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Ceramide-induced cell death in lens epithelial cells

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Purpose: To determine whether ceramide treatment contributes to reduced cell viability, increased apoptosis, caspase activation, and reactive oxygen species generation in lens epithelial cells.

Methods: Cell viability was determined by the 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Apoptotic cell death was determined by 4,6 diamidino-2-phenylindole (DAPI) nuclear staining. Quantitative DNA fragmentation was determined by specific determination of cytosolic mononucleosomes and oligonucleosome-bound DNA. Caspase-3/7 activation was determined by using the Apo-ONE Assay. Detection of reactive oxygen species was achieved by a carboxy-2,7-dichlorofluorescein diacetate (carboxy-H2DCFDA) staining method and lipid peroxidation assay.

Results: C2-ceramide and C6-ceramide reduced primary bovine lens epithelial cell and human lens epithelial cell survival in a dose- and time-dependent manner. The effect of ceramide on cell viability was specific since C2-dihydroceramide, a chemically similar ceramide lacking four to five double-bonds, did not adversely affect lens epithelial cell viability. Release of endogenous natural ceramides by treatment of lens epithelial cells with bacterial sphingomyelinase reduced cell viability. Ceramide-induced apoptosis in lens epithelial cells was determined by nuclear appearance and DNA fragmentation. Apoptosis was induced by exogenous C2-ceramide in a dose-dependent and time-dependent manner and ceramide-mediated apoptosis of lens epithelial cells was associated with caspase-3/7 activation. C2-ceramide treatment resulted in reactive oxygen species generation.

Conclusions: These results suggest that ceramide reduced cell viability and increased apoptosis in a dose-dependent and time-dependent manner in lens epithelial cells. Ceramide-induced oxidative stress suggests that age-related cataracts may be modulated by ceramide levels in the lens.

Apoptosis is an evolutionarily conserved and distinct form of programmed cell death characterized by chromatin condensation, DNA fragmentation, cell shrinkage, and membrane blebbing [1,2]. Ceramide, the second messenger of the sphingomyelin pathway, has emerged as a pleiotropic mediator that regulates cell cycle arrest, differentiation, and apoptosis [3-5]. Ceramide is generated from sphingomyelin by acid or neutral sphingomyelinase or by de novo synthesis [6-8]. Agonists of ceramide generation include cytokines such as tumor necrosis factor-α [9,10], interleukine-1-β [11], γ-interferon [9], and stress-inducing agents such as ultraviolet (UV) [12], ionizing radiation [13,14], and oxidation stress [15-17]. The changes in endogenous levels of ceramide in response to these agents occur before the onset of the first biochemical signs of apoptosis such as the activation of caspases [18,19].

The mechanisms of ceramide-mediated apoptosis have been under intensive scrutiny and it is believed that mitochondria play an important role in this process. Ceramide induces mitochondrial cytochrome C release before transmembrane depolarization and caspase-3 activation [20,21]. Recent evidence shows that ceramide specifically forms channels in mitochondrial outer membranes, facilitating mitochondrial protein release [22]. Ceramide induces caspase-dependent [21,23] as well as caspase-independent apoptosis [24,25]. In addition, ceramide treatment leads to activation of the stress-activated protein kinase (SAPK/JNK) [26,27]. Prosurvival pathways are also affected by elevated cellular concentration of ceramide. Ceramide suppresses Ras/Raf1/MEK1 activation [28]. Studies show that ceramide is involved in dephosphorylation and inactivation of PI3 kinase/Akt [23]. It is suggested that ceramide-mediated activation of phosphatases (ceramide-activated protein phosphatase) such as PP1 and PP2 [29-31] is involved in PI3 kinase/Akt inactivation [32].

Sphingomyelins are the major lipid components of lens membrane [33-35]. Sphingomyelinase activity is reported in bovine lens as well as human lens [36-38]. Moreover, Tao and Cotlier [39] reported that ceramide levels in cataractous lens were four times higher compared to those in aged-matched normal lens. Nevertheless, the potential role of ceramide in normal lens function and in the development of cataracts remains to be elucidated.

The purpose of this study was to determine the effect of ceramide on lens epithelial cell survival and apoptosis. The results demonstrate that ceramides reduce lens epithelial cell survival and increase apoptosis. These effects are dose-dependent and time-dependent. Further evidence is provided that ceramide-induced apoptosis mediates DNA fragmentation and caspase activation. Ceramide treatment of cells increases reactive oxygen species (ROS) and lipid peroxidation. As a whole, these results suggest that ceramide is a key activator of apoptosis in lens epithelial cells.

