

Review

A2E and Blue Light in the Retina: The Paradigm of Age-Related Macular Degeneration

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The photoreceptors in the retina, designed to initiate the cascade of events which link the incoming light to the sensation of 'vision', are susceptible to damage by light, particularly blue light. The damage can lead to cell death and diseases. The turnover of retinal, an essential element of the visual process, is the basis of the events that lead to damage. Free retinal, absorbing in the blue region of the visible spectrum, is phototoxic, and is a precursor of the (photo)toxic compound A2E, which specifically targets cytochrome oxidase and thereby induces cell death by apoptosis. Cell death induced by A2E in the dark is prevented by negatively charged phospholipids. The blue light-filtering molecules lutein and zeaxanthin are tailor-made substances protecting the retina. *In vitro*, they protect cytochrome oxidase against the permanent damage caused by A2E in combination with light. These novel findings should enable us to prevent or cure the dry form of age-related macular degeneration, the leading cause of severe visual impairment in humans living in developed countries.

Key words: A2E/Antioxidants/Apoptosis/Carotenoids/Mitochondria/Retinoids.

Introduction: Light Exposure Causes Damage to the Retina

In Europe, considerable public excitement arose in anticipation of the full solar eclipse on 11 August 1999. Most people remember the fact that exposure to intense light is a threat to the eye when they prepare for watching a solar eclipse (Michaelides *et al.*, 2001). People were urged not to look directly into the sun with unprotected eyes because this can result in visual morbidity. But not only intense light is dangerous. Also long-term exposure to normal light levels is harmful as learned from epidemiological studies (Taylor *et al.*, 1990; Cruickshanks *et al.*, 1993). Exposure to intense blue light for minutes to hours

is much more damaging than exposure to light of longer wavelengths (Ham *et al.*, 1976; Gorgels and van Norren, 1995), while exposure to low levels of light for weeks or months causes damage which relates to the 500 nm region.

The photoreceptors in the retina, designed to initiate the cascade of events which link the incoming light to the sensation of 'vision', are particularly susceptible to damaging light. The damage can lead to cell death and diseases. Recent findings provide explanations why blue light is particularly damaging, and identify cellular components that participate in the induction of cell death. These findings should enable us to prevent or cure the dry form of age-related macular degeneration (AMD), the leading cause of severe visual impairment in humans living in developed countries (Berger *et al.*, 1999; Sun and Nathans, 2001a).

The Retina: Detailed Anatomy

The retina is located in the back of the eye and is about 200–250 nm thick. It contains many layers of different cell types (Figure 1).

The cells of the retinal pigment epithelium (RPE) have vital support functions. They maintain the correct ionic composition in the fluid surrounding the photoreceptors, filter nutrients that arrive from the choroidal blood vessels, and provide photoprotection because of their deep pigmentation. Phagocytosis, degradation and recycling by RPE cells of outer segment material (see below) are pivotal for the physiology and pathology of the retina.

Photoreceptor outer segments are renewed at a constant rate throughout life. In the course of photoreceptor renewal, rod outer segment tips are shed and phagocytosed by the underlying cells of the RPE (Young and Bok, 1969; Feeney, 1978). Each RPE cell serves about 50 photoreceptors and engulfs and degrades 10% of 50 outer segments per day. Indeed, RPE cells are by far the most active phagocytotic cells in the body. It is generally believed that the accumulation of the autofluorescent age pigment lipofuscin in RPE cell phagolysosomes constitutes a predictor for the development of AMD. Lipofuscin accumulation and the formation of drusen and other deposits in the region of Bruch's membrane, a thin sheet of extracellular proteins and polysaccharides which separates the pigment epithelium from the underlying choroidal blood vessels, are considered initial steps in the patho-

