Ca^{2+} channel blockers verapamil and nifedipine inhibit apoptosis induced by 25-hydroxycholesterol in human aortic smooth muscle cells

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Abstract. We have characterized the death of human aortic smooth muscle cells induced by 25-hydroxycholesterol, an oxidation product of cholesterol. Chromatin condensation characteristic of apoptosis was observed by enzymatic (TUNEL) staining of chromatin, and by electron microscopy. Fourteen percent of cells treated with 5 μg/ml of 25-hydroxycholesterol for 24 h displayed chromatin degradation as determined by positive TUNEL staining. Addition of TNFα (10 ng/ml) and IFNγ (20 ng/ml) increased the proportion of TUNEL positive cells to 30%, whereas the cytokines alone were without effect. After 48 h, 40% of the cells treated with 5 μg/ml of 25-hydroxycholesterol were TUNEL positive, and 21% of the cells displayed chromatin condensation. Oligonucleosomal DNA fragmentation typical of apoptosis was demonstrated by agarose gel electrophoresis. Furthermore, activation of the ICE-like protease caspase 3 (CPP32) was observed in cells treated with 25-hydroxycholesterol. Addition of the Ca^{2+} entry blockers verapamil or nifedipine to the culture medium inhibited apoptosis by more than 70% and reduced cytotoxicity, while removal of Ca^{2+} from culture medium reduced apoptosis by 42%. Within a few minutes after addition, 25-hydroxycholesterol induced intracellular Ca^{2+} oscillations with a frequency of approximately 0.3–0.4 min^{-1}. Thus it appears that Ca^{2+} influx through plasma membrane channels is an important signal in oxysterol-induced apoptosis. Addition of TNFα and IFNγ enhanced cytotoxicity and resulted in a higher proportion of apoptotic cells, suggesting that inflammatory cytokines can increase the cytotoxicity of lipid oxidation products.—**Ares, M. P. S., M. I. Pörn-Ares, J. Thyberg, L. Juntti-Berggren, P-O. Berggren, U. Diczfalusy, B. Kallin, I. Björkhem, S. Orrenius, and J. Nilsson.** Ca^{2+} channel blockers verapamil and nifedipine inhibit apoptosis induced by 25-hydroxycholesterol in human aortic smooth muscle cells. *J. Lipid Res.* 1997. **38:** 2049–2061.

Supplementary key words oxysterol • chromatin condensation • DNA fragmentation • CPP32 • caspase 3 • Ca^{2+}

Oxysterols (oxidized cholesterol derivatives) are known to be toxic to many cell types (1–3). 25-Hydroxycholesterol, an auto-oxidation product of cholesterol, has been shown to enhance the activity of the cholesterol esterifying enzyme ACAT (acyl-CoA:cholesterol O-acyltransferase, EC 2.3.1.26) both in intact cells and in microsomes (4–6). Other effects of 25-hydroxycholesterol include suppression of lipoprotein lipase mRNA and enzyme activity (7), enhancement of tissue factor activity (8), inhibition of cellular cholesterol efflux (9), elevation of intracellular Ca^{2+} concentration (10), and inhibition of gap junctional communication between hepatocytes (11). 25-Hydroxycholesterol can be produced endogenously from cholesterol (12). Low levels of 25-hydroxycholesterol have been detected in the plasma of experimental animals and humans (13), in cultured fibroblasts (14), and in human atherosclerotic arteries (15).

Apoptosis is characterized by nuclear and cytoplasmic condensation, membrane budding, formation of apoptotic bodies, and a non-random fragmentation of DNA (16, 17). In contrast to apoptosis, necrosis is the term frequently used to denote a passive death process, characterized by swelling and cytolysis of the dying cells. Proteases related to the mammalian interleukin-1β converting enzyme (ICE) and to nematode CED-3 appear to play an essential role in the apoptotic process. Of the family of ICE-like proteases, caspase 3 (CPP32, apo-

Abbreviations: MDEM, Dulbecco's modified essential medium; LDL, low density lipoprotein; ICE, interleukin-1β-converting enzyme; IFNγ, interferon gamma; SMC, smooth muscle cell; TNF, tumor necrosis factor; TUNEL, terminal deoxytransferase-mediated dUTP nick end labeling.

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pain) seems to be a key mediator of the apoptotic cell death in many systems (18, 19).

Apoptosis occurs during embryogenesis, in the course of normal tissue turnover, and in immune cell killing by glucocorticoids and certain cytokines such as TNFα (20) and IFNγ (21). Human atheromas are characterized by an increased frequency of apoptosis as compared to non-atherosclerotic arteries (22, 23). Similarly, human vascular smooth muscle cells derived from coronary plaques are characterized by increased frequency of apoptosis (24).

It has been shown that 25-hydroxycholesterol induces oligonucleosomal DNA fragmentation in murine thymocytes and RDM4 lymphoma cells (25), HL-60 cells, and U937 cells (26). However, DNA ladders have also been obtained from primarily necrotic cells, indicating that oligonucleosomal DNA fragmentation alone is not a sufficiently specific marker of apoptosis (27). In the present study, we demonstrate that the 25-hydroxycholesterol-induced death of human aortic smooth muscle cells occurs by apoptosis, and that it is mediated by an uptake of extracellular Ca^{2+} and associated with the activation of the ICE-like protease caspase 3 (CPP32). The findings that the inflammatory cytokines TNFα and IFNγ enhanced cytotoxicity of 25-hydroxycholesterol and stimulated apoptotic cell death when given in combination with the oxysterol suggest that the effects of lipid oxidation products on cells in atherosclerotic lesions may depend on other stimuli in the local microenvironment.

MATERIALS AND METHODS

Materials

Biotin-16-dUTP and terminal deoxytransferase were from Boehringer-Mannheim (Mannheim, Germany). Ca^{2+}-free medium, cholesterol, cholesterol-5α,6α-epoxide, ExtrAvidin-FITC, Hoechst 33342, 7β-hydroxycholesterol, 25-hydroxycholesterol, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), nifedipine, paraformaldehyde, *p*-phenylenediamine, and verapamil were purchased from Sigma Chemical Co. (St. Louis, MO). Na-cacodylate (dimethylarsinic acid sodium salt trihydrate) was obtained from Merck (Darmstadt, Germany). TNFα was purchased from R&D Systems (Minneapolis, MN), and IFNγ was kindly provided by Boehringer Ingelheim (Ingelheim, Germany). Fura-2 was obtained from Molecular Probes (Eugene, OR). Anti-human Fas monoclonal antibody (IgM clone CH-11) was purchased from Medical and Biological Laboratories Co., Ltd. (Nagoya, Japan). Caspase 3 (CPP32) anti-p17 antibody was a kind gift from Dr. Donald W. Nicholson (Merck Frosst Center for Therapeutic Research, Quebec, Canada).

Cell culture

Human aortic SMCs were purchased from Cytotech (Symbion Science Park, Copenhagen, Denmark). The cells were grown in DMEM supplemented with 13% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells in the 7th through the 18th passages were used in the experiments. During treatments with 25-hydroxycholesterol, cells were incubated in DMEM/F-12 (1:1) without phenol red, supplemented with 5% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were subconfluent at the time of experiments.

