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Antonio E. Rusiñol
Quillen-Dishner College of Medicine, rusinol@etsu.edu

Lin Yang
Quillen-Dishner College of Medicine

Douglas Thewke
Quillen-Dishner College of Medicine, thewke@etsu.edu

Sankhavaram R. Panini
Quillen-Dishner College of Medicine

Marianne F. Kramer
Quillen-Dishner College of Medicine

See next page for additional authors

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Rusiñol, Antonio E.; Yang, Lin; Thewke, Douglas; Panini, Sankhavaram R.; Kramer, Marianne F.; and Sinensky, Michael S.. 2000. Isolation of a Somatic Cell Mutant Resistant to the Induction of Apoptosis by Oxidized Low Density Lipoprotein. *Journal of Biological Chemistry*. Vol.275(10). 7296-7303. https://doi.org/10.1074/jbc.275.10.7296 PMID: 10702300 ISSN: 0021-9258

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 10, Issue of March 10, pp. 7296–7303, 2000
Printed in U.S.A.
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Isolation of a Somatic Cell Mutant Resistant to the Induction of Apoptosis by Oxidized Low Density Lipoprotein*

(Received for publication, September 16, 1999, and in revised form, December 2, 1999)

Antonio E. Rusiñol, Lin Yang, Douglas Thewke, Sankhavaram R. Panini, Marianne F. Kramer, and Michael S. Sinensky‡

From the Department Of Biochemistry and Molecular Biology, James H. Quillen College of Medicine, East Tennessee State University, Johnson City, Tennessee 37614-0581

Oxidized low density lipoprotein (oxLDL) induces apoptosis in macrophages, smooth muscle cells, and endothelial cells. To elucidate the molecular mechanism of oxLDL-induced cytotoxicity and determine its tissue specificity, we have used Chinese hamster ovary (CHO)-K1 cells expressing human CD36 (CHO/CD36). Expression of CD36 rendered these cells susceptible to killing by oxLDL. This cytotoxicity was due to the induction of apoptosis. Therefore, CD36 expression is the only requirement for oxLDL-induced apoptosis. Oxysterols apparently mediate the cytotoxicity of oxLDL in macrophage foam cells and endothelial cells. 25-Hydroxycholesterol, at concentrations higher than 1 μg/ml, killed CHO-K1 cells, by apoptosis, in medium supplemented with serum as a source of cholesterol. These effects were not seen in a 25-hydroxycholesterol-resistant CHO/CD36 mutant (OX^R), which was otherwise capable of undergoing apoptosis in response to staurosporine. This mutant was also resistant to killing by oxLDL, suggesting that oxysterols are at least partially responsible for the toxic effects of oxLDL. Oxysterol-induced apoptosis did not involve regulation of sterol regulatory element-binding protein proteolysis or the cholesterol biosynthetic pathway. 25-Hydroxycholesterol stimulated calcium uptake by CHO-K1 cells within 2 min after addition. Treatment of CHO or THP-1 (macrophage) cells with the calcium channel blocker nifedipine prevented 25-hydroxycholesterol induction of apoptosis. OX^R showed no enhanced calcium uptake in response to 25-hydroxycholesterol.

Oxidized low density lipoprotein (oxLDL)[1] plays an important role in atherogenesis (1, 2). oxLDL can be generated *in vivo* by at least three classes of mechanisms: 1) autoxidation in the presence of transition metals (3, 4); 2) cell-mediated oxidation (5–7); and 3) plasma enzyme-mediated oxidation (8–11). oxLDL plays a role in many early events of atherosclerosis; it induces the expression of adhesion molecules on endothelial cells (12), the transformation of macrophages and smooth muscle cells to foam cells (13), the production of various proinflammatory cytokines and growth factors by almost all vascular cells (14, 15), the proliferation and migration of vascular cells (16–18), and the retardation of endothelial regeneration (19), and it increases the procoagulant activity on the vascular cells (20). These changes eventually result in the formation of atheromatous lesions. Local oxidative degradation of trapped LDL may generate lipid-derived inflammatory mediators, such as oxysterols, lysophospholipids, and fatty acid peroxides.

oxLDL and its lipid components have also been shown to be cytotoxic for cultured vascular smooth muscle cells, endothelial cells, macrophages, fibroblasts, and central nervous system cells (5, 21–23). Recently, this cytotoxicity has been partly attributed to induction of apoptosis. oxLDL induces both the morphological changes and DNA fragmentation characteristic of apoptosis in cultured smooth muscle cells (24), macrophages (24, 25), endothelial cells (26, 27), and lymphoid cells (29). It has been shown that the apoptosis-inducing activity was recovered in the neutral lipid fraction of oxLDL, and various oxysterols in this fraction induce apoptosis in endothelial cells (26), monocytic cell lines (30), thymocytes (31), and smooth muscle cells (32). However, it is not clear *how* oxLDL or its active components induce apoptosis in these and other vascular cells. Different agents, such as tumor necrosis factor, γ radiation, UV radiation, hydrogen peroxide (33), and growth factor removal (34), induce apoptosis in many cell types by both unique and common mechanisms. For example, Fas and tumor necrosis factor receptor family members transduce the signal of apoptosis through death domain-containing molecules, such as FADD (35), whereas many other agents induce apoptosis by pathways that do not involve protein molecules with a death domain. On the other hand, almost all known agents that induce apoptosis share the activation of caspases and the execution phase of the death program (36).

CHO cells are well suited for mutational analysis of complex cellular pathways (37). Because of this, it is possible to use transfected or mutant CHO cell lines for genetic analysis of the gene products and functions involved in the programmed cell death induced by oxLDL or its components. In this study, we have used CHO-K1 cells stably transfected with CD36 to study the cytotoxic effects of oxLDL and 25-hydroxycholesterol. We have also generated a CHO/CD36 mutant cell line resistant to killing by these two agents.

EXPERIMENTAL PROCEDURES

Materials—CHO-K1 cells were purchased from the American Type Culture Collection (Manassas, VA). All cell culture reagents were obtained from Life Technologies, Inc. 25-Hydroxycholesterol was from Steraloids Inc. (Wilton, NH). $^{45}Ca^{2+}$ was from Amersham Pharmacia Biotech. DiI and fluorescein isothiocyanate- or Texas Red-conjugated secondary antibodies were from Molecular Probes, Inc. (Eugene, OR). Anti-CD36 monoclonal antibody was from Pharmingen. Horseradish peroxidase-conjugated goat anti-mouse IgM and IgG and the micro-BCA protein assay kit were from Pierce. The plasmid pcDNA3 carrying

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‡ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, East Tennessee State University, James H. Quillen College of Medicine, Box 70581, Johnson City, TN 37614-0581. Tel.: 423-439-8016; Fax: 423-439-8010; E-mail: sinensky@etsu.edu.