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METHODS

Chemical and materials: Dulbecco's modified Eagle's medium (DMEM), EDTA-trypsin (1X), 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide, antibiotic antimycotic solution, 100X (10,000 U/ml, penicillin, 10 mg/ml streptomycin, 25 µg/ml amphotericin B), gentamicin (5 mg/ml), bacterial sphingomyelinase, hydrogen peroxide, and paraformaldehyde were purchased from Sigma Chemical Co. (St. Louis, MO). C2-Ceramide, C6-ceramide, and C2-dihydroceramide were obtained from Biomol (Plymouth Meeting, PA). 4,6 Diamidino-2-phenylindole (DAPI) and 2,7-dichlorofluorescein diacetate (DCFH-DA) were purchased from Molecular Probe (Eugene, OR). Fetal bovine serum (FBS) was purchased from HyClone Laboratories (Logan, UT)

Cell culture conditions and ceramide treatment: Bovine lenses were obtained from cattle of varying age at local abattoirs. Bovine eyes were kept on ice from the time of slaughter until enucleation, usually less than 4-5 h. For primary cultures of lens epithelial cells, bovine lens were dissected under sterile conditions and the anterior capsules with attached epithelium were cut along the equator and cultured in Dulbecco's modified Eagle's medium (DMEM), pH 7.4, containing 0.5% (v/v) antibiotic and antimycotic solution, gentamicin 5 µg/ml, and supplemented with 10% FBS. Cells were maintained at 37 °C in humidified atmosphere containing 5% CO_2 and observed daily under inverted phase-contrast microscope (Olympus BX41; Melville, NY) [40]. When the cultured cells reached confluence, subcultures were prepared using 0.05% trypsin/0.02% EDTA solution. The cells were grown on tissue culture plates and were used one to two days after plating when a subconfluent monolayer culture was achieved. A human lens epithelial cell line established by transformation of primary cultured human lens epithelium with a DNA plasmid containing the large T antigen of Simian virus (SV) 40 (SRA 01/04) [41] was kindly provided by Dr. Venkat N Reddy (Kellogg Eye Institute, University of Michigan, Ann Arbor, MI) and cultured at 37 °C in DMEM supplemented with 15% FBS containing 0.5% (v/v) antibiotic and antimycotic solution, and gentamicin 5 µg/ml in a humidified 5% CO_2 atmosphere.

Natural long-chain ceramides are extremely hydrophobic and are frequently replaced in experiments in vitro by short chain ceramides. C2-ceramide and C6-ceramide which are more soluble were used in this study. C2-dihydroceramide was used as a biologically inactive ceramide analog (Figure 1). Ceramide stock solutions were prepared in ethanol. After overnight incubation in 1% FBS-DMEM, lens epithelial cells were treated with ceramides in 1% FBS-DMEM in desired concentrations unless it was described otherwise. Vehicle-treated controls contained less than 0.1% ethanol.

Cell viability assay: Cell viability was evaluated by the reduction of 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [42]. MTT is a water-soluble tetrazolium salt that is reduced by metabolically viable cells to a colored, water insoluble formazon salt. Cells were grown in a 96 well plate ($1.5x10^4$ cells/ml) and treated with ceramide as above. In separate experiments, BLECs and HLECs were treated with bacterial sphingomyelinase enzyme at various concentrations for 24 h. Cells were then washed with 1X phosphate buffered saline (PBS; pH 7.4) and MTT (0.5 mg/ml final concentration) was added. Cells were incubated for three h at 37 °C. The assay was stopped by replacement of the MTT-containing solution with 100 µl isopropanol. The absorbance of each well was measured at 570 nm against reference wavelength (690 nm) with ELISA Reader (SLT-Spectra; Salzberg, Austria).

Apoptosis assays: Apoptosis was determined by evaluation of nuclear condensation after staining cell nuclei with DAPI and by quantification of DNA fragment formation using Cell Death Detection ELISA^{PLUS} (Roche Molecular Biochemicals; Indianapolis, IN). For DAPI staining, cells were washed two times with 1X PBS (pH 7.4), fixed with 4% paraformaldehyde for 20 min, then labeled with DAPI (300 nM). After labeling, apoptotic cells were visualized using an Olympus BX41 (Olympus, Melville, NY) microscope under light or filter designed for DAPI fluorescence (Chroma, 82000 series; Olympus). Digital imaging was performed with Scion CFW-1310C digital camera and Scion VisiCapture Application software (version 1) and analyzed using ImageJ. Cells were considered to be apoptotic when they showed either fragmented or condensed (pyknotic) nuclei. At least 300 cells were counted in each experiment. The data show the mean±SEM of at least three independent experiments.

For quantification of DNA fragmentation, specific determination of cytosolic mononucleosomes and oligonucleosome-bound DNA was performed according to the manufacturer's instructions. In short, after ceramide treatment, the cells were lysed and the DNA fragments in the lysate were bound to a microtiter plate coated with monoclonal anti-histone antibodies. The bound DNA fragments were then detected by peroxidase-conjugated monoclonal anti-DNA antibodies and 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate]. Optical density was measured at 410 nm (SLT-Spectra; Salzberg, Austria) and results are expressed as fold increase compared to vehicle-treated cells. Incubation buffer (instead of the sample solution) and DNA-histone complex included in the kit were used as the background and the positive control, respectively. The positive control was used as validity internal control of the technique.

Caspase assay: Activation of caspase-3/7 was determined using the Apo-One Homogeneous Caspase-3/7 Assay (Promega, Madison, WI) following the protocol provided by the manufacturer. In brief, after treatment, cells were trypsinized and 40,000 cells/100 ml were mixed with the same volume of the Apo-One Homogeneous Caspase-3/7 reagent. After incubation at room temperature for three h, caspase-3/7 activation were estimated from fluorescence of sample at the excitation wavelength of 492 nm and the emission wavelength of 521 nm using fluorescence reader FL_x800 (BIO TEK Instruments, Winooski, VT).

