Activation of Caspase-8 and Caspase-12 Pathways by 7-Ketocholesterol in Human Retinal Pigment Epithelial Cells

Saurabh Lutbra, Babak Fardin, Joyce Dong, Dieter Hertzog, Sami Kamjoo, Simon Gebremariam, Virit Butani, Raja Narayanan, Jeannie K. Mungcal, Baruch D. Kuppermann, and M. Cristina Kenney

PURPOSE. To determine whether caspase or cathepsin pathways are activated in human retinal pigment epithelial cells (ARPE-19) after exposure to 7-ketocholesterol (7kCh).

METHODS. ARPE-19 cells were exposed to 7kCh with or without z-VAD-fmk, a pan-caspase inhibitor. Caspase-3, -8, and -9 activities were measured by a fluorochrome inhibitor of caspase (FLICA) assay. Caspase-12 activity was detected by Western blotting. RT-PCR was performed for 18s, mortalin-2, cathepsins B, D, and L/V2.

RESULTS. At 24 hours, 7kCh-treated cultures had increased caspase-8 (P < 0.001) and caspase-3 (P < 0.001) activities compared with vehicle-treated cultures. 7kCh-induced caspase-3 activation was blocked by z-VAD-fmk (P < 0.001). Caspase-9 was not activated by 7kCh treatment (P > 0.05). Procaspase-12 was cleaved into its active form after treatment with 7kCh for 24 hours. At 6 hours, the RNA level for mortalin-2, a pro-survival gene, was upregulated. ARPE-19 cells did not express RNA for cathepsins B, D, or L/V2 under any conditions.

CONCLUSIONS. In ARPE-19 cells, 7kCh-induced apoptosis uses the receptor-mediated caspase-8 pathway and the endoplasmic reticulum stress-induced caspase-12 pathway but not the mitochondrial caspase-9 pathway. The cathepsin pathways are not involved in 7kCh-induced cell death. These data demonstrate that 7kCh causes a loss of cell viability through caspase-dependent apoptosis and can act as an oxidative stressor leading to retinal pigment epithelial cell atrophy. Elucidating the specific apoptotic pathways involved may have therapeutic potential for AMD and other retinal diseases. (Invest Ophthal Vis Sci. 2006;47:5569–5575) DOI:10.1167/iovs.06-0333

Age-related macular degeneration (AMD) is the leading cause of irreversible central vision loss in persons 65 and older in developed countries. Oxidative stress is thought to be an important contributing factor to the initiation and progression of AMD. The retina is vulnerable to oxidative stress because of its exposure to light and oxygen. Therefore, antioxidants have received significant attention recently for the treatment and management of AMD. In particular, damage to the retinal pigment epithelium by oxidative stress is an early event in AMD. This damage involves the mitochondria, specifically mtDNA, leading to destabilization of mitochondrial function and induction of apoptosis. In addition, analysis of postmortem eyes of patients with AMD suggests that retinal pigment epithelial cells (RPE), photoreceptors, and inner nuclear layer cells die by apoptosis.

Increasing evidence indicates that cholesterol plays a putative role in the pathogenesis of AMD, and studies suggest that antichocholesterol medications may decrease the risk for AMD. Oxidized cholesterol (oxysterols) increase the formation of reactive oxygen species (ROS). In human cataractous lenses, 7-ketocholesterol (7kCh) is the predominant oxysterol, but the physiological implications of this accumulation are unknown. Moreover, 7kCh with or without low-density lipoprotein (LDL) can induce apoptosis, but the caspase pathways involved are unknown.

Apoptosis refers to programmed cell death that does not result in the activation of an inflammatory response. It involves complex signaling cascades that result in the breakdown and packaging of cellular components and their subsequent removal by surrounding structures. The execution phase of apoptosis is triggered and mediated by a family of cysteine proteases known as caspases, which are categorized into initiator caspases (caspase-2, -8, -9, -12) and effector (downstream) caspases (caspase-3, -6, -7).

