



Phosphatidylglycerol Potently Protects Human Retinal Pigment Epithelial Cells Against Apoptosis Induced by A2E, a Compound Suspected to Cause Age-related Macula Degeneration

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Age-related macular degeneration (AMD) 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. The endogenous lipophilic and cationic compound N-retinyl-N-retinylidene ethanolamine (A2E) is suspected to cause the dry form of the disease, which currently cannot be treated. The authors recently reported that A2E induces apoptosis in several cell types including porcine retinal pigment epithelial cells, detaches pro-apoptotic proteins from mitochondria, and inhibits cytochrome c oxidase. A2E acts primarily at the level of cardiolipin/cytochrome c oxidase, which in the light becomes permanently inactivated by A2E. The authors now report that A2E at low concentrations causes apoptosis in cultured human retinal pigment epithelial cells. These cells are more sensitive to A2E in the light than in the dark. Phosphatidylglycerol, a negatively charged phospholipid and immediate biosynthetic precursor of cardiolipin readily inhibits apoptosis. Exposure of cells to A2E results in the formation of reactive oxygen and nitrogen species, and exposure of mitochondria to A2E results in oxidative stress. Accordingly, the potent antioxidant coenzyme Q also protects cells against A2E-induced apoptosis. These findings are highly relevant for the treatment and/or prevention of AMD.

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Key words: age-related macular degeneration; A2E; apoptosis; cardiolipin; coenzyme Q; cytochrome c; cytochrome c oxidase; light-induced damage; oxidative stress; phosphatidylglycerol; reactive nitrogen species; reactive oxygen species; retinal pigment epithelial cells.

1. Introduction

Age-related macular degeneration (AMD) 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. 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. The primary lesion in AMD appears to reside in the retinal pigment epithelium (RPE), possibly because of its high rate of degradation (Berger et al., 1999).

Cells of the RPE accumulate the autofluorescent age pigment lipofuscin in their phagolysosomes, and lipofuscin is a predictor for the development of AMD. Lipofuscin harbors two retinoids, the lipophilic cations N-retinyl-N-retinylidene ethanolamine (A2E) and its isoform, *iso*-A2E, first isolated from eyes of old humans (Eldred and Lasky, 1993; Parish et al.,

1998). The molecules are side products in the visual cycle (Sun and Nathans, 2001), and accumulate about 10-fold in the eye with advanced age (Parish et al., 1998; Suter et al., 2000).

Mitochondria are of central importance for the execution of apoptosis. In response to their membrane potential, negative inside, they avidly take up lipophilic cations. The authors therefore argued that A2E induces apoptosis due to its interaction with mitochondria. It is shown that A2E indeed does so in cultures of various mammalian cell types, including RPE cells (Suter et al., 2000). A2E-induced apoptosis is preceded by a decline in mitochondrial activity and accompanied by a translocation of cytochrome c and apoptosis inducing factor into the cytoplasm and nucleus. A2E, but not *iso*-A2E, targets the function of cytochrome c oxidase (COX) by neutralizing the negatively charged phospholipid cardiolipin, which mediates the binding of cytochrome c to COX. Respiratory chain activity upstream of cytochrome c is not affected by A2E. The authors next found (Shaban et al., 2001) that the inhibition of COX by A2E is highly specific for the lipophilic cationic A2E. In the dark, negatively charged phospholipids reactivate A2E-inhibited COX. Upon illumination, inhibition becomes more and more pronounced and can

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no longer be reversed. Here it is reported that A2E causes apoptosis in cultured human RPE cells. These cells are more sensitive to A2E in the light than in the dark. Phosphatidylglycerol, a negatively charged phospholipid and immediate biosynthetic precursor of cardiolipin potently inhibits apoptosis. Exposure of cells or mitochondria to A2E results in oxidative stress, and the antioxidant coenzyme Q protects cells against A2E-induced apoptosis. These findings suggest ways for the prevention and/or treatment of dry AMD.

2. Materials and Methods

Synthesis of A2E

A2E was synthesized from all-*trans* retinal and ethanolamine as described (Parish et al., 1998; Suter et al., 2000).

Cell Culture

Human RPE cells were obtained from the eyes of a 53 year old donor after cornea transplantation through the courtesy of Ms Daniela Zürcher, Department of Ophthalmology, Inselspital, CH-3010 Bern, Switzerland. Experiments were performed with cells of passages three to six maintained in Iscove's Dulbecco's modified Eagle's medium containing 10 % fetal calf serum, GLUTAMAX, L-glutamine, and 50 µg gentamycin ml⁻¹.

Apoptosis Assay

A2E, dissolved in ethanol, was diluted into the RPE cell culture medium to concentrations ranging from 5 to 40 µM; phosphatidylglycerol and staurosporine were dissolved in dimethylsulfoxide (DMSO); 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 solvent control, and with 1 µM staurosporine as positive apoptosis control. Incubation was at 37°C in humidified air with 5 % CO₂. Cells were illuminated every 2 hr for 30 min. Dark incubation was done by wrapping the cell culture plates in aluminum foil, with sham handling of the plates.

Cells were incubated at room temperature for 20 min in a solution containing 10 mM Hepes (pH 7.4), 150 mM NaCl, 2 mM CaCl₂, 5 mM KCl, 1 mM MgCl₂ and Annexin V-FITC (Roche Molecular Biochemicals (1 µl ml⁻¹)), with combinations of propidium iodide (Molecular Probes, Inc., Eugene, OR, U.S.A. (1 µg ml⁻¹)) and Hoechst-33342 (Molecular Probes, Inc., Eugene, OR, U.S.A. (10 µg ml⁻¹)) (Bossy-Wetzel and Green, 2000). Apoptotic cells were detected with an inverted Zeiss fluorescence microscope connected to an extended charge-coupled device camera. Hoechst-33342 stains all types of cell

chromatin blue, which can be detected at 350 nm, propidium iodide stains cells red, detected at 535 nm, in late apoptosis or undergoing secondary necrosis, and Annexin V-FITC stains early apoptotic cells green, detected at 450 nm, due to externalisation of phosphatidylserine to the cell surface.