Measurement of reactive oxygen generation: The intracellular generation of reaction oxygen species (ROS) was measured using 2',7'-dichlorodihydrofluorescein (H2-DCFH-DA) [43]. Lens epithelial cells were cultured in a 96 well plate ($1.5x10^4$ cells/ml) in DMEM containing 10% FBS. Cells were

loaded with 50 μM DCFH-DA for 60 min. DCFH-DA was removed, and cells were washed twice with 1X PBS (pH 7.4) and treated with C2-ceramide (30 μM) in DMEM containing 10% FBS for indicated time. DCFH-DA fluorescence was determined at an excitation of 485 nm and an emission of 538 nm by fluorescence reader FL_x800 (BIO TEK Instruments, Winooski, VT). Hydrogen peroxide (100 μM) was used as the positive control (data not shown). Values were normalized to the percentage in untreated control groups. It should be noted that the nonfluorescent ester H2-DCFH-DA penetrates into cells and undergoes deacetylation to DCFH by the cellular esterases. The DCFH probe is rapidly oxidized to the highly fluorescent compound 2',7'-dichlorofluorescein (DCF) by ROS such as hydrogen peroxide or fatty acid peroxides.

Measurement of thiobarbituric acid reactive substances: Lipid peroxidation is an indicator of oxidative stress. It is determined by the production of thiobarbituric acid reactive substances (TBARS) [44]. In brief, cells were exposed to C2-ceramide for 24 h, washed with PBS, then resuspended in PBS. An aliquot was taken for a protein assay, and the remaining solution was mixed with a solution containing 15% trichloroacetic acid, 0.375% thiobarbituric acid, and 0.25 N hydrochloric acid. The mixture was heated for 30 min in a boiling water bath, cooled on ice, and centrifuged to remove the precipitate. The absorbance of the sample was determined at 535 nm (ε(malondialdehyde)=1.56x10^5 M^{-1} cm^{-1}) against a blank containing all the reagents minus the cell extract.

Statistical analysis: Experimental data are presented as the mean±SEM of the mean, and the statistical significance was determined by student's t-test. Differences were considered significant when $p<0.05$.

RESULTS

Ceramides inhibit cell viability in lens epithelial cells: Lens epithelial cells were plated at 60%-80% confluency and subsequent treatments were in low serum-containing media (1% FBS) since it is well recognized that biologically active lipids, such as ceramide, can be sequestered by lipid-binding serum proteins [45]. In this defined system, the control lens epithelial cells remained attached to the culture substratum and viability was greater than 90% for at least 24 h. Primary bovine lens epithelial cells (BLEC) and human lens epithelial cell line SRA01/04 (HLEC) were exposed to various concentrations of exogenous ceramides for 24 h and cell survival was measured using the MTT method. Survival of BLEC was reduced to 68%±9.6% and 12.3%±5.9% at 10 μM and 20 μM of C2-ceramide, respectively (Figure 2A). C6-ceramide also reduced BLEC survival. However C6-cermaide appeared to be slightly less potent than C2-ceramide (Figure 2A). The IC_{50} for C6-ceramide is between 20 μM and 30 μM. C2-dihydroceramide (Figure 1), which has a similar chemical structure, uptake, and metabolism to that of ceramide but lacks its biological action [20,46], had very little effect on BLEC survival (Figure 2A).

Addition of ceramides also reduced the survival of HLEC. C2-ceramide was less potent on HLEC compared to BLEC (Figure 2B). The IC_{50} for C2-ceramide on HLEC was between 30 μM and 40 μM. However, C6-ceramide was more potent on HLEC. C6-ceramide at 10 μM reduced the survival to 40% ±7.7% and at 20 μM, the survival was reduced to 23% ±11.8%. The IC_{50} for C6-ceramide was between 0 μM and 10 μM (Figure 2B). C2-dihydroceramide did not affect viability of HLECs (Figure 2B).

To evaluate the time-dependent effect of ceramides on lens epithelial cells, BLECs were treated with 20 μM of C2-ceramide or C6-ceramide. C2-ceramide reduced the survival of BLEC to 50% after eight h of treatment (Figure 2C) while C6-ceramide did not affect cell survival during the first six h of treatment. Survival was reduced, however, at longer treatment intervals.

