Ceramide is a Mediator of Apoptosis in Retina Photoreceptors

Olga L. German, Gisela E. Miranda, Carolina E. Abrahan and Nora P. Rotstein

Purpose. The precise mechanisms involved in photoreceptor apoptosis are still unclear. In the present study, the role of ceramide, a sphingolipid precursor that induces apoptosis on cellular stress, was investigated in relation to the activation of cell death in photoreceptors.

Methods. Rat retina neuronal cultures, with or without docosahexaenoic acid (DHA), were treated with the ceramide analogue acetylsphingosine (C_2-ceramide), and with a glucosylceramide synthase inhibitor. Ceramide synthesis in cultures treated with the oxidant paraquat was evaluated with [^{3}H]palmitate. The effect of inhibitors of ceramide de novo synthesis, fumonisin B1 and cycloserine, on photoreceptor apoptosis was investigated. Apoptosis, mitochondrial membrane potential, and Bcl-2 expression were determined.

Results. Addition of C_2-ceramide induced photoreceptor apoptosis. Paraquat increased formation of [^{3}H]ceramide in photoreceptors, compared with the control, whereas inhibition of ceramide synthesis, immediately before paraquat treatment, prevented paraquat-induced photoreceptor apoptosis. Fumonisin also reduced photoreceptor apoptosis during early development in vitro. DHA, the retina major polyunsaturated fatty acid, which protects photoreceptors from oxidative stress-induced apoptosis, completely blocked C_2-ceramide-induced photoreceptor death, simultaneously increasing Bcl-2 expression. Inhibiting glucosylceramide synthase, which catalyzes ceramide glucosylation, before ceramide or paraquat treatment blocked DHA's protective effect.

Conclusions. The results suggest that oxidative stress stimulated an increase in ceramide levels that induced photoreceptor apoptosis. DHA prevented oxidative stress and ceramide damage by upregulating Bcl-2 expression and glucosylating ceramide, thus decreasing its intracellular concentration. This shows for the first time that ceramide is a critical mediator for triggering photoreceptor apoptosis in mammalian retina and suggests that modulating ceramide levels may provide a therapeutic tool for preventing photoreceptor death in neurodegenerative diseases. (*Invest Ophthalmol Vis Sci.* 2006;47: 1658–1668) DOI:10.1167/iovs.05-1310

Apoptosis of photoreceptors leads to retinal dysfunction in neurodegenerative disorders, such as retinitis pigmentosa,[1,2] by mechanisms still ill-defined. Oxidative stress has been proposed to have a decisive role in activating this death; it has been related to cone cell death in retinitis pigmentosa[3]; antioxidants can ameliorate the progression of retinal neurodegeneration[4,5]; an early and sustained increase in intracellular reactive oxygen species accompanies photoreceptor apoptosis in vitro,[6] whereas an increased expression of oxidative stress-related genes is observed during the progression of retinal neurodegeneration.[7]

We have recently shown that oxidative stress induced with the oxidant paraquat (PQ), an anion superoxide generator, triggers photoreceptor apoptosis in culture, and that loss of mitochondrial membrane potential accompanies apoptotic death.[8] Avoidance of mitochondrial depolarization is crucial for the prevention of photoreceptor death by trophic factors such as docosahexaenoic acid (DHA).[8–10]

DHA is the most abundant polyunsaturated fatty acid in the retina and is particularly enriched in photoreceptors. Essential for proper development of vision,[11] its deficiency leads to loss of visual acuity[12] and alterations in the electroretinogram.[13–15] DHA has been shown to protect retina photoreceptors from apoptosis during early development in culture[9,10,16,17] and on oxidative stress.[8] DHA prevents oxidative stress-induced apoptosis of photoreceptors by increasing antiapoptotic protein Bcl-2 levels. A similar mechanism is involved in DHA protection of retinal pigment epithelial cells from oxidative stress.[18] In addition, DHA modulates Akt and downregulates caspase-3 activity to prevent apoptosis in a neuronal cell line.[19,20]

Information on which mediators participate in the induction of photoreceptor cell death is still lacking. Ceramide, a sphingolipid metabolic precursor, is a bioactive lipid that has been proposed to be an endogenous mediator of apoptosis.[21–23] Ceramide is actively involved in the triggering of apoptosis in many cell systems, including neurons.[24,25] An increase in ceramide intracellular levels[26,27] has been shown to occur on hypoxia, trophic factor removal, treatment with chemotherapeutic agents, heat, UV radiation, and other stress signals, such as oxidative stress.[28,29] Ceramide levels depend on the activity of several enzymes that participate in its synthesis and catabolism. The increase in ceramide in response to apoptotic stimuli may arise from hydrolysis of sphingomyelin due to stimulation of sphingomyelinases,[25,30,31] through de novo biosynthesis[27,32] or from the combined stimulation of both pathways.[33] In turn, several biochemical reactions lead to the disappearance of ceramide: its hydrolysis catalyzed by ceramidases, sphingomyelin resynthesis, or ceramide glucosylation.

Evidence linking ceramide with activation of apoptosis in photoreceptors is scarce and indirect. Increased ceramide levels have been found in brains of patients with the juvenile form of Batten disease, in which neuronal apoptosis in the retina and brain lead to blindness, seizures, and cognitive decline.[34] Modulation of enzymes involved in sphingolipid metabolism suppressed retinal degeneration in *Drosophila* translation mutants.[35] Of note, a mutation in a novel ceramide kinase gene has been recently established as a cause of an autosomal recessive form of retinitis pigmentosa,[36] suggesting a direct link between sphingolipid metabolism and human retinal degeneration.

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In this study, we investigated the involvement of ceramide in the induction of photoreceptor apoptosis. Exogenous addition of C_2-ceramide, a ceramide analogue, triggered photoreceptor apoptosis, whereas inhibition of de novo synthesis of ceramide protected photoreceptors from oxidative stress-induced apoptosis. We also demonstrated that DHA blocked ceramide-induced apoptosis, and this protection not only involved an increase in Bcl-2 expression but also a decrease in ceramide levels through its metabolization to glucosylceramide. As a whole, our results suggest that ceramide is a key activator of photoreceptor apoptosis and that avoiding a ceramide increase or blocking ceramide's effects may prevent photoreceptor death after oxidative damage.

Materials and Methods

Albino Wistar rats bred in our own colony were used in all the experiments. All proceedings involving animals were performed 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 (Naperville, IL). Dulbecco's modified Eagle's medium (DMEM) was from Invitrogen-Life Technologies (Gaithersburg, MD). 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, polyornithine, selenium, gentamicin, 4,6-diamidino-2-phenylindole (DAPI), monoclonal anti-syntaxin clone HPC-1, docosahexaenoic acid, and paraformaldehyde were from Sigma-Aldrich (St. Louis, MO). C_2-acetylsphingosine (C_2-ceramide), fumonisin B1 (FB), dihydroceramide, DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol-HCl (PPMP), cycloserine, and kainic acid were from Biomol (Plymouth Meeting, PA). A tyramide signal amplification kit and [9,10-^{3}H]palmitic acid (47 mCi/mmol) were from NEN (Boston, MA). The secondary antibodies, Alexa 488-conjugated-goat anti-mouse and a mitochondrial probe (MitoTracKer Red CMXRos) were from Molecular Probes, Inc. (Eugene, OR). The monoclonal antibody to Bcl-2 (sc-7382) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The monoclonal antibody against rhodopsin, Rho4D2 was generously supplied by Robert Molday (University of British Columbia, Vancouver, British Columbia, Canada). Solvents were HPLC grade, and all other reagents were analytical grade.

Neuronal Cultures

Purified cultures of rat retinal neurons were prepared as previously described.[16,37,38] Approximately 0.5×10^5 cells/cm^2 were seeded on 35-mm diameter dishes that had previously been sequentially treated with polyornithine and Schwannoma-conditioned medium.[39]

Addition of Docosahexaenoic Acid

Docosahexaenoic acid (DHA), at a 6.7-μM final concentration, was added at day 1 in culture, complexed with bovine serum albumin (BSA), in a 2:1 (fatty acid: BSA) molar ratio.[38] The same volume and concentration of a BSA solution was simultaneously added to control cultures.