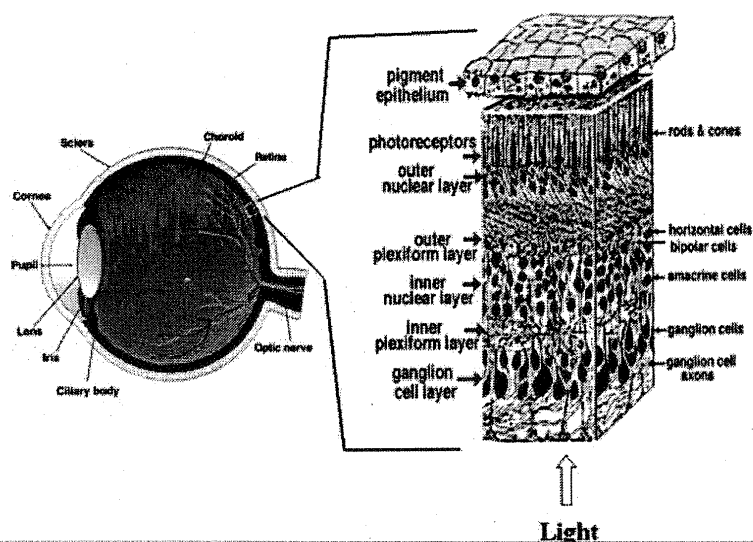


Fig. 1 The Anatomy of the Mammalian Eye. Not all layers are annotated. The Henle's fibers layer is formed by the photoreceptors' axons.

genesis of AMD. Drusen are deposits that can accumulate underneath the RPE, and their formation may be the result of progressive death and/or exocytosis of RPE cells in the central retina (Ishibashi *et al.*, 1986; Eldred, 1995).

Apoptosis in the Retina

Apoptosis (programmed cell death) is a form of cell death that is accomplished by a special cellular machinery (Hengartner, 2000). Mitochondria have a central role in apoptosis (Richter, 1993) because they respond to apoptosis-signaling molecules and release apoptosis-inducing factors (Green and Reed, 1998). Animal models of retinal diseases revealed that photoreceptors can die apoptotically (Remé *et al.*, 1998), but until recently it was not clear how apoptosis in the retina is induced. Now at least two low molecular weight-compounds, retinal and A2E, and three proteins (rhodopsin, the Rim protein, and the Rpe65 protein) are known to be intimately involved in the apoptosis of RPE cells (see below).

Mitochondria and Apoptosis

The respiratory chain, which transports electrons and protons, is located in the inner mitochondrial membrane and is composed of four complexes (I-IV; see also Figure 7). At the level of complex IV (cytochrome oxidase), mitochondria reduce molecular oxygen to water and thereby convert energy. This energy is used to produce ATP and to transport molecules across the inner mitochondrial membrane. Until recently mitochondria were exclusively viewed as life-supporting organelles.

A few years ago it was realized that mitochondria also

actively participate in the demise of cells because they are engaged in the execution of apoptosis (Richter, 1993; Hengartner, 2000). They contain proteins which, when released into the cytosol, help to selectively destroy vital cellular components such as enzymes and DNA. Presently four such proteins have been identified: cytochrome c, apoptosis inducing factor (AIF), SMAC/Diablo, and endonuclease D.

The Visual Cycle

Vision is initiated when photons strike rhodopsin, the visual pigment composed of opsin and 11-*cis*-retinal and located in the photoreceptor disc membrane. Light absorption causes an isomerization of 11-*cis*-retinal to the all-*trans*-form and its release from opsin. All-*trans*-retinal is exported from the disc by the Rim transporter (see below) and then reduced to all-*trans*-retinol by retinol dehydrogenase. These events take place in the photoreceptor outer segments. All-*trans*-retinol is subsequently transported into RPE cells where it is converted in several not totally clarified steps involving the protein Rpe65 (see below) to 11-*cis*-retinal. Thereafter 11-*cis*-retinal is attached to opsin. (For an illustration of the visual cycle, see Figure 2).

Optical Properties of Retinoids

The human lens protects the retina from UV damage because the lens efficiently absorbs light of <410 nm. Rhodopsin, of course, interacts with visible light, with a peak around 500 nm. Free all-*trans*-retinal absorbs maximally at 380 nm but is nevertheless excitable in the eye because of its absorption shoulder in the visible region,

