Published in final edited form as:

Curr Eye Res. 2013 November ; 38(11): 1159–1171. doi:10.3109/02713683.2013.811257.

Differential Effects of the Estrogen Receptor Agonist Estradiol on Toxicity Induced by Enzymatically-Derived or Autoxidation-Derived Oxysterols in Human ARPE-19 Cells

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Abstract

Purpose/aim of the study—Disturbances in cholesterol metabolism and increased levels of cholesterol oxidation products (oxysterols) in retina may contribute to age-related macular degeneration (AMD). The role of oxysterols or of their target receptors liver $\times$ receptors (LXRs) and estrogen receptors (ERs) in the pathogenesis of MD is ill-known. The purpose of this study is to determine the extent to which the oxysterols 27-hydroxycholesterol (27-OHC), 25-hydroxycholesterol (25-OHC) and 7-ketocholesterol (7-KC) affect the transcriptional activity of LXR and ER.

Materials and methods—ARPE-19 cells, untreated or incubated with 27-OHC, 25-OHC or 7-KC for 24 h were harvested. We used Western blot analyses for detecting ERs and LXRs expression, dual luciferase assays for measuring LXRs and ERs transcriptional activity, cytotox-ONE homogeneous membrane integrity assay for measuring cytotoxicity, JC-1 method for measuring mitochondrial membrane potential changes and ELISA for measuring cytokine levels.

Results—Both LXRs and ERs are expressed and are transcriptionally active in ARPE-19 cells. 27-OHC, 25-OHC and 7-KC inhibited ER-mediated transcriptional activity, whereas 27-OHC and 25-OHC increased LXR-mediated transcription. E2 reduced 25-OHC and 27-OHC-induced cytotoxicity, mitochondrial permeability potential decline, and cytokine secretion. The LXR agonist GW3965 or the LXR antagonist 5 α -6 α epoxycholesterol-3-sulfate (ECHS) did not offer protection against either 27-OHC and 25-OHC or 7-KC.

Conclusions—Increased levels of oxysterols can decrease ER and increase LXR signaling. ER agonists can offer protection against cytotoxic effects of 27-OHC and 25-OHC, two oxysterols derived by enzymatic reactions. Although they exert similar toxicity, the cellular mechanisms

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

involved in the toxic effects of oxysterols whether derived by enzymatic or autoxidation reactions appear to be different.

Keywords

Age-related macular degeneration; estrogen receptor; IL-6; liver $\times$ receptor; oxysterols; TNF- α

INTRODUCTION

Age-related macular degeneration (AMD) is the most common cause of irreversible vision loss in elderly population^{1,2} and accounts for half of all new cases of registered blindness.³ AMD progressively abolishes sharp, central vision and is a global epidemic with an estimated incidence of 30–50 million.^{4–7} AMD targets choroid, retinal pigment epithelium (RPE) and retina. RPE plays critical role in the physiology of the underlying photoreceptors and is a crucial target of injury in AMD.⁸

The causes of AMD are yet to be determined but high intake of cholesterol and saturated fat are suspected to be associated with increased risk of AMD.⁹ Free and esterified cholesterol are highly distributed in the drusen.^{10–12} The source of the cholesterol that accumulates in the retina is suggested to derive from both local cells and plasma origins.^{13–16} 7-Ketocholesterol (7-KC) is the major oxysterol generated by autoxidation on the B hydrocarbon ring of cholesterol, whereas 25-hydroxycholesterol (25-OHC) and 27-hydroxycholesterol (27-OHC) are major oxysterols produced by enzymatic oxidation on the lateral chain of the cholesterol. Oxysterols are suggested to contribute to the pathophysiology of AMD as increased levels of these cholesterol metabolites are toxic to photoreceptors and RPE cells and attract macrophages when accumulating on Bruch's membrane.¹⁷

We showed previously that cholesterol-enriched diets cause retinal degeneration and is associated with increased oxysterol levels in retinas of cholesterol-fed rabbit.¹⁸ We also demonstrated that incubation of ARPE-19 cells, a retinal derived cell line that has structural and functional properties of retinal pigmented epithelial cells, with 27-OHC, the most abundant oxysterol in circulation,¹⁹ causes oxidative stress and apoptosis.²⁰ 25-OHC and 7-KC are major oxysterols in oxidized low-density lipoproteins.²¹ 7-KC and 25-OHC were shown to have cytotoxic, pro-oxidative and/or angiogenic activities on ARPE-19 cells.²² Presence of 7-KC in lipid deposits from primate retina suggest the involvement of this oxysterol in AMD development.²³ 7-KC-treated ARPE-19 cells showed characteristics of apoptosis,²⁴ and cytotoxicity of oxLDL on RPE cells was shown to be dependent on 7-KC formation.²⁵ However, although oxysterols are cytotoxic and may be involved in the pathogenesis of AMD, the cellular pathways and mechanisms that underlie their deleterious effects are yet to be determined.