Addition of C_2-Ceramide

Because of its extremely hydrophobic characteristics, natural, long-chain ceramides are frequently replaced in experiments in vitro by short acyl chain ceramides such as acetylsphingosine (C_2-ceramide), which is more soluble and cell permeable. C_2-ceramide is naturally synthesized in cells, its addition to cell cultures has been shown to produce apoptosis in several cell systems,[40,41] and the use of inhibitors that lead to ceramide intracellular accumulation have produced similar results.[42] C_2-ceramide was added to the cultures at day 3 in vitro, in concentrations ranging from 5 to 25 μM (final medium concentration), in an ethanol-calcium (1:8) magnesium-free solution. The same volume of the vehicle used for solubilizing C_2-ceramide, or the same concentration of dihydroceramide was added to the control. Cultures were then incubated for 24 hours before fixation. In every case, ethanol final concentration in the incubation medium was lower than 0.1%. In subsequent experiments, a 10-μM C_2-ceramide concentration was used.

Inhibition of Ceramide Synthesis

To test whether ceramide participated in oxidative stress-induced apoptosis, we blocked the synthesis of sphingolipids with FB, a well-known and extensively used inhibitor of ceramide synthase. Two different series of experiments were performed. First, to deplete the neurons of sphingolipids, FB was added to the culture medium at day 0, 3 hours after the cells were seeded, at concentrations of 25- and 50-μM.[25] To induce oxidative stress, the oxidant PQ, at a 48-μM concentration, was added to the cultures at day 3.[8] In a second series of experiments, designed to block only de novo synthesis of ceramide, 25 μM FB was added at day 3. Cultures were returned to the incubator for 30 minutes and then treated with PQ.

To establish further the role of ceramide in triggering photoreceptor apoptosis, additional experiments were performed with cycloserine, a widely used inhibitor of serine palmitoyltransferase, the enzyme catalyzing the first step in ceramide de novo synthesis. Concentrations ranging from 0.06 to 2 mM were evaluated, and a 0.25-mM concentration was chosen for the experiments, because higher cycloserine concentrations had a toxic effect and lower concentrations had less or no inhibitory effect. Cycloserine was added to the cultures at day 3, and PQ was added 2 hours later.

To evaluate whether ceramide production was involved in apoptosis induction during development, 25 μM FB was added to the cultures 3 hours after the cells were seeded. After 6 days in vitro, cultures were fixed, and the amount of apoptotic photoreceptors was determined.

Inhibition of Glucosylceramide Synthase

To determine whether this enzyme was involved in the protective effect of DHA, DL-threo-1-phenyl-2 palmitoylamino-3-morpholino-1-propanol-HCl (PPMP), an inhibitor of glucosylceramide synthase, was added at a 10-μM concentration to 3-day cultures, with or without DHA. After a 30-minute incubation, cultures were treated with 10 μM C_2-ceramide for 24 hours. Cultures having PPMP, PPMP plus DHA or PPMP plus dihydroceramide were used as the control. In a different series of experiments, cultures with or without DHA were treated with PPMP and after 30 minutes with PQ.

Paraquat Addition

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

Neuronal Incubation with [^{3}H]palmitate

To determine whether endogenous ceramide levels would increase after PQ addition, neuronal cultures were incubated with [^{3}H]palmitate. At day 3, neuronal cultures were treated with kainic acid (0.25 mM) for 2 hours, to induce amacrine cell death.[43] [^{3}H]palmitate (0.7 μCi/dish) was dried in nitrogen, resuspended in the medium used for neuronal incubation, complexed with BSA (fatty acid-BSA, 2:1 molar ratio), and added to the cultures. After 30 minutes, cells were treated with PQ for 2 or 4 hours. The incubation medium was then removed, and neuronal lipids were extracted.

Lipid Extraction and Separation

Cells were washed twice with 1 mL ice-cold phosphate-buffered saline (PBS; 0.9% NaCl in 0.01 M NaH_2PO_4 [pH 7.4]), and then scraped and transferred in PBS and to a glass tube. Cells were centrifuged at 1000 rpm, for 10 minutes, the supernatant was removed, and the lipids were extracted according to Folch et al.[44] Mild alkaline treatment was then

performed, incubating lipid extracts with 1 M NaOH in a methanol solution for 10 minutes at 50°C. Incubation was stopped by adding an equal volume of 1 N HCl, and chloroform. Lipids were then separated by thin-layer chromatography (TLC), using a combination of two solvent systems, chloroform-methanol-acetic acid- H_2O (20:15:1.4:0.8) up to one third of the plate's length, and chloroform-methanol-acetic acid (190:9:1) to the top. Unlabeled lipids prepared from bovine retina were added as carriers to the samples. Lipid spots, visualized with I_2 vapors, were identified with standards and then scraped to vials. The incorporated radioactivity was determined by liquid scintillation counting.

Immunocytochemical Methods

Cultures were fixed for at least 1 hour with 2% paraformaldehyde in PBS, at room temperature, followed by permeation with Triton X-100 (0.1% in PBS) for 15 minutes. Photoreceptor cells were identified by immunocytochemistry with the monoclonal antibody Rho4D2, by their morphology, and other criteria as previously described.[9,10] Amacrine cells were identified with the anti-syntaxin monoclonal antibody, HPC-1, as described.[38] Alexa 488-conjugated goat anti-mouse was used as the secondary antibody. Tyramide signal amplification was occasionally used to improve visualization, according to the manufacturer's procedure. Controls for immunocytochemistry were performed by omitting either the primary or the secondary antibody. Apoptosis was determined by evaluating integrity of the nuclei after staining cell nuclei with DAPI, a fluorescent dye that binds to DNA. Briefly, cells were permeated with 0.1% Triton X-100 in PBS, washed with PBS and incubated with DAPI for 20 minutes. 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 unambiguously the cells as either photoreceptors or amacrine neurons, respectively, and thus establish the total amount of each cell type. The percentage of apoptotic photoreceptors and amacrine neurons was then calculated, taking into account the percentage of Rho4D2- and HPC-1-labeled cells, respectively. To assess the amount of cells preserving their mitochondrial membrane potential, cultures were incubated for 30 minutes before fixation with the fluorescent probe (0.1 μg/mL; MitoTracker; Molecular Probes), and the amount of photoreceptors displaying fluorescent mitochondria with respect to the total number of photoreceptors was determined. The number of photoreceptors expressing Bcl-2 with respect to the total number of photoreceptors was quantified by immunocytochemistry with a specific monoclonal antibody.

Statistical Analysis

For cytochemical studies, 10 fields per sample, randomly chosen, were analyzed in each case. Each value represents the average of two or three experiments, with three to four dishes for each condition ± SD. Statistical significance ($P < 0.05$) was determined by Student's two-tailed *t*-test.

Results

Effect of Addition of C_2-ceramide on Induction of Apoptosis in Retinal Neurons

To investigate whether ceramide may provoke cell death in retinal neurons, we treated neuronal cultures with 10 μM C_2-ceramide for 24 hours. This treatment induced the apoptosis of both photoreceptors (Fig. 1) and amacrine neurons. Although most photoreceptors in control conditions had intact nuclei and showed a normal morphology (Figs. 1A, 1C, 1E), the addition of ceramide prompted large alterations in neuronal morphology, retraction and loss of neurites, and nuclear fragmentation (Figs. 1B, 1D, 1F). The percentage of apoptotic photoreceptors doubled on addition of ceramide, increasing from approximately 27% in the control to almost 50% in ceramide-treated cultures (Fig. 2A). C_2-ceramide also triggered apoptosis in amacrine neurons, which increased from 5% to >35%, in control and ceramide-treated cultures, respectively (Fig. 2A). Dihydroceramide, which has a similar chemical structure, uptake, and metabolism to that of ceramide, but lacks its biological action, had no effect on neuronal apoptosis.