[1] The abbreviations used are: oxLDL, oxidized low density lipoprotein; LDL, low density lipoprotein; SRE, sterol regulatory element; SREBP, sterol regulatory element-binding protein; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; LDL, low density lipoprotein; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; TUNEL, terminal deoxyuridine nick end labeling; 25-OHC, 25-hydroxycholesterol; AMC, 7-amido-4-methylcoumarin.

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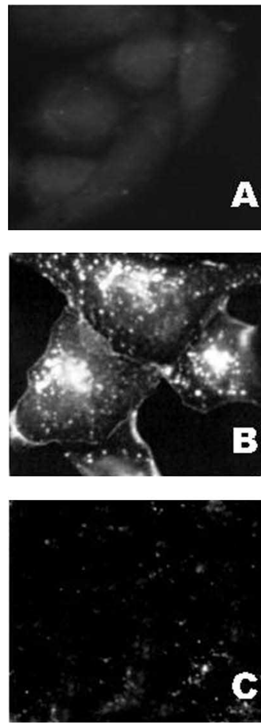


FIG. 1. **Binding and uptake of DiI-labeled oxLDL by CD36-expressing CHO-K1 cells.** CHO-K1 cells were transfected with the vector pcDNA3 carrying the human CD36FLAG cDNA (CHO/CD36). Transfected cells were grown on glass coverslips and then incubated with 10 μg/ml DiI-oxLDL for 1 h at 37 °C. After washing with PBS, cells were fixed in 4% formaldehyde in PBS and mounted. Cell association of DiI-labeled oxLDL was observed under fluorescence microscopy. *A*, CHO-K1 cells; *B*, CHO/CD36 cells; *C*, CHO/CD36 cells in the presence of a 50-fold excess of unlabeled oxLDL.

human CD36FLAG cDNA was generously provided by Dr. D. M. Lublin (Department of Pathology, Washington University School of Medicine, St. Louis, MO). SREBP constructs were kindly provided by Dr. T. F. Osborne (Department of Molecular Biology and Biochemistry, University of California, Irvine, CA). pTK(K×3)CAT was provided by Dr. David Russell (University of Texas, Southwestern, Dallas, TX).

THP-1 cells (TIB-202) were obtained from the American Type Culture Collection and were grown in RPMI 1640 medium supplemented with 10 mM HEPES, 2 mM glutamine, 10% fetal bovine serum, 50 μM 2-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin (RPMI medium) under a humidified 5% CO_2 atmosphere. For experiments, cells were plated on coverslips at 1×10^6 cells/cm^2 in RPMI medium containing 100 nM phorbol 12-myristate-13-acetate (Alexis, San Diego, CA). The cells were allowed to attach and differentiate into macrophages for 72 h.

Cell Culture and Transfection—For isolation of permanent transfectants expressing CD36, CHO-K1 cells were grown in Ham's F-12 medium containing 5% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (F12FC5) at 37 °C and 5% CO_2 and transfected with pcDNA3, carrying human CD36FLAG cDNA and neomycin resistance, by the lipofectam method using a mammalian transfection kit (Stratagene, La Jolla, CA). The neomycin-resistant cells were selected using 500 μg/ml G418 (Life Technologies, Inc.) in F12FC5. Resistant colonies were isolated and assayed for the presence of CD36 and DiI-oxLDL binding, and internalization activity as described below. Colonies expressing CD36 activity were expanded and maintained in F12FC5 containing 500 μg/ml G418. For 25-hydroxycholesterol cytotoxicity assays, CHO-K1 cells were seeded at a density of 500 or 1000 cells/35- or 60-mm dish in F12FC5 on day 0. On day 1, the cells were rinsed with phosphate-buffered saline (PBS) twice and then fed either Nutridoma-SP (1% in Ham's F-12) or F12FC5 containing oxysterols or oxLDL as described in the figure legends. Following incubation, cells were fed fresh F12FC5 and allowed to grow for 5 days. The surviving colonies were then fixed and stained with crystal violet as described (38).

Transient transfections of pTK(K×3)CAT (3 μg) were performed on 5×10^4 cells/60-mm plate using the Stratagene Transfection MBS Mammalian Transfection Kit according to the instruction manual with an internal standard expressing β-galactosidase (2 μg), pCMVβ-gal

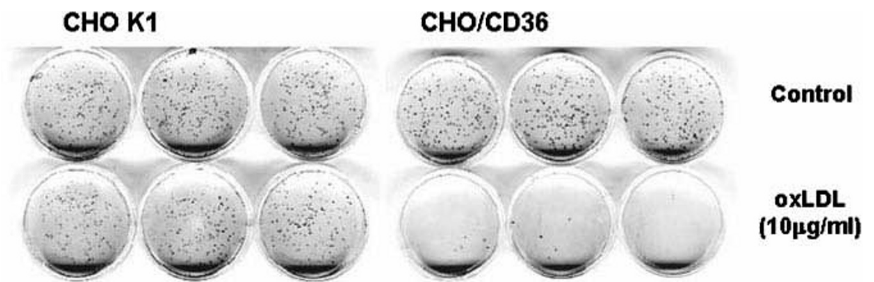
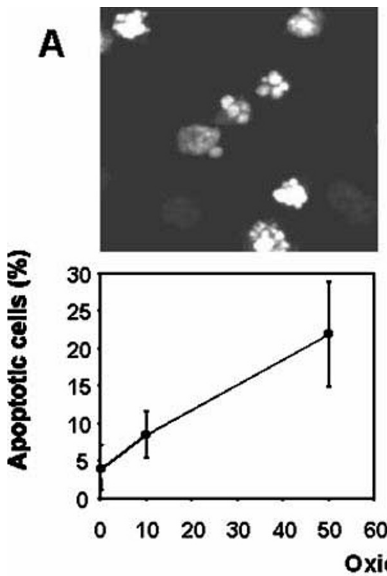


FIG. 2. **Effect of oxLDL on CHO-K1 and CHO/CD36 cells.** Cells were seeded at 500 cells/35-mm culture dish in F12FC5 and incubated overnight. The culture medium was then changed to F12FC5 containing 10 μg/ml oxLDL or F12FC5 (control). The cells were incubated 5 days and fixed, and colonies were stained as described under "Experimental Procedures."