The initiator caspases depend on two well-described pathways involved in activating apoptosis: the intrinsic (mitochondrial) pathway and the extrinsic (receptor-mediated) pathway. The extrinsic pathway involves the activation of cell-surface receptors such as Fas and Fas-ligand and subsequently activates caspase-8. Mitochondrial activation of apoptosis, on the other hand, involves the activation of caspase-2 and -9 as a result of mitochondrial stress, not cell surface receptor activity. Intrinsic and extrinsic pathways lead to the activation of downstream effector caspases (caspase-3, -6, -7) and subsequently to cell death. It is unknown which caspase pathways are activated by 7kCh treatment in human RPE cells.

Caspase-12 is a recently described initiator caspase. Once activated, it induces the cleavage of caspase-3 in a cytochrome c-independent manner. Caspase-12 is specifically activated in cells subjected to endoplasmic reticulum stress. Of note, endoplasmic reticulum stress has received growing attention because it causes pathologically relevant apoptosis and is implicated in neurodegenerative disorders. Until now, caspase-12 activation in human RPE cells in vitro has not been investigated.

Cathepsins can activate caspases and initiate apoptosis. Cathepsins B and L (cysteine proteases) and cathepsin D (aspartic protease) are abundant in cellular lysosomes. Extracts of these lysosomes can cleave Bid, leading to subsequent mitochondrial dysfunction and cytochrome c release. With ox-
ative stress, cathepsin D can translocate to the cytosol and cause apoptosis. To date, no study has examined the cathepsins in ARPE-19 cells that are undergoing oxidative stress-related apoptosis.

In the past, using an apolipoprotein E (Apo-E)–deficient mouse model, our laboratory had demonstrated that a high-cholesterol diet caused apoptosis, loss of retinal morphology (EM), and decreased retinal function. In human ARPE-19 cell cultures, 7kCh causes apoptosis in a time- and dose-dependent manner. However, the specific apoptotic pathways involved in these events remain unknown. In this study, using 40 μg/mL 7kCh—a dose of 7kCh that induces apoptosis—we demonstrate that caspase-8, -12, and -3 but not caspase-9 are activated in the treated ARPE-19 cultures. In addition, the expression of mortalin-2, a heat shock protein (HSP) prosurvival gene, is upregulated temporarily, but the expression of cathepsins B, D, and L/V2 is unchanged. These data support our hypothesis that oxysterols can damage RPE cells through apoptosis and may play a role in retinal diseases.

**Materials and Methods**

**Cell Culture**

Cells from the human retinal pigment epithelial line ARPE-19 were obtained (ATCC, Manassas, VA) for study. Cells were grown in a 1:1 mixture (vol/vol) of Dulbecco’s Modified Eagle’s Medium with Ham’s F-12 nutrient medium (DMEM F-12; Gibco, Carlsbad, CA), nonessential amino acids 10 mM 1×, 0.37% sodium bicarbonate, 0.058% t-glutamine, 10% fetal bovine serum, and antibiotics (penicillin G 100 U/mL, streptomycin sulfate 0.1 mg/mL, gentamicin 10 μg/mL, amphotericin B [Fungizone] 2.5 μg/mL). The cells were plated in 24-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) at 2 × 10⁵ cells per well and were incubated at 37°C in 5% CO₂ to reach confluence before they were exposed to 7kCh (Sigma Aldrich, St. Louis, MO).

**Exposure to 7-Ketocholesterol**

ARPE-19 cells were incubated for 2, 6, and 24 hours with 7kCh (40 μg/mL) dissolved in dimethyl sulfoxide (DMSO). One hour before exposure to 7kCh, some cells were pretreated with 20 μM z-VAD-fmk (N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; Calbiochem, San Diego, CA), a pan-caspase inhibitor.

**Caspase Detection**

Caspase-3, -8, and -9 activities were detected with the use of detection kits (Carboxyfluorescin FLICA Apoptosis Detection Kits; Immunochemistry Technologies LLC, Bloomington, MN). ARPE-19 cells containing the bound fluorochrome-inhibitor of caspase (FLICA) were analyzed by fluorometry for quantitation and fluorescence microscopy for qualitative analysis. The FLICA reagent has an optimal excitation range from 488 to 492 nm and an emission range from 515 to 535 nm. Apoptosis was quantified as the level of fluorescence emitted from FLICA probes bound to caspases. Nonapoptotic cells appeared unstained, whereas cells undergoing apoptosis fluoresced brightly.