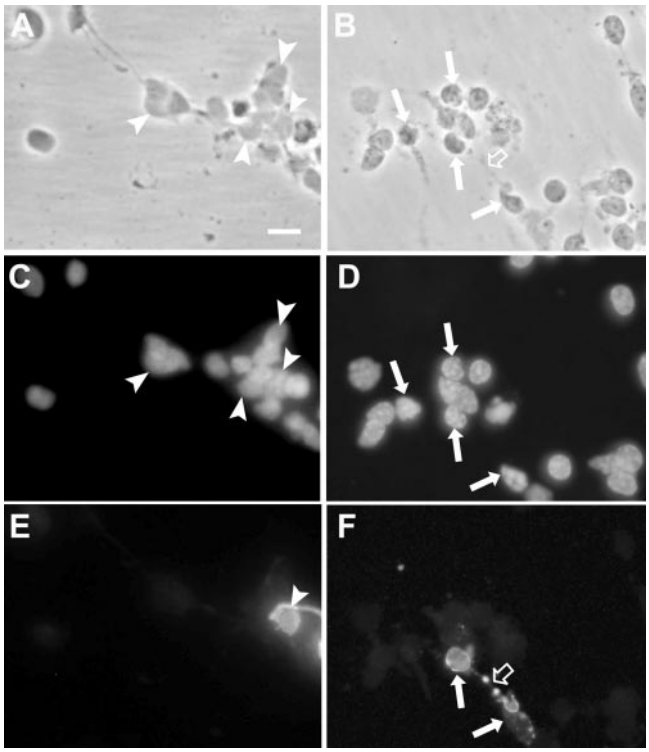

Figure 1. Addition of C_2-ceramide to retina photoreceptors in culture. (**A**, **B**) Phase and (**C–F**) fluorescence photomicrographs of neuronal cultures treated at day 3 (**A**, **C**, **E**) without or (**B**, **D**, **F**) with 10 μM C_2-ceramide for 24 hours. (**C**, **D**) Nuclei labeled with the DNA probe DAPI. (**E**, **F**) Photoreceptors identified with the monoclonal antibody Rho4D2. Most photoreceptors in control cultures had intact nuclei (**A**, **C**, **E**, *arrowheads*), whereas C_2-ceramide increased the amount of photoreceptors with fragmented nuclei (**B**, **D**, **F**, *arrows*). Note the retracting neurite in an apoptotic photoreceptor after ceramide treatment (**B**, **F**, *open arrows*). Scale bar, 10 μm.

C_2-ceramide also affected mitochondrial membrane potential. In control and dihydroceramide-treated cultures, approximately 60% of photoreceptors retained mitochondrial membrane potential, whereas after treatment with 10 μM C_2-ceramide only 30% still had intact, functional mitochondria (Fig. 2B). Amacrine neurons retaining mitochondrial membrane potential decreased from approximately 90% in the control to almost 50% in ceramide-treated cultures.

Retina neurons, cultured in a chemically defined medium without photoreceptor trophic factors, were prone to ceramide-induced apoptosis. Even a small (5 μM) concentration led to neuronal death. Neuronal apoptosis increased with higher ceramide concentrations (Fig. 2C). Apoptotic photoreceptors increased from less than 40% to more than 80%, and apoptotic amacrine cells augmented from 20% to approximately 60% when C_2-ceramide concentration increased from 5 to 25 μM. At a 50-μM C_2-ceramide concentration, almost all neurons were apoptotic and detached from the substratum.

Effect of PQ on [^{3}H]ceramide Synthesis in Photoreceptors

To evaluate directly whether oxidative stress increases endogenous ceramide levels, we measured ceramide production in

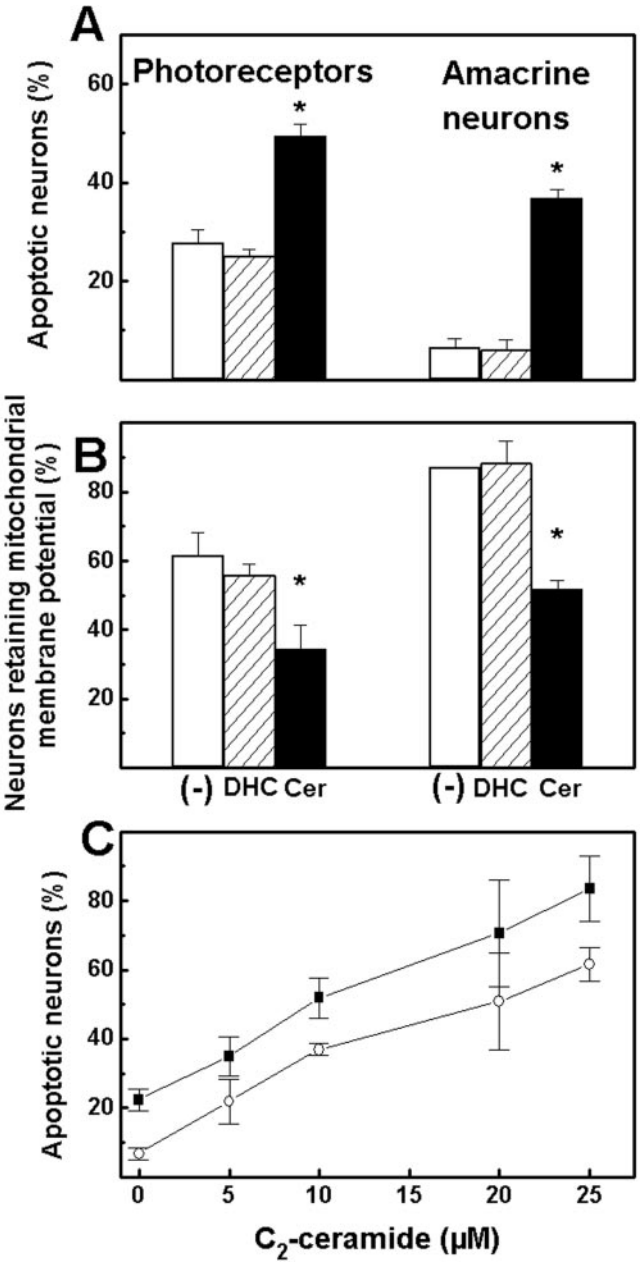

FIGURE 2. Effect of C_2-ceramide on apoptosis and mitochondrial membrane potential in photoreceptors and amacrine neurons. Three-day neuronal cultures were treated with 10 μM C_2-ceramide (Cer) or dihydroceramide (DHC), or with the solution used as a vehicle for preparing the sphingolipid solutions (−) for 24 hours. (**A**) The percentage of apoptotic photoreceptors and amacrine neurons was determined by analyzing nuclei integrity with DAPI. (**B**) The percentage of neurons preserving mitochondrial membrane potential was quantified with a fluorescent probe. *Statistically significant differences, compared with the control ($P < 0.05$). (**C**) Concentrations of C_2-ceramide ranging from 5 to 25 μM were added to neuronal cultures, and the percentage of apoptotic photoreceptor (■) and amacrine neurons (○) was determined after 24 hours.

photoreceptors on PQ treatment by pulse-labeling neuronal cells with [^{3}H]palmitate. Cultures were treated with kainic acid for 2 hours, to eliminate most amacrine neurons[43] and establish ceramide synthesis only in photoreceptors. We then added [^{3}H]palmitate, and 30 minutes later, PQ. Similar amounts of [^{3}H]palmitate were rapidly incorporated in the cells and esterified in neuronal lipids in PQ-treated and untreated cultures (not shown). Two hours after PQ addition, a clear increase was observed in labeled ceramide (Fig. 3A). The amount of [^{3}H]ceramide in PQ-treated cultures was approximately twice that in the control. This difference persisted after 4 hours of PQ treatment. In contrast, the incorporation of [^{3}H]palmitate into sphingomyelin was very low, as had been observed.[17] Both in controls and PQ-treated cultures, [^{3}H]sphingomyelin (SPM) levels were unaffected by the addition of PQ (Fig. 3B). Previous findings from our laboratory showed that after 24 hours of PQ treatment, the amount of apoptotic photoreceptors significantly increased.[8] Evaluation of photoreceptor apoptosis performed in parallel at the incubation times used for analyzing ceramide labeling showed that the increase in [^{3}H]ceramide corresponded with the onset of photoreceptor apoptosis (Fig. 3C). After 2 or 4 hours of PQ, the percentage of apoptotic photoreceptors in PQ-treated cultures was already slightly but significantly higher than in the control.