Cytotoxicity assay

Cytotoxicity was analyzed using the MTT assay (Sigma Chemical Co., St. Louis, MO). MTT was dissolved in DMEM/F12 (1:1) without phenol red at a concentration of 5 mg/ml. An amount of this solution equal to 10% of the culture medium volume was added to cell cultures. After 1 h, cultures were removed from the incubator and the formazan crystals solubilized by adding solubilization solution (10% (v/v) Triton X-100 and 0.1 N HCl in isopropanol) equal to the original culture medium volume. Metabolic activity was quantitated by measuring light absorbance at 570 nm.

TUNEL (terminal deoxytransferase-mediated dUTP nick end labeling) assay

Cells cultured on glass coverslips were fixed in 100% methanol at −20°C for 30 min. The coverslips were air-dried, rinsed twice with water, and transferred to cell culture dishes covered with wet tissue paper. Seventy-five μl of the following solution was added to each coverslip: 20 μM biotin-16-dUTP, 200 U/ml terminal deoxytransferase, 300 mM Tris-HCl (pH 7.2), 10 mM $CoCl_2$, and 300 mg/ml freshly added cacodylate. The samples were incubated at 37°C for 60 min. The coverslips were transferred to TB buffer (300 mM NaCl, 30 mM Na-citrate) for 15 min, rinsed twice with PBS, incubated in 2% BSA for 10 min, and rinsed 2 × 5 min with PBS. 100 μl of ExtrAvidin-FITC diluted to 15 μg/ml in PBS was added to each cover slip. After 30 min at 37°C, the samples were washed three times with PBS and once with PBS containing 0.1% Triton X-100. The coverslips were mounted on slides with anti-fading solution (1 mg/ml *p*-phenylenediamine, 10% (v/v) PBS, 90% (v/v) glycerol). At least 200 cells were counted for quantitative analyses. TUNEL positive cells had brightly stained nuclei, showing either homogeneous staining of intact nuclei, or condensation of chromatin into distinct, brightly stained fragments.

Electron microscopy

Primary fixative (2% glutaraldehyde, 2% formaldehyde, 0.1 M cacodylate, 0.05 M sucrose, pH 7.3) was added directly to cell culture dishes. Post-fixation was done with 1% osmium tetroxide in cacodylate buffer with 0.5% potassium ferrocyanate (2 h at 4°C). The samples were dehydrated in graded ethanol (70–100%), stained with 2% uranyl acetate in ethanol for 30 min, and embedded in Spurr low viscosity epoxy resin. The sections were cut with a diamond knife using an LKB Ultratome IV, picked up on carbon-coated formvar films, stained with alkaline lead citrate (3 min), and finally examined in a JEOL EM 100 CX operated at 60 kV.

Analysis of oligonucleosomal DNA fragmentation

Cells (10^6) were harvested by scraping and lysed in 20 µl of 10 mM EDTA, 50 mM Tris, pH 8, 0.5% Triton X-100. The samples were heated to 70°C for 5 min. RNase A (0.5 mg/ml) was added and the incubation was continued at 37°C for 1 h. The samples were then treated with proteinase K (0.5 mg/ml) at 50°C for 1 h. Aliquots of the samples were run on a 1.8% agarose gel with 0.5 µg/ml ethidium bromide.

Detection of caspase 3 by Western blotting

Cells were scraped from dishes into 4 ml of ice-cold PBS, pH 7.1, containing 100 µM phenylmethylsulfonyl fluoride (PMSF) and pelleted at 300 *g*. Cell pellets were then resuspended in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.1 mM PMSF) and boiled in a water bath for 5 min. Proteins were separated under reducing conditions at 120 V in 15% SDS-polyacrylamide gels and electroblotted at 100 V for 2 h. Blots were blocked overnight in a high-salt buffer (50 mM Tris-base, 500 mM NaCl) containing 5% dried milk and 1% bovine serum albumin, and then incubated for 1.5 h with an antibody against the p17 fragment of caspase 3 (1:10000). After washing the blots 4 times for 10 min in high-salt buffer with Tween 20 (0.05%), they were incubated with a peroxidase-conjugated secondary antibody (Pierce, Rockford, IL), and bound antibody was detected by enhanced chemiluminescence (Amersham).

Measurement of intracellular Ca^{2+}

Subconfluent SMCs on glass coverslips were loaded with 3 µM fura-2/AM for 30 min in culture medium (DME/F-12 (1:1), 5% FCS, without phenol red). The coverslips were transferred to a thermostatically controlled (37°C), open perifusion chamber placed in a holder on the stage of an inverted epifluorescence microscope (Zeiss Axiovert 35 M). Fura-2 fluorescence in single cells was measured using a SPEX fluorolog-2 CM1T11I system for dual-wavelength excitation fluorimetry, essentially as previously described (28). The excitation wavelengths were 340 and 380 nm and the emitted light, selected by a 510 nm filter, was monitored by a photomultiplier attached to the microscope. Data from all measurements are presented as 340/380 fluorescence ratios directly representative of changes in intracellular Ca^{2+}.

Statistical analysis

Mean values and standard deviations were calculated for triplicate determinations. Unpaired Student's *t* test was used to determine the statistical significance of the differences between treatments with 25-hydroxycholesterol alone and in combination with TNFα and IFNγ. For experiments with Ca^{2+}-free medium, the statistical significance of the difference between treatments with and without Ca^{2+} was determined.

RESULTS

Chromatin condensation

In apoptotic cells, the chromatin typically acquires a marginated or fragmented appearance, caused by its condensation into one or several clumps inside the nucleus. Chromatin condensation can be detected by staining with various DNA-binding dyes, such as the fluorescent dye Hoechst 33342. Human aortic SMCs with condensed chromatin were seen after treatment with 5 µg/ml of 25-hydroxycholesterol for 24 h and staining with the Hoechst dye 33342 (data not shown). Further work was done using the TUNEL assay, which detects cells at earlier stages of apoptosis, when distinct chromatin condensation has not yet occurred. Cells in which chromatin degradation but not chromatin condensation has started display uniformly and brightly stained nuclei, whereas normal non-apoptotic cells remain weakly stained (**Fig. 1**). The TUNEL method should not label necrotic cells until in late stages of necrosis (29), when the cells are characterized by diffusely distributed chromatin or totally degenerated appearance. In cell cultures, such cells can be identified and have not been counted as TUNEL positive cells in our experiments.