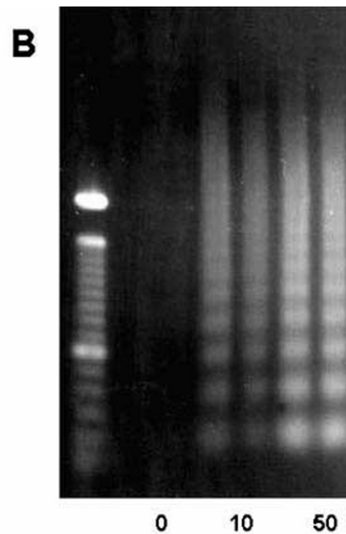


FIG. 3. **oxLDL induces apoptosis in CHO/CD36 cells.** *A*, cells (50,000) were plated on glass coverslips and incubated for 48 h in F12FC5 containing 0, 10, or 50 μg/ml oxLDL. After washing with PBS, the cells were fixed, and apoptosis was evaluated by an *in situ* TUNEL reaction as described under "Experimental Procedures." The graph represents the average percentage of TUNEL-positive cells in 10 40× fields ± S.D. *B*, cells at an initial density of 5×10^5 were cultured for 24 h in F12FC5, with 0, 10, or 50 μg/ml oxLDL, and then harvested, lysed, and fragmented DNA was detected on agarose gels as described under "Experimental Procedures."

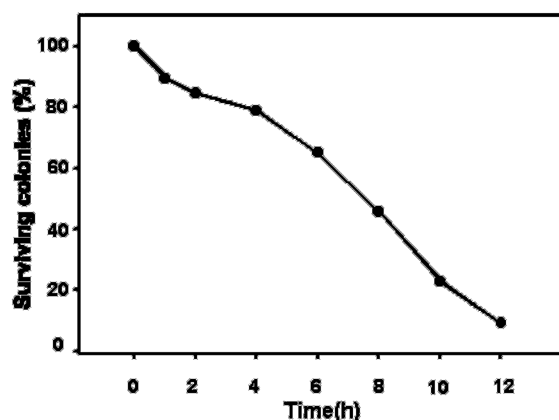


FIG. 4. **Time course killing of CHO-K1 cells by 25-hydroxycholesterol.** CHO-K1 cells were seeded at 500 cells/35-mm culture dish in F12FC5 and incubated overnight. The culture medium was then changed to F12FC5 supplemented with 0.03% EtOH (control) or 3 μg/ml 25-hydroxycholesterol. Cells were then incubated for 0, 1, 2, 4, 6, 8, 10, or 12 h. After each time point, the medium was removed, the cells were rinsed and refed F12FC5 for 5 days, and then surviving colonies were fixed, stained, and counted.

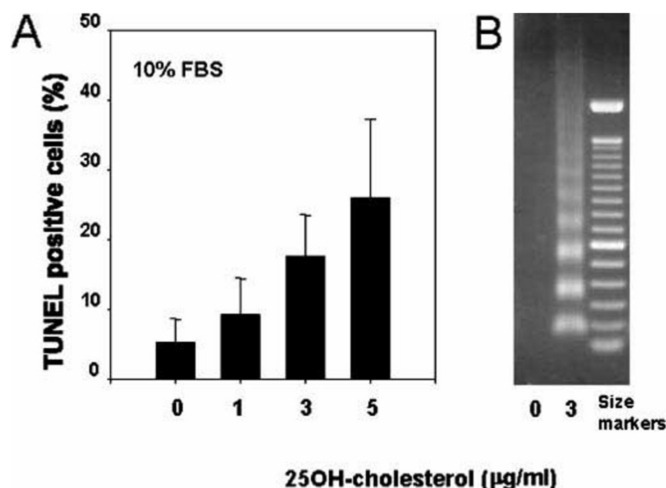


FIG. 5. **25-Hydroxycholesterol induces apoptosis in CHO/CD36 cells.** *A*, apoptosis assay. Cells (30,000) were plated on glass coverslips and incubated for 24 h in F12FC5 containing 0, 1, 3, or 5 μg/ml 25-hydroxycholesterol. Apoptosis was measured by TUNEL as described in the legend to Fig. 3. *B*, gel electrophoresis of DNA. Cells at an initial density 5×10^5 were cultured for 24 h in F12FC5, with or without 3 μg/ml 25-hydroxycholesterol, and incubated for 24 h and then harvested and lysed, and DNA fragments were detected as described in the legend to Fig. 3.

(Promega), to correct for transfection efficiency. Chloramphenicol acetyltransferase assays were as described previously (57).

TUNEL Assay—Cells (50,000) were plated on glass coverslips and incubated for different periods of time in F12FC5 with or without 10–50 μg/ml oxLDL. After washing with PBS, the cells were fixed in 4% buffered paraformaldehyde for 30 min at room temperature. Coverslips were rinsed, and cells were permeabilized with 1% Triton X-100 in 100 mM sodium citrate buffer and incubated for 1 h at 37 °C with terminal deoxynucleotidyltransferase and fluorescein isothiocyanate-dUTP to label the fragmented DNA. After completion, coverslips were mounted in anti-fade mounting solution and observed under a Nikon Diaphot-200 microscope.

Preparation of Modified LDL—LDL ($1.019 < d < 1.063$) was prepared from normal human serum by sequential ultracentrifugation as described previously (39). Extensively oxidized LDL was prepared by incubation with $CuCl_2$ as described previously (40). Oxidation of LDL was evaluated by agarose gel electrophoresis. The oxLDL had 3–5-fold higher R_F values on agarose gel electrophoresis compared with native LDL. DiI-labeled oxLDL was prepared as described elsewhere (41). Essentially, oxLDL was incubated with the DiI probe in human lipoprotein-deficient serum for 12 h at 37 °C, using the following relative amounts: 300 μl (300 μg) of DiI, 3 mg of lipoprotein lipid, 2 ml of lipoprotein-deficient serum. Subsequently, the labeled lipoproteins are reisolated by ultracentrifugation for 2.5 h at 99,000 rpm in a TL-100 centrifuge. Labeled lipoproteins were stored at 4 °C in the dark and used within 2 weeks after their preparation.

Detection of CD36 in CHO/CD36—Transfected cells were grown overnight on glass coverslips and then incubated with 10 μg/ml DiI-oxLDL for 1 h at 37 °C. After washing with PBS, cells were fixed in freshly prepared 4% formaldehyde in PBS and mounted. Cell association of DiI-labeled oxLDL was observed by fluorescence microscopy using a Texas Red filter cube. Alternatively, after fixation, standard immunofluorescence was performed with anti-CD36- or anti-FLAG-specific monoclonal antibodies.