Experiments adding [^{3}H]palmitate at day 1 in culture also showed that the amount of labeled sphingomyelin was much smaller than that of labeled ceramide, and was not diminished by PQ addition (not shown).This suggests that, in basal conditions, synthesis of ceramide in retina cultured neurons was more active than that of sphingomyelin. PQ induced a rapid accumulation of ceramide, which was consistent with the activation of photoreceptor apoptosis, without decreasing [^{3}H]sphingomyelin levels, suggesting that ceramide is formed

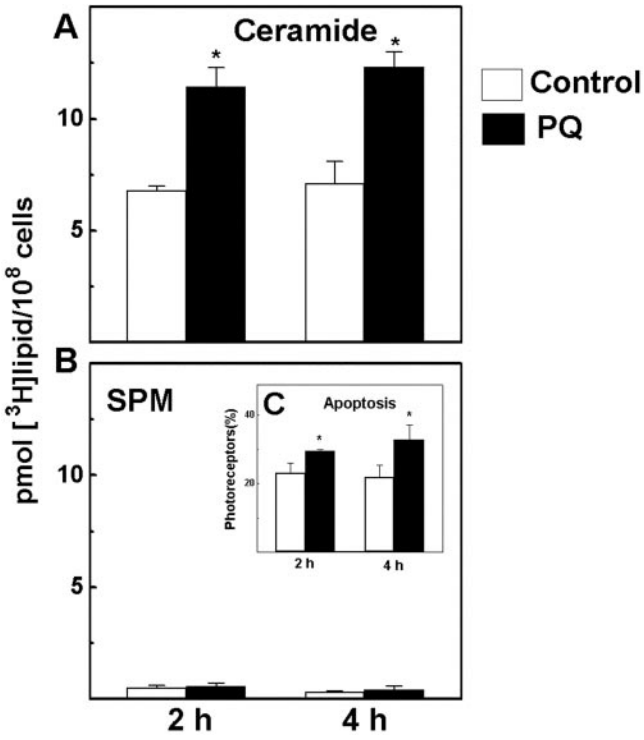

FIGURE 3. Accumulation of [^{3}H]ceramide in photoreceptors induced by PQ treatment. Three-day neuronal cultures were treated for 2 hours with 0.25 mM kainic acid, to eliminate amacrine neurons, and then were supplemented with 0.7 μCi/dish [^{3}H]palmitate. After 30 minutes, the cultures were left without (Control) or treated with 48 μM paraquat (PQ) for 2 or 4 hours. Cells were then scraped, for lipid extraction and separation by TLC. Simultaneously, photoreceptor apoptosis was determined at both incubation times. (**A**, **B**) The amount of [^{3}H]ceramide and [^{3}H]sphingomyelin (SPM) formed in control and PQ-treated cultures. (**C**) Photoreceptor apoptosis (%) after 2 and 4 hours of incubation without or with PQ. Each bar is the average of results obtained from three different samples. Data are representative of three separate experiments. *Statistically significant difference, compared with the control ($P < 0.05$).

by de novo biosynthesis and not from breakdown of sphingomyelin.

Effect of Inhibiting Ceramide Synthesis on PQ-Induced Apoptosis

To evaluate whether this increase in ceramide levels participated in triggering oxidative stress-induced apoptosis, the effect of different inhibitors of ceramide synthesis on photoreceptor apoptosis was evaluated separately. In initial experiments, cells were treated with FB, an inhibitor of ceramide and sphingolipid de novo synthesis, approximately 2 to 3 hours after they were seeded. Because neuronal cells still proliferate for the first 2 days in culture,[45] FB causes a depletion of sphingolipids, both sphingomyelin and ceramide, in these cells. Hence, if PQ induced the production of ceramide either by de novo synthesis or by sphingomyelin hydrolysis, this production would be substantially reduced. After a 24-hour treatment with PQ, cultures lacking FB showed the dramatic changes in cell morphology that accompany oxidative injury (Figs. 4A, 4C, 4E), as previously described.[8] Cells lost their characteristic morphology and acquired a round appearance, with almost no neurites (Fig. 4A). They showed pyknotic or fragmented nuclei, and most of them lost mitochondrial membrane integrity (Figs. 4C, 4E). In control cultures, FB addition had no toxic effects and did not affect neuronal survival (Fig. 4G), suggesting that lack of sphingolipid synthesis had no effect on early neuronal development. In both control and FB-supplemented cultures, less than 25% of photoreceptors were apoptotic, compared with more than 60% in PQ-treated cultures (Fig. 4G;[8]). In cultures supplemented with FB at day 0 and then treated with PQ, FB notably protected photoreceptors, which maintained their morphology, showed intact neurites, and preserved both nuclei integrity and mitochondrial membrane potential (Figs. 4B, 4D, 4F). FB addition, at either 25 or 50 μM at day 0, substantially diminished photoreceptor apoptosis. Even after PQ treatment, photoreceptor apoptosis in these cultures was the same as in control conditions (Fig. 4G).

To establish whether the increase in ceramide was due to sphingomyelin hydrolysis by sphingomyelinases or to de novo synthesis, 25 μM FB was added to the cultures at day 3, only 30 minutes before PQ treatment. This late addition would only block de novo synthesis, without decreasing sphingomyelin content, and ceramide production from breakdown of sphingomyelin would still be possible. The FB protective effect was the same as that found with addition of FB at day 0. Photoreceptor apoptosis decreased from more than 60% in PQ-treated cultures lacking FB to approximately 26% in cultures treated with FB before the addition of PQ (Fig. 5A), a value similar to that in control conditions.

In both control and FB-supplemented cultures more than 60% of the photoreceptors retained their mitochondrial membrane potential (Fig. 5B), a percentage that decreased to approximately 20% with PQ treatment. Addition of FB prevented mitochondrial depolarization after PQ treatment. The percentage of photoreceptors retaining mitochondrial membrane potential after addition of PQ to FB-supplemented cultures was similar to that in the control. Hence, blocking ceramide production completely inhibited photoreceptor apoptosis induced by oxidative stress, precluding mitochondrial depolarization.

FB was not so effective in preventing amacrine cell apoptosis induced by oxidative stress. Apoptotic amacrine cells, less than 5% of total amacrine neurons in the control, increased to 46% after PQ treatment. When FB was added at day 0, apoptosis of amacrine neurons decreased to approximately 30% with PQ treatment (Fig. 6A), but did not reach the level in control conditions, as was the case with the photoreceptors (Fig. 5A). The addition of FB at day 3, just before PQ treatment, had no protective effect on PQ-induced apoptosis of amacrine cells (Fig. 6B). These results suggest that ceramide is not the major mediator in the triggering of amacrine cell apoptosis.