The inflammatory cytokines TNFα and IFNγ have been shown to be expressed in atherosclerotic vessels (30, 31). In the present study, we added TNFα and IFNγ to oxysterol treatments to see whether they would influence the effects of 25-hydroxycholesterol. The cytokines alone were not toxic to SMCs, but they potenti-

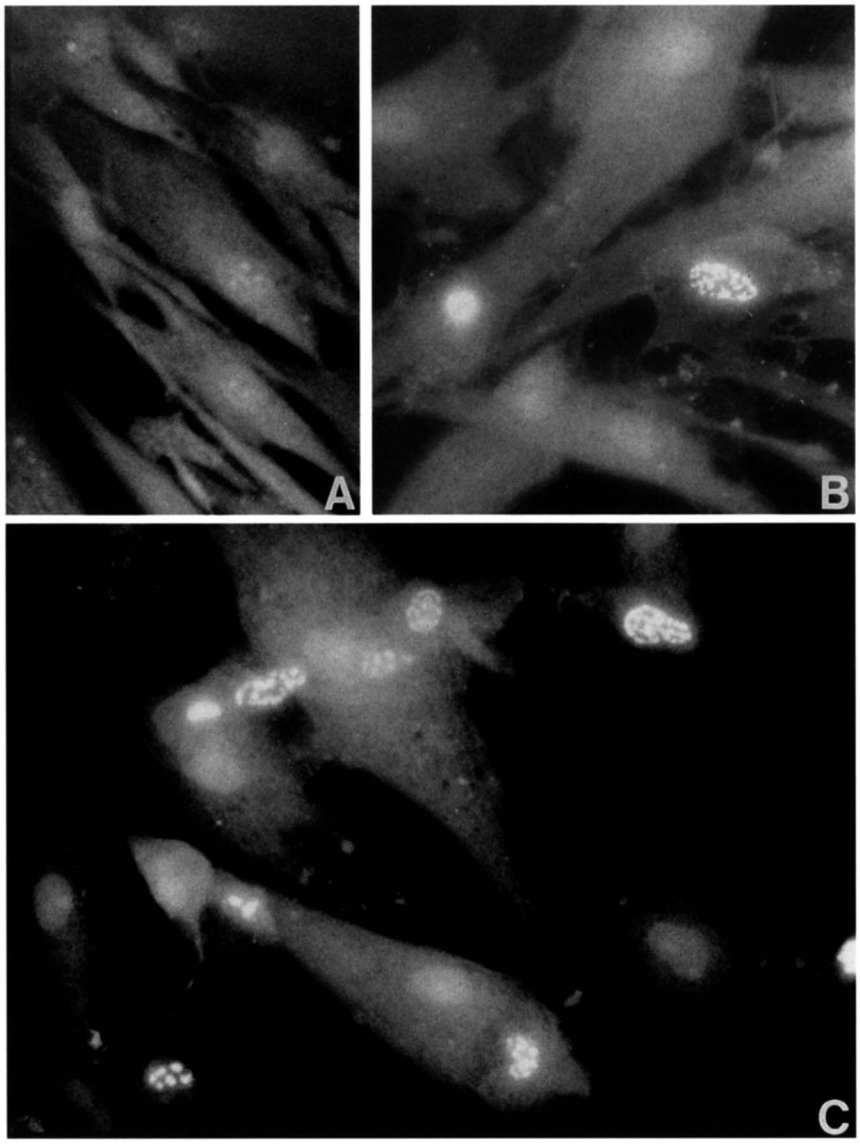

Fig. 1. TUNEL staining of SMCs showing chromatin fragmentation after treatment with 25-hydroxycholesterol. Untreated control cells (A) only showed weak background staining. After treatments with 5 μg/ml of 25-hydroxycholesterol for 24 h (B), two kinds of positive staining patterns were seen. Labeled cells with homogeneously distributed chromatin may be in the initial stage of chromatin degradation, whereas cells with condensed chromatin represent a late stage. Addition of TNFα (10 ng/ml) and IFNγ (20 ng/ml) to the treatments with 5 μg/ml of 25-hydroxycholesterol (C) resulted in a higher proportion of cells with condensed chromatin.

ated apoptosis induced by 25-hydroxycholesterol (**Fig. 2**). The percentage of TUNEL positive cells increased from 14.1% ± 3.3 to 30.1% ± 2.7 (mean ± SD; n = 3) when TNFα (10 ng/ml) and IFNγ (20 ng/ml) were added together with 5 μg/ml of 25-hydroxycholesterol (Fig. 2). The corresponding results for chromatin condensation were 3.1% ± 1.9 and 11.8% ± 3.9, respectively. In control experiments, cholesterol (up to 20 μg/ml) was completely non-toxic to SMCs, even for treatments up to 6 days. TUNEL staining was not increased in cholesterol-treated cells, as compared to nontreated controls (data not shown). Chromatin condensation (margination or fragmentation) was never observed in control cells, and the percentage of TUNEL positive cells always remained below 0.3%.

Maximal chromatin staining by TUNEL was seen 24 h after the addition of 25-hydroxycholesterol. At 48 h, cultures treated with 10 μg/ml of 25-hydroxycholesterol or 5 μg/ml of 25-hydroxycholesterol in combination with the cytokines displayed such extensive necrosis and loss of cells that quantitation of apoptosis was not possible. Concentrations of 25-hydroxycholesterol

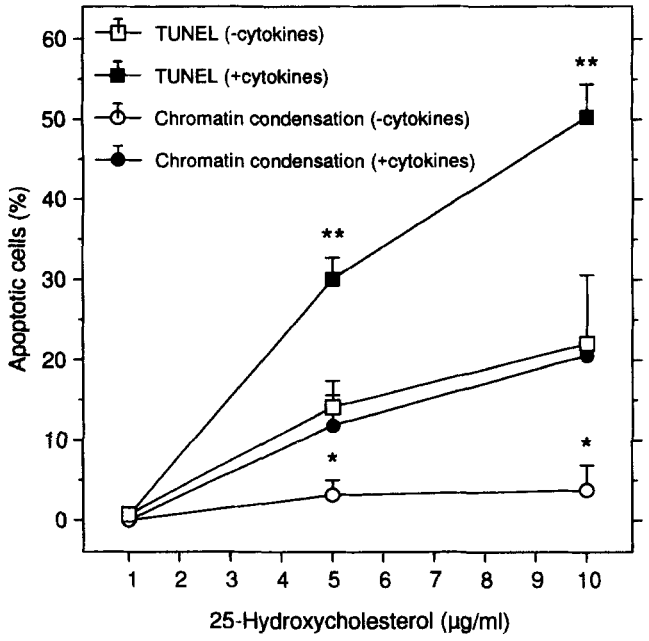

Fig. 2. TNFα and IFNγ enhance apoptosis induced by various concentrations of 25-hydroxycholesterol. SMCs were treated with 25-hydroxycholesterol ± cytokines for 24 h. Apoptosis was quantitated by the TUNEL assay, counting the percentage of cells with condensed (marginated or fragmented) chromatin and the percentage of cells that showed positively stained nuclei but not condensed chromatin. Results represent mean values ± SD ($n = 3$). Student's t test was used to compare results for treatments with 25-hydroxycholesterol alone and in combination with the cytokines ($*P < 0.05$, $**P < 0.01$).

above 1 µg/ml were needed to see significant chromatin staining. Increasing the incubation time for 1 µg/ml of 25-hydroxycholesterol ± cytokines to 96 h did not result in enhanced staining (data not shown). The optimal concentration of 25-hydroxycholesterol to induce apoptosis seemed to be 5 µg/ml. At 10 µg/ml, cell death occurred more rapidly and secondary necrosis became abundant already at 24 h. Plasma membrane blebbing was also observed in cells treated with 25-hydroxycholesterol. This was most clearly seen at 6–8 h after the addition of 25-hydroxycholesterol at a concentration of 10 µg/ml (data not shown).