27-OHC has been demonstrated to bind to both estrogen receptor (ER) and liver $\times$ receptor (LXR).^{26–30} 27-OHC is the first identified endogenous selective ER modulator that has agonist activity in MCF-7 cells, a breast cancer cell line and antagonistic activity in vascular endothelial cells and smooth muscle cells of cardiovascular system.³⁰ The IC₅₀ values for 27-OHC and 25-OHC are around 1 μ M for both ERs.³⁰ 25-OHC inhibits activation of ER by

estrogen³⁰ and was also shown to elicit estrogenic effects in cancer cells and cardiomyocytes.³¹ The two ER receptor subtypes α and β are shown to be expressed in the male and female retina.^{32,33} E2 treatment was reported to attenuate the release of cytokines, reactive oxygen species and complement proteins in microglial cells.^{34,35} E2 has been suggested as a potential therapeutic agent in the management of several neurodegenerative disorders.³⁶ E2 was shown to rescue undifferentiated neuroblastoma cells from toxicity induced by 24-OHC, another oxysterol related to 25-OHC and 27-OHC.³⁷ These protective effects of E2 prompted us to investigate whether E2 is protective against the oxysterol-induced cytotoxicity in RPE cells.

Both 27-OHC and 25-OHC are also natural endogenous ligands for LXRs.^{38,39} 7-KC, the oxysterol formed by autoxidation, is also an LXR agonist⁴⁰ as 7-KC-mediated induction of VEGF was attenuated by the LXR antagonist cholesterol sulfate.²³ 7-KC failed to antagonize ERs and E2-dependent nitric oxide synthases activity.³⁰ However, whether these oxysterols have any effect on LXR-mediated transcriptional activity in retinal pigment epithelial cells is not known.

In this study, we determined the extent to which ER and LXR are expressed and are functionally active in ARPE-19 cells. We also determined the potential beneficial or detrimental effects of the ER agonist E2, the ER antagonist ICI182780, the LXR agonist GW3965 and the LXR antagonist 5 α -6 α -epoxycholesterol-3-sulfate (ECHS) against the deleterious effects of oxysterols.

MATERIALS AND METHODS

Cell Culture and Treatments

ARPE-19 cell line at passage number 19 was purchased from ATCC (Manassas, VA), and grown in Dulbecco's modified Eagle medium: nutrient mixture F-12 (DMEM/F12) Glutamax media containing 10% charcoal dextran-stripped fetal bovine serum and 1% antibiotic antimycotic solution. All experiments were performed using cells from passage 22 to through 28. Cells were maintained in a 5% CO₂, 37°C incubator. After reaching confluency, cells were treated with vehicle (control), 10 μ M 25-OHC (Steraloids, Wilton, NH), 10 μ M 27-OHC (Medical Isotopes Inc, Pelham, NH), or 25 μ M 7-KC (Sigma Aldrich, St Louis, MO) in the presence or not of 1 μ M GW3965 (Sigma Aldrich), 1 μ M ECHS (Steraloids), 10nM E2 or 1 μ M ICI182780 (both from Sigma Aldrich), for 24 h. All oxysterols and drugs were dissolved in ethanol. In a previous study, we showed that 10 μ M 27-OHC is cytotoxic to ARPE-19 cells. In the present study, the 10 μ M of 25-OHC and 25 μ M of 7-KC were chosen after a preliminary dose-response study in which these concentrations produced the minimum significant cytotoxicity.

