Ceramide: A Potential Mediator of Apoptosis in Human Retinal Pigment Epithelial Cells

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PURPOSE. To investigate the signal transduction mechanisms involved in the cell death of human retinal pigment epithelial (RPE) cells after their exposure to either hydrogen peroxide (H2O2) or tri-butyl hydroperoxide (tBH).

METHODS. Cultured human RPE (hRPE) cells were treated with the chemical oxidants tBH and H2O2 as well as with the synthetic ceramide analogs C2, C6, and dihydroceramide for different time periods. Apoptosis was determined by TUNEL staining and annexin-V labeling of phosphatidylserine exposure. Ceramide levels were quantified by the diacylglycerol kinase assay using thin-layer chromatography.

RESULTS. H2O2 and tBH caused a high level of apoptosis in the hRPE cells. At the same time, both of these oxidants induced an early and late increase in the intracellular production of ceramide, a lipid second messenger. Moreover, addition of C2 and C6 synthetic ceramides caused a high level of apoptosis in these hRPE cells. In contrast, treatment with the immediate precursor of ceramide, dihydroceramide, resulted in no apoptotic response.

CONCLUSIONS. The results demonstrate that H2O2 and tBH induce apoptosis in hRPE cells and suggest that the underlying signaling mechanism involves ceramide generation. (Invest Ophthalmol Vis Sci. 2001;42:247–254)

Age-related macular degeneration (AMD) is the leading cause of legal blindness in patients aged 65 years or over. The retinal pigment epithelium (RPE) is considered to be the prime target for the early development of the disease.1-2 It is believed that accumulation of lipofuscin in the RPE leads to metabolic impairment of the cell, resulting in cell death and subsequent atrophy, with severe consequences for the photoreceptors whose survival depends on RPE normal functioning.3 The mechanism of cell death in macular degeneration is not known but may be the result of apoptosis.4

Apoptosis is a process of programmed cell death, and is an essential mechanism for the maintenance of homeostasis in multicellular organisms. In this orderly process of programmed cell death, the integrity of the plasma membrane is preserved until late in the death process, thus preventing the extravasation of toxic intracellular components, which may induce the inflammatory process in the tissue. Apoptotic cells or cellular fragments are eventually phagocytized by neighboring cells or macrophages without inducing an inflammatory response. Thus, apoptosis is considered to be a process in which individual cells are removed without causing tissue damage. The signal transduction cascades that activate apoptosis are present in all cells, and under normal conditions are maintained in an inactive state. The apoptotic program can be activated by a wide variety of exogenous stimuli.5-6 Potential exogenous triggers of apoptosis range from growth factor withdrawal to ligand- or antibody-mediated engagement of specific cell surface receptors capable of transducing lethal signals.7-8

Most of the signaling pathways that trigger apoptosis remain unknown; yet, during the last few years, increasing evidence has emerged demonstrating that sphingolipids may participate in these pathologic events. The term “ceramide-mediated pathway” has been coined to describe this pathway of cell signal transduction. The ceramide-mediated pathway is a signaling system that links specific cell-surface receptors and environmental stresses to the nucleus.7 This pathway is initiated by either hydrolysis of the sphingolipid, sphingomyelin, or by de novo generation of ceramide. Sphingomyelin is preferentially concentrated in the plasma membrane of all mammalian cells.10 Sphingomyelin hydrolysis occurs within seconds to minutes after stimulation via the action of sphingomyelin-specific sphingomyelinas to generate ceramide. The sphingomyelin-specific sphingomyelinas has no specific known inhibitor; thus, conducting experiments in which inhibition of endogenous ceramide production inhibits apoptosis is currently difficult to perform. De novo synthesis of ceramide occurs over a period of several hours, and several studies have suggested that ceramide synthase is the regulated enzyme in this mechanism. The ceramide, which is generated as a second messenger in this system, leads to apoptotic DNA fragmentation. Agonists of the ceramide pathway include cytokines, such as tumor necrosis factor (TNF)-α,11-12 interleukins 1,13 and γ-interferon,14 and antibodies directed against functional molecules, such as Fas/APO-115-17 or CD28 proteins,18 as well as stress-inducing agents such as UV19 and ionizing radiation20,21 and antileukemic agents.22-23 The observation that cell- permeant synthetic ceramides or natural ceramide (generated by treating cells with bacterial sphingomyelinas) could mimic the biological effects of most ceramide cycle agonists has provided significant weight to the role of ceramide in signal transduction and apoptosis.