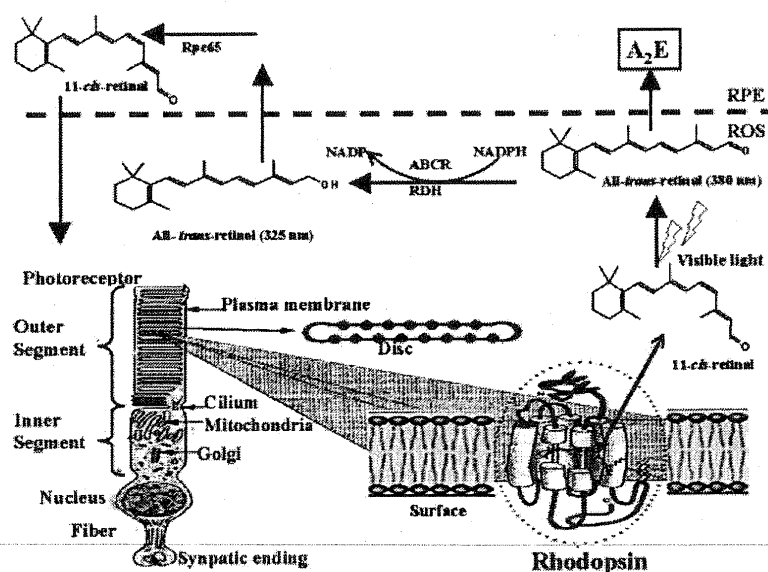


Fig. 2 The Visual Cycle.

When light has penetrated the layers of the retina and hit rhodopsin in a rod or cone photoreceptor, all-*trans*-retinal is released from opsin. All-*trans*-retinal is immediately reduced by an alcohol dehydrogenase to all-*trans*-retinol, which is subsequently transported to the retinal pigment epithelium. There, all-*trans*-retinol is esterified in the endoplasmic reticulum to long-chain fatty acids. An isomero-hydrolase then converts the retinoid of the ester to 11-*cis*-retinal. After its oxidation, 11-*cis*-retinal is transported back to the photoreceptor to be combined with opsin. ROS, rod outer segment; RPE, retinal pigment epithelium.

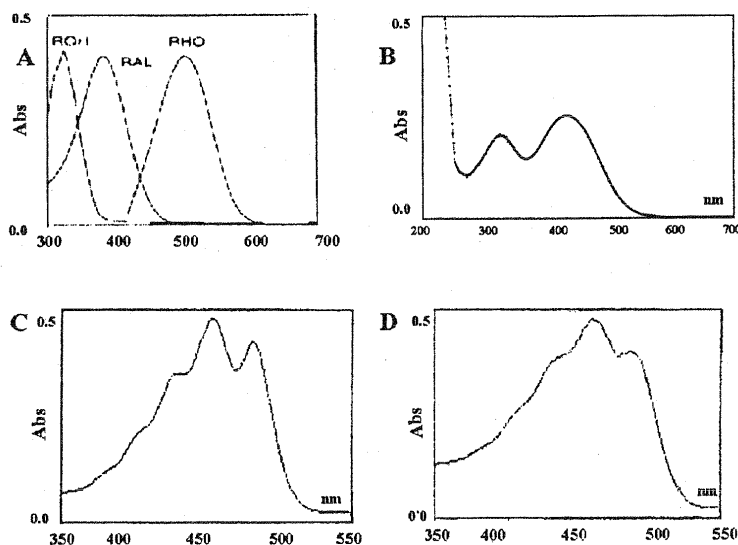


Fig. 3 Optical Spectra of Selected Retinoids and Carotenoids.

(A) Retinoids; (B) A₂E; (C) lutein; (D) zeaxanthin. ROH, retinol; RAL, retinal; RHO, rhodopsin.

which ends only around 475 nm. This contrasts with all-*trans*-retinol, which absorbs light at wavelengths <380 nm and is, therefore, not excited in the eye. Finally, A₂E (see below) has absorption maxima around 335 and 435 nm, only the latter of which potentially contributes to light toxicity in the eye. [For the optical spectra of these chromophores, and also those of lutein and zeaxanthin (see below), see Figure 3.]

Age-Related Macular Degeneration

Age-related macular degeneration affects about one fifth of the population older than 65 years and is one of the main causes of poor vision in the elderly in industrialized nations. AMD develops over many decades. The number of people affected by the disease is expected to nearly double in the next 25 years. AMD is accompanied by

photoreceptor damage in the macula. The dry form of the disease comprises about 90% of all cases. Other than vision aids (*e.g.*, glasses, magnifiers), no treatments or preventive measures are currently available for patients with dry AMD. To slow down or halt its progression would be a major achievement. The primary lesion in AMD appears to reside in the RPE, possibly because of its high rate of degradation. The wet form, which is caused by infiltration of blood vessels into the retina, can successfully be treated by photodynamic therapy (Berger *et al.*, 1999).

