Lutein and Zeaxanthin Protect Photoreceptors from Apoptosis Induced by Oxidative Stress: Relation with Docosahexaenoic Acid

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Purpose. Oxidative stress has been proposed as a major pathogenic factor in age-related macular degeneration (AMD), the leading cause of vision loss among elderly people of western European ancestry. Lutein (LUT) and zeaxanthin (ZEA), major components in macular pigment, are among the retinal antioxidants. Though xanthophyll intake may reduce the likelihood of having advanced AMD, direct evidence of neuroprotection is lacking. Prior work has shown that docosahexaenoic acid (DHA), the major polyunsaturated fatty acid in the retina, delays apoptosis and promotes differentiation of photoreceptors. This study was conducted to investigate whether LUT, ZEA, and β-carotene (BC), major dietary carotenoids protect photoreceptors from oxidative stress and whether this protection is synergistic with that of DHA.

Methods. Pure rat retinal neurons in culture, supplemented with LUT, ZEA, or BC, with or without DHA, were subjected to oxidative stress induced with paraquat and hydrogen peroxide. Apoptosis, preservation of mitochondrial membrane potential, cytochrome *c* translocation, and opsin expression were evaluated.

Results. Pretreatment with DHA, LUT, ZEA, and BC reduced oxidative stress-induced apoptosis in photoreceptors, preserved mitochondrial potential, and prevented cytochrome *c* release from mitochondria. ZEA and LUT also enhanced photoreceptor differentiation. In control cultures, photoreceptors failed to grow their characteristic outer segments; addition of DHA, ZEA, or LUT increased opsin expression and promoted the development of outer-segment–like processes.

Conclusions. These results show for the first time the direct neuroprotection of photoreceptors by xanthophylls and suggest that ZEA and LUT, along with DHA, are important environmental influences that together promote photoreceptor survival and differentiation. (*Invest Ophthalmol Vis Sci.* 2007; 48:5168–5177) DOI:10.1167/iovs.07-0037

Age-related macular degeneration (AMD) is a leading cause of irreversible vision loss and blindness in elderly people of western European ancestry.[1,2] In this multifactorial degenerative disease, the loss of visual acuity may ultimately arise from the death of retinal pigment epithelium and cone photoreceptors or choroidal neovascularization in the fovea. The fovea is an area of the macula that is highly enriched in cones; the dense packing and small aperture of foveal photoreceptors provide high spatial resolution in vision.[3] However, the initial atrophic pathologic changes in AMD seem to affect the function of rod photoreceptors surrounding the fovea,[4] and significant rod dysfunction has been reported before any noticeable anatomic degeneration or cell death.[5] Prevention of photoreceptor death is essential for slowing or avoiding progression to sight-threatening AMD, as geographic photoreceptor and retinal pigment epithelium atrophy are associated with the choroidal neovascularization that characterizes the disease.

Oxidative stress may be a major pathogenic factor in AMD (reviewed in Ref. 6), as the potency of retinal defense and repair systems necessary to operate in response to chronic environmental and metabolic energy–oxidant load is reduced in the aging eye.[7,8] The retina is particularly susceptible to oxidative damage because of its high consumption of O_2 and its constant exposure to light. Exposure to light and oxidative stress induce photoreceptor death in vitro.[9,10] This process is accompanied by an increase in reactive oxygen species (ROS) and is diminished by use of antioxidants.[11] In this context, the Age-Related Eye Disease Study (AREDS) demonstrated the effectiveness of supplementation with vitamins and minerals with antioxidant properties in preventing progression to advanced AMD among people with large drusen, geographic atrophy (GA), or neovascular (NV) AMD in the fellow eye.[12]

Several defense and repair systems may be modulated by bioactive, diet-based molecules accreted to and concentrated in the retina, such as docosahexaenoic acid (DHA). This ω-3 polyunsaturated fatty acid (PUFA) is a major structural component of photoreceptors.[13] Extensive evidence suggests that DHA may have a protective role against ischemia-, light-, oxygen-, inflammatory-, and age-associated disease of the vascular and neural retina.[14] Mechanistic studies have been the first to elucidate molecular pathways through which DHA acts in a neuroprotective role against oxidative stress in photoreceptors.[10,15,16] Epidemiologic work in AREDS[17] contributed to the body of evidence[18,19] supporting the putative protective role of DHA in advanced AMD. In AREDS, protection against GA and NV AMD was also observed to be exerted by two macular carotenoids of dietary origin: lutein (LUT) and zeaxanthin (ZEA).[20]

LUT and ZEA are oxygenated carotenoids (xanthophylls) that are present as the major diet-based compounds of macular pigment.[21] In primates, the highest concentrations of these compounds in the body are found in the central retina, with

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the highest density in the fovea. Within the macula, these xanthophylls appear to be distributed mainly in photoreceptor axons and interneurons of the inner plexiform layer.[22] In eccentric retinal areas, these compounds (mainly LUT) exist primarily in the rod photoreceptor outer segments.[23] Therefore, rod and cone photoreceptors are exposed to very high concentrations of both carotenoids. Though it is not a major constituent of retinal tissue, β-carotene (BC), a provitamin A carotenoid that contributes the most to vitamin A activity, is also a highly efficient physiological antioxidant that was included in the original AREDS formulation.[12]

In this work, we investigated whether LUT, ZEA, and BC impart direct neuroprotection to retina photoreceptors. Using retinal neurons in culture, we evaluated whether these carotenoids protect rat photoreceptors in culture from oxidative stress and whether this protection is synergistic with that of DHA. We used two different oxidants, paraquat (PQ; methyl viologen dichloride hydrate) and hydrogen peroxide (H_2O_2), as different types of oxidative stress have been shown to affect cell viability and antioxidant protection in different ways.[24] PQ, which induces anion superoxide generation, has been shown to promote photoreceptor apoptosis.[10] H_2O_2 is itself an ROS and has been widely used to induce oxidative damage in several cell types.[24,25] Our results show a direct protective effect of ZEA and LUT on photoreceptors. These xanthophylls prevented oxidative stress-induced apoptosis of photoreceptors and also promoted photoreceptor differentiation.

Materials and Methods

Albino Wistar rats bred in our own colony were used in all the experiments. All proceedings concerning animal use were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Plastic culture 35-mm diameter dishes (NUNC) were purchased from Inter Med (Roskilde, Denmark). Dulbecco's modified Eagle's medium (DMEM) was from Invitrogen-Gibco (Grand Island, NY). Bovine serum albumin (fraction V; fatty acid-free; low endotoxin, tissue culture tested), paraquat dichloride (methyl viologen, 1,1′-dimethyl-4,4′-bipyridinium dichloride [PQ]), poly-DL-ornithine, trypsin, trypsin inhibitor, transferrin, hydrocortisone, putrescine, insulin, selenium, gentamicin, 4,6-diamidino-2-phenylindole (DAPI), monoclonal anti-syntaxin clone HPC-1, DHA, propidium iodide, paraformaldehyde, and all-*trans* β-carotene (type IV, >95% purity) were from Sigma Aldrich (St. Louis, MO). Tyramide signal amplification kit was from NEN (Boston, MA). Secondary antibody, Alexa 488–conjugated goat anti-mouse, a fed flouirescent mitochondrial stain (MitoTracKer Red CMXRos), Alexa Fluor-488 conjugated annexin V, terminal deoxynucleotidyl transferase, recombinant 5-bromo-2′deoxyuridine 5′-triphosphate (BrdUTP), and terminal deoxynucleotidyl transferase (TdT) buffer were from Invitrogen-Molecular Probes (Eugene, OR). Tween-20 was from USB Corp. (Cleveland, OH). Monoclonal primary antibody anti-bromodeoxyuridine (antiBrdU) was from DSHB (Developmental Studies Hybridoma Bank; developed under the auspices of the National Institute of Child Health and Human Development [NICHD] and maintained by the University of Iowa, Iowa City, IA). Rabbit polyclonal antibody against cytochrome *c* was from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibody against rhodopsin, Rho4D2, was generously supplied by Robert Molday (University of British Columbia, Vancouver, BC, Canada). LUT and ZEA were from Indofine Chemical Co. (Hillsborough, NJ). Hydrogen peroxide 30% was from (Baker, Buenos Aires, Argentina). Solvents were HPLC grade, and all other reagents were analytical grade.