Hydrogen peroxide (H2O2), hydroxyl radicals, and superoxide anion,24-25 termed reactive oxygen intermediates (ROIs), induce oxidative stress. Collectively, these species possess significant capacity for cellular damage and have been implicated in both the aging process and the pathogenesis of chronic diseases, including atherosclerosis, cancer, and diseases of the respiratory tract.26 There is growing epidemiologic evidence that oxidative stress plays a major role in the pathogenesis of AMD. Recently, it has been shown that the oxidant tri-butyl hydroperoxide (tBH) induces cell death in the human RPE (hRPE) via the apoptotic mechanism.27 We have previously shown that H2O2 acts on cellular membranes to generate ceramide signaling and initiate apoptosis in tracheobronchial epithelial cells.28 In the present study, we evaluated whether the oxidative stress-induced apoptosis is mediated by ceramide signal transduction in hRPE cells. Because the RPE is known to contain high catalase levels,29 thus potentially being

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protected against H2O2-induced apoptosis, we also evaluated apoptosis induction after tBH treatment.

**MATERIALS AND METHODS**

**Cell Culture**

All materials in this study were obtained from Sigma (St. Louis, MO) unless stated otherwise. Immortal hRPE cells ARPE-19 were used during all experiments and grown as described. Briefly, the RPE cells were grown in growth medium DMEM/F12 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum. The cells were plated at a density of 5 × 10^4 cells/cm². Cells were incubated at 37°C with 10% CO2 atmosphere. The medium was changed every 3 days, and a final cell density of 0.7 to 1 × 10^4 cells/cm² was obtained within 5 days of incubation. Subcultures were performed as follows: near-confluent cultures were treated with trypsin (0.05%)-EDTA (1 mM) in phosphate-buffered saline (PBS), pH 7.0. After cells were detached from the plates, an equal volume of DMEM/F12 (Gibco) supplemented with 10% fetal bovine serum was added to stop the trypsination. Cells were recovered by centrifugation and resuspended in culture medium for plating. Cell numbers were determined using an improved Neubauer counter (American Optical Corp., Buffalo, NY). Cell viability was assessed by Trypan blue exclusion analysis.

**Oxidant Treatments**

For H2O2 and tBH treatments, cells were seeded into six-well plates and grown to confluence. freshly prepared H2O2, and tBH were used, as indicated in the figures. Because tBH has low solubility in water and forms micelles, it was prepared as 1000x stock solution in dimethylsulfoxide before its use. For the aminotriazole (ATZ) treatments, cells were treated with freshly prepared 300 mM ATZ 3 hours before the treatment with H2O2.

**Apoptotic Assays**

For detection of cell death, a TUNEL assay (terminal deoxy-nucleotidyl transferase [TdT]-mediated dUTP [deoxyuridine triphosphate] nick-end labeling) staining kit and an annexin V staining kit for the assay of phosphatidylserine (PS) exposure (Annexin-V-Fluos; Boehringer Mannheim, Indianapolis, IN) were used.

**TUNEL Labeling**

TUNEL staining was performed according to the manufacturer’s instruction. Briefly, after treatments, attached cells were trypsinized, as previously described in the Cell Culture subsection and combined with the cells floating in the medium. Cells were washed twice in PBS, resuspended in 10% fresh fetal bovine serum. Washed cells were analyzed per sample. An analysis region was set to exclude cell aggregates, and the green channel was set to score <1% of the signals from untreated control cells. The red (PI) and green (fluorescein) fluorescence were measured.

**Lipid Analogs**

C2-ceramide (N-acetyl sphingosine) and C6-ceramide (N-hexanoyl sphingosine) were obtained from Matreya (Pleasant Gap, PA). The polar lipids were prepared as stock solutions in 100% ethanol. The final concentrations of ethanol in the incubations were 0.2% and 0.1%, respectively, which did not induce apoptosis. All experiments involved both vehicle controls and specificity controls using biologically inactive dihydroceramide analogs.

