Cell Biology and Toxicology. 2005; **21:** 97–114.
DOI: 10.1007/s10565-005-0141-2

Comparison of the cytotoxic, pro-oxidant and pro-inflammatory characteristics of different oxysterols

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Received 2 August 2004; accepted 9 February 2005

Keywords: cell death, HMG-CoA reductase activity, IL-8, oxysterols, superoxide anions

Abstract

Oxidized low-density lipoproteins play important roles in the development of atherosclerosis and contain several lipid-derived, bioactive molecules which are believed to contribute to atherogenesis. Of these, some cholesterol oxidation products, refered to as oxysterols, are suspected to favor the formation of atherosclerotic plaques involving cytotoxic, pro-oxidant and pro-inflammatory processes. Ten commonly occurring oxysterols (7α-, 7β-hydroxycholesterol, 7-ketocholesterol, 19-hydroxycholesterol, cholesterol-5α,6α-epoxide, cholesterol-5β,6β-epoxide, 22*R*-, 22*S*-, 25-, and 27-hydroxycholesterol) were studied for both their cytotoxicity and their ability to induce superoxide anion production ($O_2^{\cdot-}$) and IL-8 secretion in U937 human promonocytic leukemia cells. Cytotoxic effects (phosphatidylserine externalization, loss of mitochondrial potential, increased permeability to propidium iodide, and occurrence of cells with swollen, fragmented and/or condensed nuclei) were only identified with 7β-hydroxycholesterol, 7-ketocholesterol and cholesterol-5β,6β-epoxide, which also induce lysosomal destabilization associated or not associated with the formation of monodansylcadaverine-positive cytoplasmic structures. No relationship between oxysterol-induced cytotoxicity and HMG-CoA reductase activity was found. In addition, the highest $O_2^{\cdot-}$ overproduction quantified with hydroethidine was identified with 7β-hydroxycholesterol, 7-ketocholesterol and cholesterol-5β,6β-epoxide, with cholesterol-5α,6α-epoxide and 25-hydroxycholesterol. The highest capacity to simultaneously stimulate IL-8 secretion (quantified by ELISA and by using a multiplexed, particle-based flow cytometric assay) and enhance IL-8 mRNA levels (determined by RT-PCR) was observed with 7β-hydroxycholesterol and 25-hydroxycholesterol. None of the effects observed for the oxysterols were detected for cholesterol. Therefore, oxysterols may have cytotoxic, oxidative, and/or inflammatory effects, or none whatsoever.

Abbreviations: AO, acridine orange; MDC, monodansylcadaverine; HE, hydroethidine; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA reductase; PE, phycoerythrin

Introduction

Oxysterols constitute an important family of molecules resulting from the auto-oxidation of cholesterol in air, enzymatic transformation of cholesterol in various cell species (Smith, 1987) or cholesterol peroxidation of LDL (Dzeletovic et al., 1995). Oxysterols accumulate in the