At the designated time period, the wells were rinsed briefly with fresh culture media, replaced with 300 μL/well of 1× FLICA solution in culture media, and incubated at 37°C for 1 hour under 5% CO₂. Cells were washed with phosphate-buffered saline (PBS). The following controls were included: untreated ARPE-19 cells without FLICA to exclude autofluorescence from cells; untreated ARPE-19 cells with FLICA for comparison of caspase activity of treated cells; untreated ARPE-19 cells with negative control z-Fa-fmk (N-benzyloxycarbonyl-Phe-Ala-fluoromethylketone; Calbiochem) and FLICA as a control for the pan-caspase inhibitor (z-VAD-fmk); tissue culture plate wells without cells with buffer alone to represent the background levels; tissue culture plate wells without cells with culture media + DMSO to exclude cross-reaction of FLICA with DMSO + culture media; ARPE-19 cells with DMSO and FLICA to account for any cross-fluorescence between untreated cells and DMSO.

Quantitative calculations of caspase activities were performed with a fluorescence image scanning unit instrument (FMBIO III; Hitachi, Yokohama, Japan). The caspase activity was measured as average signal intensity of the fluorescence of the pixels in a designated spot—Mean Signal Intensity (msi). Cultures were observed through an inverted fluorescent microscope (Leica, Solms, Germany) with a bandpass filter (excitation 490 nm, emission 520 nm) to view the green fluorescence of caspase-positive cells. Images were captured with a digital camera (MicroFire; Optronics, Goleta, CA).

**Western Blot Analyses**

Protein was extracted from ARPE-19 cultures by the addition of 0.3 mL 100% ethanol for 3 minutes at room temperature and then centrifugation at 420 g for 5 minutes at 4°C. Isopropanol (1.5 mL) was added to the supernatant for 10 minutes at room temperature and was followed by centrifugation at 12,900g for 10 minutes at 4°C. Protein pellets were washed 3 times in 0.3 M guanidine hydrochloride in 95% ethanol, dried under vacuum, and stored at −70°C until further use.

Protein concentrations were determined by the BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein (50 μg) were electrophoresed on precast 4% to 20% Tris-glycine sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels (Invitrogen, Carlsbad, CA) and were transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). The membranes were blocked for 1 hour at 4°C with 5% powdered milk containing Tris-saline and 0.5% Tween 20. After blocking, the membranes were incubated at 4°C overnight with the primary antibody caspase-12 (rabbit anti-human antibody, 1:500 dilution; eBioScience, San Diego, CA). Membranes were washed with Tween-Tris-buffered saline (TTBS) and were incubated for 1 hour at room temperature with horseradish peroxidase (HRP)–conjugated secondary goat anti-rabbit IgG (Chemicon, Temecula, CA). Membranes were developed with Super Signal West Femto Maximum Sensitivity Substrate solution, and bands were visualized with enhanced chemiluminescence (Pierce).

**RNA Extraction**

RNA from ARPE-19 cell cultures with and without 7kCh was extracted (RNeasy Mini kit; Qiagen, Valencia, CA) and quantified (2100 Bioanalyzer nanoRNA protocol; Agilent, Palo Alto, CA).

**Reverse-Transcribed–Polymerase Chain Reaction**

RNA (250 ng) was reverse transcribed into cDNA using random primers and Superscript II reverse transcriptase according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). PCR was carried out with various primers (Table 1), 1 μL cDNA, and Taq DNA polymerase according to the manufacturer’s protocol (Invitrogen). Amplified products were resolved by electrophoresis in 1.5% agarose gels and visualized under ultraviolet light after staining with ethidium bromide. Routine PCR controls without cDNA (water control) were negative. Gels were scanned with the fluorescence image scanning unit instrument (FMBIO III; Hitachi). The entire experiment was repeated 3 times.

