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Increased Resistance to Oxysterol Cytotoxicity in Fibroblasts Transfected With a Lysosomally Targeted *Chromobacterium* Oxidase

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ABSTRACT: 7-Ketocholesterol (7KC) is a cytotoxic oxysterol that plays a role in many age-related degenerative diseases. 7KC formation and accumulation often occurs in the lysosome, which hinders enzymatic transformations that reduce its toxicity and increase the sensitivity to lysosomal membrane permeabilization. We assayed the potential to mitigate 7KC cytotoxicity and enhance cell viability by overexpressing 7KC-active enzymes in human fibroblasts. One of the enzymes tested, a cholesterol oxidase engineered for lysosomal targeting, significantly increased cell viability in the short term upon treatment with up to 50 μM 7KC relative to controls. These results suggest targeting the lysosome for optimal treatment of oxysterol-mediated cytotoxicity, and support the use of introducing novel catalytic function into the lysosome for therapeutic and research applications.

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KEYWORDS: oxysterol; 7-ketocholesterol; lysosome; oxidase

Inherent imperfections in cellular transport and catabolic processes, especially in post-mitotic and senescent cells, result in the gradual accumulation of certain molecular species that can be detrimental to cell and tissue function. One example of this is the formation of 7-carbon (C7) modified oxysterols that are cytotoxic to mammalian cells and widely believed to contribute to the development of a number of age-related diseases (Lemaire-Ewing et al., 2005). The major intracellular oxysterol species, 7-ketocholesterol (7KC; Fig. 1A), has been associated with atherosclerosis (Brown and Jessup, 1999), age-related macular degeneration (Rodriguez and Larrayoz, 2010), and Alzheimer's disease (Casserly and Topol, 2004). Therefore, the introduction of novel sterol-transforming enzymes into affected cell types may be useful for controlling endogenous 7KC levels, and consequently help reduce the incidence and severity of these diseases (Mathieu et al., 2009).

Sterols, including oxysterols, enter the cell via receptor-mediated endocytosis of low density lipoproteins and traffic to the lysosomes, which are a major site of non-enzymatic oxysterol formation. Consequently, 7KC levels are the highest in the endosomal and lysosomal compartments (Brown et al., 2000). 7KC is known to inhibit sphingomyelinase (Maor et al., 1995) and facilitate the intralysosomal accumulation of both sphingomyelin and cholesterol, possibly leading to foam cell formation. Subsequent free cholesterol loading of lysosomes also promotes de-acidification (Cox et al., 2007), impairs organelle trafficking (Fraldi et al., 2010), and inhibits chaperone mediated autophagy (Kaushik et al., 2006). At micromolar concentrations, 7KC causes lysosomal membrane permeabilization (LMP). The cellular response to LMP depends on the degree of permeabilization, with mild LMP causing induction of apoptosis or apoptosis-like cell death and sustained LMP generally leading to necrosis.

In humans, several enzymes are known to metabolize 7KC (Supplementary Fig. S1), and may attenuate 7KC-induced cytotoxicity. However, these enzymes are localized in intracellular compartments other than the lysosome, and are limited in their ability to prevent LMP and the subsequent death-signaling cascade. Our goal, therefore, was to determine the feasibility of introducing novel catalytic function into the lysosome as a means of reducing oxysterol levels and toxicity.

To understand the mechanism of oxysterol cytotoxicity in wild type human fibroblasts, 7KC was introduced to cell cultures and subsequently evaluated using both an XTT cell viability assay and flow cytometry. A slight decrease in the average viable cell fraction was observed after 24 h at 10 μM 7KC (96 ± 11% compared to untreated cells) based upon the