**Lipid Studies**

Ceramide was quantified by the diacylglycerol kinase assay, as described previously. In brief, after incubation with the treatment drug, cells were pelleted by centrifugation (300g for 10 minutes), washed twice with ice-cold PBS, and extracted with 0.6 ml of chloroform:methanol:1 N HCl (100:100:1, v/v/v). Lipids in the organic-phase extract were dried under N2, and subjected to mild alkaline hydrolysis (0.1 N methanolic KOH for 1 hour at 37°C) to remove glycerophospholipids. Samples were reextracted, and the organic phase was dried under N2. Ceramide contained in each sample was resuspended in a 100-μl reaction mixture containing 150 μg cardiolipin (Matreya), 280 μM diethylenetriaminepenta-acetic acid (DTPA), 51 mM octyl-15-α-glucopyranoside (Calbiochem-Novabiochem Corp., San Diego, CA), 50 mM NaCl, 51 mM imidazole, 1 μM EDTA, 12.5 mM MgCl2, 2 μM dithiothreitol, 0.7% glycerol, 70 μM β-mercaptoethanol, 1 mM ATP, 10 μCi of [γ-32P]ATP (3000 Ci/mmol; Dupont New England Nuclear, Boston, MA), 55 μg/ml Escherichia coli diacylglycerol kinase (Calbiochem-Novabiochem Corp.), pH 6.5. After 60 minutes at room temperature, the reaction was stopped by extraction of lipids with 1 ml chloroform:methanol:1 N HCl (100:100:1), 170 μl buffer saline solution (135 mM NaCl, 1.5 mM CaCl2, 0.5 mM MgCl2, 5.6 mM glucose, and 10 mM Hepes, pH 7.2), and 30 μl of 100 mM EDTA. The lower organic phase was dried under N2. Ceramide 1-phosphate was resolved by thin-layer chromatography on silica gel 60 plates (MCB Manufacturing Chemicals, Cincinnati, OH) using a solvent system of chloroform:methanol:acetic acid (65:15:5) and detected by autoradiography, and incorporated 32P was quantified by liquid scintillation counting. The level of ceramide was determined by comparison with a standard curve generated concomitantly from known amounts of ceramide (ceramide type III; Sigma). Diacylglycerol was quantified in a similar manner to ceramide, except the alkaline hydrolysis step was omitted.

**ROI Production**

Time course experiments were performed to compare ROI production in RPE cells after different lengths of synthetic ceramide exposure. ROI production was detected using the dye 2',7'-dihydrochlorofluorescein (H2DCF), as previously described. H2DCF is a nonfluorescent cell permeate compound. Once inside the cell, it is cleaved by endogenous esterases and can no longer leave the cell. The hydrolyzed product, DCF, is fluorescent upon oxidation by ROIs. H2DCF (10 μM) was added to cells during dissociation with 0.05% tyrosine. The cells were then incubated at 37°C for 10 minutes and washed once in Hepes buffer supplemented with 2% dialyzed fetal bovine serum. Washed

nexin V and propidium iodide (PI; 5 μg/ml) for FACS analysis using a FACStar flow cytometer equipped with a doublet discriminating module (Becton Dickinson & Co., San Jose, CA). Cells negative for both PI and annexin V staining are live cells; π-negative, annexin V-positive staining cells are early apoptotic cells; and π-positive annexin V-positive staining cells are primarily cells in late stages of apoptosis. The data were analyzed using Cell Quest (Becton Dickinson & Co.). Ten thousand cells were analyzed per sample. An analysis region was set to exclude cell aggregates, and the green channel was set to score <1% of the signals from untreated control cells. The red (PI) and green (fluorescein) fluorescence were measured.
cells were resuspended in Hepes buffer and kept on ice until flow cytometric analysis. PI dye was used to gate for live cells and was added to each tube at a final concentration of 5 μM/mL. Data were collected with a FACScan fluorescence accelerated cell scanner using the data acquisition program CELLQuest (Becton Dickinson). DCF data were collected with the following excitation and emission wavelengths: λex = 475 nm, λem = 525 nm. Ten thousand live cells, as determined by the lack of PI fluorescence, were analyzed per sample. To the best of our knowledge, ROI production leads directly to DCF oxidation. The dissociation of cells before measuring DCF fluorescence accurately reflects amount of ROI present in the plated cells at the time just before dissociation. Visual inspection using fluorescence microscopy confirms that the in-plated cells remained attached and with good morphology and that the dye remained intracellular.