Cells in 24 well culture plates were examined with a fluorescence microscope for apoptosis by counting green- and red-stained cells. About 100–200 cells were examined in each well. The percentage of apoptosis was calculated by dividing the number of apoptotic cells by the total number of cells in each well. The assay was done with six different preparations. The average and S.D. were calculated for each plate, S.D. being always between 1–8 % of the average. Statistical analysis was done with Ms Excel ANOVA. LD50 and ED50 values were calculated by linear regression analysis. *P* values were obtained with Student's *t*-test.

Assay of Oxidative and Nitrosative Stress

Oxidative and nitrosative stress in cells was assessed using the non-fluorescent probe 2',7'-dichlorofluorescein (DCFH). The membrane-permeable diacetate form of the DCFH dye (DCFH-DA) was added to the perfusate at a final concentration of 5 µM. Within the cell, esterases cleave the acetate groups on DCFH-DA, thus trapping the probe DCFH intracellularly (Rothe and Valet, 1990). Oxidative and nitrosative stress in cells leads to the oxidation of DCFH, yielding the green fluorescent product 2'-7'-dichlorofluorescein (DCF) (Sawada et al., 1996), which can be visualized on a single-cell basis using fluorescence microscopy (Tsuchiya et al., 1994). After rinsing with PBS to remove excess probe, the generation of reactive oxygen and nitrogen species was monitored using the above fluorescence microscope (Zeiss). Dichlorofluorescein fluorescence was measured at an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

Animals

Wistar rats between 4 and 6 months of age were used. Animals were housed at constant temperature and humidity and with a 12 hr light/12 hr dark cycle. They were fed on a standard laboratory diet (containing 590 g carbohydrates, 30 g lipids, and 160 g protein per kg) and tap water ad libitum. Animals were treated in accordance with the principles of the Declaration of Helsinki for the good care of laboratory animals.

Isolation of Mitochondria

After the animals were killed by cervical dislocation, their livers were quickly removed. Liver mitochondria were obtained by differential centrifugation

(Rickwood et al., 1997). The mitochondrial protein content was determined by the Biuret method with bovine serum albumin as standard. Isolated mitochondria were pre-incubated with 12.5 μM A2E for 5 min at 37°C. Control mitochondria (without A2E) were pre-incubated under the same conditions.

Determination of Mitochondrial H_2O_2

The rate of H_2O_2 production was determined in isolated mitochondria using a modification of the method described by Barja (1999). Briefly, mitochondria were incubated at 37°C with 10 mM succinate in 2 ml of phosphate buffer, pH 7.4, containing 0.1 mM EGTA, 5 mM KH_2PO_4 , 3 mM MgCl_2 , 145 mM KCl, 30 mM Hepes, 0.1 mM homovanillic acid and 6 U ml^{-1} horseradish peroxidase. The incubation was stopped at 5, 10 and 15 min with 1 ml of cold 2 M glycine buffer containing 50 mM EDTA and 2.2 M NaOH. The fluorescence of supernatants was measured using 312 nm as excitation wavelength and 420 nm as emission wavelength. The rate of H_2O_2 production was calculated using a standard curve of H_2O_2 .

Assay of Reduced Glutathione

Mitochondrial reduced glutathione (GSH) was determined using the glutathione-S-transferase assay (Brigelius et al., 1983).

Assay of Oxidized Glutathione

Determination of mitochondrial oxidized glutathione (GSSG) was carried out by a high-performance liquid chromatography method with UV-VIS detection that was developed to measure GSSG in the presence of a large excess of GSH (Asensi et al., 1994a,b). The essence of this method consists of minimizing GSH oxidation, which otherwise results in a large increase in GSSG.

3. Results

A2E Kills Human RPE Cells Apoptotically

When cultured human RPE cells are exposed to A2E at concentrations found in the human eye (Sparrow et al., 1999) they die. Death occurs predominantly by apoptosis, as indicated by staining the cells with propidium iodide, Hoechst-33342, and Annexin V-FITC (Fig. 1(A)). With intermittent light exposure (see Materials and Methods) the cells are more sensitive to A2E than in the dark. Phosphatidylglycerol protects the cells against A2E-induced apoptosis (Fig. 1(B)) but not against staurosporine-induced apoptosis (Fig. 1(C)).

A2E Causes the Formation of Reactive Oxygen and Nitrogen Species in Human RPE Cells

The formation of reactive oxygen and nitrogen species in cells can be measured via the conversion of 2,7-dichlorofluorescein to dichlorofluorescein. As seen in Fig. 2(A), A2E at 5 μM causes the formation of reactive oxygen and nitrogen species in human RPE cells. Phosphatidylglycerol increases the chance to detect these species (Fig. 2(B)), as it protects these cells from death. In cells exposed to higher A2E concentrations (not shown) or to staurosporine (Fig. 2(C)), no dichlorofluorescein is detected probably because these cells are damaged to an extent that they are metabolically compromised.

Quantitative Analysis of Apoptosis and Protection

A2E kills human RPE cells apoptotically in a concentration-dependent manner, killing being more effective in the light than in the dark (Fig. 3). During the 6 hr incubation period A2E at lower concentrations induces predominantly early apoptosis, and at higher concentrations predominantly late apoptosis (Table I) and also detachment of some cells from the plate. Phosphatidylglycerol protects human RPE cells exposed to 10 μM A2E in a concentration-dependent manner (Fig. 4), 50 % protection being achieved in the dark with about 6 μM phosphatidylglycerol and in the light with about 8 μM phosphatidylglycerol.

The authors also tried to quantify the formation of reactive oxygen and nitrogen species over a broad range of A2E and phosphatidylglycerol concentrations. No clear picture emerged from this attempt presumably because these reactive species are formed in measurable amounts only when a certain degree of damage is induced, which is determined by the kind of exposure (dark vs light), the amount of A2E, and the amount of phosphatidylglycerol.

In experiments with the human carcinoma cell line MCF-7 the authors additionally tested the involvement of reactive oxygen and nitrogen species in A2E-induced cell death by using the antioxidant coenzyme Q. Ninety percent of the cells exposed to 50 μM A2E for 3 days survived in the presence of coenzyme Q at 500 $\mu\text{g ml}^{-1}$, supporting the notion that A2E-exposed cells are under oxidative stress when they undergo apoptosis.