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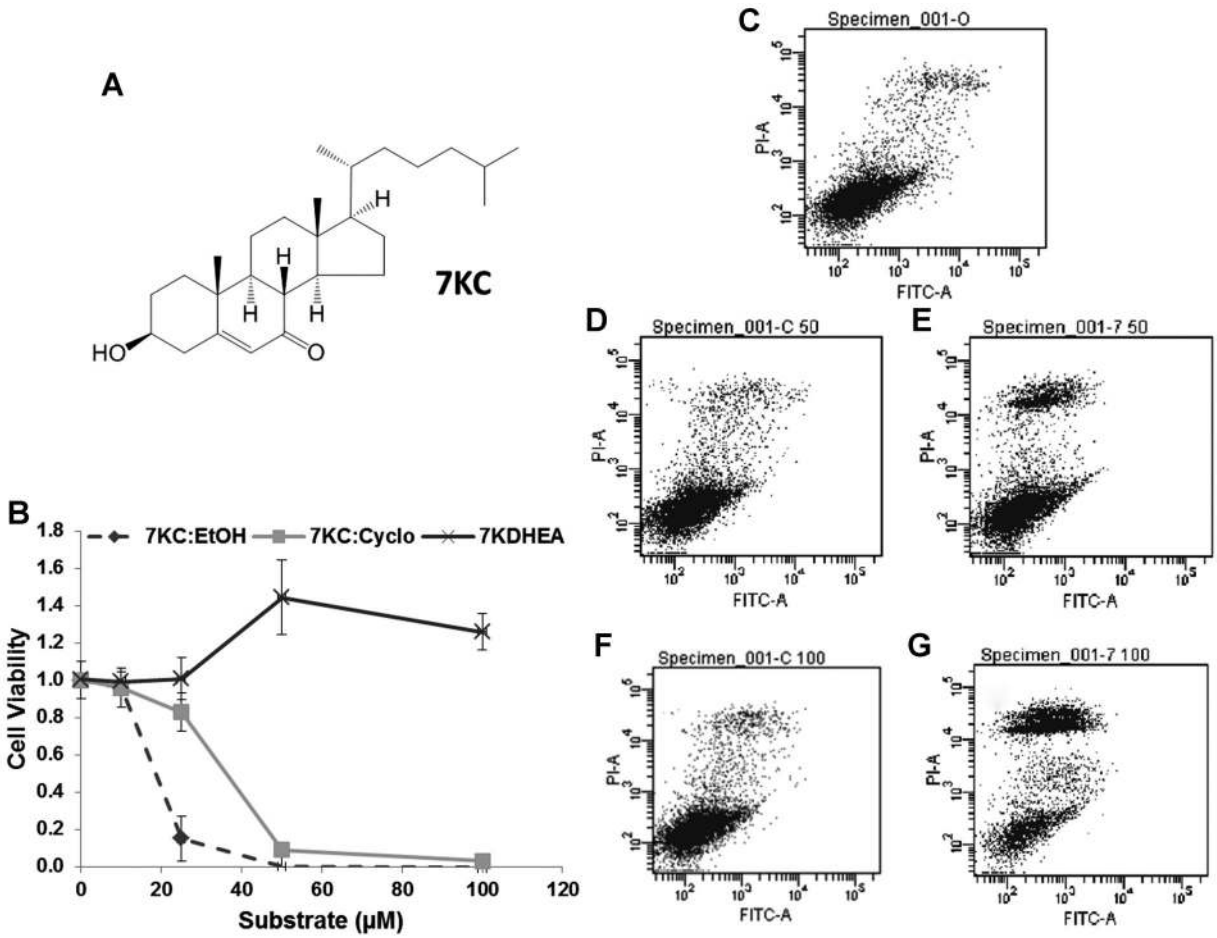

Figure 1. Cell viability of human fibroblasts exposed to 7KC. **A**: Structure of 7KC. **B**: Human fibroblasts were treated with 7KC (in ethanol), 7KC (in cyclodextrin), or 7KDHEA (in ethanol) at concentrations from 0 to 100 μM. 7KC (in ethanol) displayed the highest toxicity, while there were slight improvements in cell viability (fraction viable compared to untreated) in 7KDHEA-treated cells. Cells treated with ethanol alone showed no change in cell viability (data not shown). Cells were also treated with either cholesterol or 7KC for 24 h, stained with Annexin V-FITC and propidium iodide (PI), and analyzed by flow cytometry. **C**: Untreated control; **D**: 50 μM cholesterol; **E**: 50 μM 7KC; **F**: 100 μM cholesterol; and **G**: 100 μM 7KC. A clear increase in PI signal occurs in 7KC treatments with no corresponding increase in FITC. This is indicative of PI uptake due to membrane permeabilization, likely as a result of cell necrosis. No change occurred in cholesterol-treated cells.