Statistical Analysis

Statistical analysis was performed by Student’s t-test, and linear regression was performed by the method of least squares.

RESULTS

H2O2 and tBH Induce Apoptosis in Human RPE Cells

Human RPE cells were treated with either H2O2 (0.5–2.5 mM) or with tBH (0.3 mM) and analyzed over time for PS exposure as indicated by annexin V labeling, using flow cytometry. Figures 1B and 1C show that positive annexin V staining was detected in both H2O2- and tBH-treated cells 4 hours after treatment. Temporal analysis shows an increase in the population of apoptotic cells and of dead cells, with maximum apoptosis occurring in the 1 mM H2O2-treated group 16 hours after exposure (53% of cell annexin V or PI positive) and in the tBH-treated group at 24 hours after treatment 42% of cells were annexin V or PI positive (C). The response to fluorescein (conjugated to the annexin V antibody) is plotted on the x-axis and the response to PI is plotted on the y-axis. Cells negative for both PI and annexin V staining are live cells (bottom left); PI-negative, annexin V–positive staining cells are early apoptotic cells (bottom right); PI-positive annexin V–positive staining cells are primarily cells in late stages of apoptosis (top right) PI-positive, annexin V-negative staining cells are necrotic cells (top left).

TUNEL 24 hours after treatment. A dose response of the apoptotic process has shown that when using 0.5 mM H2O2, 12% of the treated cells were shown to be TUNEL positive at 8 hours, 25% when using 1 mM H2O2 whereas when using 2.5 mM H2O2 most cells where shown to be necrotic and dead after 8 hours.

Ceramide Mimics H2O2 in Inducing Apoptosis in RPE Cells

Because previous studies in hematopoietic cells and in tracheobronchial epithelial cells reported that apoptosis is mediated via increase of intracellular ceramide, we tested whether addition of a cell-permeable ceramide analog can cause apoptosis in hRPE cells. Figure 2 shows that treatment of RPE cells with 50 μM C2-ceramide mimics H2O2, and tBH induced effects by generating the typical apoptotic changes revealed by TUNEL staining and annexin V staining. Similar changes were also observed after exposure treatment of the cells with 50 μM C2-ceramide. Twenty-four hours after treatment with C2-ceramide, 60% of the cells showed apoptotic changes by TUNEL, and when treated with C6-ceramide for 24 hours, 50% of the cells showed apoptotic changes. In contrast, treatment with 100 μM of the immediate precursor of ceramide, dihydroceramide, which lacks the trans double bond C4–C5 of the sphingoid base backbone, resulted in no apoptotic response.

H2O2 Induces Increases in Intracellular Ceramide Levels

The ability of ceramide to mimic the action of H2O2 suggests that ceramide may be a mediator of H2O2-induced apoptosis in RPE cells. To test this hypothesis, we explored the ability of H2O2 or tBH to elevate the cellular levels of ceramide. Treatment of RPE cells with 1 mM H2O2 or 0.3 mM tBH produced both early (within minutes) and late (several hours) increases in the ceramide levels. The early increase in ceramide levels was maximized 15 minutes after H2O2 exposure, whereas in the tBH-treated cells, ceramide production reached maximal values after 30 minutes’ exposure (Fig. 3A). The rise of ceramide was twofold in the H2O2-treated cells and threefold in the tBH-treated cells. The late increase in ceramide levels was detected 3 hours after treatment with either H2O2 or tBH, and remained high for 24 hours (Fig. 3B).
FIGURE 2. Effect of treatment with synthetic ceramide analogs C2, C6 and di-hydro ceramide on apoptosis induction in hRPE cells. TUNEL (top) and annexin V assays (bottom) of hRPE cells untreated (A, control), treated with 50 µM C2-ceramide analog (B), treated with 50 µM C6-ceramide analog (C), and treated with 100 µM di-hydroceramide (D). At 24 hours after treatment with C2-ceramide, 60% of the cells showed apoptotic changes, and when treated with C6-ceramide, 50% of the cells showed apoptotic changes at the same time point. In contrast, treatment with 100 µM of the immediate precursor of ceramide, dihydroceramide, resulted in no apoptotic response. The response to fluorescein is as described in Figure 1.