subendothelial level of the arterial wall during the atheromatous process (Berliner and Heinecke, 1996) and are believed to mediate the development of atherosclerosis (Witztum and Steinberg, 2001). At the present time, a large number of studies have suggested that oxysterols are involved in the initiation and progression of atherosclerosis (Brown and Jessup, 1999), according to their wide range of biological activities. Indeed, oxysterols are frequently present at high levels in the atherosclerotic plaque (Garcia-Cruset et al., 1999) and it has been reported that 7β-hydroxycholesterol, which is a major component of oxidized LDL, can induce IL-1β secretion in vascular endothelial cells and, consequently, the expression of adhesion molecules necessary for the recruitment of monocytes and T lymphocytes found in atherosclerotic plaques (Lemaire et al., 1998). Therefore, potential roles of these molecules in the initiation of atheromatous lesions have been suggested (Colles et al., 2001). Moreover, oxysterols (especially 7β-hydroxycholesterol and 7-ketocholesterol) have been reported to be strongly toxic to a number of tumoral and normal cell species, including those of the vascular wall (Yin et al., 2000). This toxicity occurs via the induction of a complex mode of cell death chronologically associated with the rapid cytoplasmic formation of monodansylcadaverine (MDC)-positive structures (Monier et al., 2003), an overproduction of superoxide anions ($O_2^{\cdot -}$) (Miguet-Alfonsi et al., 2002), and the fragmention and/or condensation of the nuclei which is a typical morphological characteristic of apoptotically dying cells (Miguet et al., 2001). Numerous studies performed on various monocytic cell lines, including the U937 promonocytic human leukemia cell line frequently used as a macrophage model to investigate the cytotoxic properties of oxysterols (Aupeix et al., 1995; O'Callaghan et al., 2001), also underline that 7β-hydroxycholesterol and 7-ketocholesterol demonstrate the highest cytotoxicity of the tested oxysterols (Lizard et al., 1999). Moreover, Liu et al. (1997) have demonstrated that macrophages isolated from human atherosclerotic plaques overproduce IL-8 and that some oxysterols, mainly 25-hydroxycholesterol which enhances IL-8 production up to 10 times, may have a regulatory function for IL-8 production. It is noteworthy to mention that IL-8 is a cytokine of the chemokine family which might play an important role in the recruitment of T lymphocytes and monocytes into the arterial subendothelial space and which could therefore take part in the initiation and development of atherosclerotic lesions (Terkeltaub et al., 1994). In addition, IL-8 could have a potential atherogenic role by inhibiting local tissue inhibitor of metalloproteinase-1 (TIMP-1) expression, thereby leading to an imbalance between matrix-degrading metalloproteinases (MMPs) and TIMPs at focal sites of the atherosclerotic plaque and to the local extracellular degradation and ultimate rupture of atheromatous plaques (Moreau et al., 1999).

As cell death, oxidation, and inflammation are hallmarks of atherosclerosis (Mügge, 1998; Kockx and Knaapen, 2001), it is of interest to identify the molecules involved in the initiation and development of atherosclerotic plaque and to characterize their activities in order to conceive drugs capable of counteracting their side effects. Taking these considerations into account, the cytototoxic, pro-oxidant, and pro-inflammatory activities of 10 commonly occurring oxysterols (7α-, 7β-hydroxycholesterol, 7-ketocholesterol, 25-hydroxycholesterol, 19-hydroxycholesterol, cholesterol-5α,6α-epoxide, cholesterol-5β,6β-epoxide, 22*R*-hydroxycho-lesterol, 22*S*-hydroxycholesterol, and 27-hydroxycholesterol) frequently found at increased levels in the plasma of atherosclerotic patients and in atheromatous plaques (Iuliano et al., 2003) were evaluated on U937 promonocytic human leukemia cells. As free cholesterol accumulation in macrophages may result in cell death by an apoptotic process involving endoplasmic reticulum stress (Feng et al., 2003), U937 cells were also cultured in the presence of cholesterol used at concentrations similar to the oxysterols (10–20 μg/ml). The cytotoxic activity was evaluated by various assays including the externalization of

phosphatidylserines (Vermes et al., 1995), measurement of the loss of mitochondrial transmembrane potential with $DiOC_6(3)$ (Chen, 1988), and increased permeability to propidium iodide (Yeh et al., 1981). In addition, cell death was characterized by nuclei staining with Hoechst 33342, which distinguishes between necrotic and apoptotic forms of cell death according to the morphological aspect of the nuclei (Lizard et al., 1998). Lysosomal destabilization was assayed by staining with acridine orange (AO) (Yuan et al., 2000; Li et al., 2001). The formation of MDC, positive cytoplasmic structures was investigated by staining with MDC (Miguet-Alfonsi et al., 2002). The overproduction of superoxide anions was assessed with hydroethidine (HE) (Rothe and Valet, 1990) and IL-8 secretion was measured by both an ELISA method and a multiplexed particle-based flow cytometric assay (Morgan et al., 2004). Moreover, the ability of oxysterols to modulate the level of IL-8 mRNA expression was studied by reverse transcriptase–polymerase chain reaction (RT-PCR).