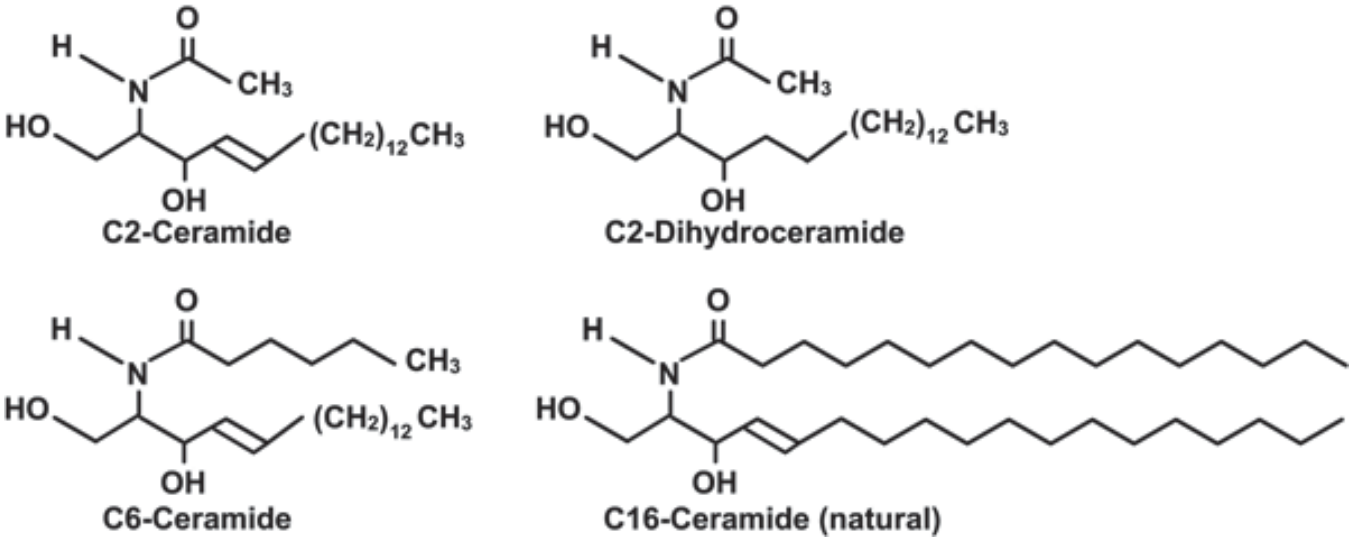

Figure 1. Synthetic and natural ceramides. Structure of ceramide analogs C2-ceramide, C6-ceramide, biologically inactive C2-dihydroceramide, and natural C16-ceramide are presented.

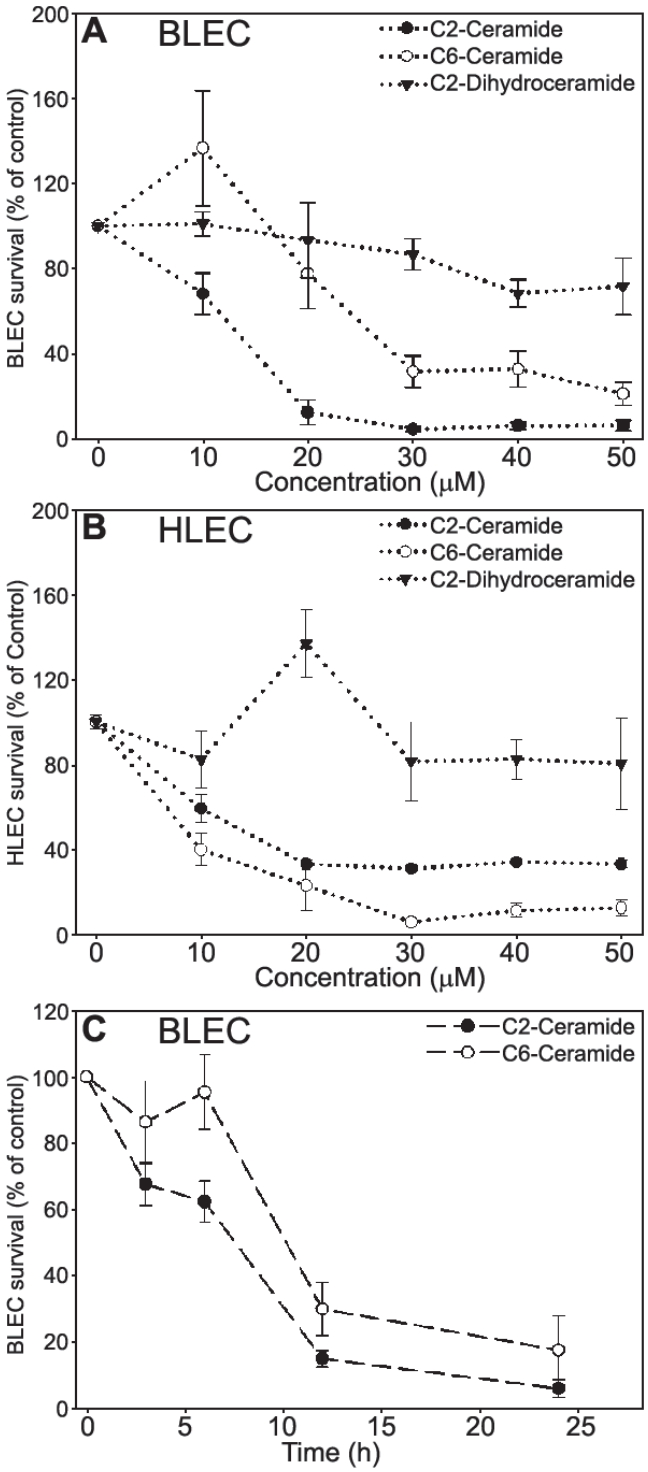

Figure 2. Ceramides reduce lens epithelial cell viability. BLECs (**A**) and HLEC (**B**) were treated with C2-ceramide, C6-ceramide, and C2-dihydroceramide (0-50 μM) for 24 h and cell viability was determined by MTT assay as described in Methods. Values expressed as percent survival of vehicle-treated controls (given as 100%). **C**: Time-dependence: BLECs were treated with either C2-ceramide or C6-ceramide (20 μM) for 3, 6, 12, and 24 h and cell viability was determined by MTT assay. Values expressed as percent survival of vehicle-treated controls (given as 100%). Data are mean±SEM of at least three independent experiments performed in triplicates. In all panels, when SEM bars are not shown, they are obscured by the symbols.