**Statistical Analysis**

Caspase data were subjected to statistical analysis by ANOVA (GraphPad Prism 3.0 version statistics program; GraphPad Software Inc., San Diego, CA). Newman-Keuls multiple comparison test was performed to compare the data within each experiment. For densitometric analysis for mortalin, we used commercially available software (ImageQuant Version 5.0; Molecular Dynamics, Inc., Sunnyvale, CA). Data are presented as mean ± SEM. Experiments were performed in triplicate. P < 0.05 was considered statistically significant.
RESULTS

Caspase-8 activity was increased in ARPE-19 cells treated with 7kCh for 2 hours, 6 hours, and 24 hours compared with vehicle-treated control cultures (26,803 ± 2541 msi vs. 2440 ± 826 msi; 22,866 ± 2955 msi vs. -899 ± 516 msi; and 37,134 ± 2910 msi vs. 1409 ± 799 msi, respectively; *P < 0.001; Fig. 1A). Cell cultures examined by fluorescence microscopy showed a significant number of caspase-8-positive cells after 24 hours (Fig. 1B).

Minimal caspase-9 activity occurred in ARPE-19 cells with or without 7kCh treatment (Fig. 2). Caspase-9 activity in ARPE-19 cells showed an increased number of cells that were FLICA positive, representing increased caspase-8 activity compared with untreated cultures. ***P < 0.001; statistically significant.

Caspase-9 activity in the 7kCh-treated ARPE-19 cultures was similar to that in the DMSO-treated ARPE-19 cultures at 2 hours (6348 ± 1280 msi vs. 7174 ± 1195 msi), 6 hours (8475 ± 1235 msi vs. 8631 ± 1978 msi), and 24 hours (6454 ± 1792 msi vs. 4740 ± 568 msi, respectively).

Caspase-3 activity increased significantly after 7kCh treatment (Fig. 3A). ARPE-19 cells treated with 7kCh for 2 hours, 6 hours, and 24 hours had elevated caspase-3 activity compared with vehicle-treated cultures (10,829 ± 352 msi vs. 1229 ± 172 msi; 12,220 ± 355 msi vs. 1959 ± 1280 msi and 13,547 ± 1043 msi vs. 2289 ± 589 msi, respectively; *P < 0.001). Untreated ARPE-19 cell cultures had negligible caspase-3 activity (68 ± 362 msi).

The 7kCh-induced caspase-3 activity was blocked by z-VAD-fmk, a pan-caspase inhibitor. ARPE-19 cells pretreated for 1 hour with 20μM z-VAD-fmk and then treated for 24 hours with 7kCh and z-VAD-fmk had a caspase-3 activity of 2025 ± 2281 msi, whereas ARPE-19 cells treated for 24 hours with 7kCh showed caspase-3 activity of 20,164 ± 2957 msi (P < 0.001; Fig. 3B). Caspase-3 activities in the untreated ARPE-19 cultures (5011 ± 2031 msi), DMSO-treated cultures (8562 ± 1757 msi), and z-FA-fmk-treated cultures, which served as the negative control (6000 ± 974 msi), were all significantly lower than the 7kCh-treated cultures (P < 0.01).

Caspase-12 was activated after 7kCh treatment in ARPE-19 cultures. Caspase-12 activation was identified using Western blot techniques because FLICA assay kits for caspase-12 were unavailable at the time. Figure 4 is a representative Western blot showing caspase-12 activation after 7kCh treatment in ARPE-19 cells. The procaspase (P)-12 appeared as a 55-kDa band and its cleaved (C) product was a 42-kDa band (Fig. 4). Untreated ARPE-19 cultures (lane 1) and ARPE-19 cultures treated with 7kCh for 2 hours (lane 2) lacked the caspase-12 band. After 24-hour 7kCh treatment, the ARPE-19 cells (lane 3) showed the 55-kDa band representing procaspase-12 and the cleaved 42-kDa band.