Formation of A2E

Cells in the retina harbor two unusual retinoids, the lipophilic cations A2E (systematic name: N-retinyl-N-retinylidene ethanolamine, 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) and its isoform, *iso*-A2E, which *in vitro* are readily synthesized by mixing 2 all-*trans*-retinals with 1 ethanolamine at slightly acidic pH (Parish *et al.*, 1998). The compounds were first isolated from eyes of old human individuals (Eldred and Lasky, 1993; Parish *et al.*, 1998) and later from rats (Suter *et al.*, 2000), and their correct structure (Figure 4) was worked out (Sakai *et al.*, 1996; Ren *et al.*, 1997). These molecules are formed in rod outer segments (Liu *et al.*, 2000). The initial reaction seems to be the condensation within the photoreceptor disc of one molecule of all-*trans*-retinal with phosphatidylethanolamine to N-retinylidene-phosphatidylethanolamine (NR-PE). The latter is either exported by the Rim transporter, or condenses with a second molecule of all-*trans*-retinal to A2-phos-

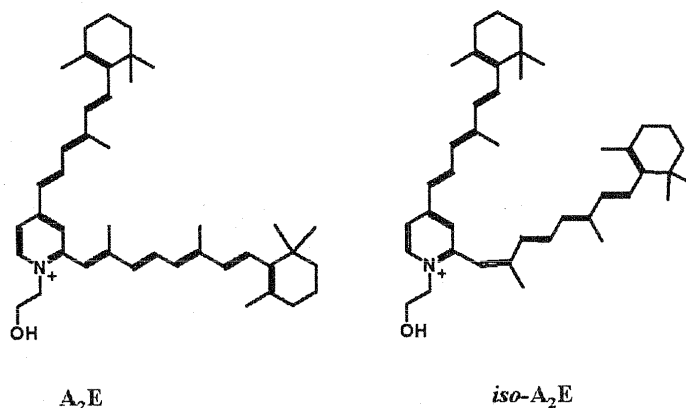


Fig. 4 The Structure of A2E and Its Isoform, *iso*-A2E.

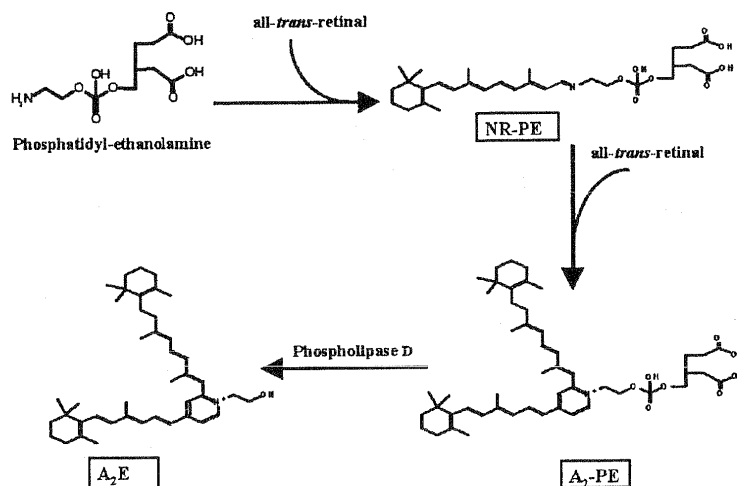


Fig. 5 The Biosynthesis of A2E.

The putative biosynthetic pathway is illustrated. All-*trans*-retinal condenses within the photoreceptor disc with phosphatidylethanolamine to form N-retinylidene-phosphatidylethanolamine (NR-PE). The latter is either exported by the Rim transporter, or condenses with a second molecule of all-*trans*-retinal to A2-phosphatidylethanolamine (A2-PE), followed by hydrolysis of the adduct by phospholipase D. The last steps of A2E formation are suggested to occur in retinal pigment epithelial phagolysosomes.

phatidylethanolamine (A2-PE), followed by hydrolysis of the adduct (Liu *et al.*, 2000; Mata *et al.*, 2000) (Figure 5). The last steps of A2E formation were suggested to occur in RPE phagolysosomes (Mata *et al.*, 2000).