Transfection and Dual Luciferase Reporter Assays

ARPE-19 cells were plated in 96-well plates (30,000 cells/well). Estrogen response element (ERE) reporter (a mixture of ERE-responsive firefly luciferase construct and renilla luciferase construct, 40:1), a negative control (a mixture of non-inducible firefly luciferase construct and constitutively expressing renilla luciferase construct, 40:1) and a positive

control (a mixture of constitutively expressing GFP, constitutively expressing firefly luciferase, and constitutively expressing renilla luciferase constructs, 40:1:1) were diluted in Opti-MEM serum free media (Invitrogen, Carlsbad, CA). For testing LXR activity, LXR reporter (a mixture of inducible LXR-responsive firefly luciferase construct and constitutively expressing renilla luciferase construct, 40:1), a negative control (a mixture of non-inducible firefly luciferase construct and constitutively expressing renilla luciferase construct, 40:1), a positive control (a mixture of constitutively expressing GFP, constitutively expressing firefly luciferase and constitutively expressing renilla luciferase constructs, 40:1:1) were diluted in OptiMEM serum free media. Lipofectamine LTX and PLUS reagents (Invitrogen) were used for transfection, carried out according to the manufacturer's protocol. After 24 h, media was replaced with DMEM/F12 media, and transfected cells were treated with 27-OHC, 25-OHC and 7-KC for ER activity and LXR activity determination. After 24 h of treatment, cells were harvested into cell lysis buffer (Promega, Madison, WI). Firefly and renilla luciferase activities were determined using Dual-luciferase assay system (Promega) and SpectraMax Gemini EM microplate reader (Molecular Devices, Menlo Park, CA). Promoter activity values were expressed as relative luciferase units which were obtained by calculating ratios from ER and LXR responsive reporter activities divided by ratios from negative control reporter activity.

Western Blotting

Cells were harvested on ice with NE-PER nuclear and cytoplasmic extraction reagents supplemented with protease and phosphatase inhibitors (Thermo Scientific, Rockford, IL). Bicinchoninic acid assay was used for determining protein concentrations. Proteins (10 µg) were separated on SDS-PAGE gels, transferred onto a polyvinylidene difluoride membrane (Biorad, Hercules, CA) and incubated with antibodies to anti-ER α rabbit antibody (1:500; Abcam, Cambridge, MA), anti-ER β rabbit antibody (1:200; Upstate, Lake Placid, NY), anti-LXR α rabbit antibody (1:500; Abcam) and anti-LXR β mouse antibody (1:500; Abcam). β -Actin (Mouse, 1:5000, Santa Cruz Biotechnology, Santa Cruz, CA) and Lamin-A (Rabbit, 1:500, Cell Signaling Technology, Inc. Danvers, MA) were used as gel loading controls for cytosolic and nuclear fractions, respectively. For antibodies of mouse origin, goat anti-mouse secondary antibody conjugated with horseradish peroxidase (HRP) was used (1:5000; Biorad), for antibodies of rabbit origin, goat anti-rabbit secondary antibody conjugated with HRP (1:5000, Biorad) was used. The blots were developed with enhanced chemiluminescence (Immun-star HRP chemiluminescent kit, Bio-Rad, Hercules, CA). Bands were visualized on a PVDF membrane and analyzed by LabWorks 4.5 software on Ultra Violet Products (UVP) Bioimaging System (Upland, CA).

Cell Viability Assay

ARPE-19 cells were seeded in 96 well plates (30,000 cells/well). Cells were treated with oxysterols in combination with ER agonist E2 or antagonist ICI, LXR agonist GW3965 or antagonist ECHS for 24 h. Lactate dehydrogenase released from cells was measured by CytoTox-ONE homogeneous membrane integrity assay (Promega). Manufacturer instructions were followed to perform the assay. SpectraMax Gemini EM microplate reader (Molecular Devices, Sunnyvale, CA) was used for measuring fluorescence.

Mitochondrial Membrane Potential Detection

The fluorescent cationic dye JC-1 (Biotium, Hayward, CA) was used to measure mitochondrial membrane potential.⁴¹ The cells were seeded in 96-well plates (30,000 cells/well). Cells were treated with oxysterols in the presence or absence of ER and LXR agonists/antagonists for 24 h. After treatment, manufacturer instructions were followed to perform the assay and fluorescence was measured by SpectraMax Gemini EM microplate reader (Molecular Devices).

Measurement of IL-6, IL-8, MCP-1, PDGF, VEGF and TNF- α Secretion by ELISA

Interleukin-6 (IL-6), IL-8, monocyte chemoattractant protein-1, platelet derived growth factor, vascular endothelial growth factor and tumor necrosis factor- α secretion were measured in the culture medium of ARPE-19 cells treated with oxysterols 27-OHC, 25-OHC and 7-KC in the presence or absence of E2, ICI, GW and ECHS. After treatment, media was collected and analysis was done by ELISA according to manufacturer's instructions (Peprotech, Rocky Hill, NJ).