Cytotoxicity of 25-hydroxycholesterol

The MTT assay provides a measure of metabolic activity that is closely correlated with the number of living cells. Toxicity of 25-hydroxycholesterol to SMCs (**Fig. 3**), as estimated by the MTT assay, always exceeded the proportion of TUNEL positive cells (Fig. 2 and data not shown). The difference is at least partly explained by the tendency of apoptotic cells to detach from the culture dish. Detached cells were not examined in the TUNEL assay and were thus excluded from quantitative evaluations. Therefore, the real proportions of apoptotic cells are likely to be higher than those reported. When 25-hydroxycholesterol-treated cells were counted with an electronic cell counter, the number of remaining cells generally exceeded the metabolic estimate obtained with the MTT test, even though de-

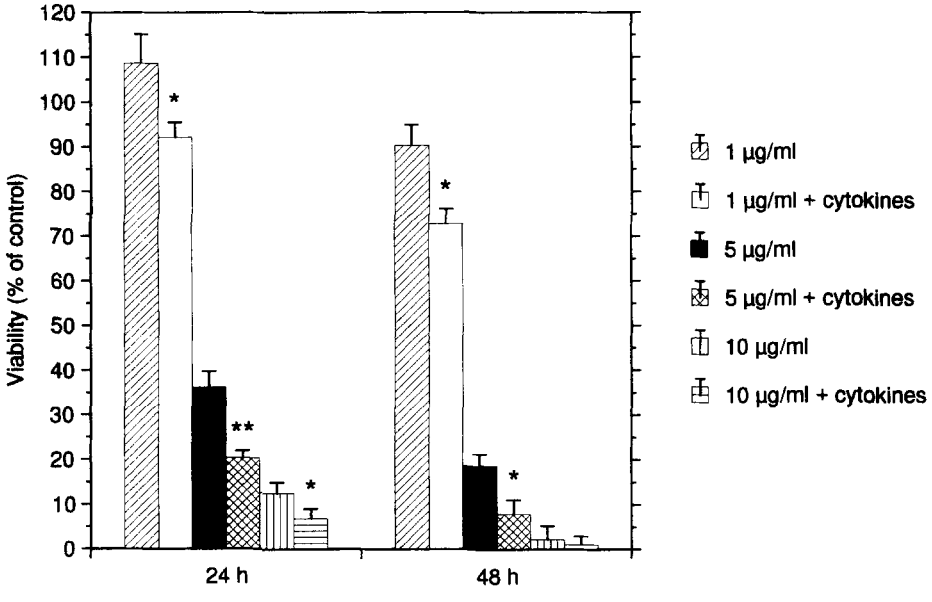

Fig. 3. Toxicity of 25-hydroxycholesterol to SMCs as measured by MTT assay. SMCs were treated with 25-hydroxycholesterol ± TNFα and IFNγ for 1 or 2 days. Lowest values indicate highest toxicity. Results represent mean values ± SD ($n = 3$). Results for treatments with 25-hydroxycholesterol alone or in combination with the cytokines were compared ($*P < 0.05$, $**P < 0.01$).

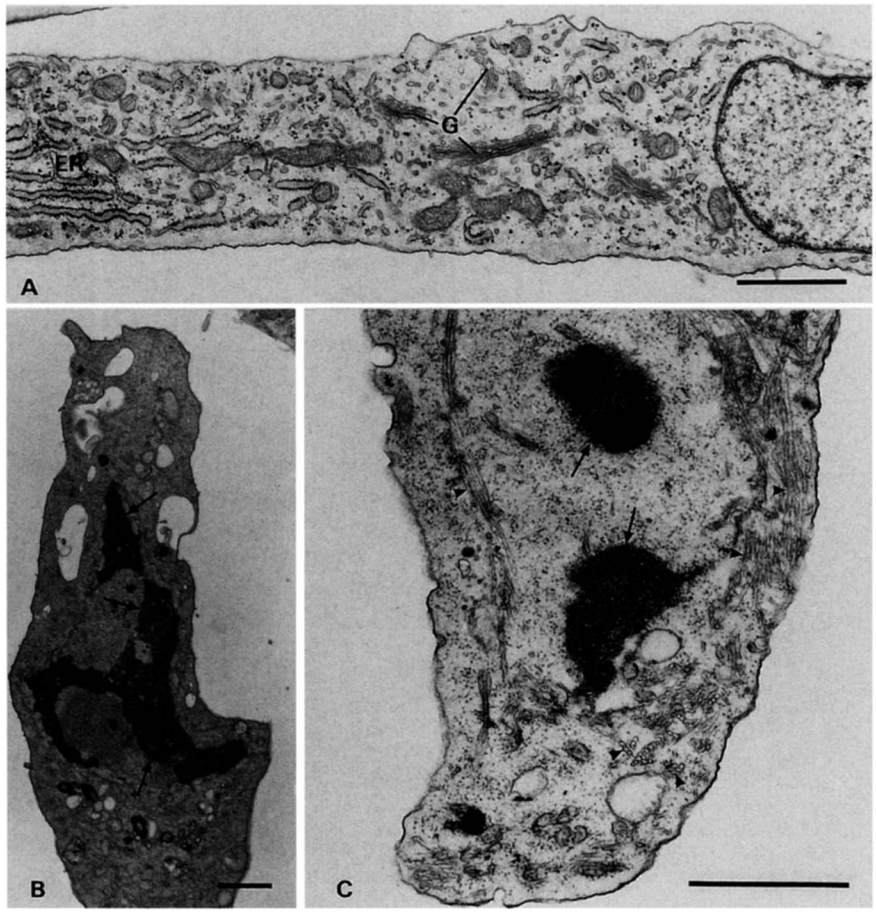

Fig. 4. Electron micrographs showing ultrastructural changes in SMCs treated with 25-hydroxycholesterol in the presence of 5% FCS. Untreated control cells (A) had abundant ER and Golgi membranes (G), typical of SMCs in the synthetic phenotype. Chromatin condensation (arrows) and tubular arrays (arrowheads) were seen in 25-hydroxycholesterol-treated cells (B and C). Cells were incubated with 5 μg/ml of 25-hydroxycholesterol for 24 h (B) or with 10 μg/ml of 25-hydroxycholesterol + TNFα (10 ng/ml) and IFNγ (20 ng/ml) for 8 h (C). Bars mark 1 μM.

tached cells had not been counted (data not shown). This suggests that many adherent, metabolically inactive apoptotic or necrotic cells were electronically counted but did not contribute to formazan formation in the MTT test. We therefore regard the MTT test to be the most suitable method for determining the toxicity of 25-hydroxycholesterol. Increasing the incubation time for treatment with 1 μg/ml of 25-hydroxycholesterol ± cytokines to 4 days did not result in increased toxicity (data not shown).