A2E Causes Oxidative Stress in Isolated Mitochondria

A2E specifically targets mitochondria and detaches cytochrome c from the inner mitochondrial membrane (Suter et al., 2000; Shaban et al., 2001). Due to the disruption of electron flow more oxygen radicals should now be formed (Cai and Jones, 1998). The authors therefore tested with isolated mitochondria whether A2E induces oxidative stress in these organelles. Indeed, there is an increased H_2O_2 level

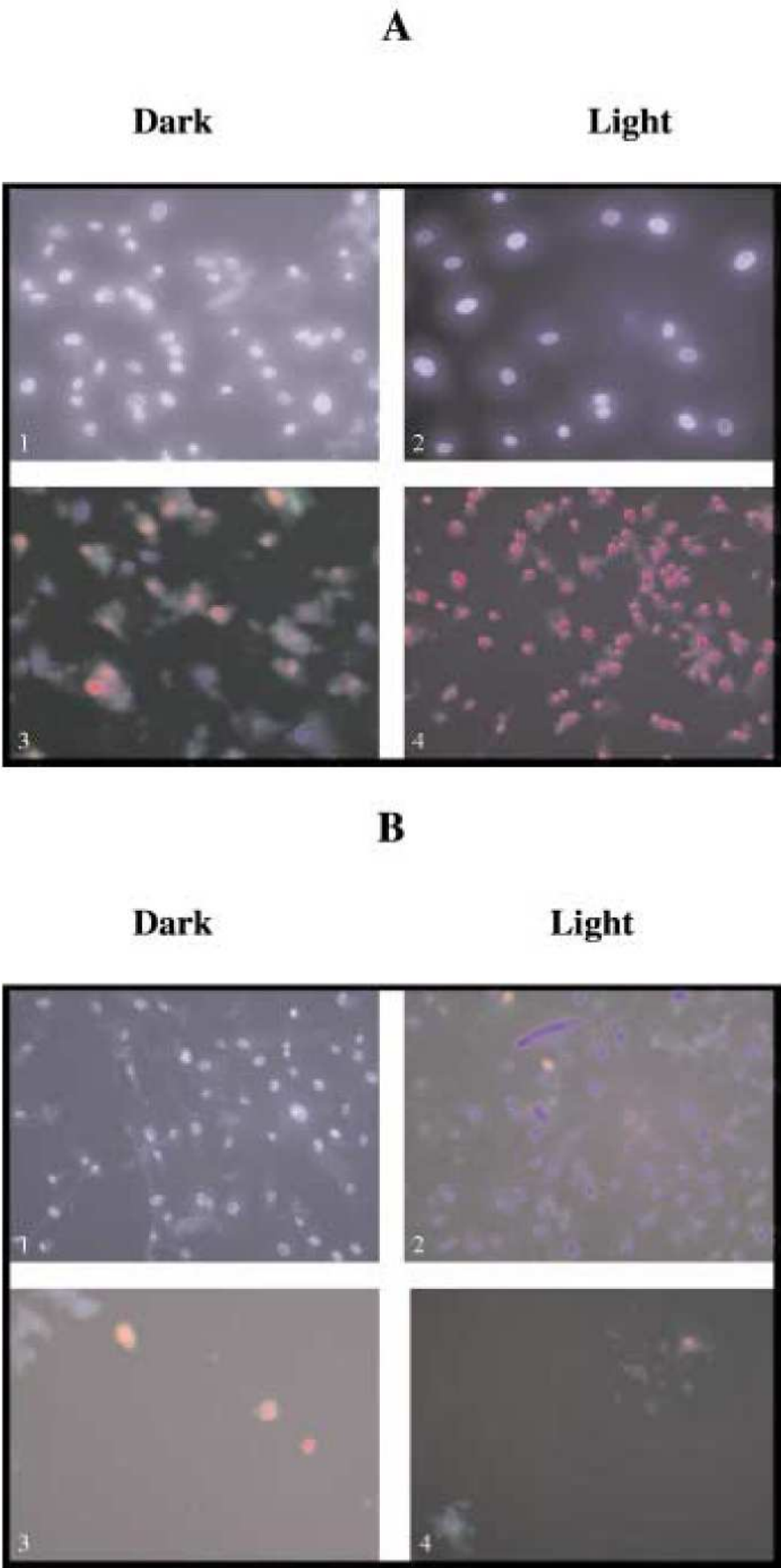


FIG. 1. Caption on next page.