Statistical Analysis

GraphPad Prism software 4.01 was used for statistical analysis. The significance of differences among the treatments was assessed by one-way analysis of variance (one-way ANOVA) followed by Tukey's post hoc test. Obtained *p* values are for two tailed outcome. All values obtained from the three different experiments were expressed as mean value $\pm$ SEM. *p* < 0.05 was considered statistically significant. Because multiple comparisons are made, multiplicity adjusted *p* values are reported.

RESULTS

ER α , ER β , LXR α and LXR β are Expressed and are Transcriptionally Active in ARPE-19 Cells

Both cytosolic and nuclear fractions were used for probing of ER α and ER β expression. ER α band was detected approximately at 68 kDa, and ER β band was detected at approximately at 53 kDa (Figure 1A). Our data confirm that ER α and ER β are expressed in both the cytoplasm and the nucleus of ARPE-19 cells. Transfection of ERE-luciferase construct and luciferase activity measured by dual luciferase assay elucidated functional activity of ER. Treatment with the ER agonist E2 caused an increase in ERE-luciferase activity confirming ER functionality (Figure 1B). An LXR α band was detected at approximately 50 kDa, and LXR β band was detected at approximately 55 kDa. Both the LXR α and LXR β were detected in the nuclear as well as the cytosolic fractions (Figure 1C). β -Actin and lamin were used as loading controls for cytosolic and nuclear fractions, respectively. Transfection of LXRE-luciferase construct and dual luciferase assay upon treatment with LXR agonist GW3965 elucidated functional activity of LXR. GW3965 caused an increase in LXRE-luciferase activity confirming LXR functionality (Figure 1D).

25-OHC, 27-OHC and 7-KC Inhibited ER Mediated Transcriptional Activity

Cells were transfected with ERE-luciferase reporter and upon treatments with 25-OHC, 27-OHC and 7-KC, luciferase activity was measured by dual luciferase assay. All three oxysterols reduction in ERE-driven luciferase activity is statistically significant ($p = 0.016$ for 7-KC, $p = 0.001$ for 27-OHC and $p < 0.0001$ for 25-OHC), indicating suppressive effects of oxysterols on estrogen transcriptional response element mediated transcription (Figure 2A).

25-OHC and 27-OHC Increased LXR Mediated Transcriptional Activity

Transfection of LXRE-luciferase construct and luciferase activity measurement by dual luciferase assay upon treatment with oxysterols 27-OHC, 25-OHC and 7-KC showed statistically significant LXR-mediated transcriptional activity increase in response to 25-OHC ($p = 0.031$) and 27-OHC ($p = 0.040$). The increase in the transcriptional activity is evidenced with an increase in LXR response element driven luciferase activity (Figure 2B). On the other hand, 7-KC mediated LXRE-luciferase activity change is not statistically significant ($p = 0.061$).

E2 Protected Cells from 25-OHC and 27-OHC-Induced Cytotoxicity

Treatments with 27-OHC, 25-OHC and 7-KC were cytotoxic to cells and the results are statistically significant ($p = 0.001$, $p < 0.0001$, $p < 0.00001$, respectively). 25-OHC and 27-OHC-induced cytotoxicity was reduced when cells were treated with E2 and results are statistically significant ($p = 0.012$, 0.019 , respectively) and the significance was shown by # symbol (Figure 3A, B). Conversely, 7-KC-induced cytotoxicity is appeared to be reduced by E2, but there was no statistically significant difference ($p = 0.114$) between treatments (Figure 3C). ER antagonist ICI incubation along with 27-OHC, 25-OHC and 7-KC failed to affect oxysterol-induced cytotoxicity significantly ($p = 0.993$, 0.299 , 0.932 , respectively, Figure 3A, B, C). We then determined whether the LXR agonist GW3965 or the LXR antagonist ECHS offer any beneficial effects in oxysterol-induced cytotoxicity. Neither the LXR agonist GW3965 nor the LXR antagonist ECHS reduced oxysterol-induced cytotoxicity (Figure 3D, E, F).