XTT assay (Fig. 1B). A noticeable divergence occurred upon treatment with 25 μM 7KC, with cells undergoing a reduction of the viable cell fraction to $15 \pm 12\%$ compared to untreated cells. Treatment with 50 μM 7KC resulted in complete elimination of viable cells. Cells treated with only ethanol at the concentrations used for sterol amendment displayed no toxicity (data not shown).

The mechanism of toxicity in cells treated with 7KC was further evaluated by flow cytometry using the annexin V and propidium iodide (PI) assays (Fig. 1C–G). PI is a membrane impermeant, intercalating dye that is normally excluded from viable cells. Upon binding to nucleic acids, PI exhibits a shift in its fluorescence absorption and emission maxima, and allows quantification of changes in membrane permeabilization, which is a marker of late apoptosis or necrosis (Krishan, 1975). We observed an increase in PI staining that positively correlated with 7KC concentration. A 63% increase in PI signal was detected in fibroblasts treated with 25 μM 7KC compared to cholesterol-treated cells, indicating a loss of membrane integrity and the presence of dead or necrotic cells. The increase in PI signal was 115% for 50 μM and 549% for 100 μM sterol concentrations. No change in Annexin V-FITC binding, which is measured as an indication of membrane rearrangement and early apoptosis, was detected at the 7KC concentrations tested. Treatment with the same concentrations of cholesterol (up to 100 μM) did not result in increased staining of either PI or FITC, suggesting that 7KC may cause necrosis or necroptosis of human fibroblasts, rather than apoptosis. This is supported by previous work that found differences in the mode of death initiated by oxysterols that were dependent on cell type, with 7KC causing necrotic cell death in human fibroblasts (Lizard et al., 1999).

Although it has been repeatedly reported that oxysterols are potent initiators of apoptosis and necrosis (Lemaire-Ewing et al., 2005), strategies to control their levels and promote their clearance in vivo have not been extensively investigated. Overexpression of cholesterol sulfotransferase reduced oxysterol levels, and moderately reduced 7KC toxicity in 293T cells (Fuda et al., 2007). To our knowledge, this is the only evidence reported thus far of an enzyme overexpressed in vivo for the purpose of reducing oxysterol toxicity. Moreover, there is no evidence for the lysosomal transformation of oxysterols. Sterols are typically exported from the lysosome via the sterol transport proteins NPC1 and NPC2. However, in certain situations sterol transport may be impaired, leading to pathology. For example, in oxLDL-loaded macrophage foam cells, which are a primary component of atherosclerotic plaques, approximately 54% of the free 7KC is inside the endocytic/lysosomal compartment (Brown et al., 2000). To determine the potential effect of reducing 7KC levels inside the lysosome, we first needed to identify an enzyme capable of transforming 7KC that might maintain activity within the confines of the lysosome.

Previous work has shown that 3β-oxidation of 7KC significantly decreases its cytotoxicity (Schloendorn et al., 2009). Hence, we hypothesized that oxidation of 7KC would prevent induction of the LMP-mediated death-signaling cascade in human cell cultures. Unfortunately, most enzymes that are capable of initiating transformation of cholesterol are redox enzymes and not likely to function in the lysosomal lumen due to the low pH, high concentration of proteases, and lack of appropriate cofactors. However, a novel cholesterol oxidase from *Chromobacterium* sp. DS1 was recently isolated that lacks isomerization activity, forming cholest-5-en-3-one in lieu of cholest-4-en-3-one (Doukyu et al., 2009). Unlike cholest-4-en-3-one, cholest-5-en-3-one does not increase membrane permeability (Ghoshroy et al., 1997). Additionally, DS1 cholesterol oxidase (DS1 ChOx) is extremely stable; maintaining activity in a variety of solvents, detergents, and at high temperature. But while *Chromobacterium* sp. DS1 cholesterol oxidase is known to oxidize cholesterol, activity against 7KC has not been previously assessed. Furthermore, despite the high degree of similarity between the 7KC and cholesterol chemical structures, the activity of most cholesterol oxidases against 7KC has not been investigated (Pollegioni et al., 2009), and the majority of cholesterol oxidases that were previously tested in our laboratory were inactive against 7KC. Although we found *Rhodococcus equi* cholesterol oxidase able to catalyze oxidation of 7KC, this enzyme quickly loses activity at low pH (Watanabe et al., 1989), and commercially available cholesterol oxidases (Sigma–Aldrich, St. Louis, MO) from *Cellulomonas* sp., *Nocardia erythropolis*, and *Streptomyces sp*. were inactive against this substrate (data not shown). Since oxidation of 7KC has the potential to eliminate its toxicity, we measured the ability of DS1 ChOx to oxidize 7KC.