FIGURE 3. Effects of H2O2 and tBH treatment on the early (A) and delayed (B) ceramide levels of hRPE cells. Human RPE cells were incubated at 37°C with either H2O2 (1 mM) or with tBH (0.3 mM). The incubations were terminated and ceramide quantification was performed, as described in Materials and Methods. A twofold increase of the original ceramide levels were detected 15 minutes after H2O2 exposure. In the tBH-treated cells, a threefold increase was also reached 30 minutes after exposure. The late increase in ceramide levels was detected 3 hours after treatment with either H2O2 or tBH and remained high for 24 hours (B).
Treatment of RPE cells with the cell membrane permeable C2- and C6-ceramide analogs also resulted in two-phase intracellular accumulation of ceramide (data not shown), providing evidence that short-chain ceramide analogs do have access to the epithelial cell interior, consistent with results in other cells.11

Addition of Aminotriazole Facilitates Apoptosis and Ceramide Production at Lower Concentrations of H2O2

We found that relatively high concentrations of H2O2 cause apoptosis (Fig. 1). RPE cells have high concentrations of catalase, which may protect the cells from the oxidative stress-induced apoptosis. Therefore, we chose to treat the hRPE cells with the catalase inhibitor ATZ to test the protective effect of catalase in oxidative stress-induced ceramide production and apoptosis. Three hours before treatment with H2O2, 30 mM ATZ was added to the cells. The addition of ATZ caused a sixfold increase (from 3% to 18%) in the amount of apoptotic cells detected by TUNEL staining 8 hours after treatment with 0.5 mM H2O2. The increase in the apoptotic activity coincided with early and late increase in the ceramide production, which was detected after the addition of ATZ. (Fig. 4).

ROI Production by Synthetic Ceramides

Previous studies in mouse hippocampal cell line36 and in hematopoietic cells37 reported that one of the early changes during apoptotic cell death is the interference with mitochondrial activity. This results in rapid accumulation of reactive oxygen intermediates (ROI). We evaluated whether the addi-
tion of a cell permeable ceramide analog can cause the accumulation of ROI. Figure 5 demonstrates the accumulation of ROI after treatment with C2-ceramide. A twofold increase in the production of ROI was observed 30 minutes after treatment with C2-ceramide, which decreased to baseline levels only 2 hours after treatment. The increase in ROI production provides additional evidence that short-chain ceramides have access to the epithelial cell interior to induce apoptosis, consistent with results in other cell types.

**DISCUSSION**

Our present studies show that H2O2 and tBH induce apoptotic signaling in hRPE cells. The immediate event in this pathway involves the generation of ceramide, which is initiated within minutes of exposure to H2O2 or tBH. The hypothesis that ceramide acts as a second messenger in the pathway of oxidative stress-induced apoptosis is supported by the fact that the C2- and C6-ceramide analogs were capable of mimicking H2O2 and tBH as inducers of the apoptotic response, as has been previously shown in TNFα-induced apoptosis.38 The specificity of various lipids in inducing apoptosis in retinal pigment epithelial cells was determined by treatments with various cell-permeable ceramide synthetic analogs. The specific analog dihydro C6-ceramide, which lacks the 4,5 double bond did not elicit apoptosis.

We have concluded that ceramide is a potential mediator of apoptosis because of the fact that we could not perform an experiment in which inhibition of endogenous ceramide production inhibits apoptosis. The sphingomyelin-specific sphingomyelinase has no specific known inhibitor, and therefore, experiments in which inhibition of early endogenous ceramide production inhibits apoptosis are difficult to perform.