Detection of Fragmented DNA on Agarose Gels—Detection of oligonucleosomal DNA fragments was done as described previously (42). Basically, cells at an initial density of 5×10^5/100-mm dish were cultured for 24 h in F12FC5 plus oxLDL or oxysterols and then harvested, lysed, and centrifuged to separate DNA fragments from intact chromatin. Supernatants were precipitated overnight at −20 °C with 2 volumes of ethanol and centrifuged at 13,000 × *g* for 15 min. Then pellets were incubated for 30 min at 37 °C in 500 μl of Tris-EDTA (TE) buffer supplemented with 100 μg/ml RNase A. Samples were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and once again with chloroform/isoamylalcohol (24:1). DNA was precipitated, and pellets were recovered by centrifugation (13,000 × *g*, 15 min), air-dried, resuspended in 10 μl of TE buffer, supplemented with 2 μl of sample buffer (0.25% bromphenol blue, 30% glycerol), and electrophoretically separated on a 1.3% agarose gel. After electrophoresis, they were stained in ethidium bromide (1 μg/ml). Pictures were taken by UV transillumination.

TABLE I
Spectrofluorometric analysis of caspase-3 activity for CHO cells treated with and without 25-hydroxycholesterol

CHO-K1 cells were treated with 0 or 3 μg/ml 25-hydroxycholesterol for 48 h, and 250 μg of total cell lysate protein were assayed for caspase-3 as described under "Experimental Procedures." Data are the average of triplicates ± S.D.

CHO-K1 Cell lysate	Ac-DEVD-AMC	Ac-DEVD-CHO	Relative AMC fluorescence	Net Relative AMC fluorescence
Untreated	+	−	0.267 ± 0.015	0.063 ± 0.021
	+	+	0.203 ± 0.012	
25-OHC (48 h)	+	−	0.560 ± 0.020	0.457 ± 0.025
	+	+	0.103 ± 0.005	

Caspase 3 Assay—CHO-K1 cells were plated and treated as described above. After treatment, both treated and nontreated cells were harvested and collected by centrifugation (800 × *g* for 10 min). Cells were washed twice with ice-cold PBS and lysed with cell lysis buffer (10 mM Tris (pH 7.5), 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM NaP_i, and 10 mM $NaPP_i$). Samples were incubated on ice for 10 min, passed through a 21-gauge syringe 15 times, and then centrifuged at 15,000 × *g* for 20 min at 4 °C. Protein concentration in the supernatant (total cell lysate) was measured by the micro-BCA kit. Triplicates of 250 μg of total cell lysate protein were incubated with Ac-DEVD-AMC (20 μM) caspase-3 substrate or substrate plus the specific inhibitor Ac-DEVD-CHO (100 nM) for 1.5 h at 37 °C in protease assay buffer (20 mM HEPES, pH 7.5, 10% glycerol, 2 mM dithiothreitol). Liberated AMC from Ac-DEVD-AMC was measured on a spectrofluorometer with an excitation wavelength of 380 nm and an emission wavelength of 440 nm.

25-Hydroxycholesterol Esterification—Cells (1.5×10^5/35-mm plate) were incubated with 3 μg/ml 25-[26,27-^{3}H]hydroxycholesterol (20,000 dpm/μg) for 18 h. The dishes were extracted twice with 3 ml of hexane/isopropyl alcohol (60:40). The fixed cells were digested for 3 h in 1 ml of 0.1 N NaOH, and 0.1-ml aliquots were assayed for protein (BCA, Pierce). The lipid extract was concentrated to 0.1 ml by evaporation under nitrogen and spotted onto silicic acid TLC plates, and the components were separated with the solvent system hexane/diethyl ether/HAc (80: 20:1). The 25-hydroxycholesterol ester was visualized by fluorography, and the bands were scraped and quantitated by liquid scintillation counting.

Calcium Uptake Assay—-Kinetic measurements of calcium were performed by a dipping technique as described previously (58). Briefly, cells were plated on glass coverslips at a density of 5×10^5 cells/35 mm

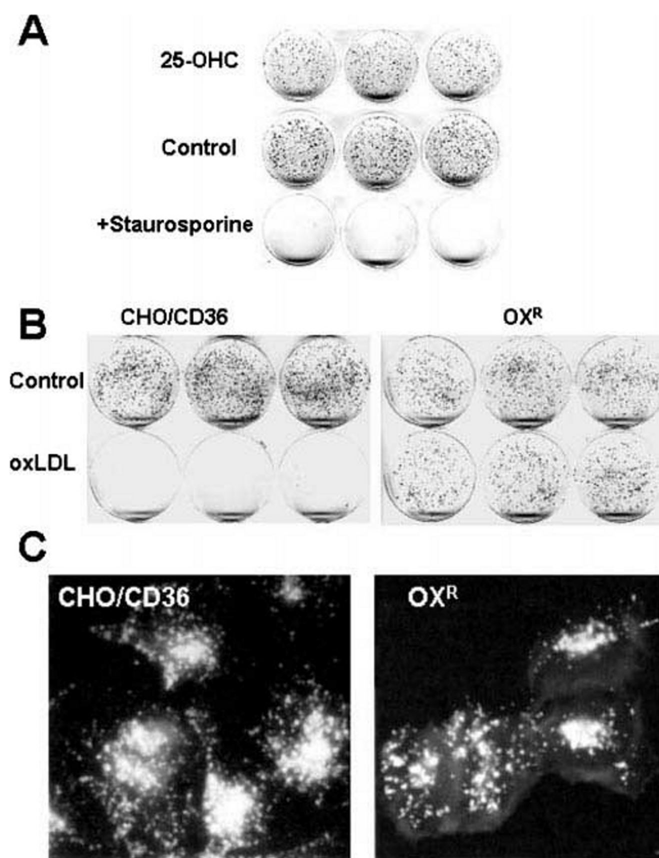


FIG. 6. **Isolation of a 25-hydroxycholesterol-resistant CHO/CD36 mutant.** CHO-K1 cells were mutagenized by treatment with methanesulfonic acid ethyl ester. Mutants were selected from the colonies that survived in 3 μg/ml 25-OHC. *A*, OX^R cells were seeded at 500 cells/35-mm culture dish in F12FC5 medium and incubated overnight. The medium was then changed to F12FC5 and 0.03% EtOH (control), 3 μg/ml 25-OHC, or 1 μM staurosporine, as indicated to the *left*. After 24 h, the medium was removed, and the cells were rinsed and refed F12FC5 for 5 days and then fixed and stained. *B*, OX^R cells were plated in F12FC5 at a density of 1000 cells/60-mm plate. On day 2, cells were treated with or without 10 μg/ml oxLDL for 6 days; cells were then washed with PBS, and the medium was changed back to F12FC5. Colonies were allowed to grow for another 2 days and then fixed and stained. *C*, CHO/CD36 and OX^R cells were grown on glass coverslips and then incubated with 10 μg/ml DiI-oxLDL for 1 h at 37 °C. After washing with PBS, cells were fixed in 4% formaldehyde in PBS and mounted. Cell association of DiI-labeled oxLDL was observed by fluorescence microscopy.