The sequence encoding the mature protein was fused to a 6xHis tag, expressed in batch, and purified to homogeneity using affinity purification. The enzyme proved active against both cholesterol and 7KC in detergent or mixed micelles. Overnight incubations of 7KC with DS1 ChOx resulted in complete elimination of 7KC as measured by HPLC (235.8 nm), with concomitant formation of a new product (319 nm). Indirect assay by measurement of hydrogen peroxide production revealed an activity of 4.75 U/mg enzyme using cholesterol as a substrate at a mole fraction of 0.50, while only 0.13 U/mg were measured using 7KC as the substrate (1 U converts 1 μmol of substrate per min; Fig. 2A and B). Enzyme activity against both cholesterol and 7KC increased at higher substrate mole fractions. GC–MS of the HPLC fractions from the DS1 ChOx-catalyzed 7KC reaction revealed the presence of two large peaks, one at 472, which corresponds to the molecular weight of 7KC after derivatization, and the other with a molecular weight of 470 (Fig. 2C). This is supportive of 3β-oxidation, which is expected to result in the loss of two hydrogen atoms (Fig. 2D).

In order to assay the potential benefit of reducing intralysosomal 7KC levels, we sought to target DS1 ChOx to the endosomal and lysosomal compartments. To achieve this, we fused the signal sequence and transmembrane domain of the lysosomal membrane protein LAMP1 to the N- and C-termini of DS1 ChOx, and cloned this construct into pEGFP-N3 for mammalian expression (pEGFP-COXL1, Fig. 3A). To test for proper localization, this plasmid was transfected into fibroblasts amended with acridine orange, a membrane-permeable dye commonly used to visualize acidic organelles. As shown in Figure 3B–D, the GFP fused construct was correctly localized to the lysosomes. However, we did notice morphological changes that were absent from non-transfected cells (Fig. 3E). These changes only became obvious in cells that had been incubating with 7KC longer than 24 h (Fig. 3F–I). Since lysosomal structure appeared intact, we speculated that these changes could be due to excessive production of hydrogen peroxide as a result of enzymatic activity.

To assay the efficacy of DS1 ChOx at mitigating 7KC cytotoxicity, as well as to establish the role of subcellular localization, fibroblasts were transiently transfected for the expression of either sterol 27-hydroxylase (pCMV6-XL4-CYP27, mitochondrial expression), 11β-hydroxysteroid dehydrogenase (pMEV-HSD, ER expression), or DS1 ChOx targeted to either the cytoplasm (pMEV-COX) or lysosome (pEGFP-COXL1). Sterol 27-hydroxylase and 11β-hydroxysteroid dehydrogenase are both known to transform 7KC, producing metabolites that are less and more toxic, respectively, than 7KC. Additionally, overexpression of the lysosomal membrane protein LAMP1 was tested as a control for the DS1 ChOx/LAMP1 fusion. The XTT cytotoxicity assay was used to determine cell viability 18 h after the amendment of various concentrations of 7KC to the cultures. Transient transfection of fibroblasts with pEGFP-COXL1, which encodes a lysosomally targeted DS1 ChOx, resulted in a significant reduction of the cytotoxicity exerted by up to 50 μM 7KC in comparison to mock transfected cells