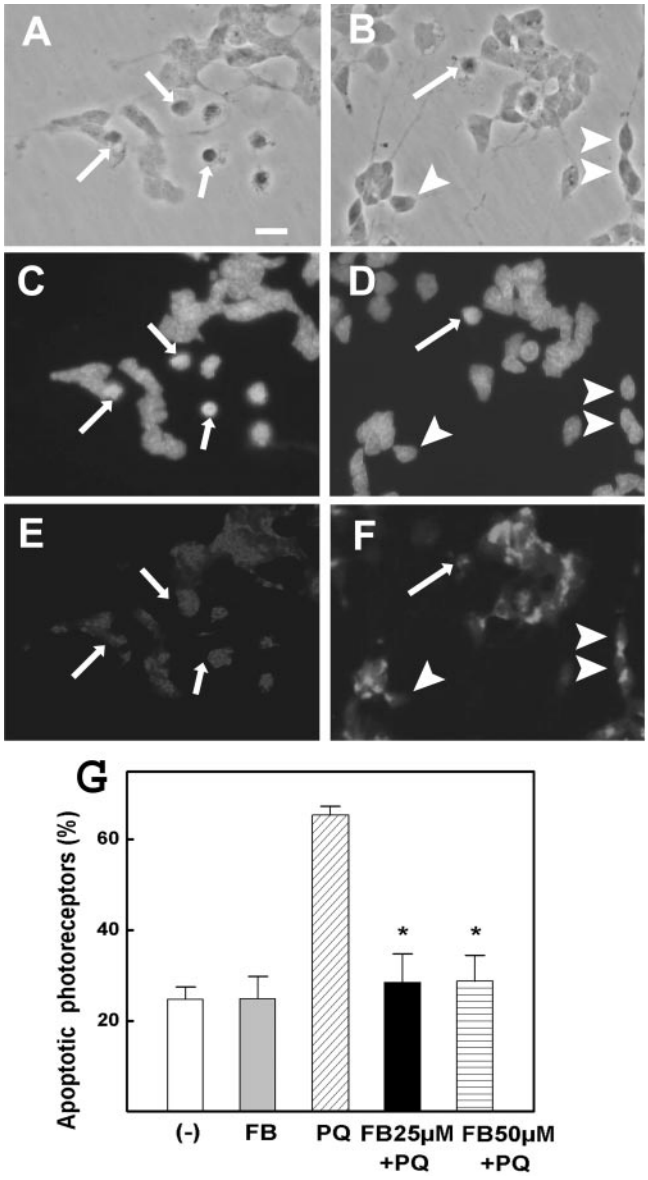

Figure 4. The effect of the addition of FB at day 0 in vitro on photoreceptor apoptosis induced by oxidative stress. Neuronal cultures were left without (*left*) or treated with (*right*) 25 μM FB, 3 hours after the cells were seeded, and at day 3 with 48 μM PQ for 24 hours. (**A**, **B**) Phase contrast and (**C–F**) fluorescence micrographs showing (**C**, **D**) nuclei labeled with DAPI and (**E**, **F**) mitochondria labeled with a mitochondrial probe. In PQ-treated cultures lacking FB (**A**, **C**, **E**) most of the photoreceptors were apoptotic (*arrows*), showed fragmented nuclei (**C**), and had a pale, fluorescent appearance, indicating mitochondrial depolarization (**E**). (**B**, **D**, **F**) The addition of FB promoted photoreceptor survival (*arrowheads*), shown by intact nuclei (**D**) and intensely fluorescent mitochondria, indicating the preservation of their transmembrane potential (**F**). Scale bar, 10 μm. (**G**) Neuronal cultures were left without (−) or treated with 25 and 50 μM FB 3 hours after the cells were seeded, and then were treated at day 3 with PQ (FB+PQ). Apoptosis was determined, as previously described. *Statistically significant difference, compared with the control ($P < 0.05$).

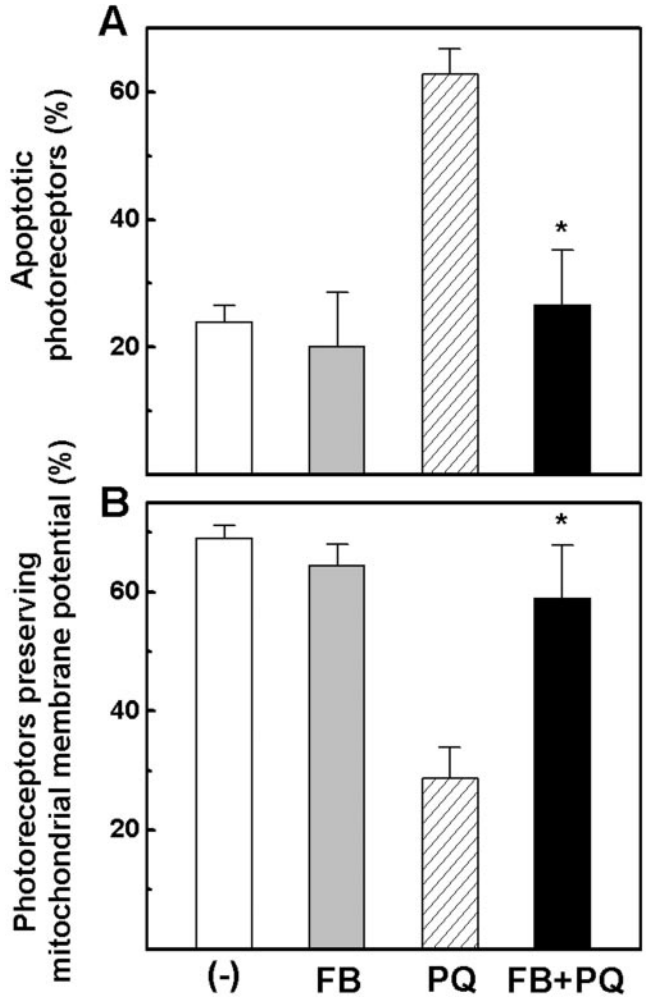

FIGURE 5. The effect of FB added at day 3 on oxidative stress-induced photoreceptor apoptosis. Three-day cultures were left without (−) or supplemented with 25 μM FB and 30 minutes later were treated with 48 μM PQ (PQ or FB+PQ, respectively) for 24 hours. (**A**) Percentage of apoptotic photoreceptors. (**B**) Percentage of photoreceptors preserving mitochondrial membrane potential. *Statistically significant difference, compared with the control ($P < 0.05$).

To confirm that ceramide accumulation mediated PQ-induced apoptosis, and that this ceramide is produced by de novo biosynthesis, we assessed the effect of inhibiting the first step of this pathway on PQ-treated cultures. De novo synthesis of ceramide starts with palmitate conversion to palmitoyl-CoA, which condenses with serine to form keto-dihydrosphingosine, a reaction catalyzed by serine palmitoyltransferase. Dihydrosphingosine and dihydroceramide are subsequently formed, the latter through the activity of (dihydro)ceramide synthase, which is inhibited with FB. However, ceramide may also be synthesized by the recycling/salvage pathway, by direct N-linkage of palmitoyl-CoA to sphingosine, also through the activity of ceramide synthase. Hence, the blockade of ceramide synthase cannot discriminate which of these two pathways is involved in ceramide synthesis in photoreceptors. To distinguish between them, cultures were treated with cycloserine, an inhibitor of serine palmitoyltransferase. Addition of cycloserine completely prevented photoreceptor death on treatment with PQ, strikingly reducing the percentage of apoptotic photoreceptors, when compared with PQ-treated cultures without cycloserine (Fig. 7A). Photoreceptor apoptosis in cultures with cycloserine and PQ was virtually the same as that in the control. This suggests that PQ stimulated de novo synthesis, increasing ceramide levels, and this increase induced photoreceptor apoptosis. Cycloserine also prevented the loss of mitochondrial membrane potential in PQ-treated photoreceptors (Fig. 7B), supporting the hypothesis that the increase in ceramide levels was upstream of mitochondrial alterations.

Effect of FB on Photoreceptor Apoptosis during Development In Vitro

In the culture conditions described herein, photoreceptors developed normally for 3 to 4 days, and then started a degenerative pathway that proceeds by apoptosis, leading to the death of more than 60% of these cells by day 6.[16,38] Addition of FB 3 hours after the cells were seeded markedly diminished photoreceptor apoptosis by day 6. The amount of fragmented or pyknotic nuclei in FB-supplemented cultures (Figs. 8C, 8D) was much smaller than in controls lacking FB (Figs. 8A, 8B); the percentage of apoptotic photoreceptors was reduced from more than 66% in control conditions to approximately 44% in FB-supplemented cultures (Fig. 8E). This suggests that inhibition of ceramide synthesis may prevent photoreceptor death during early development in vitro.

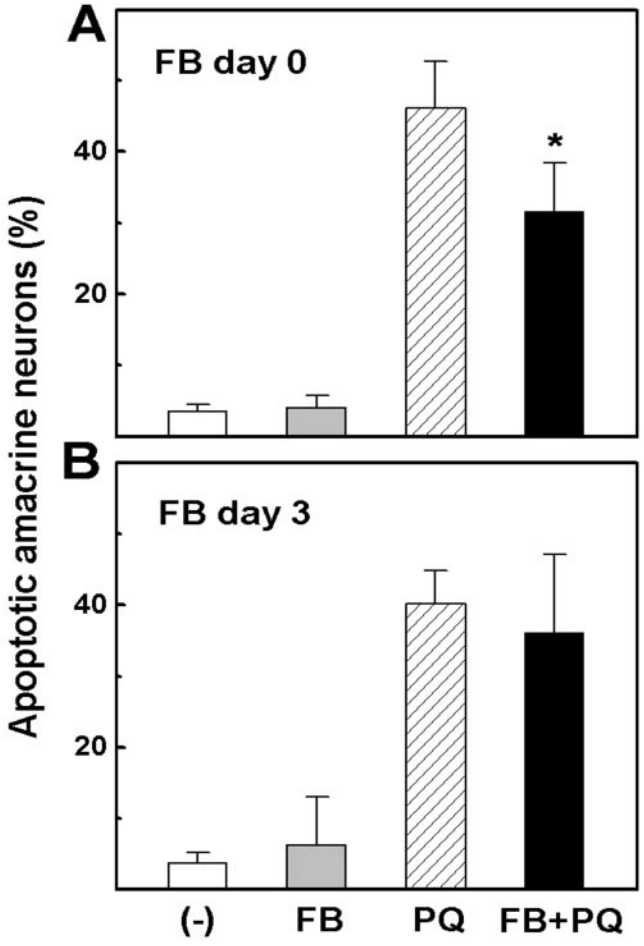

FIGURE 6. Partial prevention of amacrine neuronal apoptosis by early addition of FB. Neuronal cultures were left without (−) or were treated with 25 μM FB (**A**), 3 hours after the cells were seeded, or (**B**) at day 3 with FB, PQ, or FB+PQ or nothing (−). Amacrine neurons were identified with HPC-1, an anti-syntaxin monoclonal antibody, and the percentage of apoptotic cells was then determined. *Statistically significant difference, compared with the control ($P < 0.05$).