Electron microscopy

SMCs can express a range of phenotypes (32). At one end of this range is the cell whose function is mainly that of contraction (contractile state). At the opposite end of the range is the synthetic state, in which the muscle cell is engaged in proliferation and the production of extracellular matrix. Untreated, subconfluent control cells in the present study were in the synthetic state, characterized by abundant ER and Golgi membranes and few lysosomes (**Fig. 4A**). Very few dead cells were observed in these rapidly growing populations. Once the cells reached confluency, the number of lysosomes increased and occasional dying cells could be found. Confluent cells still had abundant ER and Golgi membranes typical of the synthetic phenotype. Apart from chromatin condensation (Fig. 4B, 4C) in SMCs treated with 25-hydroxycholesterol, interesting early changes were observed. After incubation for 4 h in the presence of 5–10 μg/ml of 25-hydroxycholesterol, the cells had an increased number of lysosomes but very few dead cells were found. In some cells, arrays of tubular membrane structures started to emerge (**Fig. 5A**). At 8–16 h, many cells had lost most of their ordered ER and Golgi membranes (Fig. 5B, 5C). Concomitant with this, the cells shrank. General swelling, a distinct feature of passive cell death, was not observed in cells treated with 5–10 μg/ml of 25-hydroxycholesterol. Swelling also did

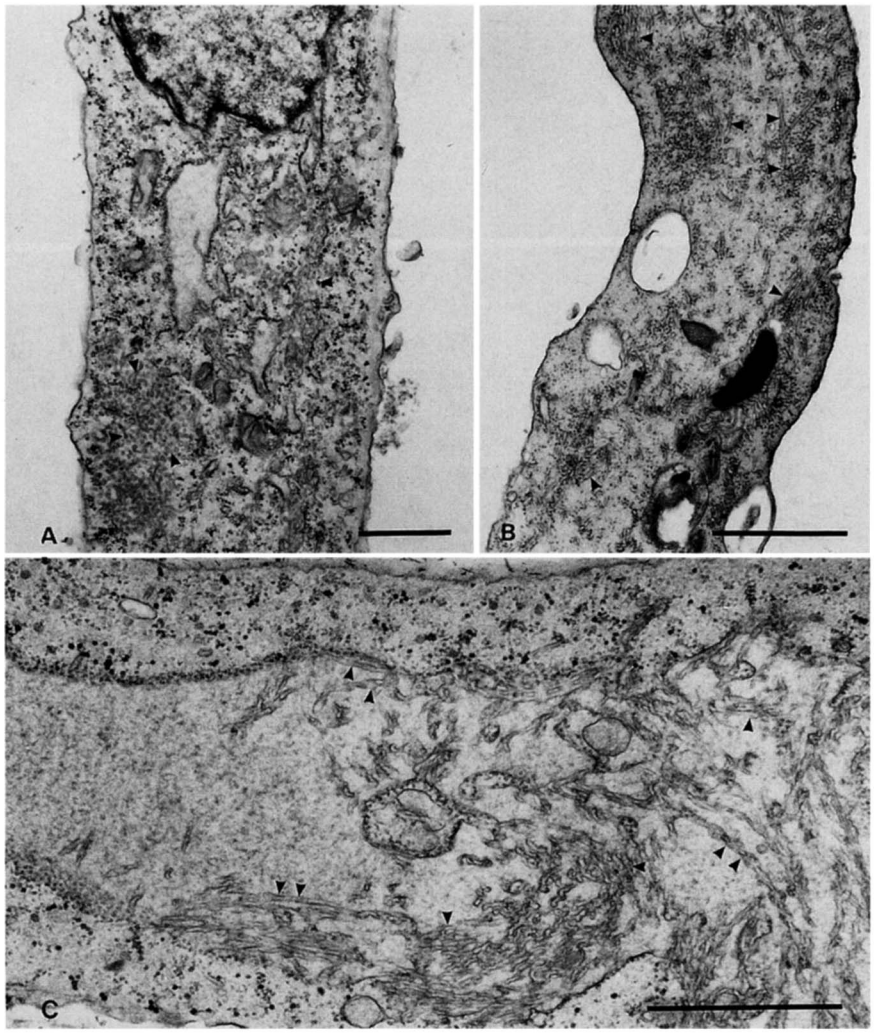

Fig. 5. Electron micrographs showing early ultrastructural changes in SMCs treated with 25-hydroxycholesterol in the presence of 5% FCS. Four hours after addition of 10 μg/ml 25-hydroxycholesterol + TNFα (10 ng/ml) and IFNγ (20 ng/ml), arrays of tubular membrane structures started to emerge in some cells (A; arrowheads). These changes gradually became more distinct (B and C; treatment with 10 μg/ml of 25-hydroxycholesterol for 16 h). It is not known whether the apparent loss of organized ER and Golgi membranes is a characteristic of apoptosis or primary necrosis in 25-hydroxycholesterol-induced cell death. However, it is important to note that swelling does not seem to be a dominant feature of these changes. Bars mark 1 μM.

not seem to account for the loss of ordered ER and Golgi membranes. Rather, these membranes were replaced by less organized arrays and networks of tubular membranes and multivesicular inclusions. Mitochondria were the last organelles to degenerate. Cell death increased with time so that at 48 h, most cells were necrotic and the occasional surviving cells had an increased number of lysosomes (**Fig. 6**). The remains of lysed cells were characterized by fragments of condensed chromatin, swollen mitochondria, and arrays of membrane stacks that resembled those membrane structures that started to form within 4 h after addition of 25-hydroxycholesterol (data not shown). Necrotic cells may have been apoptotic before plasma membrane breakdown, but a definitive distinction between primary and secondary necrosis is difficult to make on the basis of the ultrastructural analysis. Whether the apparent loss of organized ER and Golgi membranes is an indication of apoptosis or primary necrosis remains to be determined. However, swelling was not a dominant feature of these changes.

DNA fragmentation

Analysis of DNA prepared from SMCs treated with 25-hydroxycholesterol (5 μg/ml) produced a DNA ladder (**Fig. 7**), which is generally considered to be a hallmark of apoptosis. The bands represent multiples of approximately 180–200 base pairs, demonstrating oligo-