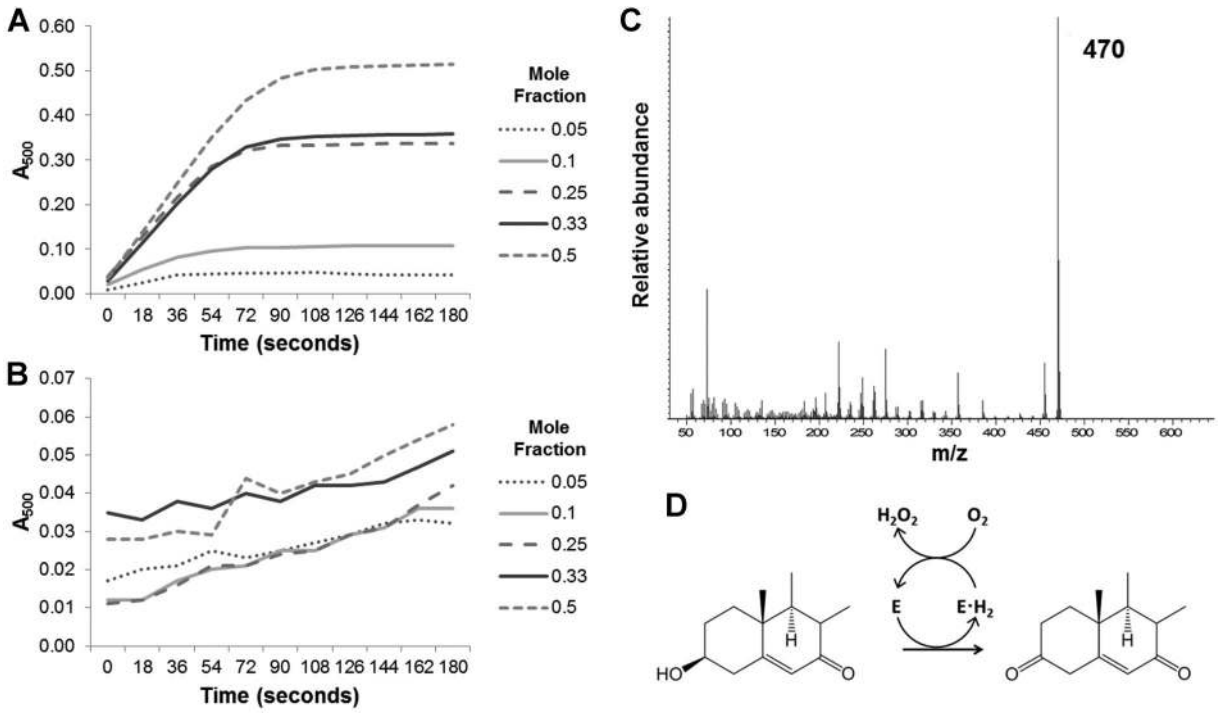

Figure 2. DS1 cholesterol oxidase activity assays. Enzyme activity against either (**A**) cholesterol or (**B**) 7KC was measured indirectly by monitoring oxidation of *o*-Dianisidine at 500 nm in 100 mM potassium phosphate buffer (pH 7). Substrate mole fraction in detergent micelles (0.1% Triton X-100) varied between 0.05 and 0.50. **C**: GC–MS spectra of 7KC metabolite produced by incubation with *Chromobacterium* sp. DS1 cholesterol oxidase. Fractions from the HPLC-based assay of DS1 cholesterol oxidase were screened using GC–MS. Two major peaks were observed with derivatized masses of 472 and 470. These correspond to non-derivatized molecular weights of 400 and 398, which represent the molecular weight of 7KC and that of its 3β-oxidized diketone respectively, providing strong evidence for the activity of the DS1 oxidase on 7KC. **D**: Reaction catalyzed by *Chromobacterium* sp. DS1 cholesterol oxidase. Oxidation of the 7KC 3β-hydroxyl group occurs through the transfer of two electron equivalents to the coenzyme FAD, producing enzyme-bound $FADH_2$ ($E \cdot H_2$). $FADH_2$ is re-oxidized with dioxygen, forming hydrogen peroxide and FAD. DS1 cholesterol oxidase lacks the isomerization activity common in other cholesterol oxidases, forming cholest-5-en-one (instead of cholest-4-en-one) from cholesterol. Cholest-5-en-one is subject to auto-oxidation, forming 6β-hydroperoxycholest-4-en-3-one. Based on the molecular weights identified using mass spectrometry, 7KC does not undergo auto-oxidation after 3β-hydroxyl dehydrogenation. Only steroid rings A and B are shown. FAD, flavin adenine dinucleotide.