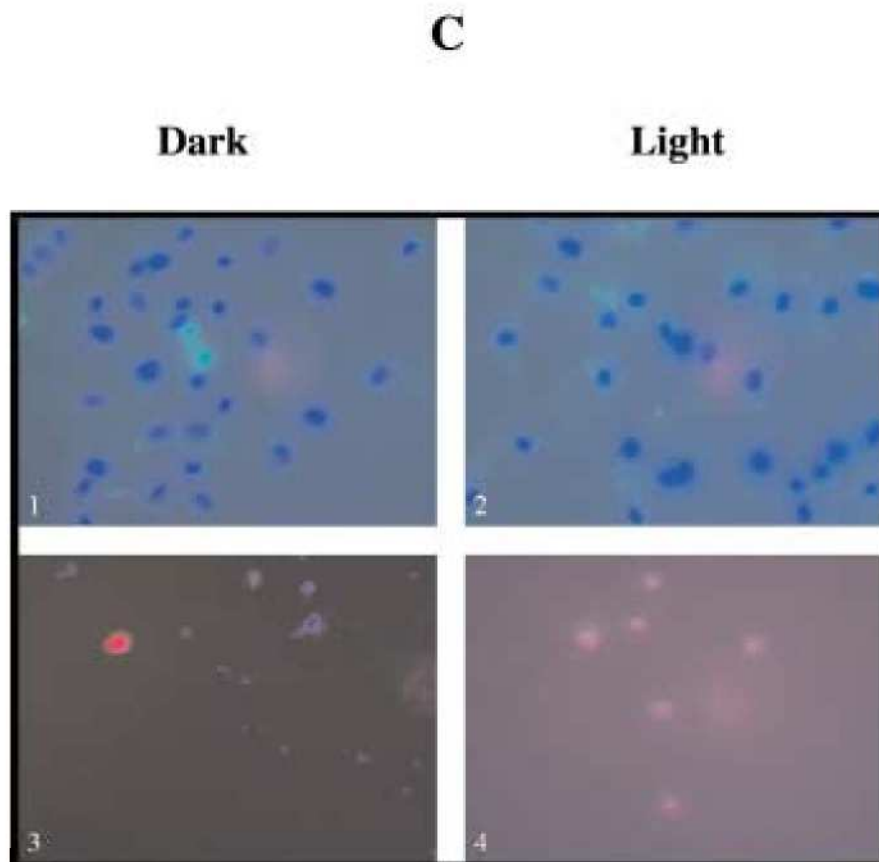


FIG. 1. *Apoptosis induced by A2E in human RPE cells.* Human RPE cells were incubated in the absence or presence of 10 μM A2E in culture medium in the dark and in the light for 6 hr. Thereafter, cells were stained with Hoechst-33342 (blue), Annexin V-FITC (green), and propidium iodide (red), as described in *Materials and Methods*. Hoechst stains chromatin, normal or condensed, in normal or apoptotic cells; Annexin V-FITC, which binds to phosphatidylserin in the plasma membrane, is a marker for early apoptosis; propidium iodide stains chromatin of late apoptotic and/or necrotic cells due to damage of the nuclear membrane. (A) Picture 1, control in the dark; no apoptotic cells are detectable. Picture 2, as picture 1 but in the light. Picture 3, 10 μM A2E in the dark; cells are Annexin- and propidium iodide-positive, i.e., early and late apoptotic. Picture 4, as picture 3 but in the light; cells are propidium iodide-positive, i.e. late apoptotic. (B) (for interpretation of the colours, see (A)): Picture 1, 10 μM A2E plus 20 μg phosphatidylglycerol ml^{-1} in the dark. Picture 2, as picture 1 but in the light. Picture 3, 1 μM staurosporine plus 100 μg phosphatidylglycerol ml^{-1} in the dark. Picture 4, as picture 3 but in the light. Note that in the presence of staurosporine fewer cells are detected because this compound causes them to detach from the surface of the plate. (C) (for interpretation of the colours, see (A)): Picture 1, 1% DMSO in the dark. Picture 2, as picture 1 but in the light. Picture 3, 1 μM staurosporine in the dark; cells are late apoptotic or detached. Picture 4, as picture 3 but in the light.

in mitochondria respiring on succinate in the presence of A2E (Fig. 5), and the redox state of the intramitochondrial antioxidant glutathione shifts from the more reduced form (GSH) to the oxidized form (GSSG) (Fig. 6).

4. Discussion

AMD is a common eye disease that gradually destroys sharp, central vision. It occurs in two forms, wet and dry, 90% of all people with AMD having the latter type. The greatest risk factor in AMD is age. Although AMD may occur during middle age, people over age 60 are clearly at greater risk than other age groups. Thus, people in middle age have about a 2% risk of getting AMD, but this risk increases to nearly 30% in those over age 75.

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 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 to 11-*cis*-retinal. Thereafter, 11-*cis*-retinal is attached to opsin, which completes the visual cycle (Berger et al., 1999).

A2E and iso-A2E are readily synthesized in vitro by mixing 2 all-*trans*-retinals with 1 ethanolamine at slightly acidic pH (Parish et al., 1998). It is presently

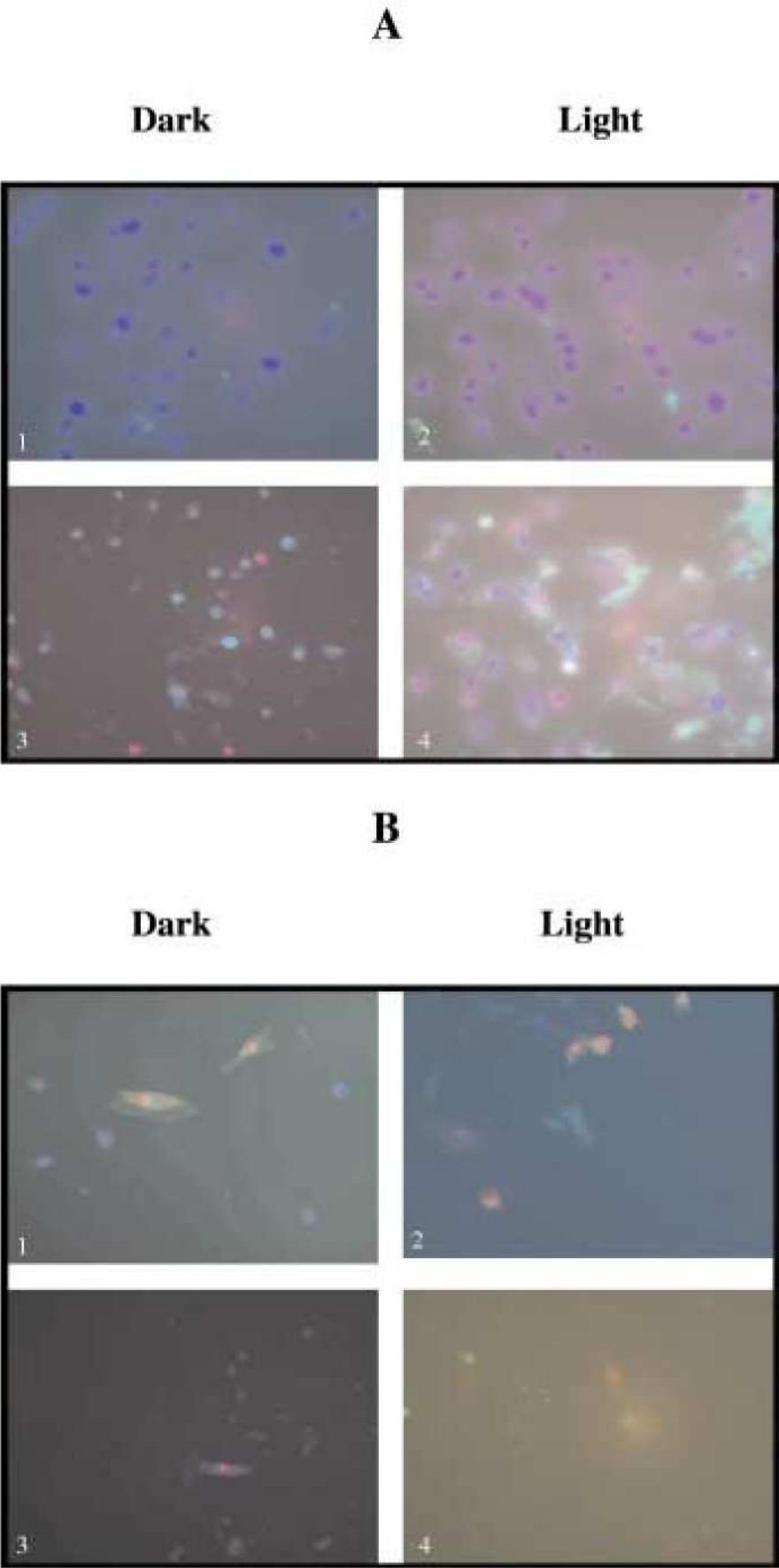


FIG. 2. Caption on next page.