Neuronal Cultures

Purified cultures of rat retinal neurons were prepared as previously described.[26,27] In brief, 2-day rat retinas were dissected and dissociated by trypsin digestion followed by mechanical dissociation. After dissociation, the cells were resuspended in a chemically defined medium, lacking the specific trophic factors required for photoreceptor cells, as previously described. Approximately 1 to 1.2 × 10^6 cells/dish were seeded on 35-mm diameter dishes, which had previously been sequentially treated with polyornithine and Schwannoma conditioned medium.[28] Cultures were incubated at 36°C in a humidified atmosphere of 5% CO_2.

Photoreceptors and amacrine neurons were the two major cell types in the culture. Photoreceptors have a small, round cell body 5 to 8 μm in diameter, with a single neurite at one end, which usually ends in a conspicuous synaptic "spherule." Sometimes they display a connecting cilium at the opposite end, but they fail to develop their characteristic outer segments; opsin is diffusely distributed over their cell body, which is usually darker than that of amacrine neurons. To be identified as photoreceptors, the cells had to display at least three of these criteria. Amacrine neurons are larger than photoreceptors (8–20 μm) and have multiple neurites.

Addition of Carotenoids

ZEA, LUT, and BC in a 0.05% Tween solution were added to the cultures, immediately after the cells were seeded. The effect of increasing concentrations of these carotenoids on photoreceptor apoptosis was evaluated, finally choosing 170, 140, and 80 nM, for ZEA, LUT, and BC, respectively. Control cultures were treated with or without the same volume of a 0.05% Tween solution. This addition had no effect on neuronal apoptosis (not shown).

Addition of DHA

DHA, at a 6.7 μM final concentration in the culture, was added at day 1 in culture, in a complex with bovine serum albumin (BSA), in a 2:1 (fatty acid and BSA) molar ratio.[26] The same volume and concentration of a BSA solution was simultaneously added to control cultures. Concentrations of DHA below 2 μM or above 10 μM have been shown to have no protective effect or to induce cell death, respectively.[26]

PQ Treatment

PQ (48 μM final concentration in the incubation medium, in a calcium-magnesium–free solution) was added to 3-day cultures.[10] Neurons were then incubated for 24 hours before fixation.

H_2O_2 Treatment

The cultures were treated with H_2O_2 essentially according to the method published by Cookson et al.,[29] with slight modifications. Briefly, 1 mL of the neuronal medium was removed from the dishes at day 3 in culture and different amounts of a H_2O_2 solution were added to obtain concentrations ranging from 1 μM to 1.0 mM (final concentrations in the incubation medium). The cultures were incubated for 30 minutes at 36°C. The medium was then removed and replaced with 2 mL fresh, neuronal medium, and the cultures were returned to the incubator for 8 hours before fixation. A 10 μM H_2O_2 concentration was chosen to be used in further experiments, since a lower H_2O_2 concentration had negligible effects on apoptosis and higher H_2O_2 concentrations, which led to increasing amounts of apoptotic neurons and induced significant cell detachment, which made results difficult to reproduce.

Immunocytochemical Methods

Neurons were fixed with 2% paraformaldehyde (PF) in phosphate-buffered saline (PBS) for 15 minutes, usually followed by permeation with Triton X-100 (0.1% in PBS). Neuronal cell types were identified by immunocytochemistry, using the monoclonal antibodies syntaxin (HPC-1) and Rho4D2, which selectively react with amacrine and photoreceptor neurons, respectively,[30–32] and by their morphology using phase-contrast microscopy. Alexa 488-conjugated goat anti-mouse was used as the secondary antibody. Tyramide signal amplification was occasionally used to improve visualization, according to the procedure

described by the manufacturers. Controls for immunocytochemistry were performed by omitting either the primary or the secondary antibody.

Cell Viability and Apoptosis

Cell viability was determined by the exclusion of propidium iodide (PI), 0.5 μg/mL in culture, after a 30-minute incubation.[33] Surviving cells were quantified, taking into account their simultaneous absence of PI-labeling plus a healthy morphologic appearance.

Apoptosis was determined by three different methods: DAPI, TUNEL, and Annexin V staining.

Nuclear integrity was evaluated after staining cell nuclei with DAPI, a fluorescent dye that binds to DNA. Briefly, cells were permeated with Triton X-100 in PBS, washed with PBS, and incubated with DAPI for 20 minutes. The cells were considered to be apoptotic when they showed either fragmented or condensed (pyknotic) nuclei. The amount of apoptotic photoreceptors or amacrine cells was counted in cultures double-labeled with DAPI and with either Rho4D2 or HPC-1, to identify cells unambiguously as either photoreceptors or amacrine neurons, respectively, and thus establish the total number of each cell type. The percentage of apoptotic photoreceptors or amacrine neurons was then calculated, taking into account the percentage of Rho4D2-labeled cells or HPC-1 labeled cells, respectively.

For TUNEL staining, the cells were fixed at day 4 with 2% PF for 15 minutes and then stored in 70% ethanol for 48 hours at −20°C. Before labeling, the cells were washed twice with PBS for 5 minutes each at room temperature. The samples were preincubated with 1× TdT buffer for 15 minutes and then incubated with the TdT reaction mixture (0.05 mM BrdUTP, 0.3 U/μL TdT in TdT buffer) at 37°C in a humidified atmosphere for 1 hour. The reaction was stopped by a 15-minute incubation with stop buffer (300 mM NaCl, 30 mM sodium citrate; pH 7.4) at room temperature. Negative controls were prepared by omitting TdT. The presence of BrdU was determined with an anti-BrdU monoclonal antibody, according to standard immunocytochemical techniques.

For Annexin V staining, the incubation medium was removed, and the dishes were washed twice with ice-cold PBS. The cells were then incubated with a 1:4 dilution of Annexin V conjugate in Annexin-binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl2 [pH 7.4]) at room temperature in the dark for 15 minutes. PI was added immediately after, and cells were incubated for another 15 minutes in the same conditions. The cells were then washed twice in cold PBS, fixed in 1% PF in Annexin-binding buffer for 1 hour and washed in cold PBS. Labeling with Annexin V, PI, or both was then analyzed by fluorescence and confocal microscopy.

Evaluation of Mitochondrial Membrane Potential

To assess the amount of cells with preserved mitochondrial membrane potential, cultures were incubated for 30 minutes before fixation with a fluorescent mitochondrial probe (0.1 μg/mL MitoTracker; Invitrogen-Molecular Probes). The amount of photoreceptors displaying fluorescent mitochondria with respect to the total number of photoreceptors was determined.