Protective Effect of DHA on Ceramide-Induced Apoptosis of Photoreceptors

Because DHA protects photoreceptors from PQ-induced apoptosis,[8] if increased ceramide levels activate PQ-induced apoptosis, DHA should also prevent photoreceptor apoptosis induced by the addition of ceramide. In DHA-supplemented cultures, either without C_2-ceramide, or with dihydroceramide, approximately 25% of photoreceptors were apoptotic. The addition of C_2-ceramide doubled the amount of apoptotic photoreceptors in DHA-lacking cultures; however, when we added C_2-ceramide at day 3 to cultures supplemented with DHA from day 1, photoreceptor apoptosis was completely blocked (Fig. 9A).

In addition, whereas ceramide markedly reduced the percentage of photoreceptors preserving mitochondrial membrane potential, in DHA-supplemented cultures, mitochondrial depolarization was prevented, despite ceramide addition (Fig. 9B). Hence, the protective effect of DHA on photoreceptors correlated with its preservation of their mitochondrial membrane potential.

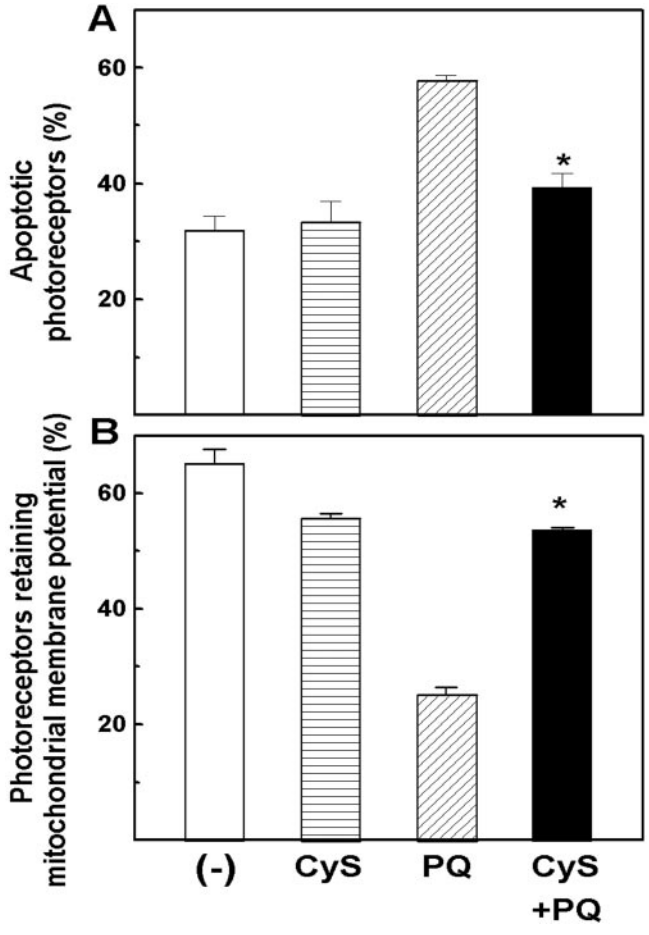

FIGURE 7. Protective effect of cycloserine on PQ-induced apoptosis of photoreceptors. Three-day neuronal cultures were left untreated (−) or were supplemented with 0.25 mM cycloserine (CyS), and, after 2 hours, they were treated with PQ (CyS+PQ) for 24 hours. (**A**) The percentage of apoptotic photoreceptors and (**B**) the percentage of photoreceptors preserving mitochondrial membrane potential was determined. *Statistically significant difference, compared with the control ($P < 0.05$).

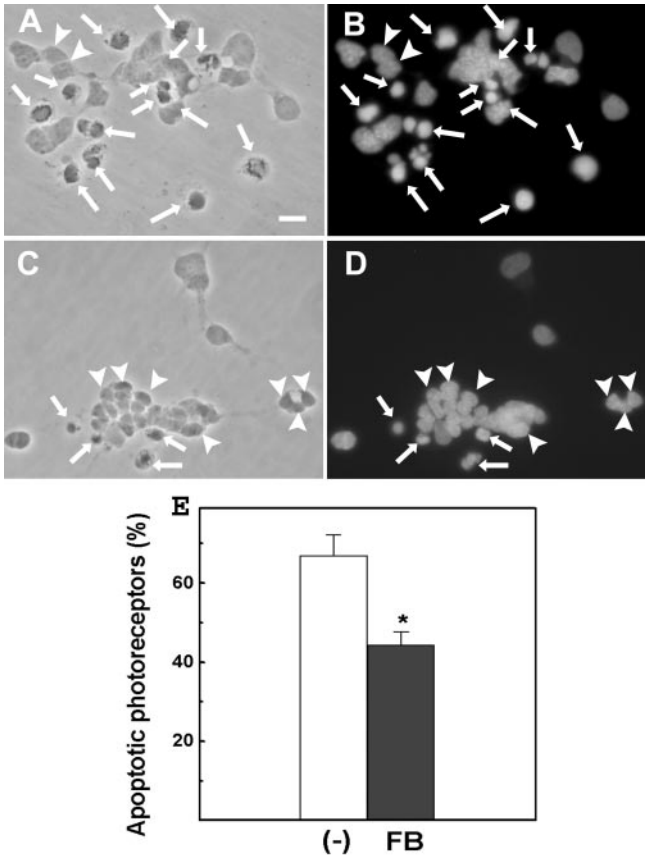

FIGURE 8. FB prevention of photoreceptor apoptosis during development in vitro. Cultures were left without (**A**, **B**) or treated with (**C**, **D**) FB, 3 hours after the cells were seeded and then incubated for 6 days. (**A**, **C**) Phase micrographs showing apoptotic (*arrows*) and surviving (*arrowheads*) photoreceptors. (**B**, **D**) Fluorescence micrographs showing nuclei labeled with DAPI. (**E**) The percentage of apoptotic photoreceptors in control (−) and FB-supplemented (FB) cultures. *Statistically significant difference, compared with the control ($P < 0.05$). Scale bar, 10 μm.

Effect of DHA on Bcl-2 Expression in Photoreceptors

We have shown that DHA upregulates the expression of Bcl-2 in photoreceptors.[8] We investigated in the present study whether ceramide treatment blocks the increase in Bcl-2 expression or whether this increase also participates in the DHA-protective effect on ceramide-induced apoptosis. Evaluation of Bcl-2 expression in cultures without or with DHA showed that DHA increased Bcl-2 levels in every culture condition (Fig. 10). DHA-lacking cultures (−), treated with the vehicle used for ceramide addition, with 10 μM dihydroceramide or with 10 μM C_2-ceramide showed approximately 36% of photoreceptors expressing Bcl-2. In DHA-supplemented cultures, this percentage increased to approximately 60% of the total photoreceptors, either with or without C_2-ceramide (Fig. 10). Hence, DHA upregulation of Bcl-2 levels persisted despite ceramide addition, probably contributing to the antiapoptotic effect of DHA.