There are conflicting results concerning the intracellular localization of A2E. It was detected in lysosomes of cells exposed in culture to the free compound or to A2E coupled to low-density lipoprotein particles (Holz *et al.*, 1999a; Sparrow *et al.*, 1999). Detailed near-field scanning optical microscopy measurements (Krogmeier *et al.*, 2001) suggest that A2E is not a lipofuscin component, and the compound does not affect the activity of lysosomal hydrolases (Bermann *et al.*, 2001). Energized mitochondria establish across their inner membrane an electric potential, negative charge inside, which drives sufficiently lipophilic cations non-specifically into the organelle (Chen, 1988). Confocal microscopic studies revealed the predominant presence of A2E in mitochondria (Suter *et al.*, 2000). The presence of A2E in lysosomes but not in mitochondria reported by others (Holz *et al.*, 1999a,b; Sparrow *et al.*, 1999), and the mitochondrial localization reported by Suter *et al.* (2000) suggests that after damaging lysosomes A2E can be released and subsequently taken up by mitochondria. Alternatively, A2E may initially accumulate in and be retained by mitochondria, which when damaged may be engulfed by lysosomes. In contrast to these presumably physiological intracellular steps of A2E distribution, pathogenic steps leading to cell death may be mainly due to overloading of mitochondria with this lipophilic cation.

Retinoid Metabolism as the Basis of Light-Induced Damage to the Retina

The use of genetically modified animals or cells gave unequivocal proof that light-induced damage to the retina is related to the activity of proteins engaged in the visual cycle, and involves all-*trans*-retinal and derivatives thereof.

Mice lacking rhodopsin are completely resistant against damage of bright light, as are mice deficient in the gene Rpe65 (Grimm *et al.*, 2000). The latter is specifically expressed in the pigment epithelium, is essential for the re-isomerization of all-*trans*-retinol in the visual cycle and therefore for the regeneration of rhodopsin after photobleaching. The Rim protein, which is coded for by the ABCR gene and acts as a N-retinylidene exporter ('flippase') in the disc membrane (see above), is itself damaged by the combination of light and retinal, as demonstrated in cells containing a mutated ABCR gene (Sun and Nathan, 2001b).

Mutations in the Rim protein drastically augment the level of A2E in cells. Rim^{-/-} mice accumulate A2E (and retinal) in RPE cells more than 20-times than wildtype animals (Weng *et al.*, 1999). The authors concluded that the 'flippase' may be important in the protection of RPE cells against A2E and retinal accumulation because the 'flipp-

ase' cleans the disc interior of A2E's precursor. They proposed that in mutant mice (i) NR-PE accumulates in discs, (ii) discs are engulfed by RPE cells, and NR-PE is formed in RPE phagolysosomes, which (iii) causes death of RPE cells and loss of their supporting role. Similar findings were reported by Mata *et al.* (2000), who in addition made two important observations: In the dark, no A2E is formed, but when formed in the light A2E is very stable. The same group (Mata *et al.*, 2001) very recently reported that in Rim^{+/-} mice A2PE and A2E accumulate in dependence of light exposure.

Oxidative Damage

The retina is exposed to light, relatively high oxygen levels, and contains high concentrations of unsaturated fatty acids. These factors render it prone to oxidative stress (Winkler *et al.*, 1999). The latter has mitochondria as the main target (Cai *et al.*, 2000). Epidemiological studies suggest that oxidative stress is a contributing factor of AMD, and because the antioxidant capacity decreases with age, prevention of AMD by the use of antioxidants is indicated (Cai *et al.*, 2000).

Zeaxanthin and lutein are constituents of the *Macula lutea*. They are present in Henle's fibers and in rod outer segments. Of the about ten carotenoids found in the blood these two are specifically concentrated in the macula. This is probably not accidental because these dietary carotenoids are antioxidants (they scavenge oxygen free radicals and singlet oxygen; Schalch, 1992), and they act as blue light filters (Junghans *et al.*, 2001). (For their optical spectra, see Figure 3). A high intake of food rich in lutein and zeaxanthin is correlated with a lowered risk for AMD (Seddon *et al.*, 1994), and a low plasma level is associated with a higher risk for AMD (EDCC Study Group, 1993). Antioxidants including lutein and zeaxanthin reduce lipofuscin formation in cultured RPE cells (Sundelin and Nilsson, 2001), which are particularly sensitive to blue light (Seko *et al.*, 2001).