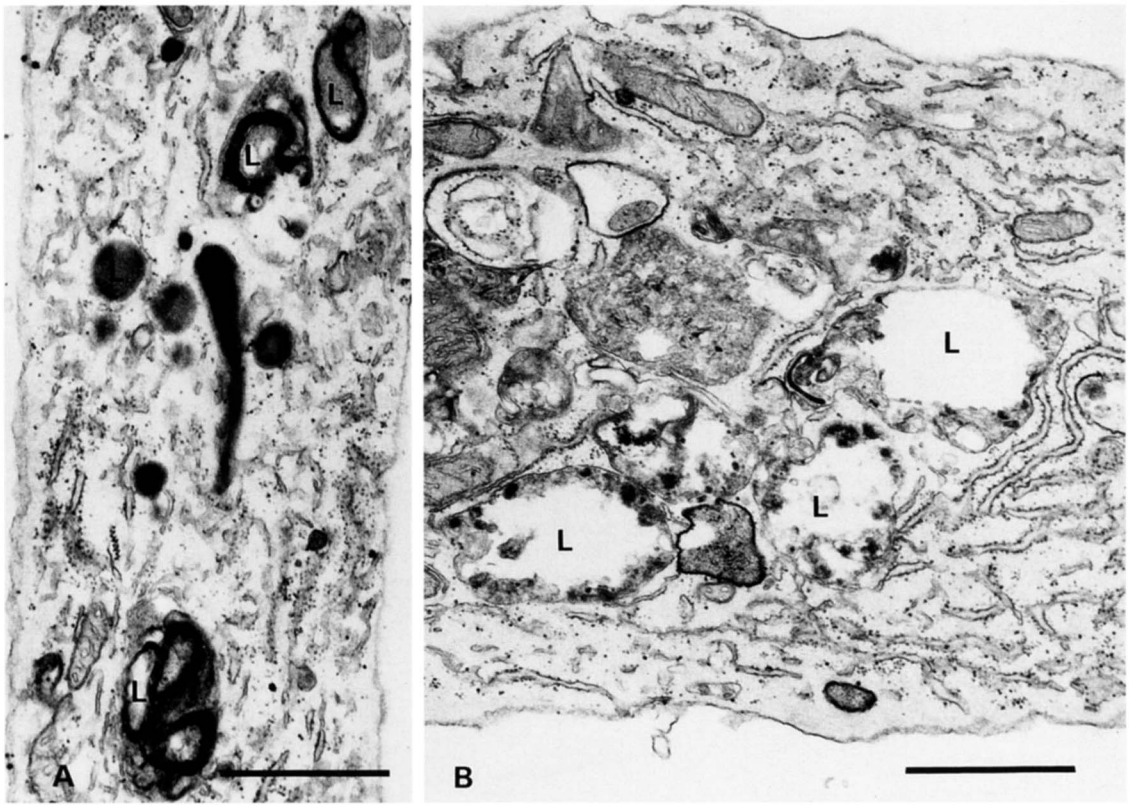

Fig. 6. Electron micrographs showing the accumulation of lysosomes (L) in cells that had survived long treatments with 25-hydroxycholesterol + TNFα (10 ng/ml) and IFNγ (20 ng/ml). Many of the inclusions may be remains of other cells that had been taken up from the culture medium. Incubation times were 40 h (A) and 48 h (B). Bars mark 1 μM.

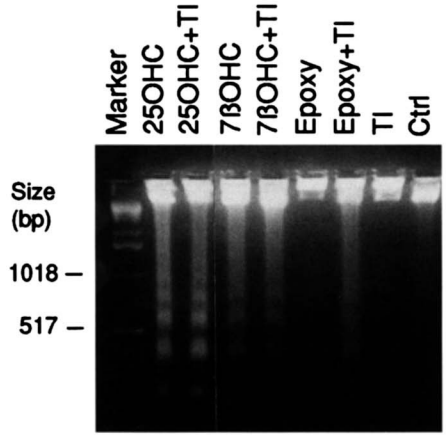

Fig. 7. Treatment with 25-hydroxycholesterol leads to oligonucleosomal DNA fragmentation. Cells were harvested by scraping, lysed, and treated with RNase and proteinase K as described in Methods. DNA fragmentation was analysed by agarose gel electrophoresis. Lane 1: marker; lane 2: 25-hydroxycholesterol (5 μg/ml, 24 h); lane 3: 25-hydroxycholesterol + TNFα (10 ng/ml) and IFNγ (20 ng/ml), 24 h; lane 4; 7β-hydroxycholesterol (5 μg/ml, 72 h); lane 5: 7β-hydroxycholesterol (5 μg/ml) + cytokines (48 h); lane 6: cholesterol-5α,6α-epoxide (10 μg/ml, 96 h); lane 7: cholesterol-5α,6α-epoxide (10 μg/ml) + cytokines (96 h); lane 8: TNFα (10 ng/ml) and IFNγ (20 ng/ml), 72 h; lane 9: control (no additions, 72 h).

nucleosomal DNA fragmentation. Addition of cytokines resulted in a higher proportion of low-molecular weight fragments. High-molecular weight DNA fragments could also be demonstrated (data not shown). After addition of 25-hydroxycholesterol to SMCs, high-molecular weight DNA fragments formed within 16 h, whereas the formation of low-molecular weight DNA fragments required a somewhat longer treatment (24 h). DNA fragmentation was also seen in cells treated with 7β-hydroxycholesterol or cholesterol-5α,6α-epoxide (Fig. 7). These oxysterols were less toxic than 25-hydroxycholesterol and required a longer incubation time. Cytokines alone did not induce DNA fragmentation. After prolonged incubations, when the cell populations were predominantly necrotic, no ladder pattern but only a smear of randomly cleaved DNA was seen (data not shown).

Ca^{2+} plays a critical role in 25-hydroxycholesterol-induced apoptosis

A sustained elevation of cytosolic free Ca^{2+} is known to induce apoptosis in several experimental systems (33–35). 25-Hydroxycholesterol has been shown to increase the cellular uptake of Ca^{2+} (10). Therefore, we

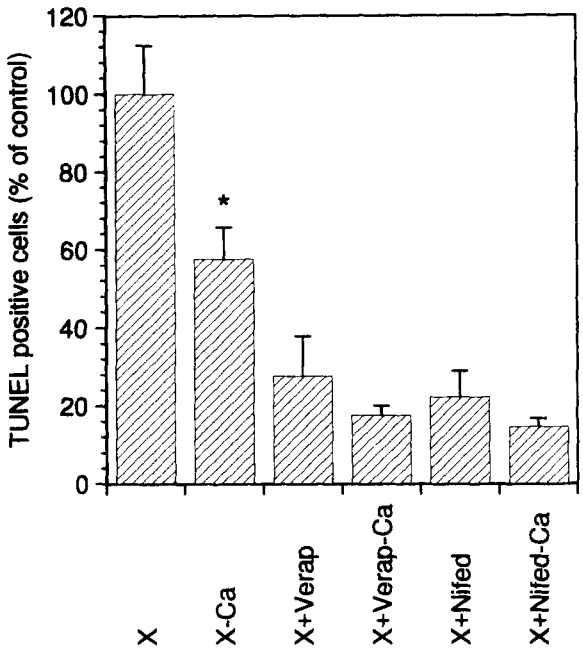

Fig. 8. Ca^{2+}-free medium and Ca^{2+} channel blockers inhibit apoptosis induced by 25-hydroxycholesterol. SMCs incubated in the presence of 5% FCS were treated with 25-hydroxycholesterol (5 μg/ml) + TNFα (10 ng/ml) and IFNγ (20 ng/ml) for 24 h (all treatments, denoted by X). Apoptosis was quantified by TUNEL assay. X: normal Ca^{2+}-containing culture medium; X-Ca: Ca^{2+}-free medium; X+Verap: 1 μM verapamil; X+Verap-Ca: 1 μM verapamil in Ca^{2+}-free medium; X+Nifed: 1 μM nifedipine; X+Nifed-Ca: 1 μM nifedipine in Ca^{2+}-free medium. Results represent mean values ± SD (n = 3). Student's *t* test was used to compare results obtained with or without Ca^{2+} ($^*P < 0.05$).