Bacterial sphingomyelinase (bSMase) has been shown to increase natural intracellular ceramide levels by hydrolyzing membrane sphingomyelin into ceramide and phosphorylcholine [7,47]. Treatment of BLEC (Figure 3A) and HLEC (Figure 3B) for 24 h with bSMase resulted in a significant, concentration-dependent suppression of cell survival. Overall, these data show that exogenous ceramide reduces the viability of lens epithelial cells in a time-dependent and concentration-dependent manner. The action of ceramides on cell viability is specific since C2-dihydroceramide had little effect on cell viability. In addition, the release of endogenous natural ceramides from lens epithelial cell membrane by bSMase treatment produced the same reduction of cell viability.

C2-ceramide induces apoptosis in lens epithelial cells: To determine the effect of ceramide on apoptosis in lens epi-

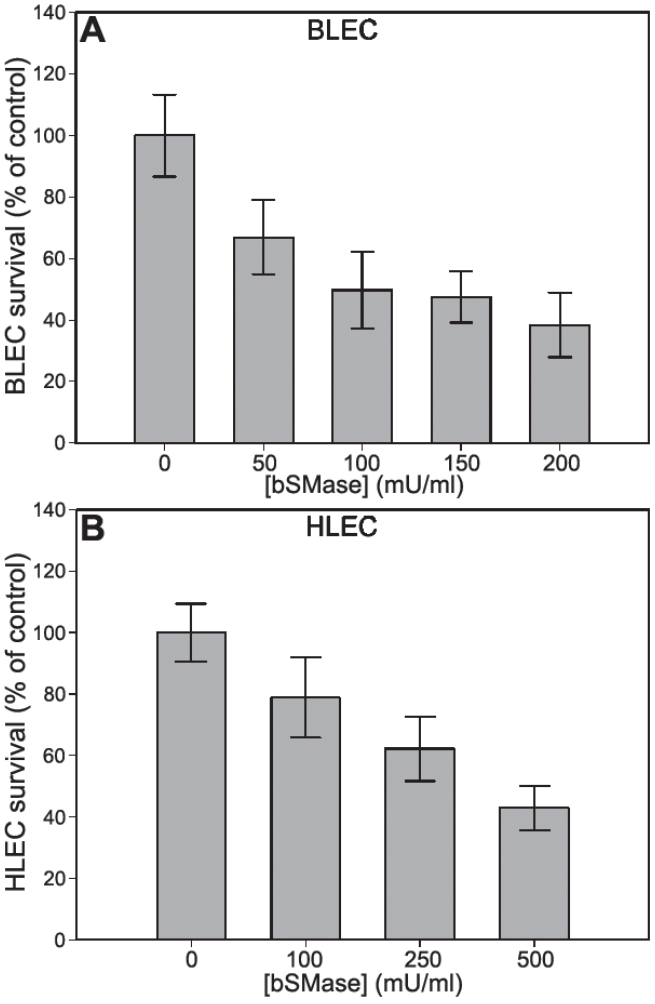

Figure 3. Bacterial sphingomyelinase suppresses lens epithelial cell survival. BLEC (**A**) and HLEC (**B**) were treated with vehicle (zero concentration) or different concentrations of bSMase for 24 h. Cell viability was determined by MTT assay as described in Methods. Values expressed as percent survival of vehicle-treated controls (given as 100%). All values reflect the mean±SEM of at least three independent experiments.