E2 Stabilized 27-OHC and 25-OHC-Induced Mitochondrial Membrane Potential Decline

In healthy cells, JC-1 accumulates as aggregates in the mitochondrial membranes, resulting in red fluorescence (590 nm).⁴² In dead cells and in cells undergoing apoptosis, JC-1 exists in the green fluorescent (529 nm) monomeric form because of decreased mitochondrial membrane potential. 27-OHC, 25-OHC and 7-KC all significantly reduced the red to green fluorescence ratio ($p = 0.022$, $p = 0.025$ and $p = 0.034$, respectively), indicating a decrease in the mitochondrial membrane potential (Figure 4A, B, C). E2 counteracted 27-OHC ($p = 0.022$) and 25-OHC ($p = 0.040$) induced mitochondrial decline and statistical significance was shown by # symbol (Figure 4A, B). But, E2 counteraction from 7-KC-induced mitochondrial membrane potential decline was not statistically significant ($p = 0.892$; Figure 4C). ER antagonist ICI incubation along with 27-OHC, 25-OHC and 7-KC, however, did not counteract oxysterol-induced mitochondrial membrane potential decline significantly (p

= 0.992, 0.912, 0.996; Figure 4A, B, C). Neither GW3965 nor ECHS protected cells from oxysterol-induced mitochondrial membrane potential decline (Figure 4D, E, F).

27-OHC-Induced Increase in TNF- α and IL-6 Secretion is Reduced by E2, GW3965 and ECHS

IL-6 and tumor necrosis factor (TNF)- α secretion was increased in 27-OHC-treated cells ($p = 0.023$, 0.008 , respectively; Figure 5A and D). There is no statistically significant difference in IL-6 and TNF- α secretion upon treatment with 25-OHC ($p > 0.99$, $p = 0.732$, respectively) and 7-KC ($p = 0.836$, $p > 0.99$, respectively; Figure 5B, C, E and F). Incubation of cells with the ER agonist E2 reduced 27-OHC-induced IL-6 secretion ($p = 0.001$). ER antagonist ICI182780 incubation failed to affect significantly ($p = 0.205$). On the other hand, both the LXR agonist GW3965 and the antagonist ECHS significantly reduced 27-OHC-induced IL-6 secretion ($p < 0.0001$, $p = 0.001$). The ER agonist E2 as well as the LXR agonist GW3965 or the LXR antagonist ECHS all significantly reduced 27-OHC-induced TNF- α secretion ($p < 0.0001$, $p < 0.0001$, $p = 0.0005$, respectively; Figure 5D). Other cytokines secretion measured, including IL-8, MCP-1, PDGF and VEGF were not significantly affected by oxysterol treatment (data not shown).

DISCUSSION

In this study, we confirm that both ER α/β and LXR α/β are present and are transcriptionally active in ARPE-19 cells. We also demonstrate that oxysterols, whether derived from cholesterol by enzymatic reaction, such as 25-OHC and 27-OHC, or derived by autoxidation such as 7-KC, all inhibit transcriptional activity of ERs. Additionally, while 25-OHC and 27-OHC increased transcriptional activity of LXRs, 7-KC appeared to reduce LXR mediated transcription, but was not statistically significant. We used dual luciferase reporter assays to probe transcriptional activity changes as this reporter technology simultaneously expresses both firefly luciferase and renilla luciferase in each cell. The constitutively expressing renilla luciferase acts as an internal control to normalize cytotoxicity as we probed transcription activity in presence of toxic molecules. This ensures that the reduced activity obtained is not because of dying cells. These results are in agreement with previous studies showing involvement of ERs in oxysterol-induced effects and further suggest that LXRs underlie effects of oxysterols formed by enzymatic reactions (i.e. 25-OHC and 27-OHC) but not those formed by autoxidation (7-KC). We also found that 25-OHC, 27-OHC and 7-KC induce cytotoxicity in ARPE-19 cells. Treatment of cells with the ER agonist E2 significantly reduced 25-OHC and 27-OHC-induced cytotoxicity. On the other hand, treatment with the LXR agonist GW3965 or the LXR antagonist ECHS did not affect the cytotoxicity induced by 25-OHC, 27-OHC or 7-KC. Treatment with E2 also reversed the decline in mitochondrial membrane potential induced by 25-OHC and 27-OHC but 7-KC-induced mitochondrial membrane potential counteraction by E2 was not significant. Treatment with either the agonist or antagonist of LXRs did not reduce membrane potential decline triggered by 25-OHC, 27-OHC or 7-KC. All together our results suggest that ER agonists can reduce cytotoxicity triggered by the oxysterols derived by enzymatic reaction. Although oxysterols are also modulators of LXRs, agonists and antagonists of these receptors do not appear to regulate oxysterol toxicity.