dish. After an overnight incubation in F12FC5, coverslips were sequentially washed in a reference buffer. At time 0, the coverslips were dipped in a beaker containing Ca^{2+} and $^{45}Ca^{2+}$ in reference buffer with or without 25-hydroxycholesterol. After the indicated period of time, cells were washed and solubilized in 1% SDS, and radioactivity was measured by liquid scintillation.

RESULTS

Transfection of CD36 into CHO-K1 Cells Confers the Ability to Take Up oxLDL—Chemical modifications of LDL, such as oxidation, convert LDL into a high affinity ligand of scavenger receptors. oxLDL is cytotoxic for many cell types in the vessel wall, and its interaction with the macrophage scavenger receptors and subsequent toxic effects play a crucial role in the initiation of the atherosclerotic lesion. To test if uptake of oxLDL is sufficient to elicit cytotoxic effects in a fibroblastic cell line, we have transfected CHO-K1 with CD36, a scavenger receptor structurally related to SR-BI and its human counterpart CLA-I. This receptor has been reported to bind oxLDL and acetylated LDL. (43, 53). Cell association of oxLDL to CHO-K1 expressing CD36 was examined by fluorescence microscopy after incubating DiI-labeled oxLDL with CHO-K1 stably transfected with a vector carrying CD36FLAG (CHO/CD36). Untransfected CHO cells displayed a diffuse light staining, possibly due to traces of free DiI or uptake through other scavenger receptors (Fig. 1*A*). However, when CHO/CD36 cells were incubated with DiI-oxLDL, a subset of cells, corresponding to the number of cells expressing CD36, as determined by immunofluorescence with monoclonal antibodies specific against CD36 or FLAG (results not shown), showed both an internal punctuated staining pattern (probably endosomes and lysosomes) and an intense plasma membrane staining (Fig. 1*B*). Specificity of the interaction was proven by the ability of unlabeled oxLDL (50-fold excess) to inhibit the DiI-oxLDL staining (Fig. 1*C*). These findings confirm previous reports (44) that CD36 when expressed on CHO cells binds and allows internalization of oxLDL.

oxLDL Is Cytotoxic to CHO/CD36—Using a single-cell plating assay to determine cytotoxicity, we found that expression of CD36 in CHO cells renders the cells susceptible to killing by oxLDL (Fig. 2). In this assay, 500 cells are plated on 35-mm dishes, subjected to different treatments, and allowed to form colonies, which can be stained and counted. Fig. 2 shows that treatment of CHO/CD36 with 10 μg/ml of oxLDL for 5 days eliminates formation of colonies, indicating cytotoxicity, whereas untransfected CHO-K1 cells were not affected by this treatment.

oxLDL Induces Apoptosis in CHO/CD36—oxLDL induced apoptosis in a time- and dose-dependent manner (Fig. 3). Approximately 10 or 20% of the CHO/CD36 cells became TUNEL-positive after a 48-h incubation with 10 or 50 μg of protein/ml of oxLDL, respectively (Fig. 3, *A* and *B*). Commensurate with the results derived from the TUNEL assay, DNA ladders were present in genomic DNA extracted from CHO/CD36 cells incubated with similar oxLDL concentrations (Fig. 3*C*). The fragmented DNA showed the distinct pattern of oligonucleosomes found in apoptotic cells. oxLDL (10 μg/ml) induced significant apoptosis in CHO/CD36 cells as early as at 16 h of incubation (data not shown), and the number of apoptotic cells increased up until 48 h of incubation. Incubation of CHO/CD36 with native LDL or incubation of CHO-K1 with oxLDL did not induce apoptosis beyond the control level (less than 1% of total cells) (data not shown). These findings indicate that oxLDL is able to induce apoptosis in cultured fibroblasts when the cells are able to bind and/or internalize oxLDL.

25-Hydroxycholesterol Induces Apoptosis in CHO-K1 Cells—Several recent studies have shown that much of the cytotoxicity of oxLDL is associated with the neutral lipid components, in particular the oxysterols (45, 46). The cytotoxicity of oxysterols may be evidenced by a number of mechanisms, from effects on cholesterol synthesis to effects on cell membranes to induction of apoptosis. (30, 47). In the next set of experiments, we addressed the following two main questions. 1) Are oxysterol-resistant mutants cross-resistant to oxLDL? 2) Does transcriptional down-regulation of cholesterol synthesis by oxysterols play a role in their induction of apoptosis?

Fig. 4 shows the results of a representative single-cell plating experiment as a function of time of exposure to 3 μg/ml 25-hydroxycholesterol in the presence of serum as the exogenous source of cholesterol. A significant reduction (~50%) in the number of viable cells was seen after a 6-h incubation. Furthermore, after a 12-h incubation, virtually all treated cells were dead. The mode of cell death was confirmed to be through apoptosis by TUNEL assay (Fig. 5, *A* and *B*), oligonucleosomal DNA laddering assay (Fig. 5*C*), and caspase 3 activation assay (Table I).