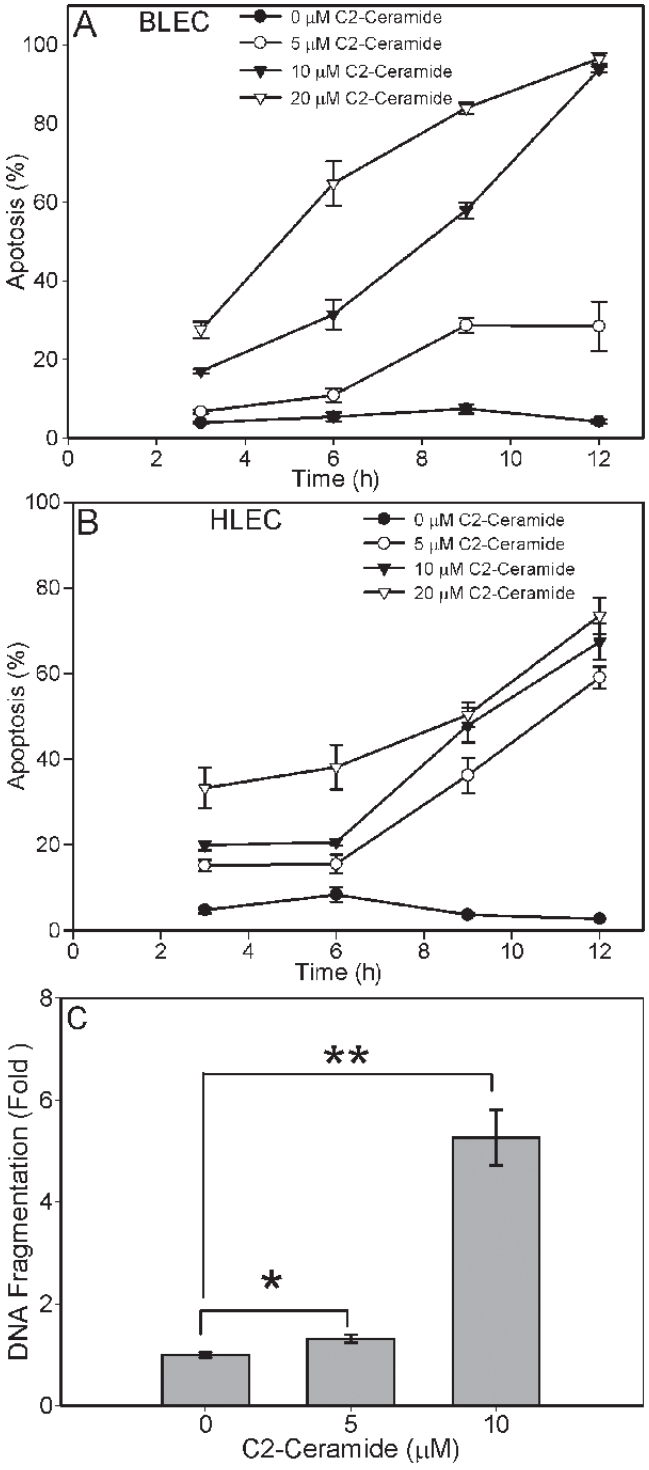

Figure 4. Ceramide induces apoptosis in lens epithelial cells. BLEC (**A**) and HLEC (**B**) were treated in the absence or presence of C2-ceramide (5-20 μM) for indicated times. Levels of apoptosis were quantitatively determined by DAPI nuclear staining as described in Methods. Values are expressed as percentage of apoptotic cells. **C** illustrates the effect of ceramide-induced apoptosis determined by Cell Death Detection^{PLUS} ELISA assay as described in Methods. BLEC were treated with C2-ceramide (0, 5, and 10 μM) for 24 h. In each case, the data represent mean±SEM from three separate experiments. The single asterisk denotes a $p<0.05$ and the double asterisk indicates a $p<0.001$.

thelial cells, BLECs and HLECs were treated with C2-ceramide (0, 5, 10, and 20 μM) and levels of apoptosis were assessed after 3 h, 6 h, 9 h, and 12 h (Figure 4). Basal levels of apoptosis was low (>10%) in vehicle-treated cells. Apoptosis was not markedly increased in BLECs treated with 5 μM C2-ceramide (Figure 4A). The maximum rate of apoptosis (26%) was achieved after 9 h of treatment and the rate did not change up to 12 h of exposure to C2-ceramide. However, addition of 10 μM, and 20 μM C2-ceramide increased apoptosis by 31.4% and 64.7%, respectively, after 6 h of treatment. Longer incubation times resulted in higher rates of apoptosis. These treatments produced characteristic morphologic changes including cell shrinkage, rounding of cells, blebbing of the cell membrane, detachment from the substratum, and cell death. Cell detachment was only observed at the higher concentration of ceramide (>20 μM) or long-term ceramide treatment (>48 h; data not shown).

Similarly, treatment of HLEC with C2-ceramide increased apoptosis. Treatment of HLECs with 5 μM C2-ceramide induced a minimal level of apoptosis at 3 h and 6 h of incubation; however treatment with 5 μM C2-ceramide increased apoptosis to 36.2% and 59.1% by 9 h and 12 h, respectively (Figure 4B). Similarly, apoptosis was increased markedly with 10 μM C2-ceramide by 9 h and 12 h of treatment. Treatment of HLECs with 20 μM C2-ceramide increased apoptosis at all time intervals (Figure 4B).

To confirm a proapoptotic action of ceramide, nuclear fragmentation was measured by Cell Death Detection ELISA^{PLUS} assay. BLECs were treated with various concentration of C2-ceramide (0, 5, and 10 μM) for 24 h. C2-ceramide increased cytoplasmic histone-associated DNA fragments of lens epithelial cells 1.3- and 5.3 fold at 5 and 10 μM C2-ceramide concentrations, respectively (Figure 4C). These results indicate that ceramide is a potent inducer of apoptosis in