Freshly isolated human retinal pigment epithelial cells and ARPE-19 cells were shown to express ER α and ER β .^{43,44} In one recent study, ER α gene expression was observed in freshly isolated RPE cells but not in ARPE-19 cell culture and primary RPE culture.⁴⁵ In another study, ER α mRNA but not ER β mRNA was detected in ARPE-19 cells.⁴⁶ As cell line establishment and different growth conditions may be responsible for these differences in ER expression, we sought to determine ERs levels in ARPE-19 cell line we used. Transfection with ERE-luciferase constructs or LXRE-luciferase constructs and dual luciferase assays upon treatments with the ER agonist E2 and the LXR agonist GW3965 confirmed ER and LXR transcriptional activity.

An endoplasmic reticulum localized sterol 27-hydroxylase (CYP27A1) catalyzes the conversion of cholesterol to 27-OHC.⁴⁷ A strong stoichiometric correlation has been recognized between circulating levels of 27-OHC and total cholesterol in humans.^{48,49} 25-OHC is produced from cholesterol primarily by the action of cholesterol 25-hydroxylase, an endoplasmic reticulum and Golgi localized enzyme. The ability to produce 25-OHC from cholesterol was also reported for sterol 27-hydroxylase and mitochondrial enzyme cholesterol 24-hydroxylase (CYP46A1).^{50,51} CYP46A1 and CYP27A1 expression have been identified in the RPE cells.^{52,53} 25-OHC has also long been recognized as a cholesterol autooxidation product. 7-KC is commonly found in oxLDL associated with atherosclerotic plaques. Light is involved in mediating the oxidation of cholesterol to 7-KC in retina.⁵⁴ Here, we determined whether oxysterols have any effect on ER and LXR-mediated transcriptional activity. 27-OHC, 25-OHC and 7-KC inhibited the ERE-luciferase activity. 27-OHC, 25-OHC activated LXRE-luciferase activity, whereas 7-KC did not modify LXRE-luciferase activity. Numerous studies suggested that cytotoxic effects of oxysterols are associated with human diseases including AMD.⁵⁵ Increased oxysterol concentrations subsequent to increased cholesterol levels may contribute to the generation of AMD-like pathological hallmarks. 27-OHC, 25-OHC and 7-KC were shown to be cytotoxic to RPE cells by us and others.^{20,24,56} As these oxysterols also function as ligands of ER and LXR, we sought to study any potential protective or destructive effects of ER and LXR ligands in combination with the oxysterols.

Some epidemiologic studies suggest that the risk of AMD is increased in women undergoing menopause and that women on hormone-replacement therapy have a reduced risk of having the disease.^{1,57} A relationship between the use of postmenopausal exogenous E2 and a lower risk of AMD also suggests a role for estrogen in the pathogenesis of the pathophysiology of this disease.⁴⁴ However, there are conflicting reports about AMD risk and history of estrogen exposure.^{58–60} It is to be noted the multiple systems of the classification of AMD (for example, The Wisconsin Age-Related Maculopathy Grading System, The Chesapeake Bay Water Men Grading System, The Age-Related Eye Disease Study Grading System) used for population studies that vary in AMD features description. Both ER subtypes are expressed in human RPE cells and regulated in a dose-dependent fashion by E2.⁴⁴ E2 has been suggested as a potential therapeutic agent in the management of several neurodegenerative disorders because of its actions as an anti-oxidant, anti-apoptotic and anti-inflammatory agent.^{61,62} Furthermore, E2 can stimulate repair of injured neuronal tissue by enhancing neurogenesis, remyelination and regeneration of neuronal processes and by decreasing reactive gliosis.⁶³

Our study demonstrates that 27-OHC, 25-OHC and 7-KC were cytotoxic to ARPE-19 cells and E2 protected against 25-OHC and 27-OHC-induced cytotoxicity. However, 7-KC-induced cytotoxicity was not significantly reduced by either the ER agonist E2 or the antagonist ICI. Cell death comprises several events including the loss of mitochondrial membrane potential.⁶⁴ A recent study⁶⁵ reported 7-KC causes mitochondrial dysfunction in ARPE-19 cells. 25-OHC, 27-OHC and 7-KC caused mitochondrial membrane potential decline, which is usually observed during apoptosis. E2 treatment counteracted mitochondrial potential decline in cells triggered by 27-OHC and 25-OHC, but not in cells treated with 7-KC. Estrogens inhibit cellular apoptosis through several mechanisms, including acting as nonspecific antioxidants, modulating gene expression⁶⁶ and activating nongenomic signaling pathways.^{67–69} These mechanisms are dependent on the cell type and pathophysiological situation of the cell. Several in vitro studies have shown that E2 prevents oxidative stress-induced apoptosis of retinal neurons,⁷⁰ RPE cells⁴³ and human lens epithelial cells.⁷¹ LXR agonist GW3965 or antagonist ECHS did not offer any protection against cytotoxicity or mitochondrial membrane potential decline in oxysterol-treated cells.