Cytochrome *c* Translocation

To evaluate whether cytochrome *c* was released from mitochondria, the cultures were first incubated with the mitochondrial tracer and then fixed with methanol for 15 minutes. Immunocytochemical labeling of cytochrome *c* was then performed, with a polyclonal antibody.

Statistical Analysis

For cytochemical studies, 10 fields per sample, randomly chosen, were analyzed in each case. Each value represents the average of at least three experiments, with three dishes for each condition ± SD, except when indicated otherwise. Statistical significance was determined by Student's two-tailed *t*-test.

Results

Protective Effect of Carotenoids against Death Induced by Oxidative Stress

In pure retinal neuron cultures most photoreceptors survived for 4 days in vitro (Fig. 1). Only 18% of the photoreceptors showed PI labeling, which indicates cell death. Supplementation with either ZEA or LUT at day 0 in vitro showed no significant changes in the percentage of photoreceptors labeled with PI, when compared with control cultures. Generation of oxidative damage with H_2O_2 induced a 2.5-fold increase in photoreceptor cell death, compared with the control (Fig. 1A). Carotenoids efficiently prevented this death. Cultures supplemented with ZEA, LUT, or BC showed no significant increase in photoreceptor death after H_2O_2 treatment when compared to cultures not subjected to oxidative stress (Fig. 1A).

Treatment with 48 μM PQ for 24 hours increased the amount of PI-labeled cells to 45%, almost three times higher than in control conditions (Fig. 1B). This increase was similar

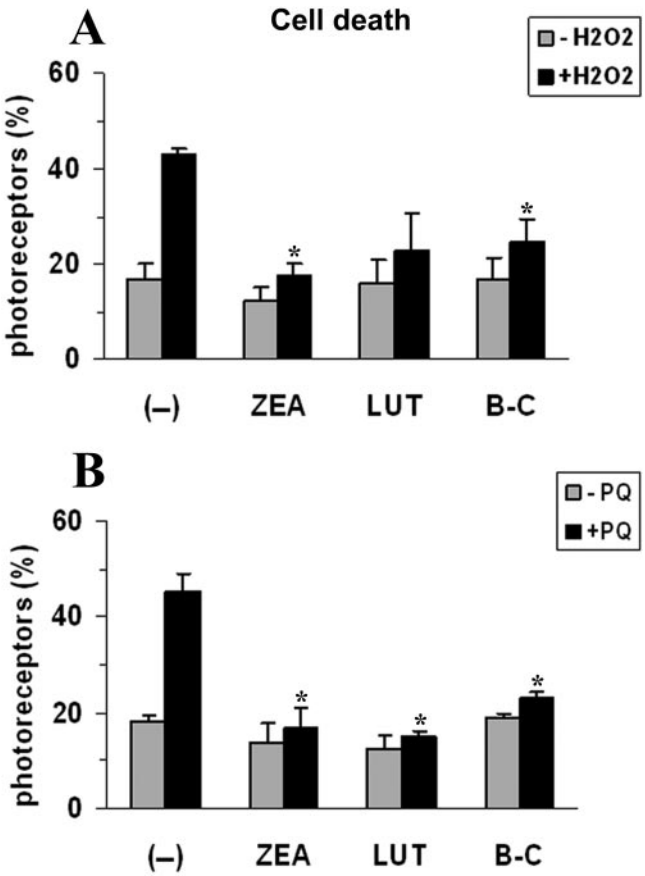

Figure 1. Effect of carotenoids on photoreceptor death induced by oxidative stress. Pure retina neurons were supplemented with 170 nM ZEA, 140 nM LUT, or 80 nM BC or with the 0.05% Tween solution used as a vehicle for the carotenoid compounds (−) at day 0, immediately after the cells were seeded. At day 3, the cultures were treated with 10 μM H_2O_2 (**A**) for 30 minutes or with 48 μM PQ for 24 hours (**B**). The cells were incubated with PI for 30 minutes immediately before fixation with 2% paraformaldehyde. Data are the percentage of photoreceptors that were labeled with PI. Total number of cells per dish was $1.4 \times 10^6 \pm 112{,}376$ ($n = 3$) in (**A**) and $1.10 \times 10^6 \pm 45{,}500$ ($n = 5$) in (**B**). *Statistically significant differences, compared with either H_2O_2- or PQ-treated cultures lacking carotenoids ($P < 0.05$ and $P < 0.01$, respectively).

to that induced by H_2O_2. ZEA and LUT proved to be as efficient in preventing this death in PQ- as in H_2O_2-treated cultures. These xanthophylls completely blocked the increase in photoreceptor death induced by PQ. With either ZEA or LUT, the percentage of PI-labeled photoreceptors was the same in cultures with or without PQ (Fig. 1B). BC addition also diminished PQ-induced photoreceptor death, though it was slightly less protective than ZEA and LUT. PQ treatment induced a small though significant increase in the percentage of dead photoreceptors, from approximately 18.9% ± 1.1% in BC-supplemented cultures to 23.2% ± 1.2% after PQ treatment of these cultures (Fig. 1A).

Effect of Carotenoids and DHA in the Prevention of Photoreceptor Apoptosis Induced by Oxidative Stress

Since studies have shown that PQ induces photoreceptor death through an apoptotic pathway,[10] we wanted to evaluate whether carotenoids protect photoreceptors from this apoptosis. In control cultures, most of the photoreceptors showed intact nuclei (Fig. 2B). Addition of PQ markedly increased the amount of pyknotic or fragmented nuclei in the photoreceptors (Fig. 2H). However, PQ did not induce a similar increase in neuronal apoptosis in ZEA-supplemented cultures. The addition of ZEA resulted in markedly preserved nuclear integrity in the photoreceptors, preventing apoptosis (Fig. 2K).

While in control and ZEA-supplemented cultures photoreceptors showed brightly labeled mitochondria, indicating that they maintained their mitochondrial membrane potential (Figs. 2C, 2F), PQ treatment promoted mitochondrial membrane depolarization (Fig. 2I). ZEA supplementation prevented this depolarization as photoreceptor mitochondria maintained their membrane potential, even after PQ treatment (Fig. 2L).

H_2O_2 treatment also induced the apoptosis of photoreceptors, doubling the amount of apoptotic photoreceptors from approximately 22% in control cultures to almost 50% in H_2O_2-treated cultures (Fig. 3A). This apoptosis was prevented in cultures supplemented with ZEA, LUT, or BC before H_2O_2 treatment (Fig. 3A). The three carotenoids were equally protective, as no significant increase in the percentage of apoptotic photoreceptors was observed in cultures supplemented with either of them.