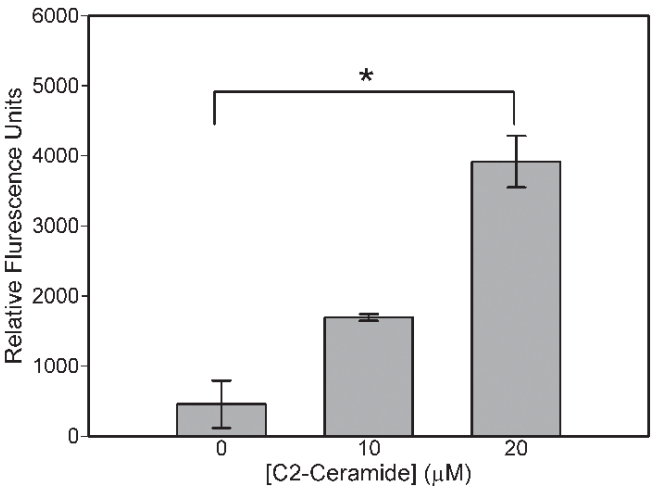

Figure 5. C2-ceramide increases caspase-3/7 activity in bovine lens epithelial cells. Cells were exposed to C2-ceramide (0-20 μM) for 12 h. Caspase-3/7 activity was measured as described in Methods. Data shown are mean±SEM values of three separate experiments. The asterisk denotes a $p<0.05$.

lens epithelial cells and its effect is concentration-dependent and time-dependent. DAPI as well as DNA fragmentation studies show that C2-ceramide at low concentrations (5 μM) have a limited apoptotic effect in BLECs whereas at higher concentrations, it significantly stimulates apoptosis in BLECs.

Ceramide induces caspase activation: Next, activation of caspase in ceramide-mediated apoptosis was determined. Treatment of BLEC with 10 μM and 20 μM of C2-ceramide for 12 h significantly increased caspase activity 3.6 fold and 7.4 fold (Figure 5). These results suggest that ceramide-mediated apoptosis of lens epithelial cells induces caspase activity and C2-ceramide activates caspase 3/7 at relatively higher concentration.

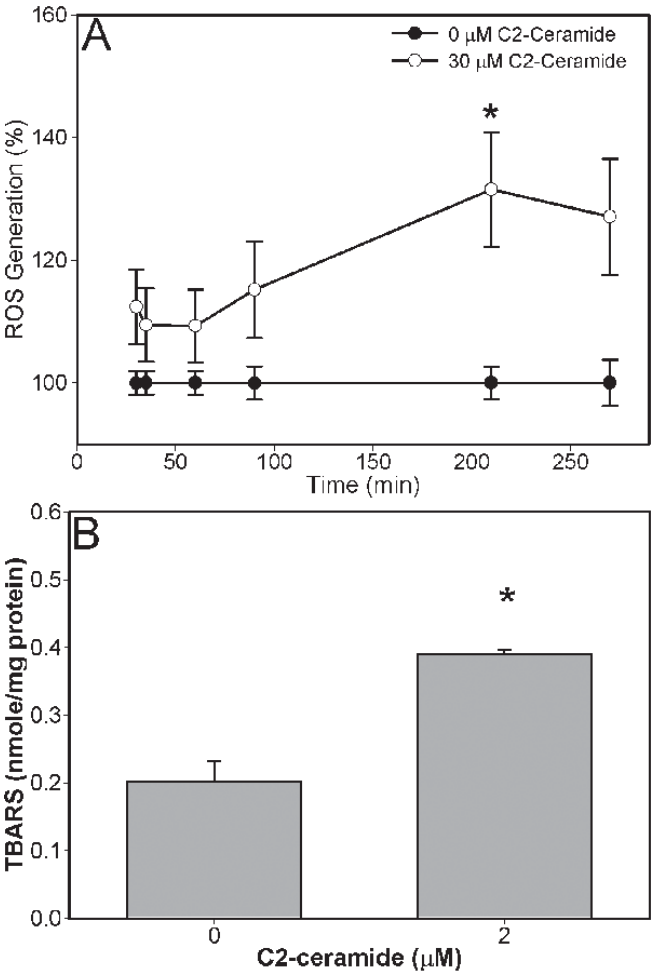

Figure 6. Ceramide increases reactive oxygen species accumulation and lipid peroxidation in bovine lens epithelial cells. **A**: BLECs were loaded with DCFH-DA at final concentration of 50 μM for 60 min. After incubation, DCFH-DA was removed, and the cells were treated with C2-ceramide (30 μM) for 30-270 min as described in Methods. The control group was treated with vehicle after loading with DCFH-DA. Data shown are mean±SEM (n=8) percentages of DCF fluorescence normalized to the control (no C2-ceramide) at each sampling time. **B**: C2-ceramide induces production of TBARS in BLECs. The cells were incubated in complete DMEM containing C2-ceramide for 24 h. Cells were washed with PBS, and TBARS assay was carried out as described in Methods. The TBARS concentration was expressed as malondialdehyde (MDA) equivalents and normalized to protein amount in the cells. Results are the average±SEM of three experiments. The asterisk denotes a $p<0.05$.

Ceramide increases oxidation stress in lens epithelial cells: To investigate whether ceramide increases the level of ROS, BLECs were exposed to 30 μM C2-ceramide for various time intervals. The ROS in the cells was made visible by 2',7'-dichlorodihydrofluorescien diacetate (H_2-DCFH-DA). C2-ceramide induced time-dependent increases in DCF staining (Figure 6A). Additionally, ceramide-mediated oxidation was determined by lipid peroxidation. Oxidation results in the production of lipid radicals which subsequently produces complex mixture of lipid degradation products (malondialdehyde; MDA, and other aldehydes). Lens epithelial cells exposed to C2-ceramide induce lipid peroxidation production (Figure 6B). These results indicate that ceramide induces oxidative stress in lens epithelial cells.