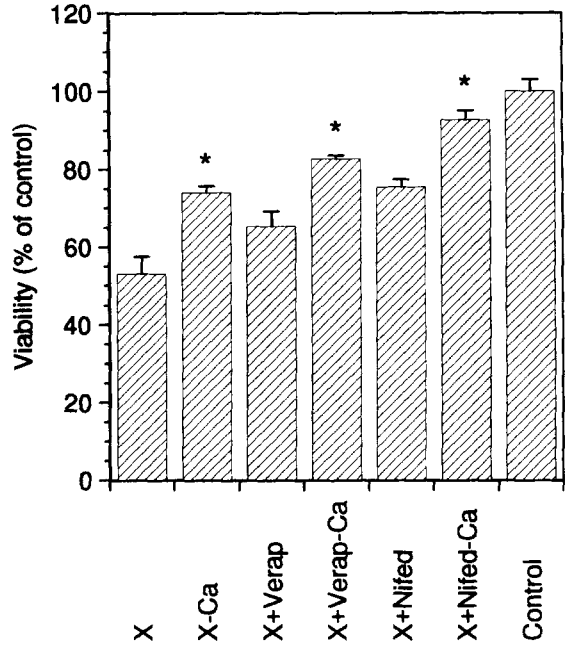

Fig. 9. Ca^{2+}-free medium and Ca^{2+} channel blockers reduce the cytotoxicity of 25-hydroxycholesterol. SMCs incubated in the presence of 5% FCS were treated with 25-hydroxycholesterol (5 μg/ml) + TNFα (10 ng/ml) and IFNγ (20 ng/ml) for 24 h (denoted by X). Viability was determined by MTT assay. X: normal Ca^{2+}-containing culture medium; X-Ca: Ca^{2+}-fee medium; X+Verap: 1 μM verapamil; X+Verap-Ca: 1 μM verapamil in Ca^{2+}-free medium; X+Nifed: 1 μM nifedipine; X+Nifed-Ca: 1 μM nifedipine in Ca^{2+}-free medium; Control: nontreated cells. Results represent mean values ± SD (n = 3). Student's *t* test was used to compare results obtained with or without Ca^{2+} ($^*P < 0.05$).

investigated the possible involvement of Ca^{2+} in 25-hydroxycholesterol-induced apoptosis. In SMCs incubated in nominally Ca^{2+}-free medium, TUNEL staining of SMCs treated with 25-hydroxycholesterol (5 μg/ml), TNFα, and IFNγ was reduced by 42% (**Fig. 8**). The L-type Ca^{2+} channel blockers verapamil and nifedipine (1 μM) inhibited apoptosis by more than 70% (Fig. 8). Addition of EGTA to sequester the trace amounts of free Ca^{2+} in the culture medium (contributed mainly by 5% FCS) did not result in any further inhibition of apoptosis (data not shown). Ca^{2+}-free medium, verapamil, and nifedipine also reduced cytotoxicity of 25-hydroxycholesterol alone (as measured by MTT assay; data not shown) and in the presence of TNFα and IFNγ (**Fig. 9**).

Caspase 3 is activated in cells treated with 25-hydroxycholesterol

The ICE-like protease caspase 3 (CPP32) is regarded as an important regulator of apoptosis (18, 19). Activation of this protease involves processing of the 32 kDa proenzyme to a p17 fragment (36). Western blot analysis of total cell extract from SMC using an antibody against the p17 fragment of caspase 3 showed cleavage of the 32 kDa proenzyme after 24 h of 25-hydroxycholesterol treatment (**Fig. 10**), indicating activation of caspase 3.

25-Hydroxycholesterol induces Ca^{2+} oscillations

In order to confirm that oxysterol treatment affects the intracellular concentration of Ca^{2+}, we directly measured Ca^{2+} in single smooth muscle cells. Addition of 25-hydroxycholesterol (10 μg/ml) induced Ca^{2+} oscillations, that continued for at least 60–120 min (**Fig. 11**). The first spike typically occurred 2–3 min after the addition of 25-hydroxycholesterol, but in some cases (Fig. 11B) regular Ca^{2+} oscillations started at a later time point, reflecting differences between single cells. The oscillations were slow with a frequency of approximately 0.3–0.4 min^{-1}. Ca^{2+}-free medium inhibited the oscillations (data not shown). Further experiments are being done to find out whether the Ca^{2+} signals originate from the culture medium only or whether intracellular Ca^{2+} pools are involved as well.

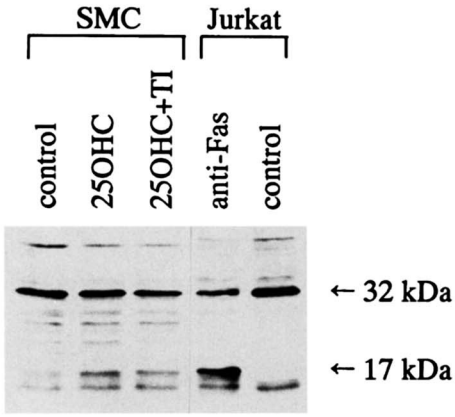

Fig. 10. 25-Hydroxycholesterol induces cleavage of caspase 3 (CPP32). SMCs were treated for 24 h with 10 μg/ml of 25-hydroxycholesterol (25OHC) alone or in combination with 10 ng/ml of TNFα and 20 ng/ml of IFNγ (TI). The cells were harvested by scraping in ice-cold PBS, and the caspase 3 content of lysed cells was analyzed as described under Methods. Extracts from Jurkat T cells incubated with or without anti-Fas monoclonal antibody (250 ng/ml) were run in parallel as a positive control for cleavage of caspase 3. The blot is representative of three similar experiments.

DISCUSSION

The present findings show that smooth muscle cells exposed to 25-hydroxycholesterol undergo cell death by apoptosis. TNFα and IFNγ potentiated 25-hydroxycholesterol-induced cell death, but were not toxic in the absence of 25-hydroxycholesterol at the concentrations used. Both TNFα and IFNγ have been shown to be produced in atherosclerotic vessels (30, 31). Their presence in atherosclerotic lesions is also suggested by studies demonstrating that oxidized LDL can induce production of IFNγ in human lymphocytes (37) and expression and secretion of TNFα in adherent human monocytes (38). Moreover, mononuclear leukocytes from patients with ischemic heart disease (39) and macrophages from atherosclerotic lesions have been reported to produce more TNFα than the relevant controls (40).

We show here for the first time that treatment of SMC with 25-hydroxycholesterol causes caspase 3 activation, a key event in the induction of apoptosis. This suggests that the oxysterol is capable of triggering a specific and highly conserved pathway for the induction of cell death.

The importance of Ca^{2+} signals for triggering apoptosis has been demonstrated in many experimental systems (33–35, 41). A sustained elevation of Ca^{2+} can activate degradative enzymes such as Ca^{2+}-dependent proteases (e.g., calpain) and endonuclease(s) responsible for DNA fragmentation (42). Our results show that the Ca^{2+} channel blockers nifedipine and verapamil diminished apoptosis induced by 25-hydroxycholesterol. Both DNA fragmentation and cytotoxicity decreased in the presence of nifedipine and verapamil. Similarly, apoptosis was inhibited when nominally Ca^{2+}-free medium was used for the treatments. Within a few minutes after addition, 25-hydroxycholesterol induced intracellular Ca^{2+} oscillations with a frequency of approximately 0.3–0.4 min^{-1}. These findings strongly suggest that increased intracellular Ca^{2+} is a critical mediator of oxysterol toxicity.

Oxysterols have several effects that can be expected to influence maintenance of intracellular membranes. They inhibit cholesterol synthesis (43) and may thus prevent membrane formation, particularly in proliferating cells. Many cholesterol derivatives have been shown to block lysosomal cholesterol transport (44, 45), even to such a high degree that efflux of cholesterol to extracellular acceptors such as HDL is significantly diminished (9). Disturbances in intracellular lipid transport could contribute to the loss of organized ER and Golgi membranes observed by electron microscopy in the present study.