or pEGFP-LAMP1 ($P \leq 0.05$; Fig. 4). Particularly, pEGFP-COXL1 transfected populations displayed 120% (25 μM 7KC) and 160% (37.5 μM 7KC) increases in cell viability over mock transfected cells. Interestingly, there was also an increase of 44% in cell viability in pEGFP-COXL1 transfected cells that were not treated with 7KC compared to mock transfections. pMEV-COX, containing the DS1 ChOx lacking a signal sequence and LAMP1 transmembrane domain, was also protective at both 37.5 and 50 μM, providing a 55% and 61% increase in cell viability, respectively. Additionally, transfection with pEGFP-LAMP1 resulted in a 65% increase in cell viability at 37.5 μM 7KC ($P < 0.05$). There was no discernible difference in cell viability between transfection with pCMV6-XL4-CYP27, encoding sterol 27-hydroxylase, and the mock transfected cells. However, we did notice a decrease in cell viability in populations transfected with pMEV-HSD, containing 11β-hydroxysteroid dehydrogenase. The changes were significant ($P < 0.05$) at 0, 37.5, and 50 μM 7KC, with 41%, 38%, and 58% decreases respectively. It should also be noted that the pEGFP-COXL1 construct was significantly more cytoprotective than all other treatments at 25, 37.5, and 50 μM 7KC.

In human cells, all endogenous enzymes that are capable of transforming oxysterols are localized in the ER, cytoplasm, or mitochondria, making it unlikely they would be capable of preventing lysosomal membrane permeabilization. Our results suggest that this is true; transient transfection of fibroblasts with plasmid expressing CYP27A1, a mitochondrially localized hydroxylase, did not display increased viability as we hypothesized. In contrast, the results of this study indicate that overexpression of a lysosomally targeted cholesterol oxidase is able to increase resistance to 7KC in human fibroblasts for periods of short duration. Recent results suggest that 7KC is not the only cytotoxic component of oxLDL (Rutherford and Gieseg, 2012). Nevertheless, 7KC has been shown to permeabilize the lysosomal and mitochondrial membranes in a sequential manner (Larsson et al., 2006), as well as induce monocyte differentiation and foam cell formation alone or as a component of oxLDL (Hayden et al., 2002). Experimental strategies such as we have utilized here should