The 25-Hydroxycholesterol-resistant Mutant Cell Line (OX^R)

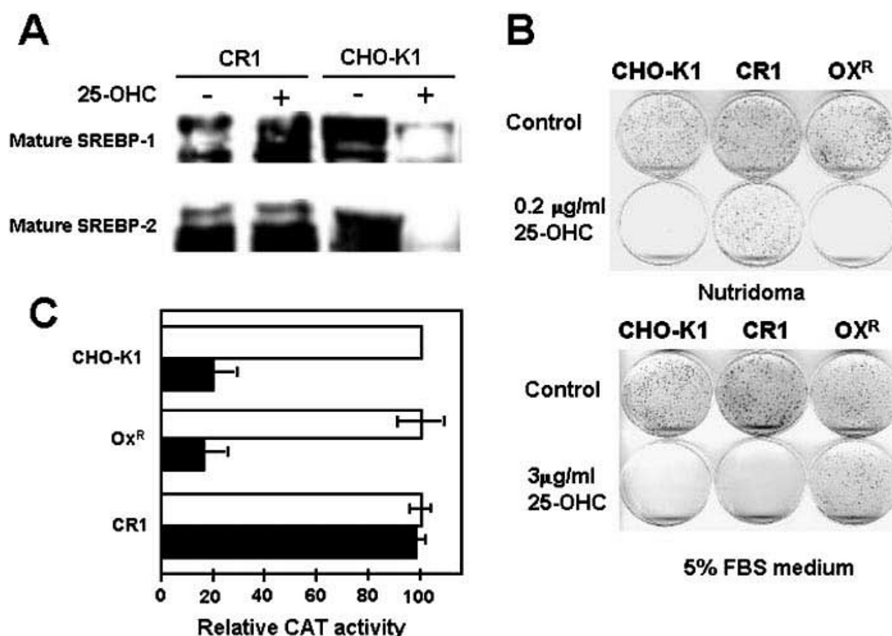


FIG. 7. **Comparison of the phenotype of a cholesterol regulatory mutant, CR1, and OX^{R}.** *A*, SREBPs are constitutively processed in CR1. Nuclear proteins were isolated from CHO-K1 and CR1, cultured for 24 h in delipidized medium supplemented with or without sterols (0.2 μg/ml 25-hydroxycholesterol and 10 μg/ml cholesterol) as indicated. Immunoblots for SREBP were performed essentially as described elsewhere (57). *B*, growth phenotype of CR1 and OXR cells. CHO-K1, OX^{R}, and CR1 cells were seeded at a density of 1000 cells/60-mm plate and permitted to attach in F12FC5 overnight. The medium was then switched to F-12 plus 1% Nutridoma (cholesterol-free medium) and 0.2 μg/ml 25-hydroxycholesterol or 3 μg/ml 25-hydroxycholesterol in F12FC5, incubated for another 5 days, and then fixed and stained. *C*, comparison of transcriptional control of SREs in OX^{R} and CR1. Cells (5×10^4 cells/60-mm plate) were incubated in growth medium overnight. Then 3 μg of pTK(K×3)CAT reporter vector and 2 μg of pCMVβ-gal were transiently transfected per 60-mm plate. The cells were assayed for chloramphenicol acetyltransferase and β-galactosidase activities after 24 h of incubation in delipidized medium (56) in the absence (*open bars*) or presence (*shaded bars*) of sterols (1 μg/ml 25-hydroxycholesterol and 10 μg/ml cholesterol).

Is Cross-resistant to oxLDL—If oxysterols are the mediators of the cytotoxic effects of oxLDL, an oxysterol-resistant mutant should be resistant to killing by oxLDL. We mutagenized CHO/CD36 with methanesulfonic acid ethyl ester and selected colonies that grew in medium containing 3 μg/ml 25-hydroxycholesterol (25-OHC). As shown in Fig. 6*A*, OX^{R} cells are resistant to killing by 3 μg/ml 25-hydroxycholesterol. These cells were also resistant to killing by 35 μg/ml oxLDL (Fig. 6*B*). Fig. 6*C* shows that the staining pattern of OX^{R} cells with DiI-oxLDL is similar to that in CHO/CD36, suggesting that binding and internalization of oxLDL in the OX^{R} cell line is unaltered from its parental cell line. A general defect in one or more steps in the apoptosis pathway *per se* (*e.g.* defective caspase 3, overexpression of Bcl-2, etc.) would produce a similar resistance phenotype. Therefore, we also confirmed that OX^{R} cells were capable of undergoing apoptosis in response to another known apoptosis inducer. Fig. 6*A* shows that OX^{R} cells are susceptible to staurosporine, a reagent that induces apoptosis through inhibition of protein kinase C, and therefore seemed to have a functioning apoptosis pathway at least in the execution phase of the death program.

Oxysterol-induced Apoptosis Does Not Involve Transcriptional Control of Cholesterol Biosynthesis—The best known biological activity of oxysterols is transcriptional repression of cholesterol biosynthesis, through inhibition of processing of the SREBPs, although other activities for these molecules have recently come to be appreciated (62). Oxysterols have been reported to initiate apoptosis in CEM leukemic cells by interfering with the synthesis of cholesterol in the absence of an exogenous cholesterol source (47). Since we carried out our incubations in the presence of serum, as an exogenous cholesterol source, we would expect that the apoptotic pathway would not be mediated by inhibition of cholesterol synthesis. However, we directly examined the possibility that oxysterol killing by apoptosis concerned regulation of SREBP proteolysis and the cholesterol biosynthetic pathway.

Somatic cell mutants of CHO cells, resistant to inhibition of growth by 25-hydroxycholesterol in the absence of an exogenous cholesterol source, have been extremely useful in defining the role of SREBPs in the regulation of cholesterol biosynthesis (37, 48). There are two classes of oxysterol-resistant mutants characterized to date: class 1 mutants, which constitutively produce a truncated SREBP-2 that acts as a mature transcription factor, and class 2 mutants, which are defective in SREBP cleavage-activating protein, which appears to transduce the oxysterol-mediated inhibition of SREBP proteolysis. Two lines of evidence using some of these mutants suggest that SREBP proteolysis regulation is not involved in oxysterol-induced apoptosis. First, CHO-K1 cells transfected with constitutively active SREBP-2 are resistant to inhibition of growth by <0.3 μg/ml 25-hydroxycholesterol in sterol-free medium but are still killed by 3 μg/ml 25-hydroxycholesterol in medium supplemented with serum (data not shown). Second, we also compared OX^{R} cells to CR1 cells, a 25-hydroxycholesterol-resistant mutant isolated in cholesterol-free medium (56), which we have recently found to be a class 2 mutant (data not shown). This line, as do other class 2 mutants, shows constitutive expression of both SREBP1 and SREBP2 in cholesterol-free medium supplemented with 25-hydroxycholesterol (Fig. 7*A*) but is still sensitive to killing by 3 μg/ml 25-hydroxycholesterol in medium supplemented with serum (Fig. 7*B*).

