-soluble nanoparticle hydroxylated fullerene [fullerol, nano-C_{60}(OH)_{22–26}] has several clinical applications. Water-soluble fullerene nanoparticles can bypass the blood–brain and –retina barriers (240). Nanoparticle delivery of photosensitizers to ocular (and dermal) cells improves photodynamic therapy of tumors (241). Nanoparticles also show promise as drug carriers to the retina (242–246) and are currently being developed as carriers for antiangiogenesis drugs and vectors for gene delivery to retinal pigment epithelial cells (247,248), for treatment of wet macular degeneration. It has been shown that a single intravitreous injection of aliphatic nanoparticles preferentially localizes in retinal pigment epithelial cells (248,249), and that these nanoparticles are retained by RPE cells for a significant period of time (248,249). Unfortunately, fullerol is both cytotoxic and phototoxic to human lens epithelial cells (HLE B-3) (250) and human retinal pigment epithelial cells (251).

Nanotoxicity studies must consider the relationship between distribution particle size, surface area, composition, reactivity, coatings, stability and propensity to agglomerate or aggregate in different media (252,253) and cytotoxicity and phototoxicity. Time-resolved microspectrofluorimetry and time-gated fluorescence imaging has been used to investigate fullerol distribution and toxicity in human lens and retina cells in vitro (254). It was found that the fluorescence lifetimes change slightly, but gradually with fullerol dose, and that the average fluorescence intensity appears to correlate with the onset of cytotoxicity. Other studies have shown that aggregation appears to decrease phototoxicity of fullerene nanoparticles (241) and silica nanoparticles (255). Studies of nano-TiO₂ of

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<th>Table 4. Properties of potential phototoxic sensitizers in the retina.</th>
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<td>Absorption spectrum</td>
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different primary particle sizes and crystal structures found that phototoxicity to human retinal cells were directly correlated with particle surface area, and indirectly correlated with primary particle size (256).

Nanoparticles have great potential for therapeutic applications, especially for drug delivery to ocular compartments. However, for nanoparticles to be of safe clinical use their physical characteristics must be correlated with cytotoxicity and phototoxicity to ocular tissues.

**St. John’s wort—hypericin**

Hypericin is a biologically active component of St. John’s wort (SJW), an over-the-counter dietary supplement that has antidepressant properties (257,258). The Hypericum perforatum extract has also been associated with side effects and interactions with other drugs (259). It crosses the blood–retinal barrier and accumulates in the human retina. When ingested or given by intravitreal injection for the photodynamic treatment of retinal neovascularization, hypericin accumulates in the retina at a concentration of 1–100 μM (260). Hypericin induces cytotoxic damage (apoptosis) in retinal pigment epithelial (261).

Hypericin is known to be a very efficient photosensitizer when irradiated with either UV or visible light (262). St. John’s wort and hypericin induce photosensitized erythema in the skin (263,264). *In vitro* studies suggest it will induce phototoxic damage to the lens (265). Fluorescence and fluorescence imaging studies have determined that hypericin is taken up by both human lens and retinal epithelial cells (266,267). *In vitro* studies have determined that hypericin damages human RPE cells by inducing oxidative stress and lipid peroxidation in these cells (268).

**Indocyanine green**

Indocyanine green (ICG) a fluorescent dye whose absorbance spectrum is above 600 nm, assists the peeling of the internal limiting membrane during vitreoretinal surgery. This dye significantly improves the internal limiting membranes’s visibility and has made this technique much easier and more accurate. Unfortunately, ICG has also been associated with phototoxic effects on cultured human RPE. However, if the RPE cells were incubated with lutein (20 μM) an endogenous quencher of free radicals ROS (270,271), before ICG and visible light irradiation, there was significantly less RPE damage.

This has direct translational applications. Clinical damage of RPE caused by phototoxic effects of ICG may be reduced or eliminated by avoidance of illumination during ICG application, reducing the light intensity while dissecting the internal limiting membrane, taking care to avoid shining the light directly at the macular hole and maintaining an adequate distance between the light source and the retinal surface; and preoperative oral administration of lutein (10 mg per day) for several days. This has been found to be very effective at eliminating phototoxic damage to RPE cells during surgery (D-N Hu, unpublished data).

**CONCLUSION**

In conclusion, we have known for many years that direct ocular exposure to sunlight is hazardous to the eye in general and the retina in particular. We now understand that it is not only UV radiation exposure that is hazardous to the eye but also visible light ambient radiation that endangers the eye, especially if the recipient is over 40 years of age. Recent research has defined the action spectrum (400–440 nm) of light that is a particular risk for triggering or exacerbating macular and retinal degeneration. After the age of 40 there is a decrease in naturally protective antioxidant systems and an increase in visible light absorbing endogenous phototoxic chromophores. The presence of drugs, herbal supplements or nanoparticles that cross blood–retinal barrier and absorb ambient light dramatically enhances visible light damage to the retina. If short blue (400–440 nm) wavelengths are filtered by sunglasses or intraocular lenses, the blue light hazard should be dramatically decreased. However, it is important not to remove 480 nm, which is essential to triggering circadian rhythm (230). Understanding the proper use and improper exposure of light directed toward the eye can significantly reduce or retard age-related ocular disease.

**AUTHOR BIOGRAPHIES**

Albert Wielgus received his PhD in Biophysics from Jagiellonian University (Poland). He then joined the National Institute of Environmental Health Sciences, NIH (USA) where he was a postdoctoral fellow. Currently he works as Research Associate Senior in Duke Eye Center at Duke University School of Medicine (USA). His scientific research interests include photodamage to the human retina induced by endogenous agents (retinoids and lipofuscin) and environmental xenobiotics (hydroquinone, nanoparticles and plant-derived photosensitizers) and a role of polyunsaturated fatty acids, cholesterol and melanin in oxidative injury in the ocular tissues.

Joan E. Roberts

Joan Roberts is a tenured Professor of Chemistry at Fordham University in the Department of Natural Sciences. Her main area of expertise is the positive and negative effects of light on the human eye. With her collaborators in New York Eye and
Ear Infirmary, New York, Milan, Italy; Shanghai, China; Brookhaven National Laboratories, Long Island and the National Institute of Environmental Health Sciences in Research Triangle Park, North Carolina, she studies how UV and blue visible light enhance the formation of cataracts and macular degeneration and studies ways of preventing these age-related diseases. Dr. Roberts also studies how modification of circadian rhythm influences the human immune system, jet lag and mood (seasonal depression). In addition to over 90 publications in peer reviewed scientific journals, Joan’s work has been published in several prominent “lay journals” including New Scientist, Eye World, Scientificamerica.com and Sky and Telescope. Joan has been featured on ABC World News Tonight with Bill Blakemore for her research on melatonin/circadian immune responses and the Brian Lehr show on NPR about her research on “Lighting and Human Health”.

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