PQ increased the percentage of apoptotic photoreceptors in cultures lacking carotenoids to approximately 50%, compared with 20% in control conditions (Fig. 3B). Addition of either ZEA or LUT to the cultures completely prevented PQ-induced apoptosis, reducing the percentage of apoptotic pho-

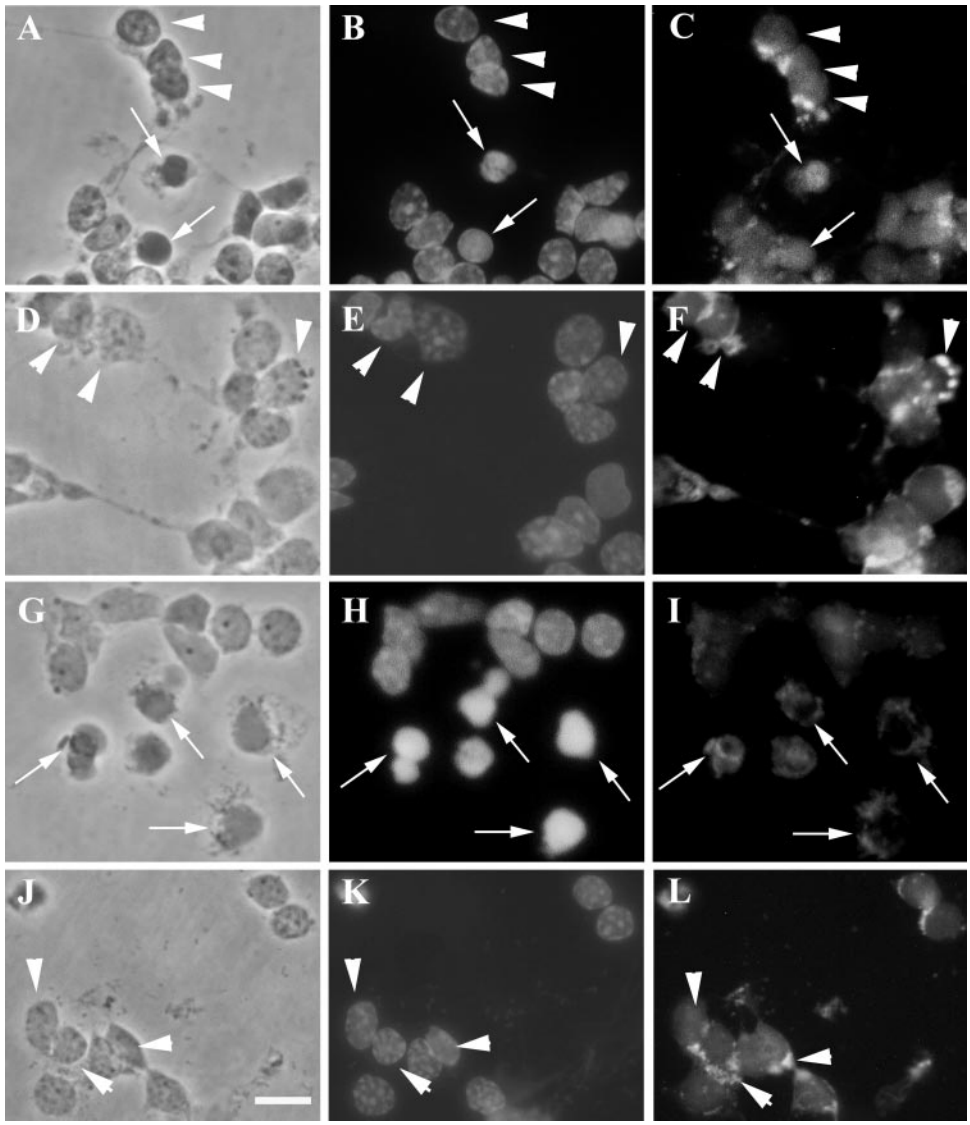

FIGURE 2. Effect of ZEA on photoreceptor apoptosis induced by PQ. Phase photomicrographs (*left column*) show neuronal cultures without (**A–C**, **G–I**) or with ZEA (**D–F**, **J–L**), and treated (**G–L**) or not (**A–F**) with PQ. Fluorescence micrographs show cultures labeled with DAPI, to analyze nuclei fragmentation (*middle column*), and with a red fluorescent tracer, to evaluate mitochondrial membrane potential (*right column*). Addition of PQ induced photoreceptor apoptosis (*thin arrows*). Note that ZEA preserved nuclei integrity and mitochondrial function (*arrowheads*) despite PQ treatment. Bar, 10 μm.

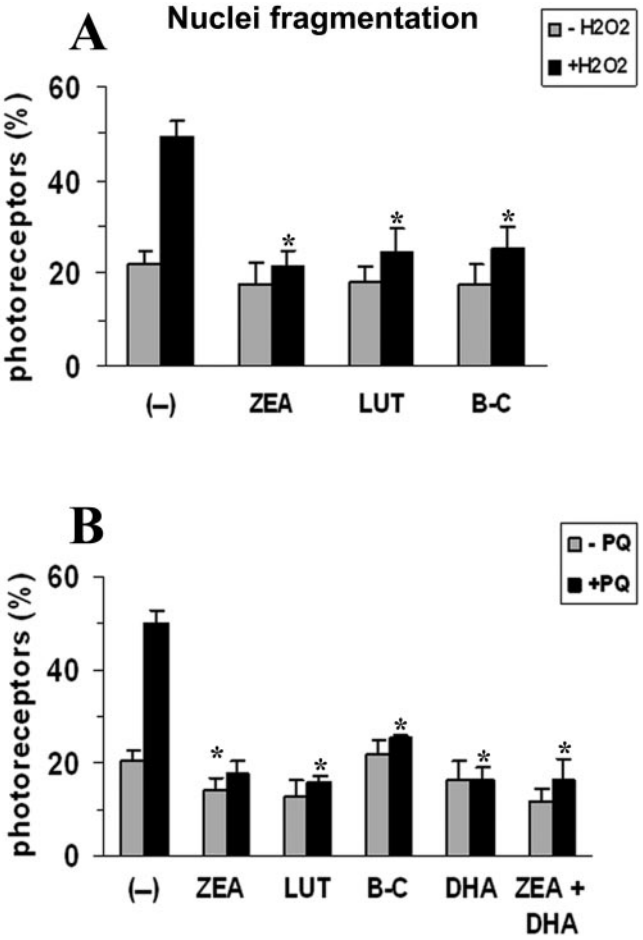

FIGURE 3. Protective effect of carotenoids and DHA on apoptosis of photoreceptors subjected to oxidative stress. Cultures without (−) or with ZEA, LUT, and BC at day 0, and 6.7 μM DHA at day 1 were treated with H_2O_2 (**A**) or PQ (**B**) at day 3. The percentage of apoptotic photoreceptors was evaluated by analyzing nuclei fragmentation, after DAPI labeling. The total number of cells per 35-mm diameter dish was $1.4 \times 10^6 \pm 112{,}376$ ($n = 3$) in (**A**) and $1.08 \times 10^6 \pm 101{,}380$ ($n = 6$) in (**B**). *Statistically significant differences compared with H_2O_2- and to PQ-treated cultures lacking carotenoids ($P < 0.05$ and $P < 0.01$, respectively).

toreceptors to levels even smaller than in control conditions. Noteworthy, addition of ZEA and LUT diminished photoreceptor apoptosis in control cultures as well. BC supplementation also decreased PQ-induced apoptosis of photoreceptors, though not so effectively as ZEA and LUT (Fig. 3B).

Evaluation of the effects of increasing carotenoid concentrations on PQ-induced photoreceptor apoptosis showed that carotenoids had a protective effect in a narrow range of concentrations, which was different for each carotenoid (not shown). For further experiments, we used the concentration at which each carotenoid showed the highest protective effect (i.e., 170, 140 and 80 nM, for ZEA, LUT and BC, respectively). Below this concentration, the protective effect decreased, and above it a deleterious effect was observed.

We then compared the protective effect of carotenoids with that of DHA. ZEA, LUT, and DHA acted similarly, preventing photoreceptor apoptosis induced by PQ (Fig. 3B). Moreover, the protective effect of the combined addition of ZEA and DHA against PQ-induced photoreceptor apoptosis was the same as that observed when either ZEA or DHA was separately added (Fig. 3B). Similarly, the combined addition of DHA and LUT did not provide additional protection (not shown).