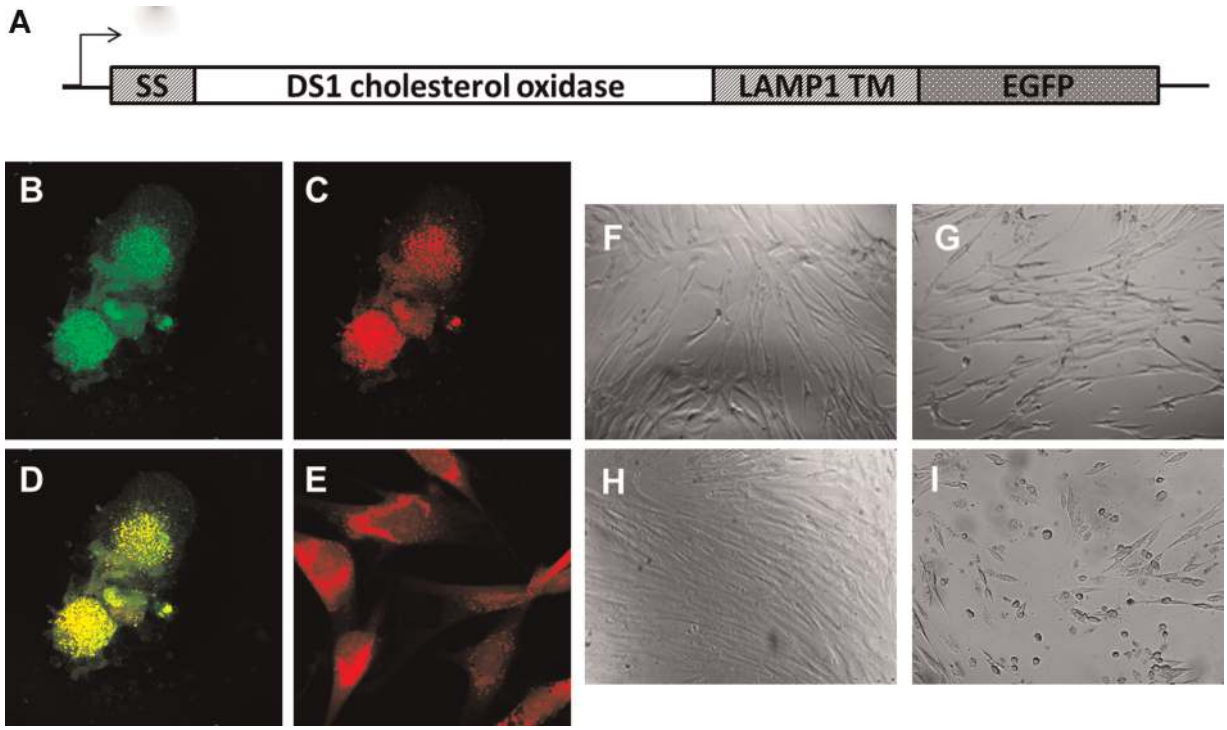

Figure 3. **A**: DS1 ChOx was fused to the signal sequence and transmembrane domain of LAMP1 and inserted into pEGFP-N3. The DS1 cholesterol oxidase/LAMP1 fusion was transfected in human fibroblasts and visualized using confocal microscopy. **B**: Detection of EGFP and (**C**) acridine orange, which partitions to the lysosome. **D**: Merge of (B) and (C), which shows co-localization of EGFP and acridine orange. Transfection of human fibroblasts with the construct produces some slight morphological changes as compared to wild type cells (**E**). Micrographs of fibroblasts transfected with the lysosomally targeted DS1 ChOx at 0 μM (**F**) and 50 μM 7KC (**G**). Mock transfected cells show greater cell densities at 0 μM 7KC (**H**), but decreased densities at 50 μM (**I**). [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com]

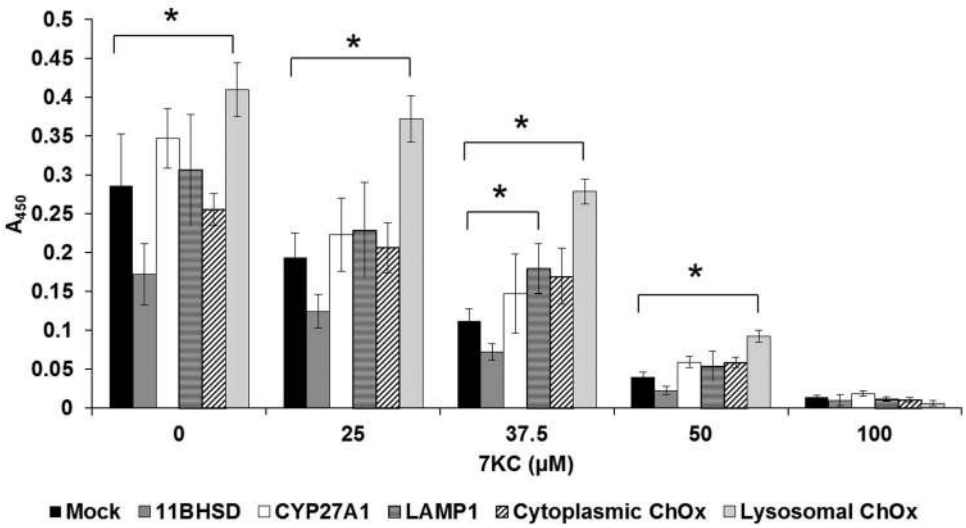

Figure 4. Cytotoxicity analysis of transfected human fibroblasts. Normalized changes in absorbance (450 nm) of XTT due to reduction coupled to mitochondrial dehydrogenase that corresponds to differences in cell viability. A greater absorbance indicates more viable cells are present. Cells were transfected and exposed to varying concentrations of 7KC for 24 h prior to the XTT assay. Significant differences existed amongst treatments up to 50 μM 7KC, with transfection by lysosomally targeted DS1 ChOx being the most cytoprotective. LAMP1 overexpression also moderately increased resistance to 7KC at 37.5 μM. However, the lysosomally targeted DS1 ChOx was significantly more protective than all other treatments at 25, 37.5, and 50 μM 7KC. Asterisks (*) denote statistical significance ($P \leq 0.05$).

help untangle the specific effects of 7KC and separate them from the other components of oxLDL.