27-OHC was shown to increase IL-6 secretion in macrophages.⁷² NF- κ B is known to be a master regulator of both innate and adaptive immunity. As NF- κ B is a central regulator of IL-6, a cytokine involved in choroidal neovascularization,⁷³ we measured the IL-6 secretion upon treatment with oxysterols. IL-6 secretion is significantly increased upon 27-OHC treatment, when compared to control cells. When E2, GW3965 and ECHS were applied along with 27-OHC, IL-6 levels were significantly decreased to levels comparable to those in control cells. Proinflammatory and proangiogenic cytokine TNF- α secretion was shown to be induced by 27-OHC in macrophages,⁷⁴ rabbit hippocampal slices⁷⁵ and RPE cells.²⁰ Here we show 27-OHC incubation increased TNF- α secretion and incubation of cells with E2, GW3965 or ICI182780 reversed the increase in TNF- α levels.

E2 effects are mostly mediated by ERs.⁷⁶ Classic ER signaling involves E2 binding to EREs in the promoter regions of target genes. ERs can also modulate non-ERE-containing genes by interacting with the DNA-bound transcription factors. A number of studies have advocated that membrane-bound ERs cause rapid non-genomic signaling that modify cellular physiology in bone, brain, cardiovascular system and mammary gland.⁷⁷ These rapid effects of estrogens are facilitated by the stimulation of signaling cascades such as nitric oxide, receptor tyrosine kinases, G-protein coupled receptors, phosphatidylinositol-3-kinase, serine threonine kinase Akt, mitogen activated protein kinase family members, protein kinase A and protein kinase C.^{78–80} The extent to which the protective effects of E2 occur through ER α , ER β or non-genomic signaling pathways is yet to be determined. Under normal conditions and when oxysterol levels are low, ER function is preserved, whereas conditions that increase these oxysterol levels, such as hypercholesterolemia or conditions that reduce estrogen levels, the ER function could be inhibited by oxysterols. This ER function compromise could explain AMD in conditions related to low levels of E2. These results emphasize the importance of potential ER agonists for counteracting oxysterol-induced toxicity, which could protect against AMD. E2, as an example, can inhibit collagen gel contraction and a drop in E2 levels was shown to decrease tensile strength in the macular area and promote a higher incidence of macular holes.^{81–84} Even though our results suggest that E2 may be protective against oxysterol-induced cytotoxicity,

mechanisms are not clear. In the future experiments, we would like to find out the mechanism, as estrogen protection could be ER-mediated or it is just acting as a general antioxidant.

In summary, our data show that both ERs and LXRs are expressed and are transcriptionally active in ARPE-19 cells, and that their transcription is modulated by ER and LXR ligands including oxysterols. Our study also demonstrates that, although oxysterols are cytotoxic, the underlying mechanisms differ between oxysterols derived by enzymatic reactions and those formed by autooxidation from cholesterol. Such results are also of seminal importance to the characterization of cellular mechanisms that are targeted by the various oxysterol groups.

Acknowledgments

This work was supported by a grant from the NIH (NIEHS, R01ES014826) to O.G.

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FIGURE 1.

(A) Western blots showing ERα and ERβ protein levels in cytosolic and nuclear fractions of untreated ARPE-19 cells. (B) Increased ERE-luciferase activity by E2 indicates that estrogen receptors are functional. (C) Western blots showing LXRα and LXRβ protein expression in cytosolic and nuclear fractions of untreated ARPE-19 cells. (D) GW3965 increased LXRE-luciferase activity indicating that liver × receptors are functional. Reporter activity values are expressed as arbitrary units. ** $p < 0.01$; *** $p < 0.001$.

FIGURE 2.