The protective effects of carotenoids was also analyzed by TUNEL staining. Very few TUNEL-positive cells were identified in control and LUT-supplemented cultures (Figs. 4A, 4D), reaching less than 15% (Fig. 4M). PQ treatment substantially increased the amount of TUNEL-positive cells, to approximately 45% (Figs. 4B, 4E, 4M). Supplementation with LUT prevented the increase in TUNEL-positive cells, confirming the antiapoptotic effect of this carotenoid (Figs. 4C, 4F, 4M).

Annexin V-PI staining provided additional support to the antiapoptotic effect of carotenoids. Control cultures showed few photoreceptors labeled with Annexin V and PI, evidencing low levels of apoptosis (Figs. 4G, 4J, 4N). PQ treatment increased the amount of double-labeled cells, evidence that approximately 40% of photoreceptors had reached an advanced stage of apoptosis (Figs. 4H, 4K, 4N). ZEA protection was corroborated by the low percentage of double-labeled photoreceptors in ZEA-supplemented cultures after PQ treatment (Figs. 4I, 4L, 4N).

Effect of Carotenoids on the Preservation of Mitochondrial Membrane Potential in Photoreceptors

The increase in apoptosis after PQ treatment was parallel to a decrease in the percentage of photoreceptors preserving their mitochondrial membrane potential, from nearly 60% in controls to approximately 30% in PQ-treated cultures (Fig. 5J). The protective effect of carotenoids on photoreceptor apoptosis induced by PQ was accompanied by the preservation of their mitochondrial membrane potential. In ZEA-, LUT- and BC-supplemented cultures, around 60% of photoreceptors maintained their mitochondrial membrane potential after PQ treatment, the same percentage found in control conditions and in cultures supplemented with ZEA, LUT, and BC without PQ (Fig. 5J). As previously shown,[10] DHA supplementation also preserved mitochondrial membrane potential in photoreceptors after PQ treatment. The combined addition of DHA and ZEA had the same effect on preventing mitochondrial membrane depolarization as DHA and ZEA when added separately (Fig. 5J). Similar results were observed with DHA and LUT (not shown).

We then investigated whether oxidative stress induces the release of cytochrome *c* from mitochondria and whether the addition of ZEA would prevent this release. Colocalization of cytochrome *c* fluorescence and red fluorescence (MitoTracker)-labeled mitochondria was usually observed in control conditions (Figs. 5A, 5D, 5G; white arrows). PQ treatment led to the loss of colocalization of cytochrome *c* fluorescence with that of the mitochondrial fluorescent tracer (Figs. 5B, 5E, 5H, arrowheads), suggesting that the translocation of cytochrome *c* from mitochondria to cytosol accompanied the onset of apoptosis. Addition of ZEA to the cultures, before PQ treatment, preserved cytochrome *c* and mitochondrial colocalization in photoreceptors (Figs. 5C, 5F, 5I, white arrows). Hence, ZEA preserved mitochondrial membrane potential and its integrity, thus avoiding the release of cytochrome *c* to the cytosol.

Effects of ZEA and LUT on Photoreceptor Differentiation

In control conditions, only a few photoreceptors showed a short cilium (Figs. 6A, 6C), and almost none of them developed outer segments resembling those found in their mature in vivo counterparts. It is noteworthy that in cultures supplemented with ZEA, many photoreceptors presented apical processes, similar to rudimentary outer segments, which were intensely labeled with Rho4D2 (Figs. 6B, 6D). LUT addition to neuronal cultures had a similar effect, promoting the development of outer-segment–like apical processes (not shown).