Inhibition of Ceramide Glucosylation and DHA's Antiapoptotic Effect

The intracellular levels of ceramide depend on the balance between its production and its further metabolization. Ceramide may be glucosylated through the activity of glucosylce-

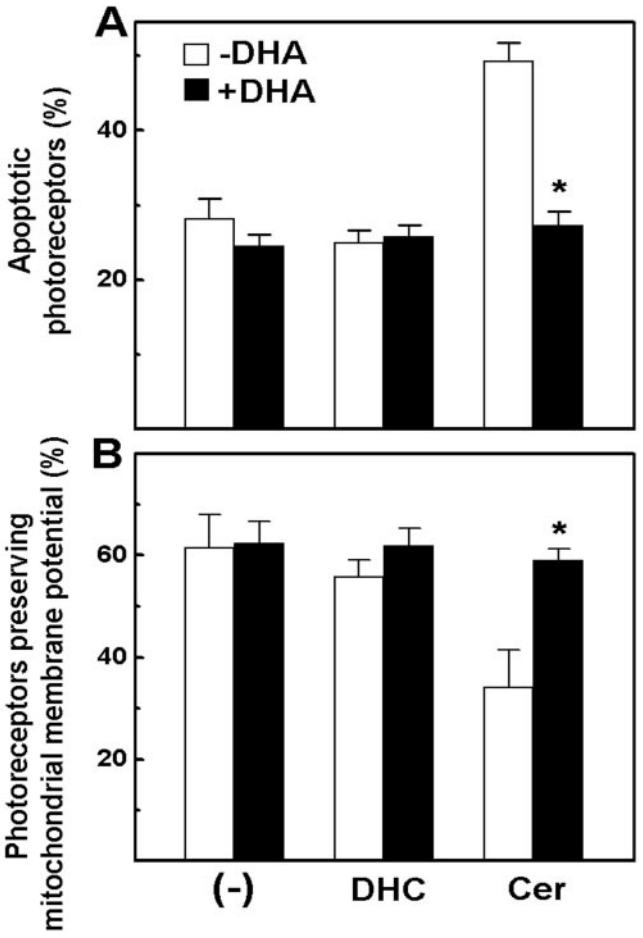

FIGURE 9. DHA protection of photoreceptors from apoptosis induced by C_2-ceramide. Cultures were supplemented at day 1 in vitro with 6.7 μM DHA (+DHA), complexed with bovine serum albumin (BSA), or with the same volume of the BSA solution (−DHA). At day 3, they were treated with 10 μM C_2-ceramide (Cer) or dihydroceramide (DHC), or the vehicle used for preparing the sphingolipid solutions (−) for 24 hours. (**A**) Percentage of apoptotic photoreceptors and (**B**) of photoreceptors preserving mitochondrial membrane potential, determined as previously described. *Statistically significant difference, compared with the control ($P < 0.05$).

ramide synthase, with increased glucosylation leading to a decrease in the size of the ceramide pool, thus reducing the deleterious effects of ceramide. To investigate whether glucosylceramide synthase participates in DHA protection against ceramide-induced photoreceptor apoptosis, we added PPMP, an inhibitor of this enzyme, to neuronal cultures, with or without DHA, before C_2-ceramide treatment. Addition of PPMP alone had no deleterious effect on neuronal cultures, the amount of apoptotic photoreceptors in these cultures being the same as in the control (Fig. 11A). Addition of PPMP followed by ceramide treatment did not increase photoreceptor apoptosis in DHA-lacking cultures, suggesting that this activity was negligible in cultures without DHA and hence did not contribute to the decrease in ceramide levels. On the contrary, DHA protection completely disappeared in cultures to which PPMP was added before ceramide treatment (Fig. 11A). In DHA-supplemented cultures without PPMP, ~25% of total photoreceptors were apoptotic after ceramide addition. With PPMP, this percentage rose to more than 45%, the same as in cultures lacking DHA, with a parallel decrease of the amount of photoreceptors having intact mitochondria (not shown). PPMP did not affect amacrine cell apoptosis on C_2-ceramide treatment, either in DHA-lacking or DHA-supplemented cultures (not shown). This suggests that inhibiting ceramide glucosylation abolishes the protective effect of DHA and, conversely, that DHA promotes ceramide glucosylation, hence decreasing ceramide levels, and this decrease prevents photoreceptor apoptosis.

DHA protects photoreceptors from PQ-induced apoptosis.[8] If PQ triggered neuronal death by increasing ceramide production and DHA prevented death by stimulating ceramide glucosylation, addition of PPMP would diminish the protective effect of DHA. When DHA-supplemented cultures were treated with PPMP and 30 minutes later with PQ, DHA's antiapoptotic effect was almost completely inhibited by PPMP (Fig. 11B). The amount of apoptotic photoreceptors in PPMP and PQ-treated cultures was the same in cultures with or without DHA, a finding that supports the hypothesis that ceramide accumulation leads to oxidative stress-induced photoreceptor apoptosis and strongly suggests that an increase in ceramide glucosylation is a critical DHA-activated mechanism to prevent ceramide induction of apoptosis.

DISCUSSION

Activation of Photoreceptor Apoptosis by an Increase in Intracellular Ceramide Levels

Ceramide has been proposed as a signaling molecule involved in the induction of cell death by oxidative stress. Exogenous oxidants or conditions that increase the intracellular levels of reactive oxygen species also increase ceramide production, whereas inhibition of this increase prevents the generation of ceramide.[46] Modulation of enzymes involved in ceramide synthesis and degradation suppress retinal degeneration and facilitate endocytosis in *Drosophila* mutants[35,47]; however, direct evidence of ceramide involvement in photoreceptor death in mammalian retina was lacking. Our results show that ceramide accumulation triggered apoptosis in photoreceptors subjected to oxidative stress. Several lines of evidence support this conclusion. An exogenously induced increase in ceramide levels, through addition of C_2-ceramide, activated neuronal death. The involvement of endogenous generation of ceramide in the

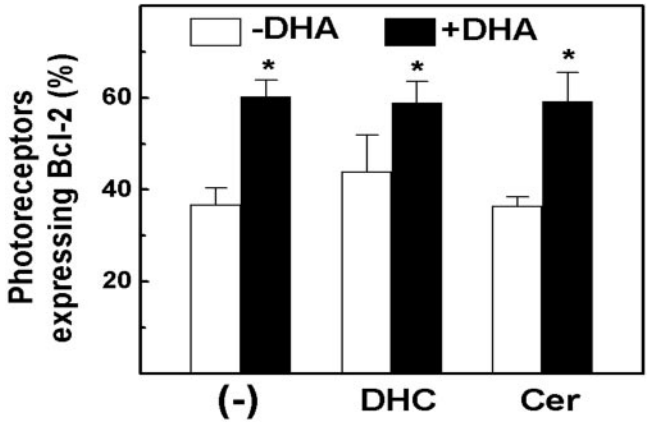

FIGURE 10. Upregulation of Bcl-2 expression in photoreceptors by DHA supplementation. Cultures supplemented at day 1 in vitro with DHA (+DHA) or with the same volume of a BSA solution (−DHA) treated at day 3 with C_2-ceramide (Cer), dihydroceramide (DHC), or the vehicle used for preparing the sphingolipid solutions (−). Bcl-2 expression was determined by immunocytochemistry, using a specific monoclonal antibody. Bars depict the amount of photoreceptors expressing Bcl-2 with respect to the total amount of photoreceptors. *Statistically significant difference, compared with the control ($P < 0.05$).

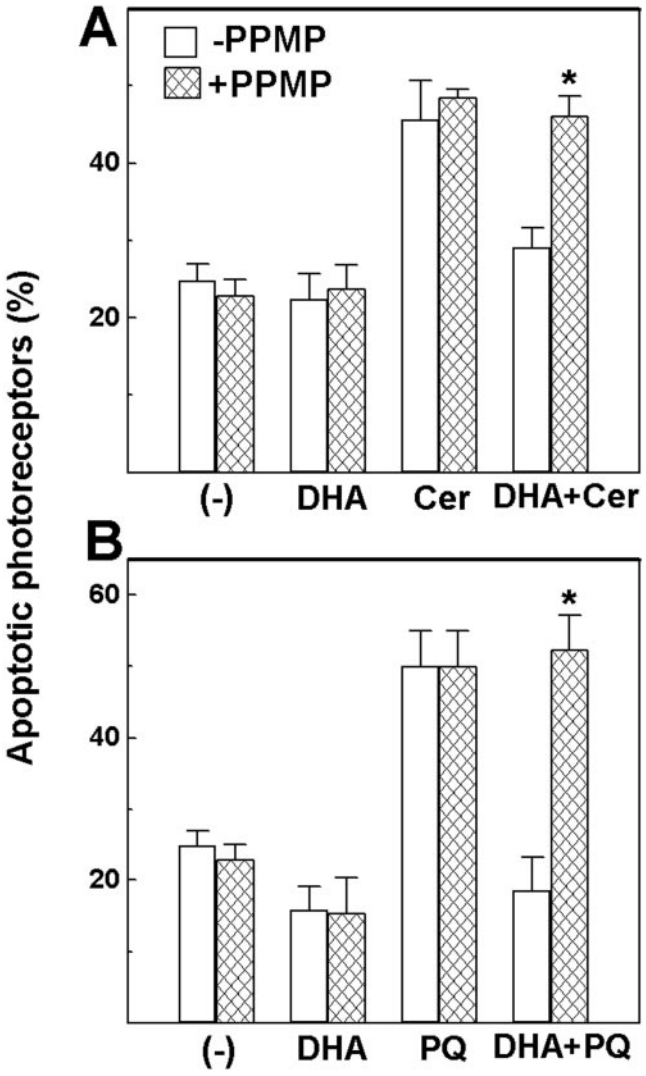

FIGURE 11. Effect of inhibition of glucosylceramide synthase on DHA protection of photoreceptors. Cultures supplemented at day 1 in vitro with DHA or with a BSA solution (−) were treated at day 3 with (+PPMP) or without (−PPMP) 10 μM PPMP, an inhibitor of glucosylceramide synthase. Thirty minutes later, cultures were treated either (**A**) with 10 μM C_2-ceramide (Cer, DHA+Cer) or (**B**) with 48 μM PQ (DHA+PQ). After a 24-hour incubation, the percentage of apoptotic photoreceptors in each experimental condition was determined. *Statistically significant difference, compared with the control ($P < 0.05$).