(Photo)toxicity of A2E

A2E at physiologically relevant concentrations is toxic already in the dark to many cell types including RPE cells (Suter *et al.*, 2000), and A2E renders cells sensitive to blue light as shown with cultured RPE cells (Sparrow *et al.*, 2000; Sparrow and Cai, 2001).

Since the molecular details of the (photo)toxicity of A2E are at least in part understood (see next paragraph) its inhibition could be attempted in a rational way. Table 1 shows that a negatively charged phospholipid (phosphatidylglycerol) and the antioxidative, blue light-filtering lutein effectively prevent cell death induced by A2E in the dark and in the light. These findings strongly suggest that it should be possible to prevent and/or treat the dry form of AMD by proper application of the above compounds.

Table 1 Prevention of Cell Death Induced by A2E.

	Apoptosis (%)			
	Dark	SD	Light	SD
Control	1.5	0.5	1.5	0.5
Control plus DMSO (1%)	1	1.0	1.5	0.5
40 μ M A ₂ E	12	2.0	22	1.5
40 μ M A ₂ E plus 150 μ g/ml PG	2.5	1.0	2	1.0
40 μ M A ₂ E plus 75 μ M lutein	4.5		6.5	

Experiments were performed with MCF-7 cells in Iscove's Dulbecco's modified Eagle's medium containing 10% fetal calf serum, GLUTAMAX, L-glutamine, and 50 μ g gentamycin/ml. The cells in culture were exposed to A2E (40 μ M) for 6 hours in the dark or in the light (30 min illumination every 2 hours with a 40 Watt white Neon lamp at a distance of 65 cm) in the presence of phosphatidylglycerol (PG) and lutein, dissolved in dimethylsulfoxide (DMSO). A2E, dissolved in ethanol, was diluted into the culture medium; ethanol never exceeded 0.1%, and DMSO never exceeded 1%. Control incubations were done with 1% DMSO or 0.1% ethanol in the absence of A2E as negative control. Incubation was at 37 °C in humidified air with 5% CO₂. Dark incubation was done by wrapping the cell culture plates in aluminum foil, with sham handling of the plates. All experiments were done six times, with the exception of the one using lutein, which was done twice; the given value is their average. SD, standard deviation.

Molecular Details of A2E's (Photo)toxicity

A2E Targets Mitochondria

The lipophilic and cation nature of A2E suggested that this compound targets mitochondria and in so doing may induce apoptosis. This was verified in RPE and other cells (Suter *et al.*, 2000). Apoptosis was accompanied by appearance of the pro-apoptotic proteins cytochrome c and apoptosis inducing factor in the cytoplasm and the nucleus. Biochemical examinations showed that A2E specifically targets cytochrome oxidase. With both, isolated mitochondria and purified cytochrome oxidase, A2E inhibits oxygen consumption synergistically with light. Inhibition is reversed by addition of cytochrome c or cardiolipin (1,3-diphosphoglycerol, 1,3-DPG), a negatively charged phospholipid that facilitates the binding of cytochrome c to cytochrome oxidase. Neither succinate dehydrogenase activity is altered by A2E (Suter *et al.*, 2000), nor the coupling of mitochondria (Shaban and Richter, unpublished data). These observations show that A2E specifically prevents the binding of cytochrome c to cytochrome oxidase and prevents electron flow down the respiratory chain. As a consequence more oxygen radicals are formed and oxidative stress increases.

Light-Dependent Inactivation of Cytochrome Oxidase and Its Prevention

The synergism between light and A2E was investigated in detail with purified cytochrome oxidase (Shaban *et al.*,