This is the first report of enzyme-mediated clearance of 7KC in the lysosome. Results suggest a key role of LMP in oxysterol mediated toxicity, and that overexpression of LAMP1 alone was moderately cytoprotective against 7KC toxicity. The LAMP proteins are suspected of playing roles in both lysosomal stability and cholesterol transport (Eskelinen et al., 2004). Our results agree with these previous findings and suggest that LAMP1 also has a protective function against oxysterol-induced cytotoxicity. In contrast, overexpression of cytoplasmically targeted DS1 ChOx, though showing some cytoprotective benefit, resulted in significantly lower protection from 7KC-induced cell death than lysosomal DS1 ChOx or LAMP1. And overexpression of HSD11β1 actually resulted in increased cell death, presumably due to formation of 7βHC. Overall, this study strongly suggests that reducing lysosomal oxysterol concentrations is promising for mitigating oxysterol toxicity.

Methods

Molecular Cloning

DS1 ChOx (provided by Dr. Noriyuki Doukyu, Toyo University) was PCR amplified and subcloned into pET101/D-TOPO (Invitrogen, Carlsbad, CA; pET-COX). For mammalian transfections, DS1 ChOx and HSD11β1 (Geneart, Regensburg, Germany) were subcloned into a pEGFP-N3 variant containing human LAMP1 (Dr. Esteban Dell'Angelica, UCLA). The LAMP1 and EGFP sequences were deleted during linearization for insertion of the HSD11β1 gene (pMEV-HSD). For the DS1 ChOx sequence, two vectors were created; one in which the LAMP1 and EGFP genes were deleted for cytoplasmic targeting (pMEV-COX), and another in which only the luminal portion of LAMP1 was deleted (pEGFP-COXL1).

Protein Expression

6xHis tagged DS1 ChOx was purified by expression of pET-COX in *E. coli* BL21 (Invitrogen). Cells were lysed with B-PER protein extraction reagent (Thermo Fisher Scientific, Waltham, MA) and extracts were subjected to Ni-NTA column purification (HisPur Ni-NTA resin, Thermo Fisher Scientific) under native conditions.

Enzyme Assays

DS1 ChOx was indirectly assayed for activity against cholesterol and 7KC by monitoring oxidation of *o*-Dianisidine at 500 nm. Lipids were extracted from separate reactions and used for HPLC analysis. The fractions from HPLC were further characterized using GC–MS.

Cytotoxicity Assays

For cytotoxicity assays, fibroblasts were seeded at approximately 2×10^5 cells per well in 6-well plates or 5×10^3 cells in 96-well plates containing Eagle's minimum essential media (EMEM) with 10% fetal bovine serum and 1% glutamine–penicillin–strepomycin solution, and allowed to recover overnight. Cells were exposed to varying concentrations of cholesterol, 7KC, or 7KDHEA for 24 h and evaluated for cytotoxicity using an XTT Cell Viability Assay Kit (Biotium, Hayward, CA). Additionally, treated fibroblasts were analyzed using the CytoGLO Annexin V-FITC Apoptosis Detection Kit (IMGENEX, San Diego, CA).

Localization

The intracellular expression of EGFP was observed using an inverted fluorescence microscope (IX-71, Olympus America, Melville, NY) with a FITC filter set. To determine proper localization of the cholesterol oxidase construct, cell cultures were rinsed with PBS and exposed to EMEM containing 5 μg/mL acridine orange (Molecular Probes, Bleiswijk, The Netherlands) for 15 min at 37°C. Co-localization of acridine orange and EGFP was determined using an Olympus IX81 confocal microscope.

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