ARPE-19 cultures (Fig. 5A) showed an approximate fourfold increase in RNA levels for mortalin-2, an HSP prosurvival gene,

<table>
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<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Size (bp)</th>
<th>PCR Annealing Temperature (°C)</th>
<th>MgCl₂ (mM)</th>
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<td>TCGGATGAGCTGGTCAACTAG</td>
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<tr>
<td>CAT-D</td>
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<td>AGGAGCTTGCTGACGGAAT</td>
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<td>CAT-L/V2</td>
<td>TACCGCTTTGAAGGAGGAAT</td>
<td>AGAATAAAGCATGACCTTTGA</td>
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<td>3</td>
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<tr>
<td>MORT-2</td>
<td>TACTACCGAACTCTGCGTGCGGGATGATGAGA</td>
<td>CAAATGGCTCATTGCGAATGAGAATA</td>
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<td>GGACATCTAAGGGCATACAGAC</td>
<td>400</td>
<td>53</td>
<td>2</td>
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</tbody>
</table>

CAT-B, cathepsin B; CAT-D, cathepsin D; CAT L/V2, cathepsin L/V2; MORT-2, mortalin-2.
induced apoptosis in human ARPE-19 cells. Using 40 μg/mL 7kCh, ARPE-19 cells had significantly higher caspase-3 activity at 2 hours (10.829 ± 352 msi vs. 1229 ± 172 msi; ***P < 0.001), 6 hours (12.220 ± 355 msi vs. 1959 ± 1280 msi; ***P < 0.001), and 24 hours (15.547 ± 1043 msi vs. 2289 ± 589 msi; ***P < 0.001) than DMSO-treated cells. Untreated ARPE-19 cells had negligible caspase-3 activity (68 ± 362 msi). (B) The 7kCh-induced caspase-3 activity after 24-hour exposure was blocked by z-VAD-fmk, a pan-caspase inhibitor. Caspase-3 activity was significantly increased in 7kCh-treated cells compared with DMSO-treated cells (20.164 ± 2957 msi vs. 8562 ± 1757 msi; ***P < 0.001). After incubation with z-VAD-fmk, caspase-3 activation was blocked (2025 ± 2281 msi; ***P < 0.001), and values were comparable to those of DMSO-treated ARPE-19 cells (8562 ± 1757 msi). Untreated ARPE-19 cells (5011 ± 2051 msi) and z-FA-fmk-treated cells, which served as the negative control (6000 ± 974 msi). ***P < 0.001; ***P < 0.001.

after 6-hour incubation with 7kCh. For this experiment, the relative densitometry values for DMSO-treated culture was 1.0, whereas for the 7kCh-treated samples were 0.93 for 2 hours, 1.2 for 6 hours, and 1.2 for the 24 hours. ARPE-19 cells lacked RNA expression for cathepsin D (Fig. 5B), cathepsin B, and cathepsin L/V2 (data not shown) when cultured with or without 7kCh.

DISCUSSION

The present study examined the pathways involved in 7kCh-induced apoptosis in human ARPE-19 cells. Using 40 μg/mL 7kCh, a dose known to induce apoptosis, we showed that the extrinsic caspase-8 pathway and the endoplasmic reticulum caspase-12 pathway were activated but that the mitochondrial caspase-9 pathway was not involved. In addition, the non-caspase cathepsin pathways were not activated by 7kCh treatment. The study is unique because it examines not only the caspase pathways involved in human ARPE-19 apoptosis but also the caspase-independent 7kCh pathways. Our data support the hypothesis that oxysterols such as 7kCh can cause loss of cell viability by caspase-dependent apoptosis and can be environmental oxidative stressors that lead to RPE cell atrophy. Furthermore, elucidating the specific apoptotic pathways involved may have therapeutic potential for AMD and other retinal diseases.