The resistance of CR1 to inhibition of growth in cholesterol-free medium (Fig. 7*B*) is at a concentration of 25-hydroxycholesterol (0.2 μg/ml), where killing of wild-type cells does not occur in medium supplemented with cholesterol (59). Resistance of CR1 to killing, under these conditions, reflects the constitutive processing of SREBPs and the resultant loss of transcriptional control of cholesterol biosynthesis (60), medi-

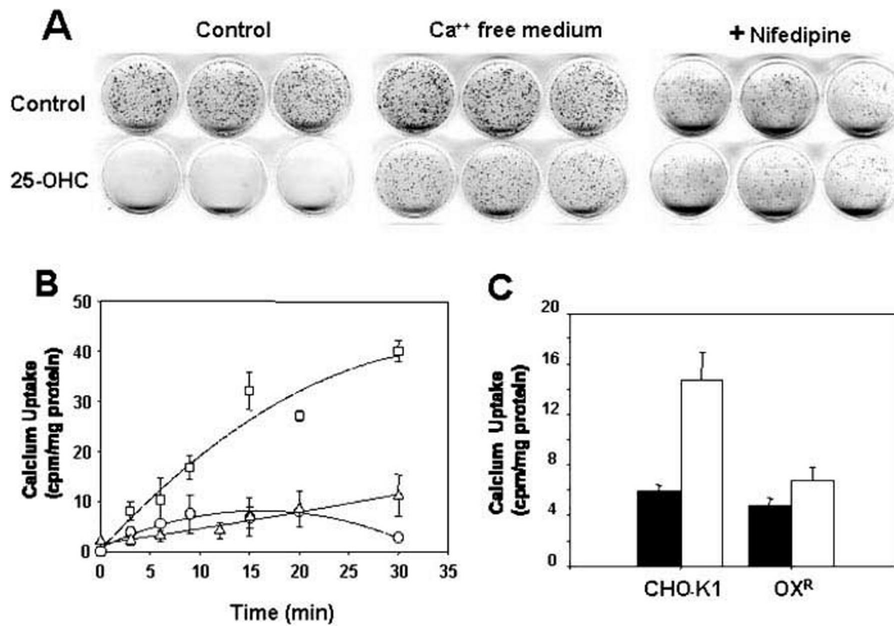


FIG. 8. **Role of Ca^{2+} uptake in the apoptosis induced by 25-hydroxycholesterol.** *A*, CHO-K1 cells were seeded at 500 cells/35-mm culture dish in F12FC5 and incubated overnight. The culture medium was then changed to Dulbecco's modified Eagle's medium, with or without Ca^{2+}, or supplemented with nifedipine (100 μM) 5% fetal bovine serum medium, and 0.03% EtOH (control) or 3 μg/ml 25-OHC and incubated for 12 h. The medium was removed, and the cells were rinsed and refed F12FC5 for 5 days before being fixed and stained. *B*, CHO-K1 cells were plated on glass coverslips at a density of 5×10^5 cells/35-mm dish. After an overnight incubation in F12FC5, $^{45}Ca^{2+}$ uptake was determined as described under "Experimental Procedures" in the absence (○) or presence (□) of 3 μg/ml 25-hydroxycholesterol or in medium supplemented with both 3 μg/ml 25-hydroxycholesterol and 100 μM nifedipine (△). *C*, CHO-K1 or OX^R cells were seeded as in *B* and incubated overnight, and $^{45}Ca^{2+}$ uptake was determined in the absence (*solid bars*) or presence (*open bars*) of 3 μg/ml 25-hydroxycholesterol.

ated by SRE1 elements. Sensitivity of OX^R to 25-hydroxycholesterol under these conditions suggests that cholesterol biosynthesis is still transcriptionally controlled in this mutant. This hypothesis was tested directly by means of an SRE1 reporter construct, pTK(K×3)CAT (61), for transcriptional control by sterols. Comparison of the response of this reporter to treatment with 25-hydroxycholesterol in CR1 and OX^R (Fig. 7*C*) indicates that regulation of SRE1 in OX^R cells is normal in these cells, in contrast to the loss of transcriptional control in CR1.

This result also suggests that uptake and intracellular transport of 25-hydroxycholesterol is unaffected in OX^R. We checked this directly by determining the incorporation of 25-[^{3}H]hydroxycholesterol into ester in OX^R cells as compared with its parental cell line, CHO/CD36, (see "Experimental Procedures"). The results indicated similar levels of incorporation of label into ester in the two cell lines (CHO/CD36, 0.18 nmol/mg of protein/h; OX^R, 0.15 nmol/mg of protein/h), confirming that uptake and transport of 25-hydroxycholesterol to the endoplasmic reticulum is not affected in the mutant.

It might be expected that there would be no role for transcriptional control of cholesterol biosynthesis in the induction of apoptosis by higher concentrations of 25-hydroxycholesterol in medium supplemented with serum and, therefore, an exogenous source of cholesterol. This expectation is confirmed by sensitivity of the transcriptionally constitutive CR1 cells to 3 μg/ml 25-hydroxycholesterol in medium supplemented with serum, in contrast to the resistance of OX^R (Fig. 7*B*) under these conditions.

Calcium Uptake Initiates the Programmed Cell Death Induced by 25-Hydroxycholesterol—High intracellular calcium levels have been reported to induce apoptosis in several experimental models (32, 47, 49, 50) including in response to oxLDL (27). 25-Hydroxycholesterol has also been shown to increase the cellular uptake of calcium in a variety of cell types (32, 51). Therefore, we investigated the possible involvement of calcium in the initial signaling events in the oxysterol-induced apoptosis. Fig. 8*A* shows that CHO-K1 cells were protected from the killing effects of 3 μg/ml 25-hydroxycholesterol when the treatment was carried out in calcium-free medium. Therefore, in order to confirm that oxysterol affects Ca^{2+} transport, we measured calcium uptake using $^{45}Ca^{2+}$. Fig. 8*B* shows that 3 μg/ml 25-hydroxycholesterol stimulates Ca^{2+} uptake in CHO-K1 with an increase of 3-fold in the first 10 min. Furthermore, when the L-type calcium channel blocker nifedipine was included during the incubations, the 25-hydroxycholesterol-induced apoptosis (Fig. 8*A*) and the calcium uptake (Fig. 8*B*) were blocked. Nifedipine had no effect on induction of apoptosis