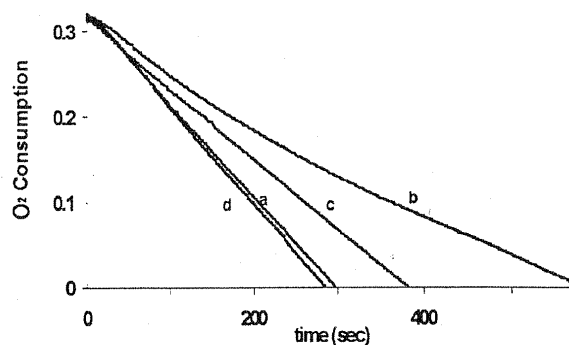


Fig. 6 Inhibition of Isolated Rat Liver Cytochrome Oxidase by A2E and Protection by Negatively Charged Phospholipids. Cytochrome oxidase activity was measured as described (Shaban *et al.*, 2001). Briefly, oxygen consumption was measured at 25°C with a Clark-type oxygen electrode (Yellow Spring Instruments, Yellow Spring, USA) under continuous stirring. Solubilized cytochrome c oxidase respired in 40 mM K⁺-phosphate, 0.05 mM EDTA (pH 7.0), 0.1% Tween 20, containing as substrate 0.02 mM cytochrome c and a mixture of ascorbate/tetramethyl-*p*-phenylenediamine (10 mM/1 mM). Trace a, control; trace b, plus 40 μ M A2E; trace c, plus 40 μ M A2E plus 300 μ g/ml 1,3-diphosphatidylglycerol; trace d, plus 40 μ M A2E plus 300 μ g/ml phosphatidylglycerol.

2001). The inhibition of cytochrome oxidase is highly specific for A2E and was observed with the solubilized and reconstituted enzyme. In the dark, 1,3-DPG or other negatively charged phospholipids overcame inhibition. With illumination, inhibition was stronger, became complete with prolonged exposure, and was thereafter no longer abrogated by 1,3-DPG. Cardiolipin effectively displaced A2E from cytochrome oxidase, suggesting non-covalent binding of A2E to the enzyme. It was concluded that A2E is a potent cytochrome oxidase-specific inhibitor, which interferes with the binding of cytochrome c to cytochrome oxidase and, in the light, causes persistent modifications of the enzyme.

The negatively charged phosphatidylglycerol is much more powerful than 1,3-DPG as protector of cytochrome oxidase in the presence of A2E (Figure 6). This *per se* interesting result is even more important in view of the fact that phosphatidylglycerol is the immediate biosynthetic precursor of 1,3-DPG. Lutein and zeaxanthin also abrogate light-dependent inactivation. Furthermore, coenzyme Q₁₀ or phosphatidylglycerol powerfully prevents A2E-induced apoptosis (Shaban *et al.*, 2002). It is worth mentioning that the level of 1,3-DPG present in old people is only about 50% of that present in young people (Ames *et al.*, 1995).

A2E Causes Oxidative Stress in Mitochondria

Release of cytochrome c from mitochondria into the cytosol has two major consequences. One is the interrup-

tion of electron flow along the respiratory chain. Single electrons are now transferred by components upstream of the cytochrome c binding site to single oxygen molecules, which results in superoxide anion formation, oxidative stress, and damage to mitochondria. The other is the stimulation of apoptosis (see above) because of the formation of an 'apoptosome' in the cytosol, a pro-apoptotic complex comprising cytochrome c, apaf-1, and procaspase-9 (Figure 7). A2E induces oxidative stress in isolated mitochondria: there is an increased H_2O_2 and decreased glutathione level in respiring mitochondria exposed to A2E (Shaban *et al.*, 2002). Furthermore, in the presence of A2E mitochondria release cytochrome c, AIF, and SMAC/Diablo into the extramitochondrial space (Garrido and Richter, unpublished data).

Outlook: New Vision in Sight

Biomedical research done during the last few years provides explanations why blue light can damage the retina. A curious scenario emerges: the turnover of retinal, an essential element of the visual process and initiated by light of about 500 nm, is the basis of events that lead to damage. Free retinal, absorbing in the blue region, in combination with light inactivates the 'flippase' and thereby augments the level of the (photo)toxic compound A2E, which itself is activated by blue light (Figure 8). As a consequence of this and possible other events such as lipofuscin activation apoptosis is initiated. The blue light-filtering molecules lutein and zeaxanthin are tailor-made substances protecting the retina.