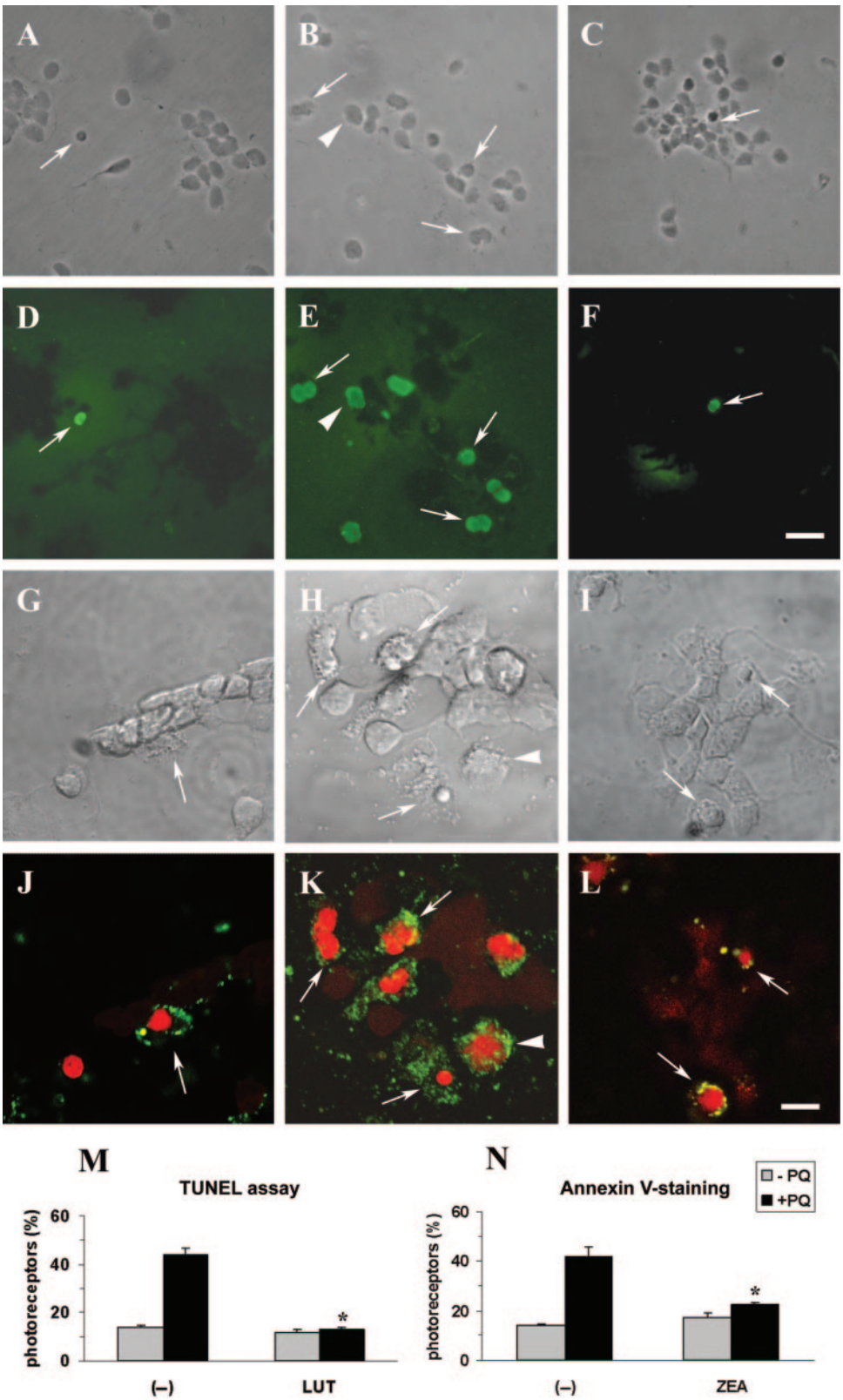

FIGURE 4. Protective effect of LUT and ZEA on PQ-induced apoptosis of photoreceptors established by TUNEL and Annexin V staining. Photomicrographs of control cultures (**A**, **D**, **G**, **J**), cultures treated with PQ without (**B**, **E**, **H**, **K**) or with LUT (**C**, **F**) or ZEA (**I**, **L**) supplementation were fixed and TUNEL (**A–F**) or Annexin V-PI (**G–L**) staining was then performed. (**D–F**) TUNEL-positive cells are stained *green*. (**J–L**) Annexin V- and PI-positive cells are stained *green* and *red*. The percentage of photoreceptors showing TUNEL or Annexin V and/or PI staining is depicted in (**M**) and (**N**), respectively. In control cultures, few photoreceptors (*thin arrows*) showed TUNEL and Annexin V/PI staining. PQ treatment dramatically increased the amount of photoreceptor (*thin arrows*) and amacrine cells (*arrowheads*) showing TUNEL and Annexin V/PI staining (*thin arrows* and *arrowheads*, respectively, in **B**, **E**, **H**, **K**). LUT and ZEA supplementation significantly reduced the number of TUNEL or Annexin V/PI-positive cells (**C**, **F**, and **I**, **L**, respectively). (**A–F**) Images were obtained by fluorescence microscopy and in (**G–L**) by confocal microscopy. *Statistically significant differences compared to PQ-treated cultures without carotenoids ($P < 0.01$). Bar: (**A–F**) 20 μm; (**G–H**) 10 μm.

At this early stage of development, 4 days in vitro, opsin levels in photoreceptors are still very low.[34] Only approximately 3% of photoreceptors expressed opsin in control conditions (Fig. 6E). Supplementation of the cultures with ZEA, LUT, BC, or DHA increased opsin expression (Fig. 6E), with DHA and ZEA being the most effective, since they showed a statistically significant increase. The combined addition of ZEA and DHA showed the same increase in opsin expression as the addition of each compound by itself. Together, these results suggest that carotenoids enhance photoreceptor differentiation.

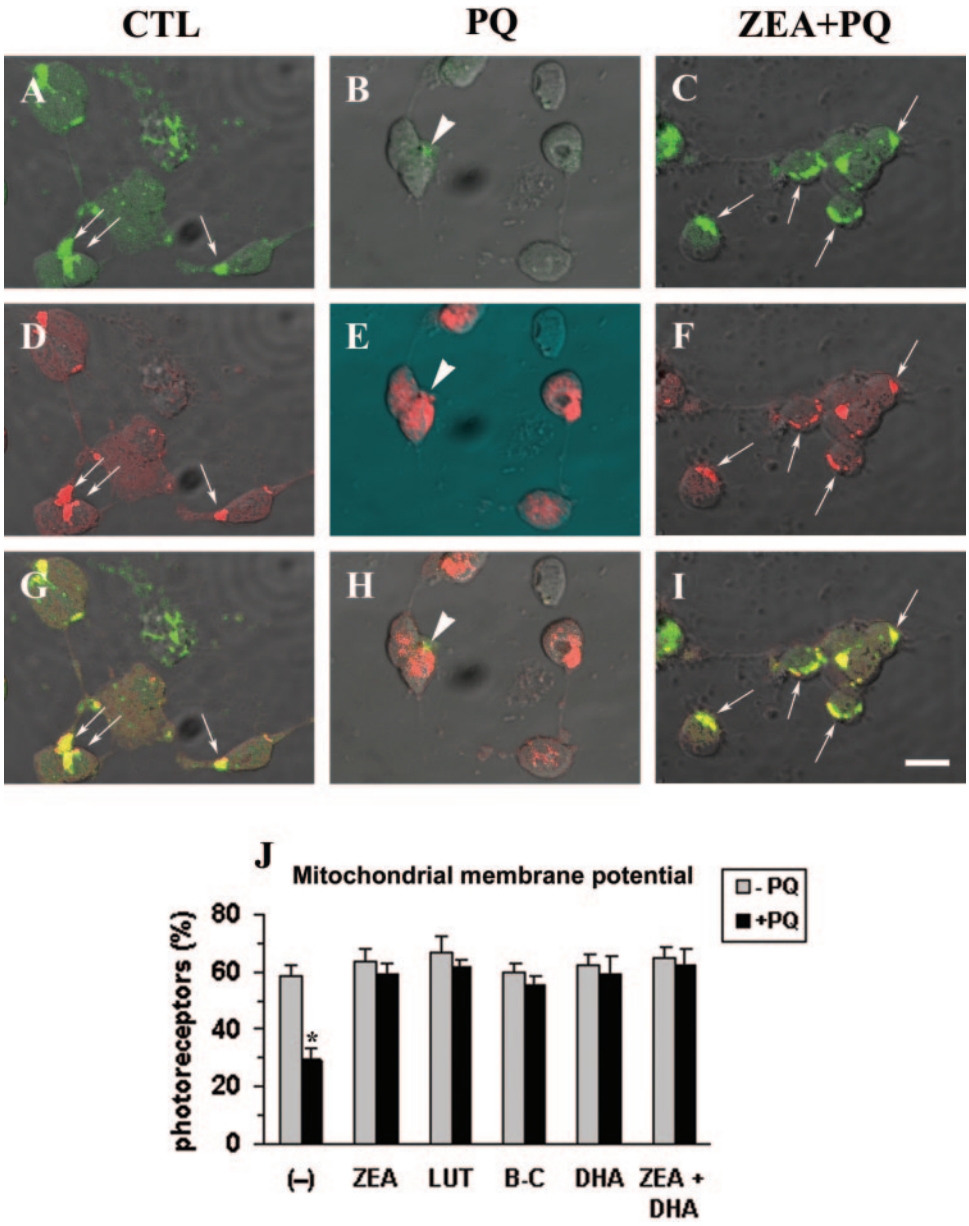

FIGURE 5. Effects of carotenoids on cytochrome *c* release and on preservation of mitochondrial membrane potential after PQ-induced oxidative stress. Cultures without (**A**, **D**, **G**) or with (**B**, **E**, **H**) PQ treatment or with ZEA supplementation before PQ treatment (**C**, **F**, **I**) were incubated with a fluorescent mitochondrial tracer (**D–F**, *red*) before fixation with methanol for 15 minutes. Immunocytochemical labeling of cytochrome *c* (**A–C**, *green*) was then performed, using a polyclonal antibody. (**G–I**) Merged photomicrographs. Note that control, and ZEA-supplemented, PQ-treated cultures showed colocalization of cytochrome *c* staining and mitochondrial fluorescence labeling (*thin white arrows*). Lack of colocalization of cytochrome *c* and red fluorescence (**H**, *arrowheads*) was visible only in PQ-treated cells, lacking ZEA supplementation. Images were obtained by confocal microscopy. Cultures without (CTL) or with ZEA, LUT, and BC at day 0, and 6.7 μM DHA at day 1, were treated with PQ at day 3. Bar, 10 μm. The percentage of photoreceptors preserving their mitochondrial membrane potential under the different culture conditions is depicted in (**J**). The number of cells per dish was $1.08 \times 10^6 \pm 101{,}380$ ($n = 6$). *Statistically significant differences compared with controls ($P < 0.01$).