Caspase-3 Activity

In response to 7kCh, human ARPE-19 cells undergo apoptosis, as reflected by increased caspase-3 activity. Significantly more caspase-3 activity was observed in ARPE-19 cells treated with 7kCh for 2, 6, and 24 hours (P < 0.001) compared with vehicle-treated samples. This is in agreement with studies of U937—promonocytic leukemia cells—showing caspase-3 activity after treatment with 100 μM (40 μg/mL) 7kCh for 24 hours. In contrast, other cell types, such as ECV304, a human bladder cancer cell line, respond to 7kCh by undergoing necrotic cell death without caspase-3 activation. In our study, caspase-3 activity could be blocked by z-VAD-fmk, providing additional evidence that the caspase pathways are involved in 7kCh-induced apoptosis. Complete inhibition of caspase-3 activity by z-VAD-fmk suggested that 7kCh-induced apoptosis in ARPE-19 cells occurs solely through the caspase pathways.

We realize that the ARPE-19 cells were not fully differentiated and that perhaps fully differentiated RPE cells or hRPE...
cells might have responded differently to 7kCh-induced apoptosis. However, the fully differentiated ARPE-19 cells required conditions of low serum levels for extended culture periods, which can upregulate proapoptotic proteins. Because our goal was to identify the apoptotic pathways for 7kCh, the additional source of stress could have confounded our results. In addition, in cultures of human microvascular endothelial cells and rat neural–retinal precursor (R28) cells, the caspase-8 and caspase-12 pathways were also activated after 7kCh treatment (data not shown). Therefore, we thought that even the differentiated RPE cells might use the same caspase pathways after 7kCh treatment.

**Caspase-8 Activity**

In human ARPE-19 cultures, caspase-8 was activated after 7kCh treatment. This is in agreement with previous findings that Fas-mediated apoptosis played a role in oxidant-induced cell death in human RPE cells. This signaling pathway of TNF-α or Fas-mediated apoptosis has been well described in numerous cell types, including RPE cells. In our study, we found both caspase-8 and caspase-12 activation, indicating that multiple pathways are involved in the response to 7kCh treatment. This is consistent with another human RPE cell culture study that showed blocking Fas/FasL interactions using an anti-Fas antibody could inhibit apoptosis, but only partially. Those findings implied that in addition to caspase-8, other apoptotic pathway(s) were involved in oxidant-induced apoptosis. Our data suggest that 7kCh-induced cell death not only occurred through the cell-surface death receptors (caspase-8 pathway) but also involved endoplasmic reticulum stress and, hence, caspase-12 activation. The interactions between caspase-8 and caspase-12 pathways that caused apoptosis in ARPE-19 cells still must be determined.

**Caspase-12 Activity**

Caspase-12 is representative of the endoplasmic reticulum stress-induced cell-signaling pathway. Inactive caspase-12 is localized to the cytosolic face of the endoplasmic reticulum and, once activated, can trigger caspase-3 activation. After 7kCh treatment, the ARPE-19 cultures showed caspase-12 activation, pointing to involvement of the endoplasmic reticulum pathway for apoptosis. This is in agreement with studies of human promonocytic U937 cells showing that 7kCh can stimulate components of the endoplasmic reticulum and apoptosis. To the best of our knowledge, this is the first study confirming caspase-12 involvement in apoptosis in human retinal cells.

**Caspase-9 Activity**

Caspase-9 activities were not changed in the 7kCh-treated ARPE-19 cultures, indicating that the mitochondrial pathway for apoptosis was not involved. Our data differ from those of primary RPE cells studies, which demonstrated that oxidant-induced apoptosis released cytochrome c and implied mitochondrial pathway involvement. In our experiments, the lack of caspase-9 activity might have been caused in part by the presence of heat shock proteins (HSPs), which are abundant in RPE cells and can interfere with caspase-9 cleavage during apoptosis assembly. For example, αβ-crystallin, an HSP, is constitutively expressed in RPE cells, and its expression is increased with oxidative stress. Furthermore, αβ-crystallin may function as a stress-inducible, antiapoptotic protein in human RPE cells. Future studies will examine whether the absence of caspase-9 activity is the result of high HSP levels in ARPE-19 cells.