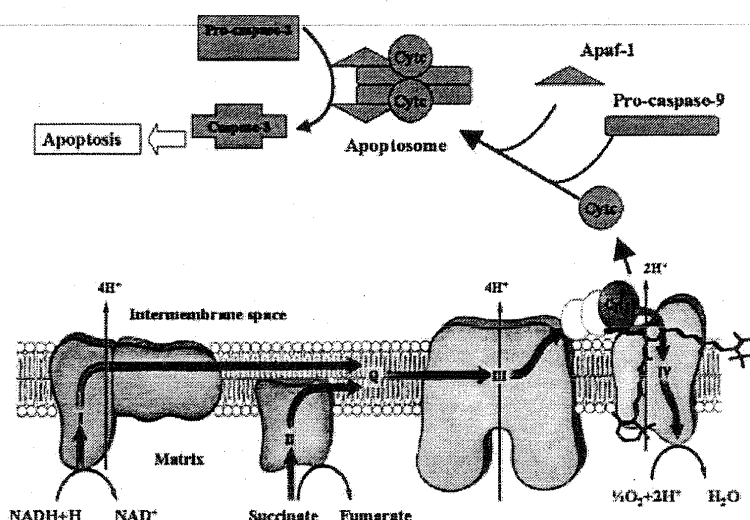


Fig. 7 The dual role of Cytochrome c.

As a member of the mitochondrial respiratory chain, cytochrome c is life supporting. Once released into the cytosol, cytochrome c combines with procaspase-9 and Apaf-1 and thereby becomes proapoptotic. A2E specifically prevents the binding of cytochrome c to cytochrome oxidase and causes release of cytochrome c into the cytosol. Note that parallel to, or as a consequence of, A2E-induced cytochrome c release also two other proapoptotic proteins, apoptosis inducing factor and SMAC/Diablo, are released from mitochondria (see text).

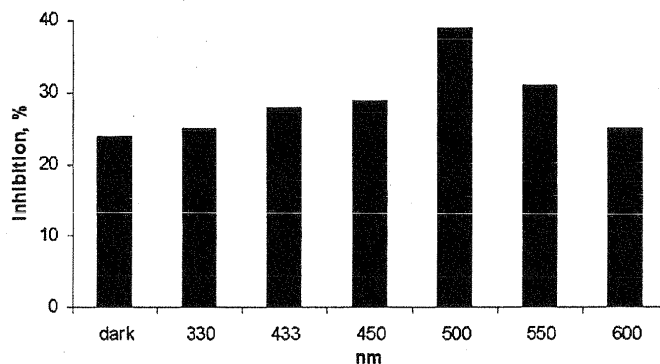


Fig. 8 Blue Light- and A2E-Induced Inhibition of Isolated Rat Liver Cytochrome Oxidase.

Cytochrome oxidase activity was measured as described in Shaban *et al.* (2001). For experimental details, see the legend to Figure 6. The action spectrum was obtained with a Varian spectrofluorometer as light source (1000 W tungsten lamp, band path 1.5–3 nm). The experiment was performed twice. In both cases maximal inhibition was seen between 450 and 550 nm.

It is highly likely but presently not proven that A2E is responsible for or involved in the etiology of the dry form of AMD. The knowledge of the molecular details of A2E's (photo)toxicity strongly suggest that AMD can be the result of A2E-induced apoptosis in RPE cells, with cytochrome oxidase as a major target of A2E. The possibility that AMD is a mitochondrial disease has been discussed previously (Richter, 2001). Loss of RPE cell viability and subsequent progressive degeneration of the central retina through inhibition of mitochondrial function provides rationales for the prevention and possibly even therapy of AMD. The level of A2E is at least ten-fold higher in old compared to young humans or rats (Parish *et al.*, 1998; Suter *et al.*, 2000). The quality of mitochondria declines with age, but can be improved by antioxidants and micronutrients (Shoffner and Wallace, 1994; Hagen *et al.*, 1998). More importantly in the context of A2E toxicity, the cellular 1,3-DPG content decreases with age (Ames *et al.*, 1995). Methods presently available for the treatment of mitochondrial diseases comprise biochemical and nutritional approaches, particularly the use of general antioxidants or specific electron transfer mediators. The damage introduced into RPE cells by A2E is augmented by blue light, goes along with oxidative stress, and can be effectively prevented by negatively charged phospholipids such as 1,3-DPG or phosphatidylglycerol. Therefore, the supplementation with negatively charged phospholipids, with blue light-filtering molecules, and with antioxidants is a promising approach to counteract the dry form of AMD.

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