Effect of Carotenoids on the Survival and Differentiation of Amacrine Cells

We then evaluated whether carotenoids would be equally effective in preventing PQ-induced apoptosis of amacrine neurons, the other major cell type in the cultures. Oxidative stress increased the apoptosis of amacrine neurons, which was augmented from less than 10% in control conditions to approximately 40% in PQ-treated cultures (Fig. 7D). ZEA, LUT, and BC partially protected amacrine neurons from PQ-induced apoptosis (Fig. 7D). In these cultures, after PQ treatment amacrine cell apoptosis was reduced by half, when compared with cultures lacking carotenoids, increasing from less than 10% to approximately 20% of total amacrine neurons (Fig. 7D). Hence, carotenoid protection was more effective for photoreceptors than for amacrine cells.

The effect of both xanthophylls on differentiation was not restricted to photoreceptors. ZEA and LUT markedly stimulated neurite outgrowth in amacrine neurons (Fig. 7). At this time in development, amacrine neurons in control cultures were still immature (Fig. 7A; white arrows). Approximately 30% of them had neurites (Figs. 7A, Fig. 7C; black arrowheads) that were usually short, thin, and still poorly developed. In contrast, after addition of ZEA, 50% of amacrine neurons extended long and thick neurites throughout the culture (Figs. 7B, 7C; black arrowheads), resembling the extensive neurite outgrowth found in these neurons at more advanced times of development. PQ treatment markedly reduced the percentage of amacrine neurons that had neurites, dropping to 15% in cultures without xanthophylls (Fig. 7C). Of note, ZEA supplementation effectively prevented neurite loss induced by PQ in most amacrine neurons (Fig 7C). LUT supplementation had a similar effect on neurite outgrowth (data not shown), suggesting that xanthophylls effectively promote differentiation of these cells.

DISCUSSION

The novel findings of the current report demonstrate a direct neuroprotective effect of ZEA and LUT on retina photorecep-

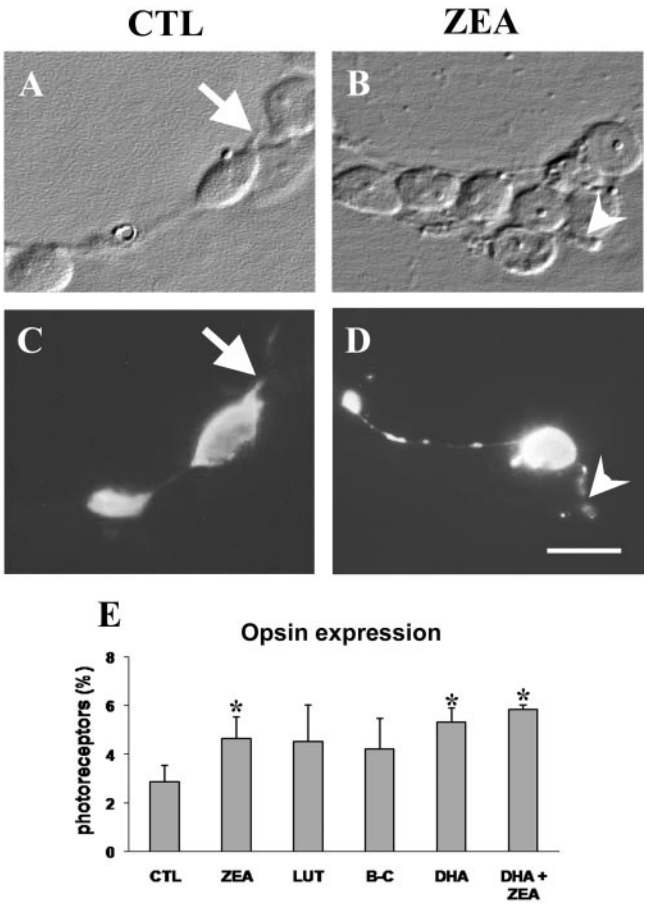

FIGURE 6. Effect of carotenoids and DHA on photoreceptor differentiation and opsin expression. Cultures were without (−) or with ZEA, LUT, or BC at day 0 and/or with DHA at day 1. Phase (**A**, **B**) and fluorescence (**C**, **D**) micrographs of 4-day neuronal cultures without (CTL) or with ZEA, labeled with the monoclonal antibody against rhodopsin Rho4D2. Whereas photoreceptors in control cultures usually lacked apical processes (**A**, **C**, *arrows*), ZEA supplementation induced the development of rudimentary apical processes (**B**, **D**, *arrowheads*), intensely labeled with the Rho4D2 antibody. Bar, 10 μm. The percentage of photoreceptors showing opsin expression in each condition is depicted in (**E**). Total number of cells per dish was $1.08 \times 10^6 \pm 101{,}380$ ($n = 4$). *Statistically significant differences ($P < 0.05$) compared to controls.

tors in culture. BC was slightly less effective than the macular xanthophylls in protecting photoreceptors from apoptosis induced by oxidative stress. In addition, both ZEA and LUT promoted the differentiation of photoreceptors, showing effects similar to those of DHA. These results suggest that ZEA and LUT may be, along with DHA, part of a diet-based system essential for maintaining the development and sustaining the viability of photoreceptors.

Several studies[20,35,36] examining the association of dietary LUT/ZEA intake with advanced AMD yielded protective relationships, indicating a reduction in the likelihood of having advanced AMD among the people reporting the highest LUT/ZEA intake (relative to people reporting lowest intake). In addition, lower levels of ZEA and LUT were found in patients with AMD.[37] Since tissue concentrations of LUT and ZEA per unit area were substantially higher in the retina than elsewhere in the body and retinal tissue status of these compounds was modifiable by and dependent on dietary intake, these findings suggested a relevant role for xanthophylls in prevention and treatment of AMD.

However, direct evidence of the protective effect of xanthophylls was almost absent. A diet enriched in xanthophyll carotenoids has been shown to protect quail photoreceptors by reducing light-induced photoreceptor apoptosis in vivo.[38] Long-term exposure to intense illumination may be among the most relevant damaging factors involved in AMD pathogenesis. A common feature between this and other well-established environmental risk factors such as, for instance, tobacco smoke is the generation of ROS that lead to cumulative oxidative damage. Different types of experimentally induced oxidative stress have been proposed to follow different patterns, affect different cellular compartments, and be counteracted to different extents by exogenous antioxidants.[24] However, these oxidants share among themselves and with light-induced degeneration a crucial feature: They induce photoreceptor cell death by apoptosis. Hence, to gain insight into the existence of a direct neuroprotective effect of ZEA and LUT on retinal neurons, we exposed these neurons in culture to two different types of oxidative stress, treating them with PQ and H_2O_2. Both oxidants induced photoreceptor death by apoptosis; however, direct supplementation of neuronal cultures with ZEA or LUT promoted the survival of photoreceptors, decreasing their apoptosis. ZEA and LUT completely blocked the deleterious effects of PQ and H_2O_2 on photoreceptors, suggesting that these xanthophylls may act at a common step in the apoptotic pathway, thus allowing cells to cope with different types of oxidative damage.