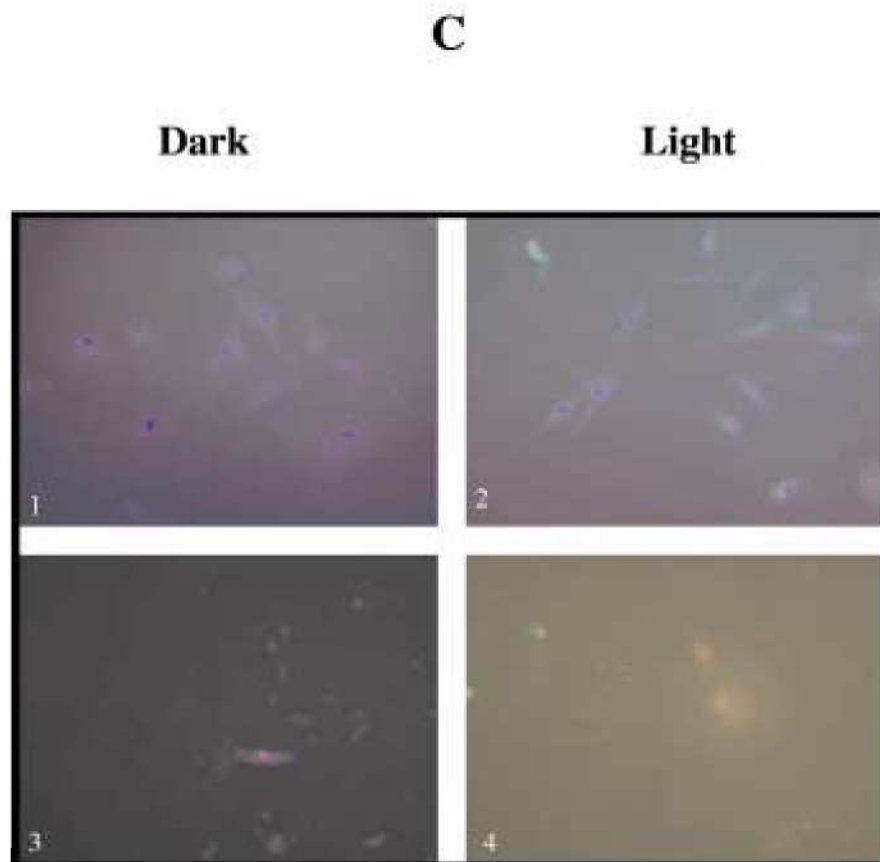


FIG. 2. Oxidative and nitrosative stress in human RPE cells exposed to A2E. Human RPE cells were incubated in the absence or presence of $5 \mu\text{M}$ A2E in culture medium in the dark and in the light for 12 hr. Thereafter, cells were stained with 2,7-dichlorofluorescein diacetate (DCF) (green), propidium iodide (red), and Hoechst-33342 (blue) as described in *Materials and Methods*. (A) Picture 1, control in the dark; very few cells are DCF-positive, i.e., under oxidative stress. Picture 2, as picture 1 but in the light. Picture 3, $5 \mu\text{M}$ A2E in the dark; many cells are DCF- and propidium iodide-positive, i.e., under oxidative stress and late apoptotic. Picture 4, as picture 3 but in the light; most cells are DCF- and propidium iodide-positive, i.e., under oxidative stress and late apoptotic. (B) (for interpretation of the colours, see (A)): Picture 1, $5 \mu\text{M}$ A2E plus $20 \mu\text{g}$ phosphatidylglycerol ml^{-1} in the dark. Picture 2, as picture 1 but in the light. Picture 3, $1 \mu\text{M}$ staurosporine plus $100 \mu\text{g}$ phosphatidylglycerol ml^{-1} in the dark. Picture 4, as picture 3 but in the light. (C) (for interpretation of the colours, see (A)): Picture 1, 1 % DMSO in the dark. Picture 2, as picture 1 but in the light. Picture 3, $1 \mu\text{M}$ staurosporine in the dark. Picture 4, as picture 3 but in the light.

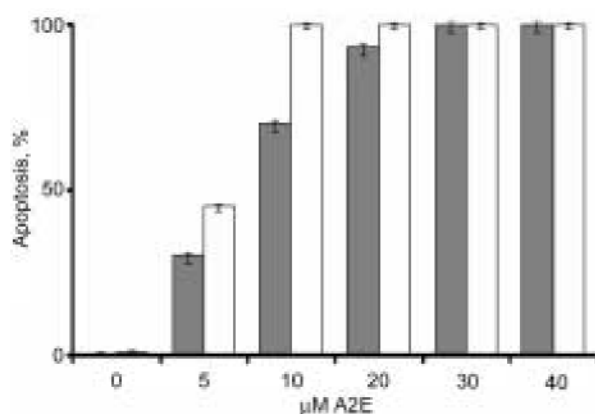


FIG. 3. Quantitative analysis of apoptosis induced by A2E in human RPE cells. Human RPE cells were incubated with different concentration of A2E (0–40 μM) for 6 hr in the dark (dark columns) and the light (light columns) and analysed for apoptosis as described in *Materials and Methods*. The mean and standard deviation are shown ($n = 6$).

uncertain where and how these molecules are formed in vivo. 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. The latter is either exported by the Rim transporter, or condenses with a second molecule of all-trans-retinal to A2-phosphatidylethanolamine, followed by hydrolysis of the latter (Liu et al., 2000; Mata et al., 2000). The last steps of A2E formation were suggested to occur in RPE phagolysosomes due to the requirement of oxidizing and acidic conditions (Mata et al., 2000).