Interestingly, we found that another HSP family member, mortalin-2, was upregulated in the 7kCh-treated ARPE-19 cultures. Mortalin-2 is an HSP70 family member inducible in response to oxidative stress, and it can promote cell survival. Mortalin-2 can inactivate the proapoptotic tumor suppressor gene p53, which thereby delays or inhibits apoptosis and extends the lifespan of the cells. Mortalin-2 is localized to subcellular sites, including endoplasmic reticulum, cytosol, plasma membranes, and mitochondria. We speculate that the increased mortalin-2 RNA expression found in 7kCh-treated ARPE-19 cultures may be a protective response by the cell to avoid apoptosis. However, further studies will be required to elucidate the role of mortalin-2 in 7kCh-induced apoptosis.

More than 60 oxyysterols have been described, but only some are cytotoxic. 7kCh is among the most toxic in various cell culture systems, causing increased ROS production and apoptosis. At lower concentrations, 7kCh (10 μg/mL) can stimulate differentiation and inhibit proliferation of bovine lens capsule epithelial cells. Rodriguez et al. showed that the oxidation of cholesterol yields oxyysterols in the 7-carbon position; these include 7kCh, one of the most toxic elements in oxidized low-density lipoprotein (oxLDL). The concentration of 100 μM (40 μg/mL) 7kCh has been reported for plasma concentrations in humans after a meal rich in oxyysterols. However, the appropriate concentration of 7kCh and whether it should be mixed with LDL are matters of controversy in vitro experiments. Ong et al. showed ARPE-19 cells underwent apoptosis after treatment with 50 μg/mL (125 μM) 7kCh. At a concentration of 40 μg/mL (100 μM) 7kCh without an LDL carrier, the U937 cells showed 30% apoptosis, and ECV304 cells showed 50% loss of cell viability. Recently, Rodriguez et al. reported that ARPE-19 cells internalized LDL and oxLDL and described a new method to study oxysterol toxicity that delivered a mixture of LDL and specific oxysterol to the culture system. The present study did not use the LDL–7kCh combination; rather, it applied 7kCh alone at concentrations known to induce apoptosis to the cells. Although some may argue that this is an unlikely in vivo mechanism, our demonstration that 7kCh increased caspase-8, -12, and -3 activities suggests that the 7kCh was internalized into the ARPE-19 cells, perhaps through carriers provided in the serum.

This study also examined the expression of cathepsins, which represent a caspase-independent pathway for apoptosis. Cathepsins are lysosomal proteases that can act as mediators of apoptosis by triggering mitochondrial dysfunction and cleavage of Bid. In the present study, ARPE-19 cells treated with or without 7kCh did not express cathepsins D, B, or L/V2. This indicates that the cathepsin involvement with apoptosis found in other cell cultures is lacking in the human ARPE-19 cell system. We were surprised that human ARPE-19 cells lacked cathepsin RNA because cathepsin activity is abundant in RPE cells of Long Evans rats and in human corneal stromal fibroblasts (data not shown).

Apoptosis plays a pivotal role in normal retinal development and in the molecular pathophysiology of various retinal conditions. Specific apoptotic pathways have been examined in multiple retinal conditions, such as AMD, retinal detachment, diabetic retinopathy, proliferative vitreoretinopathy, cytomegalovirus (CMV) retinitis, and cancer-associated retinopathy. Oxyysterols can damage RPE cells through apoptosis, and this might play a role in retinal degeneration. Furthermore, LDL, which is internalized by RPE cells, accumulates in Bruch membrane and can be oxidized into cytotoxic oxLDL, with 7kCh the predominant and most toxic form. We hypothesize that AMD has characteristics similar to those of cardiovascular diseases. We speculate that within the eye, cholesterol undergoes oxidation to oxyysterols, including 7kCh, that are cytotoxic to RPE cells, macrophages, and vascular cells and cause regions of localized inflammation. Eventually, RPE cells undergo apoptosis, leading to regional atrophy and affect-
ing the adjacent retina. Dissecting the mechanism(s) of specific apoptotic pathways involved may allow us to identify a therapeutic target for AMD and other retinal disorders.

References