PQ treatment leads to photoreceptor death in vitro, through an apoptotic pathway that entails the loss of mitochondrial membrane potential.[10] ZEA and LUT protection from PQ-induced apoptosis implicated the preservation of mitochondrial

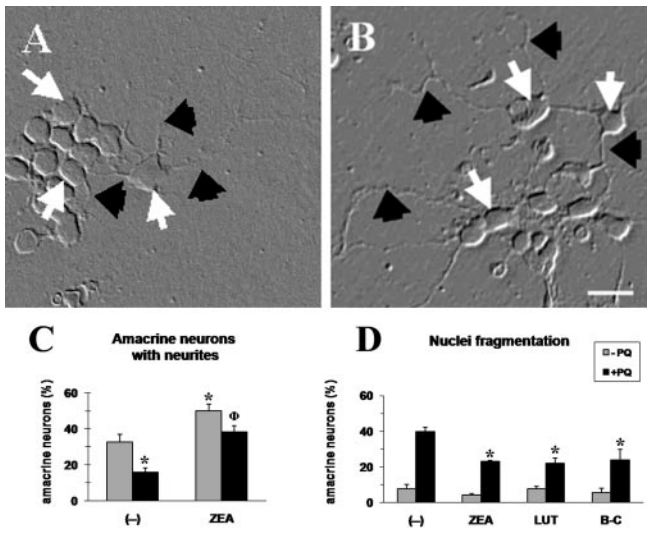

FIGURE 7. Effect of carotenoids on neurite outgrowth and oxidative stress-induced apoptosis of amacrine neurons. Phase photomicrographs of control (**A**) and ZEA-supplemented cultures (**B**) revealed that by day 4, most amacrine neurons (*white arrows*) in control conditions were still rather immature and had no or thin and short neurites (**A**, *black arrows*). In contrast, in ZEA-supplemented cultures many amacrine neurons (*white arrows*) developed long, branched, and conspicuous neurites (**B**, *black arrows*). Cultures without (−) or with ZEA, LUT, and BC at day 0 were treated at day 3 with PQ for 24 hours. Amacrine neurons were then identified with the specific monoclonal antibody HPC-1. (**C**) The percentage of amacrine neurons having neurites. (**D**) The percentage of apoptotic amacrine neurons was evaluated by analyzing nuclei fragmentation. Statistically significant differences, compared with cultures lacking ZEA, *without and Φwith PQ treatment ($P < 0.01$) (**C**). Total number of cells: $1.04 \times 10^6 \pm 41{,}885$ per dish ($n = 5$). *Statistically significant differences compared with PQ-treated cultures without carotenoids ($P < 0.01$) (**D**). Bar, 20 μm.

membrane depolarization and the avoidance of cytochrome *c* translocation in photoreceptors. Lack of release of cytochrome *c* to cytosol would prevent caspase activation, thus preventing apoptosis (reviewed in Ref. 39). This suggests that both xanthophylls may act at a stage in the apoptotic cascade that precedes mitochondrial alterations. Two main hypotheses can be proposed to explain these findings: Either both xanthophylls act as antioxidants or they activate cell signaling pathways to achieve photoreceptor protection. The most direct explanation is the well-known antioxidant activity of ZEA and LUT. Carotenoids in general, and ZEA and LUT in particular, with their high number of conjugated double bonds, can directly quench ROS.[40] Both xanthophylls have been shown to be efficient scavengers of the superoxide and hydroxyl radicals[41] that may be produced by PQ and H_2O_2, respectively. By doing so, they may reduce the levels of damaging free radicals, thus preventing the impairment of mitochondrial function and consequently avoiding the triggering of neuronal death. Studies in cats and dogs reveal that dietary LUT is significantly incorporated in mitochondria,[42] a localization that might enable carotenoids to preclude oxidative injury to these organelles.

ZEA and LUT showed the highest antiapoptotic effect and were equally effective with both oxidants. BC, which is also an effective antioxidant was slightly less effective in decreasing photoreceptor apoptosis induced by PQ. The biophysical properties of these carotenoids may explain the differences in their protective effects. ZEA and LUT are highly enriched in the macula, and the hydroxyl groups (not present in carotenes) on their ionone rings impart polar properties that affect solubility, aggregation, reactivity in membranes, and membrane disposition. These properties may allow LUT and ZEA to orient in a fashion that stabilizes the membrane structure[43] and protects both against both water- and lipid-soluble peroxy radicals.[44] The concentration of xanthophylls in the unsaturated fraction in a model of rod outer segment membranes[45] suggests that they are ideally suited for acting as endogenous antioxidants in these membranes preventing the peroxidation of their highly unsaturated fatty acids. In contrast, a recently published epidemiologic study shows a protective effect for ZEA and LUT, but not for BC, against AMD.[46]

In addition to their antioxidant properties, LUT and ZEA may protect photoreceptors via activation of intracellular signaling pathways. Carotenoids and/or their metabolites have been shown to affect cell signaling[47] and modulate the expression of genes, such as those involved in detoxification of reactive metabolites. LUT has been shown to regulate apoptosis and angiogenesis in tumor-bearing mice by controlling gene expression[42] and to decrease apoptosis in blood leukocytes.

Our novel findings that in addition to their antiapoptotic effects, ZEA and LUT promote the differentiation of photoreceptors, increasing expression of opsin and the development of outer-segment–like apical processes, support the hypothesis that xanthophylls may activate cell signaling pathways to both modulate development of new cellular structures and enhance expression of photoreceptor specific genes, such as opsin. While it is entirely possible that the antiapoptotic effects of ZEA and LUT are related to their capacity to regulate the cellular redox status, their effects on photoreceptor differentiation clearly go beyond the antioxidant capacity of xanthophylls and allow us to propose that other mechanisms may also be relevant for their effects on photoreceptors.

The finding that the combined treatment with DHA and ZEA or LUT showed no additive effect on survival and differentiation of photoreceptors suggests that these compounds may affect the same cell population and/or activate a similar signaling pathway. DHA is an efficient survival factor for photoreceptors,[26,27,48,49] which effectively protects them from oxidative stress.[10] DHA has recently been shown to activate the ERK/MAPK signaling pathway to promote photoreceptor survival,[15] thus leading to an increase in the expression of the antiapoptotic protein Bcl-2. In addition, DHA precludes the increase in ceramide levels induced by oxidative stress, thus preventing photoreceptor death.[16] DHA is also essential to advance differentiation in photoreceptors,[50] an effect that involves activation of the ERK/MAPK pathway as well.[18] Determining whether xanthophylls stimulate pathways similar to those of DHA to modulate photoreceptor survival requires further research.

Of interest, carotenoids were not as effective in protecting amacrine cells from oxidative stress. Addition of LUT, ZEA, and BC reduced amacrine cell apoptosis after PQ treatment by half, instead of completely preventing this death, as was the case with photoreceptors. Of interest, the protective effect of BC was the same as that of ZEA and LUT, suggesting that the three compounds may regulate amacrine survival by similar mechanisms, perhaps related to their antioxidant capacities. The different protective effects of carotenoids on photoreceptors and amacrine cells suggest that PQ may activate different mechanisms in both cell types to induce their apoptosis. In this context, ceramide has been shown to be a key mediator in oxidative stress-induced apoptosis of photoreceptors, but not in amacrine cells.[16] In contrast, xanthophylls advanced differentiation in both cell types. The extensive neurite network is a characteristic feature of differentiated amacrine neurons and is usually observed in long-term cultures.[26] Addition of LUT and ZEA to the cultures enhanced the differentiation of amacrine cells, markedly promoting their neurite outgrowth. Thus, after 4 days in culture, the appearance of amacrine cells already resembled that of more mature neurons.

In conclusion, our work shows that ZEA and LUT efficiently protect photoreceptors in culture from apoptosis induced by oxidative stress. In addition, LUT and ZEA concur with DHA to promote the differentiation of photoreceptors. Thus, our results provide a molecular explanation to support the epidemiologic evidence suggesting that macular xanthophylls and DHA may act as factors that modulate processes implicated in AMD pathogenesis and progression. This knowledge may eventually contribute to establish the intake of these compounds as a nonintrusive preventive intervention for neurodegenerative retinal diseases.

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