The concentration of A2E in the eyes of old individuals is estimated to be $25 \mu\text{M}$ (Sparrow et al., 1999; Suter et al., 2000). It is found that at this concentration A2E causes apoptosis in cultured porcine RPE cells (Suter et al., 2000), suggesting that this compound contributes to or is responsible for

TABLE I
Early and late apoptosis induced by A2E in human RPE cells

	Dark		Light	
	Early apoptosis average $\pm$ S.D.	Late apoptosis average $\pm$ S.D.	Early apoptosis average $\pm$ S.D.	Late apoptosis average $\pm$ S.D.
control	1 $\pm$ 0	0 $\pm$ 0	3 $\pm$ 0	2 $\pm$ 2
control+DMSO	1 $\pm$ 1	0.5 $\pm$ 0	2 $\pm$ 0	3 $\pm$ 1
5 μ M A2E	2 $\pm$ 1	10 $\pm$ 2	10 $\pm$ 1.5	35 $\pm$ 5
10 μ M A2E	30 $\pm$ 2	40 $\pm$ 1	20 $\pm$ 0	80 $\pm$ 0
20 μ M A2E	10 $\pm$ 0	83 $\pm$ 1	5 $\pm$ 0	95 $\pm$ 1
30 μ M A2E	2 $\pm$ 0	98 $\pm$ 2	3 $\pm$ 0	97 $\pm$ 1
40 μ M A2E	0 $\pm$ 0	100 $\pm$ 0	0 $\pm$ 0	100 $\pm$ 1
10 μ M A2E + 2 μ g ml ⁻¹ PG	40 $\pm$ 1	45 $\pm$ 1	4 $\pm$ 1	96 $\pm$ 0
10 μ M A2E + 4 μ g ml ⁻¹ PG	50 $\pm$ 2	30 $\pm$ 5	30 $\pm$ 5	45 $\pm$ 0
10 μ M A2E + 6 μ g ml ⁻¹ PG	30 $\pm$ 3	25 $\pm$ 2	40 $\pm$ 5	30 $\pm$ 1
10 μ M A2E + 8 μ g ml ⁻¹ PG	15 $\pm$ 5	9 $\pm$ 2	20 $\pm$ 8	25 $\pm$ 0
10 μ M A2E + 10 μ g ml ⁻¹ PG	5 $\pm$ 1	2 $\pm$ 0	5 $\pm$ 0	6 $\pm$ 0
10 μ M A2E + 15 μ g ml ⁻¹ PG	1 $\pm$ 0	0 $\pm$ 0	2 $\pm$ 2	1 $\pm$ 0
20 μ M A2E + 20 μ g ml ⁻¹ PG	0 $\pm$ 0	0 $\pm$ 0	1 $\pm$ 0	0 $\pm$ 1
40 μ M A2E + 50 μ g ml ⁻¹ PG	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 1
40 μ M A2E + 100 μ g ml ⁻¹ PG	0 $\pm$ 0	0 $\pm$ 0	1 $\pm$ 0	0 $\pm$ 1
1 μ M ST	0 $\pm$ 0	100 $\pm$ 0	0 $\pm$ 0	100 $\pm$ 0
1 μ M ST + 100 μ g ml ⁻¹ PG	0 $\pm$ 0	100 $\pm$ 0	0 $\pm$ 0	100 $\pm$ 0

The cells in culture were exposed to A2E at concentrations ranging from 0 to 40 μ M for 6 hr in the dark or in the light. Early and late apoptosis were analysed as described in *Materials and Methods*. The Table shows the raw numerical data of Figs 3 and 4. Values are apoptotic cell counts expressed as percent of total cells counted. DMSO, dimethylsulfoxide; PG, phosphatidylglycerol; S.D., standard deviation; ST, staurosporine.

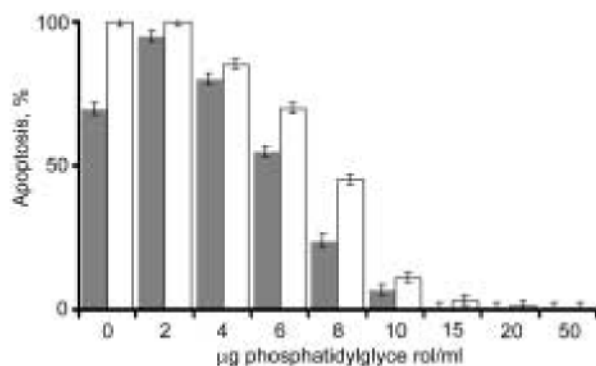


FIG. 4. Protection by phosphatidylglycerol of human RPE cells exposed to A2E. Human RPE cells were incubated with 10 μ M A2E and different concentration of phosphatidylglycerol (0–50 μ g ml⁻¹) for 6 hr in the dark (dark columns) and the light (light columns), and were analysed for apoptosis as described in *Materials and Methods*. The mean and S.D. are shown ($n = 6$).

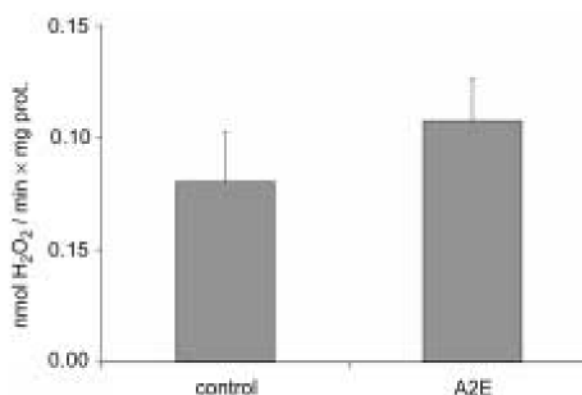


FIG. 5. Mitochondrial hydrogen peroxide levels in the absence and presence of A2E. Isolated rat liver mitochondria were incubated in the absence or presence of 12.5 μ M A2E with succinate as respiratory substrate. H₂O₂ was analysed as described in *Materials and Methods*. The mean and S.D. are shown; $P < 0.05$ ($n = 4$).