DISCUSSION

The present work shows that exogenously-supplied C2-ceramide and C6-ceramide decreased cell viability, induced apoptosis, and increased generation of ROS in human and bovine lens epithelial cells grown in culture. It was also shown that exogenously-supplied sphingomyelinase (which causes release of natural ceramides from the plasma membrane) reduced survival of cultured lens epithelial cells.

Ultraviolet radiation and oxidative stress are among the important age-associated cataract-inducing agents [48]. The lens has unusually high concentrations of glutathione and an active glutathione redox cycle is apparently important in protecting the lens from ROS [48] and in maintaining lens transparency [49]. By producing glutathione, lens epithelium and superficial cortex detoxify potentially damaging effects of H_2O_2 and dehydroascorbic acid [48]. The low ratio of glutathione to protein-SH makes the aging lens more sensitive to oxidative stress. Another important but frequently overlooked outcome of low glutathione levels and UV radiation is activation of sphingomyelinase and increased ceramide production by the lens [50-53]. Sphingomyelinase activity has been reported in the lens epithelial, cortical, and nuclear regions [36-38]. The present work would suggest that the increase in ceramide production by low glutathione and UV radiation might result in cell death through apoptosis and ROS production.

The relationship between lens epithelial cell death and cataract formation is controversial. Some reports provide evidence that apoptosis is not observed in cataractous lens [54,55] while other investigators have found lens epithelial cell death in cataractous lens and have suggested that this to be a cause of cataract formation [56,57]. Additional studies using animal models will be needed to determine the effect of ceramides on cell death and cataract formation in intact lens.

Due to their solubility, short-chain ceramides were used in this study. However, the lens epithelial cell membrane sphingomyelin contains predominantly C16 to C24 acyl groups [58,59]. Several lines of evidence suggest that our work with short-chain ceramides is applicable to the in vivo situation.

First, both short- and long-chain ceramides can cause release of cytochrome C from mitochondria and cause apoptosis [20,22,60,61]. Second, the addition of short-chain ceramide (C6-ceramide) significantly increased the amount of endogenous long-chain ceramide through recycling of the sphingosine backbone of C6-ceramide via deacylation/reacylation [62,63]. Third and most importantly, the addition of sphingomyelinase to our HLEC and BLEC cultures caused a dose-dependent decrease in cell viability (Figure 3).

Our data shows that exogenously supplied ceramide results in an increase in ROS in epithelial cells. Other reports have supported ceramide-mediated oxidative stress [64,65]. Acid sphingomyelinase (aSMase) activation in endothelial cells increased NAD(P)H oxidase fraction $gp91^{phox}$ protein levels and enzyme activity in lipid raft-enriched fractions [66,67]. Formation of a lipid raft redox signaling platform and endothelial dysfunction was significantly decreased using the siRNA strategy to reduce aSMase activity [67]. This indicated the importance of aSMase in mediating and modulating the formation of lipid raft signaling platform in coronary endothelial cells. Similarly, ceramide-mediated increase in NAD(P)H oxidase activity, and production of radical oxygen species was reported in glomerular mesangial cells [68]. Therefore, it seems that ceramide-rich membrane domains may become platforms for activation of oxidative enzymes.

Because the lens is an avascular organ, ROS produced by lens epithelial cells in response to ceramide would remain in the lens and result in a decrease in glutathione levels. The decreased glutathione levels would activate sphingomyelinase in the lens [50,51] and cause an increase in ceramide levels. Ceramides released from the cortical and nuclear region may have adverse effects on the lens epithelial region including ROS production and increased oxidative stress. The ROS produced by the lens epithelial cells might also diffuse into the cortical lens fibers and contribute to the formation of light-scattering protein aggregates [69].

In addition to induction of apoptosis, ceramides might also contribute to cataract formation by altering membrane structure and function. Sphingolipids account for greater than 50% of total human lens phospholipids and increase in aging and cataract [34,70]. Elevated sphingolipid levels in lens membrane, along with a stress-induced increase in ceramide levels, may alter membrane lipid domains. Investigators have found that ceramide stabilized lipid rafts and displaced cholesterol from these domains [71,72]. Displacement of cholesterol from lipid rafts is significant (about 50%) when levels of ceramide are increased in the total bilayers [73,74] leading to altered membrane structure and function. Digestion of 25% of plasma membrane sphingomyelin and generation of ceramide led to loss of 50% of plasma membrane cholesterol [74] enhancing glucose uptake with no significant changes in the abundance of GLUT-1 at the cell membrane. Correlation between membrane ceramide levels and glucose uptake in the lens epithelial cells and aging lens requires further study.

The present study has shown that ceramides cause decreased cell viability, increased apoptosis, and increased ROS in cultured lens epithelial cells. The relationship between these in vitro effects and the formation of cataracts in vivo needs to be studied further.

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