Christ et al. (25) reported that 25-hydroxycholesterol induced oligonucleosomal DNA fragmentation in murine thymocytes and RDM4 lymphoma cells. Cycloheximide and actinomycin D were capable of preventing the oxysterol-induced cell death in these cells. In another study by Hwang (1), various inhibitors of protein or RNA synthesis greatly increased the viability of murine lymphoid cells during oxysterol treatments. We have tested inhibitors of protein and RNA synthesis in SMCs, but as these inhibitors (cycloheximide, puromycin, actinomycin D) themselves turned out to be highly toxic to SMCs, it was not possible to evaluate the need for macromolecular synthesis for apoptosis in our system.

Death of SMCs in the fibrous cap region of the atherosclerotic plaque is believed to play an important role in plaque rupture (46). Recent studies have demonstrated a high incidence of apoptotic cell death among SMCs in this region (22, 23). Moreover, electron microscopic studies suggest that additional cell death by necrosis occurs in parallel (47). The present finding that Ca^{2+} channel blockers inhibit oxysterol-induced apoptosis and cytotoxicity may thus be of clinical relevance. Interestingly, Ca^{2+} channel blockers have been shown to increase cholesteryl ester hydrolysis in human aortic tissue (48) and they may retard the development of early atherosclerosis in humans (49, 50). Plaque rupture is known as a major cause of unstable angina and myocardial infarction (46). The use of Ca^{2+} channel blockers in treatment of unstable angina has been a matter of controversy because of reports suggesting increased mortality after dihydropyridine treatment (51). However, more recent studies using the Ca^{2+} channel blocker diltiazem have demonstrated beneficial effects

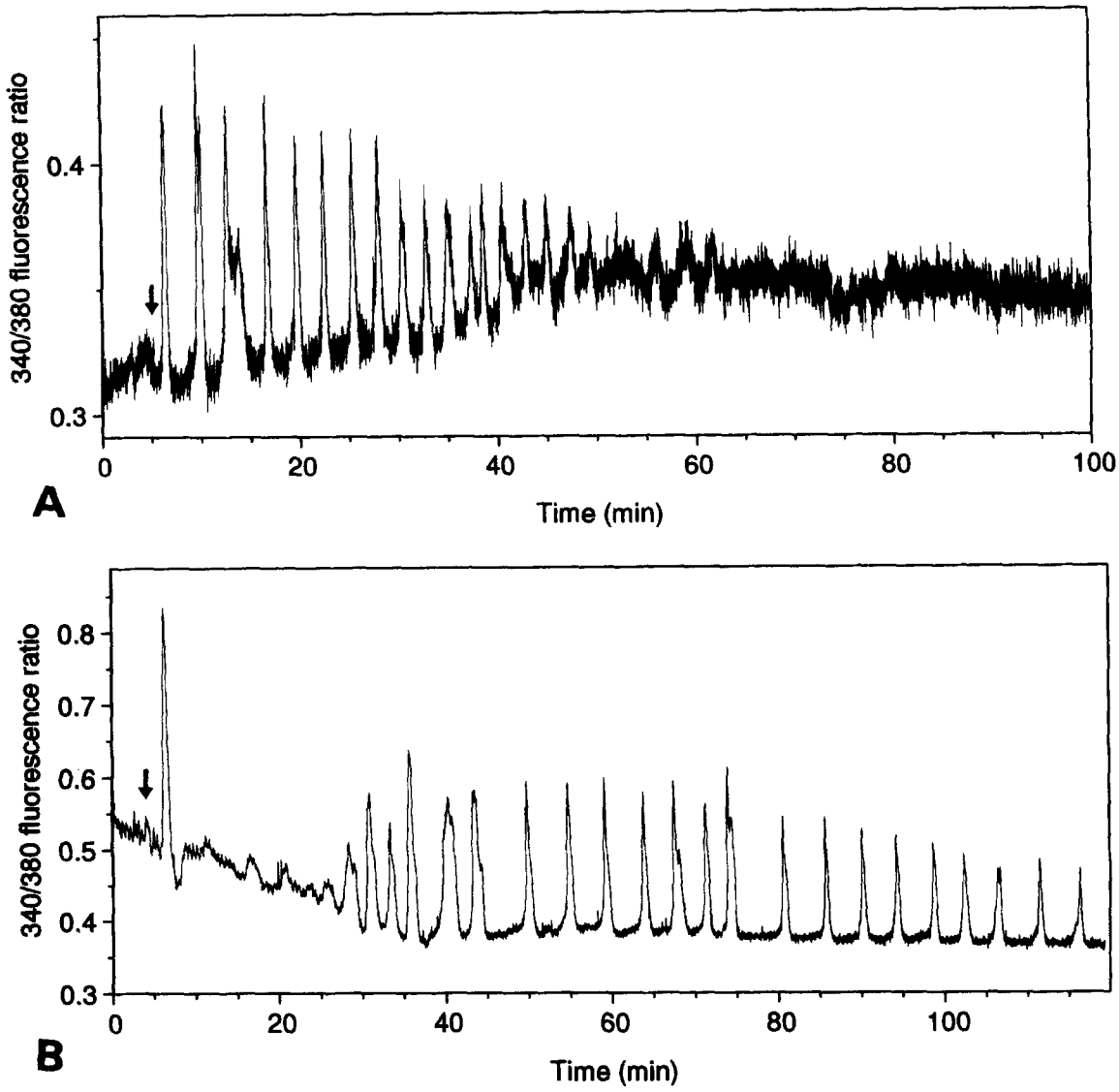

Fig. 11. Effects of 25-hydroxycholesterol on intracellular Ca^{2+} in fura-2-loaded cells. The arrowheads indicate the time of addition of 10 μg/ml 25-hydroxycholesterol (A: 5 min, B: 4 min). The induced oscillations in intracellular Ca^{2+} had a frequency of 0.3–0.4 min^{-1}. A and B are representative of six experiments.

on refractory angina and myocardial infarction in patients with unstable angina (52). It is an interesting possibility that Ca^{2+} channel blockers may stabilize unstable plaques by protecting SMCs from the cytotoxicity of oxidized lipids, thereby restoring their repair capacity.

Based on oligonucleosomal DNA fragmentation and ultrastructural findings, it has been reported that oxidized LDL induces apoptosis in macrophages (53, 54) and smooth muscle cells (55, 56). Oxysterols have been shown to account for most of the cytotoxicity of oxidized LDL (57), but oxysterols differ greatly in their cytotoxicity and potential to induce an apoptotic cell death (M. Ares, unpublished observations). The reasons for these differences are currently under investigation in our laboratory.

In conclusion, our results show that 25-hydroxycholesterol-induced apoptosis appears to be mediated by enhanced Ca^{2+} influx into the cells and to be associated with activation of the ICE-like protease caspase 3. The potentiation of 25-hydroxycholesterol-induced apoptosis by TNFα and IFNγ suggests that inflammatory cytokines may be important regulators of cellular responses to lipid oxidation products. The combination of lipid accumulation and a local inflammatory reaction in atherosclerotic plaques may produce an environment promoting cell death, even if the levels of individ-

ual toxic substances alone would not be lethal to the vascular cells.

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