AMD. In both, cells and isolated mitochondria, A2E detaches pro-apoptotic proteins including cytochrome c from mitochondria (Suter et al., 2000). The authors also showed (Shaban et al., 2001) that A2E tightly but non-covalently binds to and inhibits isolated COX in the dark. This inhibition can be overcome by negatively charged phospholipids. Light accentuates the inhibition, which is now no longer relievable. These studies showed that A2E induces apoptosis primarily by preventing the binding of cytochrome c to COX.

It is now reported that A2E at very low concentrations causes apoptosis in cultured human RPE cells. These cells are more sensitive to A2E in the light than in the dark. Phosphatidylglycerol potentially inhibits apoptosis induced by A2E but does not affect apoptosis induced by staurosporine, a protein kinase inhibitor. This shows that the inhibition is specific. Phosphatidylglycerol is the immediate biosynthetic precursor of cardiolipin. Whether phosphatidylglycerol acts itself in cell cultures or increases the level of cardiolipin is presently not known. It should be

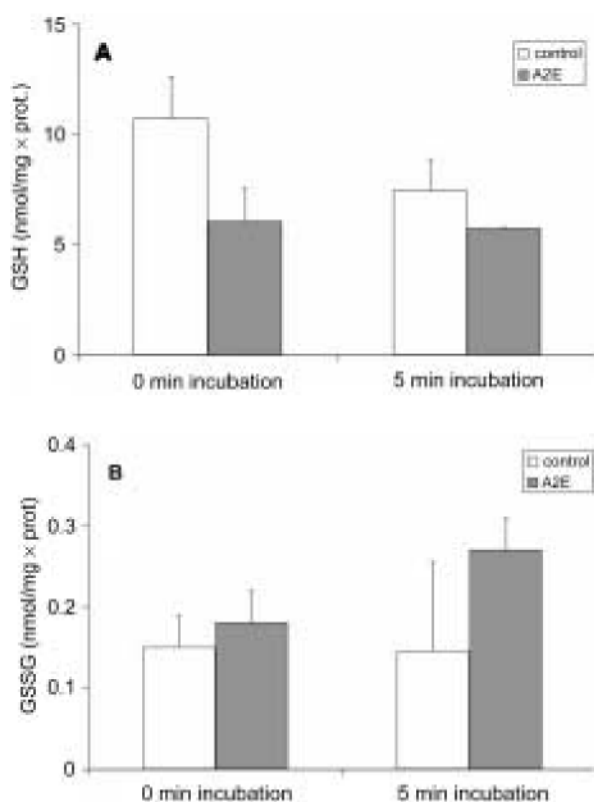


FIG. 6. Mitochondrial glutathione levels in the absence and presence of A2E. Isolated rat liver mitochondria were incubated as in Fig. 5 for 0 and 5 min. Thereafter, reduced glutathione (A) and oxidized glutathione (B) were analysed as described in *Materials and Methods*. GSH, reduced glutathione; GSSG, oxidized glutathione. The mean and standard deviation are shown; $P < 0.05$ ($n = 4$).

pointed out that isolated COX is much better protected by phosphatidylglycerol than by cardiolipin against A2E-induced inactivation (Shaban and Richter, 2002). Cells exposed to A2E form reactive oxygen and nitrogen species, and the antioxidant coenzyme Q protects cells against A2E-induced apoptosis.

The RPE cells used in this study were from a 53 year old donor and were passages three to six, in contrast to the RPE cells used in the previous work, which were freshly isolated from 6 month old pigs (Suter et al., 2000). This may explain the relative insensitivity of the porcine compared to the human cells.

How does A2E promote apoptosis? In cells, A2E causes release of the pro-apoptotic proteins cytochrome c and apoptosis inducing factor, but A2E is not a generally acting mitochondrial toxin because electron flow through the respiratory chain down to complex III is not affected by A2E (Suter et al., 2000). Furthermore, mitochondria remain tightly coupled in the presence of A2E (Shaban and Richter, Unpubl. res.). Rather, A2E specifically prevents the docking of cytochrome c to COX, which is normally mediated by cardiolipin.

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

interruption 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 (Cai and Jones, 1998). In isolated mitochondria A2E indeed causes oxidative stress as shown here by the increase of H_2O_2 production and the shift of the glutathione redox balance to the more oxidized state. The other consequence is the stimulation of apoptosis because of the formation of an 'apoptosome' in the cytosol, a pro-apoptotic complex comprising cytochrome c, apaf-1, and procaspase-9. It is found that in the presence of A2E mitochondria release besides cytochrome c two other pro-apoptotic proteins, AIF and SMAC/Diablo, into the extramitochondrial space (Garrido and Richter, Unpubl. res.).

Why do negatively charged phospholipids and an antioxidant protect against A2E-induced apoptosis? As mentioned above, the docking of the positively charged cytochrome c to COX is mediated by the negatively charged phospholipid cardiolipin, and A2E competes with cardiolipin at the level of COX (Shaban et al., 2001). It is known that the protection of COX is not specific for cardiolipin but can be afforded by other negatively charged phospholipids (Shaban et al., 2001). In the present cell culture study the authors used phosphatidylglycerol for two reasons. First, it protects isolated COX much more potently than cardiolipin against A2E-induced damage (Shaban and Richter, 2002). Second, considering the use of negatively charged phospholipids in the therapy or prevention of dry AMD, cardiolipin may be less advisable because it could stimulate the unwanted formation of anti-cardiolipin antibodies.

Since release of cytochrome c from mitochondria stimulates the formation of reactive oxygen species in mitochondria, the protection of cells against A2E-induced apoptosis by coenzyme Q is most likely related to its outstanding antioxidative properties (Frei et al., 1990). Epidemiological studies suggest that oxidative stress contributes to AMD, and mitochondria seem to be the main target of oxidative injury (Cai et al., 2000).