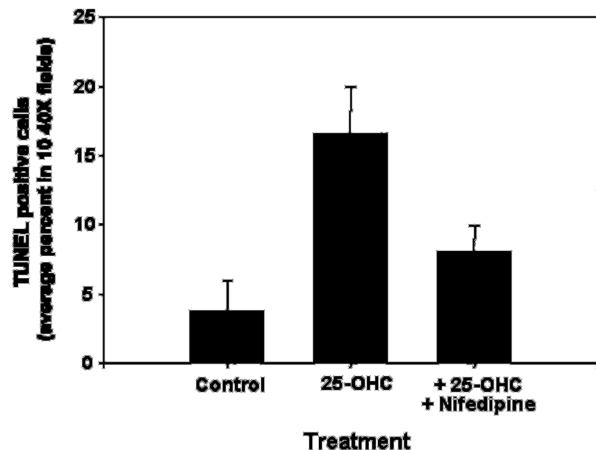


FIG. 9. **The induction of apoptosis by 25-hydroxycholesterol is inhibited in THP-1 macrophage by nifedipine.** THP-1 monocytes were differentiated into macrophage on coverslips with phorbol ester as described under "Experimental Procedures." They were then treated with 5 μg/ml 25-hydroxycholesterol plus 0 or 100 μM nifedipine. After a 24-h incubation, apoptosis was assayed by *in situ* TUNEL as described above.

by staurosporine (data not shown), a reagent that does not signal apoptosis through stimulation of Ca^{2+} uptake. Measurement of 25-hydroxycholesterol stimulation of Ca^{2+} uptake in OX^R indicated that the mutant was unresponsive (Fig. 8*C*), an observation highly consistent with oxysterol-induced apoptosis being mediated by intracellular Ca^{2+}.

To further assess the physiological relevance of these findings, we also examined the effect of nifedipine on the induction of apoptosis by 25-hydroxycholesterol in a monocyte-macrophage cell line, THP-1. THP-1 cells were differentiated into macrophage by treatment with phorbol ester. Treatment with 25-hydroxycholesterol (5 μg/ml) efficiently induced apoptosis as assayed with TUNEL. Nifedipine was shown to substantially reduce the induction of apoptosis by 25-hydroxycholesterol (Fig. 9).

DISCUSSION

Previous reports have shown that oxysterols and oxidized LDL induce apoptosis in a variety of cell types. In this study, we have confirmed these observations and shown that oxidized LDL induces apoptosis in a fibroblast cell line transfected with the human CD36 scavenger receptor. This finding indicates that the apoptotic pathway, which mediates oxidized LDL cytotoxicity, is not tissue-specific. Tissue specificity is conferred by the presence of oxidized LDL receptors, which mediate the uptake of oxidized LDL and therefore deliver large amounts of oxysterols to cells that possess them. CD36 is a glycoprotein with a molecular mass of 88 kDa. CD36 is normally expressed in platelets, monocytes, macrophages, capillary endothelial cells, and adipocytes. The physiological function of CD36 has not been completely elucidated. Although there has been some controversy concerning the forms of LDL that bind to CD36, the ability of CD36 to bind oxLDL has been well documented (43, 53, 54). Our results confirm these findings, since expression of CD36 in CHO cells was sufficient to confer on these cells the ability to take up oxLDL (Fig. 1) and to render them susceptible to killing by copper-oxidized LDL (Figs. 2 and 3).

We also showed here that oxysterols are at least partially responsible for the cytotoxic effects of oxidized LDL. Several studies have examined the toxicity of oxysterols on various cell types of the vascular wall, including endothelial cells, smooth muscle cells, and monocyte-macrophages. In these studies, a wide variety of effects and toxicity strength among the different oxysterols was observed (55). We selected 25-hydroxycholesterol for our studies, since it is widely used as a model of this class of compounds.

The single cell plating assay for cytotoxicity and the *in situ* TUNEL assay for apoptosis used in this study are dependent on cell attachment. In the former (Figs. 2, 4, and 6–8), live cells that detach before the end of the treatment will not form colonies and therefore will not be counted, producing artificially high estimations of cell death. In the apoptosis assay (Figs. 3 and 5), on the other hand, detached cells will not be subjected to the TUNEL reaction, and artificially low estimation of apoptotic cell counts may be obtained. Nevertheless, our results clearly indicate that 25-hydroxycholesterol induces apoptosis. Our finding of cross-resistance to oxysterols and oxLDL suggests that the elucidation of the precise mechanism by which oxysterols can induce apoptosis would be of interest.

The best described biological activity of oxysterols is the regulation of proteolytic maturation of the SREBPs and consequent transcriptional regulation of promoters bearing SREs. However, the apoptotic response to oxysterols does not appear to occur through their regulatory effects on SREBP processing and lipid metabolism. This notion is immediately suggested by the observation of apoptosis in the presence of exogenous lipoproteins but is also clearly supported by the differential responses of the cholesterol regulatory, CR1- and apoptosis-resistant OX^R mutants to 25-hydroxycholesterol. Particularly noteworthy is the observation of normal oxysterol regulation of an SRE reporter expressed in OX^R (Fig. 7*C*), which strongly argues against any defect in the SREBP pathway in this type of resistant mutant.

Intracellular Ca^{2+}, rather than transcriptional control of SREs, appears to mediate the apoptotic effects of 25-hydroxycholesterol. The importance of calcium signals for initiating cell death has been demonstrated in other experimental systems (49), including in response to treatment of cells with 25-hydroxycholesterol (32, 47). In work reported by others (63) and in this current study, 25-hydroxycholesterol has been shown to induce apoptosis in monocyte-macrophage. Our observation (Fig. 9) that 25-hydroxycholesterol induction of apoptosis in monocyte-macrophage is inhibited by nifedipine implicates Ca^{2+} uptake as part of the signaling process. Consistent with the proposition that oxysterols mediate its apoptotic activity, oxidized LDL has been demonstrated to induce apoptosis through a calcium-dependent pathway (27) in endothelial cells.

Our results on CHO-K1 cells show that within a few minutes after addition, 25-hydroxycholesterol induced a 3-fold increase in intracellular calcium, which could be inhibited by nifedipine. This observation of activation of Ca^{2+} uptake, through a nifedipine-inhibitable channel in CHO-K1 cells, is novel. The existence of a voltage-dependent, nifedipine-inhibitable calcium channel in CHO-K1 cells has previously been demonstrated (52) by the patch-clamp technique. However, no functional role for this channel or regulator of its activity has previously been described.

Several observations indicate that this response affects apoptosis; DNA fragmentation (not shown) and cytotoxicity (Fig. 8) decreased in the presence of nifedipine or when calcium-free medium was used for the treatments, and a 25-hydroxycholesterol-resistant mutant, OX^R, did not exhibit enhanced Ca^{2+} uptake when treated with 25-hydroxycholesterol. These findings strongly suggest that increased intracellular calcium is a critical mediator of oxysterol toxicity.

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