Cells were treated with vehicle (control), 10 μ M 25-OHC, 10 μ M 27-OHC or 25 μ M 7-KC in the presence or not of 1 μ M GW3965 (LXR agonist), 1 μ M ECHS (LXR antagonist), 10nM E2 (ER agonist) or 1 μ M ICI182780 (ER antagonist), for 24 h. (A) 27-OHC, 25-OHC and 7-KC decreased ERE-luciferase activity. ARPE-19 cells were transfected in parallel with ER reporter, and negative control and cells were subjected to 27-OHC, 25-OHC and 7-KC treatments for 24 h. Oxysterols decreased ERE-luciferase activity ($p = 0.001$ for 27-OHC, $p < 0.0001$ for 25-OHC, $p = 0.016$ for 7-KC). (B) ARPE-19 cells were transfected in parallel with LXR reporter, and negative control and cells were subjected to oxysterol treatments for 24 h. 25-OHC and 27-OHC increased LXRE-luciferase activity ($p = 0.031$, 0.040, respectively). 7-KC appeared to reduce the luciferase activity, but was not statistically significant ($p = 0.061$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

FIGURE 3.

Cells were treated with vehicle (control), 10 μ M 25-OHC, 10 μ M 27-OHC or 25 μ M 7-KC in the presence or absence of 1 μ M GW3965, 1 μ M ECHS, 10nM E2 or 1 μ M ICI182780, for 24 h. (A) 27-OHC is cytotoxic to cells ($p = 0.001$), and treatment with the ER agonist E2 reduced the cytotoxicity induced by 27-OHC ($p = 0.019$). (B) 25-OHC is also cytotoxic to cells ($p < 0.0001$) and treatment with E2 reduced the 25-OHC-induced cytotoxicity ($p = 0.012$). (C) 7-KC is cytotoxic to cells ($p < 0.00001$) but neither E2 nor ICI182780 significantly reduced cells from 7-KC-induced cytotoxicity ($p = 0.114, 0.932$, respectively).

(D) Treatment with the LXR agonist GW3965 or with the LXR antagonist ECHS did not offer any protection against 27-OHC-induced cytotoxicity. (E) Treatment with GW3965 or ECHS did not offer any protection against 25-OHC-induced cytotoxicity. (F) Treatment of GW3965 or ECHS along with 7-KC did not offer any protection against 7-KC-induced cytotoxicity. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus control; # $p < 0.05$ versus 27-OHC or 25-OHC.

FIGURE 4.

Cells were treated with vehicle (control), 10 μ M 25-OHC, 10 μ M 27-OHC or 25 μ M 7-KC in the presence or absence of 1 μ M GW3965, 1 μ M ECHS, 10nM E2 or 1 μ M ICI182780, for 24 h. JC-1 assay was used for detection of mitochondrial membrane potential. (A) 27-OHC decreased the mitochondrial membrane potential significantly ($p = 0.022$). The ER agonist E2 restored the mitochondrial membrane potential decline induced by 27-OHC ($p = 0.022$). (B) 25-OHC caused a decrease in mitochondrial membrane potential ($p = 0.025$), which is counteracted by E2 ($p = 0.040$). (C) 7-KC-induced mitochondrial membrane

potential decline ($p = 0.034$) was not significantly counteracted by E2 ($p = 0.892$) or ICI182780 ($p = 0.996$). (D–F) Neither the LXR agonist GW3965 nor the LXR antagonist ECHS counteracted the 27-OHC, 25-OHC or 7-KC-induced mitochondrial membrane potential decline. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ versus control; $\#p < 0.05$ versus 27-OHC or 25-OHC.

FIGURE 5.

ELISA was used to measure IL-6 and TNF- α secretion from ARPE-19 cells. (A) IL-6 secretion was significantly increased after 24 h treatment with 27-OHC compared to control cells ($p = 0.023$). Treatment with E2, GW3965 and ECHS significantly reduced the 27-OHC-induced increase in IL-6 secretion ($p = 0.001$, $p < 0.0001$, $p = 0.001$). (B, C) 25-OHC and 7-KC in the presence or absence of E2, ICI, GW3965 or ECHS showed no significant difference in IL-6 secretion when compared with controls. (D) TNF- α secretion was significantly increased after 24 h treatment with 27-OHC compared to control cells ($p =$

0.008). Treatment with E2, GW3965 and ECHS significantly reduced the 27-OHC-induced increase in TNF- α secretion ($p < 0.0001$, $p < 0.0001$, $p = 0.005$, respectively). (E, F) 25-OHC and 7-KC in the presence or absence of E2, ICI, GW3965 and ECHS showed no significant difference in TNF- α secretion when comparing with controls. * $p < 0.05$ versus control # $p < 0.05$, ### $p < 0.001$ versus 27-OHC.