The dry form of AMD can so far not be treated. AMD develops over many decades, and to slow down or halt its progression would be a major achievement. It is presently not proven but likely that A2E contributes to or causes dry AMD. Its level increases about 10-fold in old individuals, and the level of cardiolipin in old people goes down to about 50 % of the value found in young individuals (Ames et al., 1995). Animal models should be used to verify the participation of A2E in light-induced retinal damage. A2E absorbs light in the eye maximally around 440 nm. This, together with the fact that negatively charged phospholipids potently protect RPE cells against A2E-induced apoptosis suggests the use of these phospholipids, particularly phosphatidylglycerol, alone or in combination with

blue light-absorbing compounds such as lutein and zeaxanthin to prevent or stop AMD. In this way it may even be possible to regain acute vision in mildly affected individuals. Each cell contains many mitochondria, which turn over much more rapidly than the cell. It is conceivable that in a given cell A2E affects only a subpopulation of mitochondria. Once the action of A2E is stopped by proper medication, the healthy mitochondrial subpopulation may outgrow the affected mitochondria and thereby rescue the cell.

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References

- Ames, B. N., Shigenaga, M. K. and Hagen, T. M. (1995). Mitochondrial decay in aging. *Biochim. Biophys. Acta* **1271**, 165–70.
- Asensi, M., Sastre, J., Pallardó, F. V., Estrela, J. M. and Viña, J. (1994a). Determination of oxidized glutathione in blood: high-performance liquid chromatography. *Methods Enzymol.* **234**, 367–400.
- Asensi, M., Sastre, J., Pallardó, F. V., García de la Asunción, J., Estrela, J. M. and Viña, J. (1994b). A high-performance liquid chromatography method for measurement of oxidized glutathione in biological samples. *Anal. Biochem.* **217**, 323–8.
- Barja, G. (1999). Mitochondrial oxygen radical generation and leak: sites of production in states 4 and 3, organ specificity, and relation to aging and longevity. *J. Bioenerg. Biomembr.* **31**, 347–66.
- Berger, J. W., Fine, S. L. and Maguire, M. G., eds. (1999). Age-related Macular Degeneration. Mosby: St. Louis, MO, U.S.A.
- Brigelius, R., Muckel, C., Akerboom, T. P. M. and Sies, H. (1983). Identification and quantitation of glutathione in hepatic protein mixed disulfides and its relationship to glutathione disulfide. *Biochem. Pharmacol.* **32**, 2529–34.
- Bossy-Wetzel, E. and Green, D. R. (2000). Detection of apoptosis by annexin V labelling. *Methods Enzymol.* **322**, 15–8.
- Cai, J. and Jones, D. P. (1998). Superoxide in apoptosis: mitochondrial generation triggered by cytochrome c loss. *J. Biol. Chem.* **273**, 11401–4.
- Cai, J., Nelson, K. C., Wu, M., Sternberg, P., Jr. and Jones, D. P. (2000). Oxidative damage and protection of the RPE. *Prog. Retin. Eye Res.* **19**, 205–21.
- Eldred, G. E. and Lasky, M. R. (1993). Retinal age pigments generated by self-assembling lysosomotropic detergents. *Nature* **361**, 724–6.
- Frei, B., Kim, M. C. and Ames, B. N. (1990). Ubiquinol-10 is an effective lipid-soluble antioxidant at physiological concentrations. *Proc. Nat. Acad. Sci. U.S.A.* **87**, 4879–83.
- Liu, J., Itagaki, Y., Ben-Shabat, S., Nakanishi, K. and Sparrow, J. R. (2000). The biosynthesis of A2E, a fluorophore of aging retina, involves the formation of the precursor, A2-PE, in the photoreceptor outer segment membrane. *J. Biol. Chem.* **275**, 29354–60.
- Mata, N. L., Weng, J. and Travis, G. H. (2000). Biosynthesis of a major lipofuscin fluorophore in mice and humans with ABCR-mediated retinal and macular degeneration. *Proc. Nat. Acad. Sci. U.S.A.* **97**, 7154–9.
- Parish, C. A., Hashimoto, M., Nakanishi, K., Dillon, J. and Sparrow, J. R. (1998). Isolation and one-step preparation of A2E and iso-A2E, fluorophores from human retinal pigment epithelium. *Proc. Nat. Acad. Sci. U.S.A.* **95**, 14609–13.
- Rickwood, D., Wilson, M. T. and Darley-Usmar, V. M. (1987). In *Mitochondria: A Practical Approach*. (Darley-Usmar, V. M., Wilson, M. T. and Rickwood, D., Eds.), pp 1–16. IRL: Oxford, U.K.
- Rothe, G. and Valet, G. (1990). Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2',7'-dichlorofluorescein. *J. Leukocyte Biol.* **47**, 440–8.
- Sawada, G. A., Raub, T. J., Decker, D. E. and Buxser, S. E. (1996). Analytical and numerical techniques for the evaluation of free radical damage in cultured cells using scanning laser microscopy. *Cytometry* **25**, 254–62.
- Shaban, H., Gazzotti, P. and Richter, C. (2001). Cytochrome c oxidase inhibition by N-retinyl-N-retinylidene ethanolamine, a compound suspected to cause age-related macular degeneration. *Arch. Biochem. Biophys.* **394**, 111–6.
- Shaban, H. and Richter, C. (2002). A2E and blue light in the retina: the paradigm of age-related macular degeneration. *Biol. Chem.*, in press.
- Sparrow, J. R., Parish, C. A., Hashimoto, M. and Nakanishi, K. (1999). A2E, a lipofuscin fluorophore, in human retinal pigmented epithelial cells in culture. *Invest. Ophthalmol. Vis. Sci.* **40**, 2988–95.
- Sun, H. and Nathans, J. (2001). ABCR, the ATP-binding cassette transporter responsible for Stargardt macular dystrophy, is an efficient target of all-trans-retinal-mediated photooxidative damage in vitro. Implications for retinal disease. *J. Biol. Chem.* **276**, 11766–74.
- Suter, M., Reme, C. E., Grimm, C., Wenzel, A., Jäätelä, M., Esser, P., Kociok, N., Leist, M. and Richter, C. (2000). Age-related macular degeneration: the lipofuscin component A2E detaches pro-apoptotic proteins from mitochondria and induces apoptosis in mammalian retinal pigment epithelial cells. *J. Biol. Chem.* **275**, 39625–30.
- Tsuchiya, M., Suematsu, M. and Suzuki, H. (1994). In vivo visualization of oxygen radical-dependent photoemission. *Methods Enzymol.* **233**, 128–40.