induction of apoptosis in photoreceptors was clearly evident in the rapid accumulation of newly made [^{3}H]ceramide in photoreceptors after PQ treatment in cultures labeled with [^{3}H]palmitate, which preceded the onset of apoptosis. Inhibition of ceramide synthesis with both cycloserine and FB completely blocked PQ-induced photoreceptor apoptosis, demonstrating that an increase in ceramide is critical for triggering apoptosis. The presence of these inhibitors left the oxidant unable to induce photoreceptor apoptosis, revealing that ceramide was an essential mediator in the death process. Moreover, DHA, which protects photoreceptors from oxidative stress[8] and ceramide exogenous addition, reduced ceramide accumulation to promote survival. Inhibition of glucosylation of ceramide, with the subsequent accumulation of ceramide, led to the loss of the protective effect of DHA. As a whole, these results strongly support that an increase in ceramide intracellular levels is involved in the activation of photoreceptor apoptosis and, conversely, inhibition of this increase can prevent photoreceptor apoptosis on oxidative stress.

Oxidative stress has been shown to increase ceramide production by both sphingomyelin hydrolysis[46] and de novo biosynthesis.[48] Our findings suggest that in photoreceptors, oxidative stress increased ceramide endogenous levels by stimulating the de novo biosynthetic pathway. PQ induced a rapid accumulation of [^{3}H]ceramide, whereas radioactive sphingomyelin levels were much lower than those of ceramide and did not change after addition of PQ, suggesting that ceramide is not produced from the breakdown of sphingomyelin. The addition of two inhibitors of ceramide de novo synthesis, FB and cycloserine, which block different steps of the de novo pathway, just before PQ treatment was sufficient to inhibit photoreceptor apoptosis completely. These results strongly support that oxidative stress increases ceramide production through stimulation of the de novo pathway.

Of note, inhibition of ceramide synthesis also rescued photoreceptors from apoptosis during their early development in vitro. Previous work from our laboratory has shown that after 4 days in culture, photoreceptors start to die through an apoptotic pathway, and this death may be postponed by trophic factors such as DHA.[9,10,16,38] In the present study, even in the absence of trophic factors, inhibiting ceramide synthesis was sufficient to reduce photoreceptor apoptosis markedly. This suggests that an increase in ceramide production may contribute to photoreceptor death in the absence of trophic factors, and strongly supports that ceramide may be a common mediator of photoreceptor apoptosis in different situations of cellular stress.

Addition of C_2-ceramide also induced the apoptosis of amacrine cells. However, endogenous ceramide involvement in the activation of amacrine cell death seemed less critical than in photoreceptors. The addition of FB at day 0 only partially reduced amacrine cell apoptosis after PQ treatment, and its addition at day 3 did not prevent it at all. Amacrine cells comprise more than 20 subtypes,[49] and several of them coexist in vitro. Our results suggest that either ceramide is not the principal mediator in the activation of apoptosis in amacrine neurons or it participates only in the induction of apoptosis in one amacrine cell subtype or a few. Moreover, because only early addition of FB had a protective effect, the increase in ceramide leading to apoptosis in some amacrine cells may arise from sphingomyelin hydrolysis. The different effect of inhibitors of ceramide synthesis in photoreceptor and amacrine cell death suggests that distinct synthetic pathways lead to ceramide production in each cell type.

Apoptosis induced by ceramide involved the mitochondrial pathway, because C_2-ceramide led to a loss of mitochondrial membrane potential in photoreceptors and amacrine neurons, and this loss was prevented by inhibition of ceramide synthesis. Mitochondria are well-known targets of ceramide,[50] which induces generation of ROSs[51,52] and leads to mitochondrial perturbations and function loss.[29,53,54] An increase in ceramide increase and mitochondrial depolarization are closely connected. Inhibiting mitochondrial depolarization significantly reduces ceramide-induced cell death,[55] whereas blocking C_{16}-ceramide synthesis prevents mitochondrial depolarization and nuclear fragmentation.[56] Ceramide induces the release of proapoptotic mitochondrial proteins,[57–60] leading to activation of caspases. Our results suggest that an increase in ceramide preceded the loss of mitochondrial integrity and was involved in triggering this loss.

Effect of DHA on Ceramide-Induced Apoptosis

DHA prevents the apoptosis of photoreceptors both during their early development in culture and that induced by oxida-

tive stress.[8–10,16,38] We have shown that DHA enhances the expression of the antiapoptotic protein Bcl-2 in photoreceptors, an increase that still stood in PQ-treated cultures in the current study.[8] Consistent with the hypothesis that ceramide is the main mediator in oxidative stress–induced apoptosis of photoreceptors, DHA also precluded apoptosis triggered by exogenous addition of ceramide in these cells. As with PQ, this protection involved the pronounced upregulation of Bcl-2 expression, unaffected by ceramide addition, and prevention of the loss of integrity of the mitochondrial membrane. Overexpression of Bcl-2 interferes with the ceramide pathway.[29,61,62] Bcl-2 has been proposed to act at two levels: upstream of ceramide synthesis or downstream, preventing cytochrome *c* release in isolated mitochondria[59] and inhibiting caspase activation.[63] Because increased Bcl-2 contributes to photoreceptor survival on addition of exogenous ceramide, our results suggest that it acts at least downstream of ceramide production, probably preventing the release of death-inducing factors from mitochondria.

DHA has recently been shown to induce a differential transcription in several genes in fetal human retina,[64] among them the gene coding for glucosylceramide synthase. By promoting ceramide glucosylation, this enzymatic activity decreases ceramide levels, and consequently, its inhibition mimics many of the effects of directly increasing intracellular ceramide concentrations.[65] Conversely, an increased synthesis and accumulation of glucosylceramide has been associated with drug resistance in several cancer cell lines.[66,67] Inhibiting the activity of this enzyme abolished DHA protection after C_2-Cer and PQ treatment. This suggests that an increase in the synthesis or activity of glucosylceramide synthase was involved in DHA's antiapoptotic effect. Inhibiting this enzyme was also sufficient to block DHA protection of photoreceptors on oxidative stress. This supports the proposal that oxidative stress leads to an increased production of ceramide, and this increase triggers apoptosis. DHA, by enhancing ceramide glucosylation, hindered its accumulation, thus blocking the initiation of apoptosis. These results also suggest that an increase in Bcl-2 expression is not sufficient to guarantee photoreceptor cell survival. The decrease in ceramide accumulation by its increased glucosylation is indispensable in the protective effect of DHA.

Our findings suggest that ceramide plays a key role in the induction of photoreceptor apoptosis. Oxidative stress increased ceramide production by de novo biosynthesis, and this increase signaled photoreceptor apoptosis. Diminishing ceramide accumulation, either by inhibiting its synthesis or by promoting its metabolization prevented photoreceptor death. Regulation of ceramide levels was crucial for DHA protection, which involved a combination of mechanisms. DHA upregulated Bcl-2 expression, and simultaneously diminished ceramide accumulation. As a whole, these results suggest that modulating the activity of the enzymes involved in sphingolipid metabolism may provide a therapeutic tool for preventing photoreceptor death in retinal neurodegenerative diseases.

Acknowledgments

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