Signaling pathways involved in apoptosis induction remain largely unknown. The sphingomyelin pathway, initiated by hydrolysis of sphingomyelin in the cell membrane to generate the second-messenger ceramide35,39,40 is thought to mediate apoptosis in response to TNFα,11,38 Fas ligand,16 X-rays,20 and H2O2 in lung epithelial cells38 and in U937 human monoblastic leukemia cells.19 De novo generation of ceramide mediates apoptosis in response to daunorubicin, CPT 11, and serum withdrawal in leukemia cells.9

During normal physiological conditions hRPE cells are exposed extensively to reactive oxidants, one of which is H2O2. The oxidative stress is initiated in the RPE cells by the uptake and degradation of retinal outer segments,59 by intense illumination from light sources,40 and by the high oxygen tension in the macular area.41 Because hRPE cells are exposed extensively to reactive oxidants, we set up studies aiming to address whether these normal cells are capable of entering apoptosis when exposed to H2O2 and tBH and whether the process is mediated by ceramide as a second messenger.

Ceramide cell survival is essential for the normal function of the retinal photoreceptors. Our working hypothesis is that the pathogenesis of AMD is related to aging changes that occur in the retina, and an important part of the changes are related to the RPE cell function. Aging changes may induce apoptosis, and in the present study, evaluation of an oxidative stress model for the initiation of apoptotic changes was examined. We chose an oxidative stress model because of its possible clinical relevance in the development of aging macular degeneration.

The role of oxidative stress in apoptosis has generated considerable debate because antioxidants as well as pro-oxidants were shown to inhibit this form of cell death.42-44 There is growing evidence that ROIs play a key role in the regulation of apoptosis, although the precise nature of this control is unclear.45-48 On the other hand, there is evidence that oxidative stress, induced by overproduction or decreased elimination of H2O2, provides tumor cells with a survival advantage over normal counterparts.49 It has also been recently reported that oxidative stress may activate growth-stimulatory responses similar to those induced by hormones and cytokines.26,35,49 This mechanism may predominate at the physiologically low-dose range of H2O2, in which unrepaired lethal damage to the DNA may be less common than at the higher doses, which is rarely applicable to physiologic situations. In our model, a relatively high concentration of H2O2 was required to produce apoptotic changes in hRPE cells, yet these concentrations were sublethal in the cell line used. The same observation was previously noted, and assumptions from this experimental model to in vivo mechanisms must be made with caution.27 One possible explanation for the high concentration of H2O2 needed to induce apoptosis in vitro may be related to the high concentration of catalase that is present in hRPE cells, thus partly protecting the cells from the oxidative induced apoptosis.51 It has been previously shown that catalase activity decreases with increasing age in humans.50 Thus, it may be possible that aged RPE cells are more susceptible to oxidative stress.

We have found that the kinetics of ceramide formation in response to oxidative stress are complex in hRPE cells. Both acute and reversible elevation of ceramide levels (within minutes) and prolonged and persistent (few hours) elevations were found. Our findings are consistent with previous reports, which documented a complex and variable response of ceramide formation after stress induction.9 The most pronounced changes in the amount of ceramide occur hours after the stress induction, unlike the immediate changes, which are seen in other stress signals (adenosine 3′,5′-monophosphate [cAMP] and many of the eicosanoids). The prolonged and persistent accumulation of ceramide is most likely related to activation of a de novo pathway of ceramide generation.2 These findings were observed in many other cells examined11,44,16 and have led to the concept that ceramide may function as a component of a “biostat” that measures and initiates responses to cellular stress, much like a thermostat that measures the temperature over a long period. The cell then responds to the changes in the ceramide levels by undergoing apoptosis or cell arrest, which occur via multiple enzymatic pathways. Thus, ceramide levels may act as a general measurement for the “stress level” of the cell.
In the present study, we found that synthetic ceramide induces ROI production in RPE cells (Fig. 5). These findings are consistent with previous reports, which have shown that ceramide is not only a signaling product of oxidative stress but also mediates the production of ROI in the mitochondria. ROIs may function as early mediators of ceramide-induced apoptosis, suggesting that coupling between oxidative stress and ceramide production is bidirectional; oxidants may not only activate ceramide production, but ceramide may also induce generation of reactive oxidants (Fig. 6). In conclusion, the present studies directly demonstrate that apoptotic signaling can be produced via the sphingomyelin pathway with ceramide generation by H$_2$O$_2$ or tBH in hRPE cells. The identification of a signal transduction agent, ceramide, which is involved in the induction of apoptosis in RPE cells, may have clinical implications. Accordingly, ceramide antagonists may be used experimentally to reduce oxidative stress–induced apoptosis, thus reducing the amount of RPE cell death associated with the early changes of AMD.

References