Based on the results of the aforementioned tests, it is demonstrated in the present study that 7α-,7β-hydroxycholesterol, 7-ketocholesterol, 19-hydroxycholesterol, cholesterol-5α,6α-epoxide, cholesterol-5β,6β-epoxide, 22*R*-, 22*S*-, 25-, and 27-hydroxycholesterol may have cytotoxic, oxidative, and/or inflammatory effects, or none whatsoever, on U937 human promonocytic leukemia cells. Of note, none of the effects observed with oxysterols were identified in cholesterol-treated cells. Therefore, some of these oxysterols, which are frequently detected at enhanced levels in atherosclerotic plaques, could be strongly involved in atherogenesis, since it is known that the atherosclerotic process simultaneously involves the death of vascular and immunocompetent cells, oxidative stress, and enhanced IL-8 secretion, all of which play important roles in the recruitment of immunocompetent cells and smooth muscle cells into the subendothelial space (Kockx and Knaapen, 2001; Hansson, 2001; De Nigris et al., 2003).

Materials and methods

Reagents

7α- and 7β-hydroxycholesterol were purchased from Steraloids Inc. (Newport, RI, USA) and all other oxysterols (7-ketocholesterol, cholesterol-5α,6α-epoxide, cholesterol-5β,6β-epoxide, 19-, 22*R*-, 22*S*-, 25-, 27-hydroxycholesterol), as well as cholesterol were purchased from Sigma (L'Isle d'Abeau Chesnes, France). The purity of these different sterols was determined to be 100% by gaseous phase chromatography–mass spectrometry. Lovastatin was purchased from Sigma. [^{3}H]Acetic acid (sodium salt; specific activity 4.1 Ci/mmol) was purchased from Amersham Biosciences (Orsay, France).

Cells

Human U937 promonocytic leukemia cells were grown in suspension in culture medium consisting of RPMI 1640 medium (Gibco, Eragny, France), 2 mmol/L L-glutamine (Gibco), and antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin) (Gibco) supplemented with 10% (v/v) heat inactivated fetal calf serum (Gibco). The cells were seeded at 5×10^5 per ml of culture medium, passaged twice a week, and grown at 37°C under 5% CO_2 in a humidified incubator.

Cell treatments

For all experiments, initial sterol solutions were prepared extemporaneously at a concentration of 800 μg/ml, as previously described (Lizard et al., 1998). Briefly, to prepare these initial solutions, 800 μg of each oxysterol were dissolved in 50 μl of absolute ethanol and 950 μl of culture medium was added. To obtain 10 and 20 μg/ml final concentrations, 12.5 and 25 μl of these initial solutions were respectively introduced per milliter of culture medium at the beginning of the culture. U937 cells were adjusted to a density of 5×10^5 cells/ml and oxysterols were added to

the culture medium at final concentrations of 10 μg/ml (25 μmol/L) or 20 μg/ml (50 μmol/L) for 24 h. Lovastatin was prepared at an initial concentration of 2 mmol/L and used at a final concentration of 100 μmol/L for 24 h. When U937 cells were treated with an oxysterol or with lovastatin, these compounds were introduced in the culture medium at the beginning of the culture.

Flow cytometric detection of phosphatidylserine externalization using fluorescein-labelled Annexin V

The detection of phosphatidylserine externalization was performed on untreated and treated cells with the Annexin-V-FLUOS Staining Kit (Boehringer-Mannheim, Meylan, France) by flow cytometry. To detect phosphatidylserine, 2 μl of Annexin V fluorescein isothiocyanate (FITC) reagent and 2 μl of propidium iodide solution were added to 5×10^5 cells suspended in 100 μl of HEPES buffer. After 15 min of incubation at room temperature, the cells were immediately analyzed by flow cytometry on a GALAXY flow cytometer (Partec, Münster, Germany). The green fluorescence of Annexin V FITC was collected through a 520/10 nm band pass filter and the red fluorescence of propidium iodide was collected through a 630 nm long pass filter. The fluorescent signals were measured on a logarithmic scale consisting of 4 decades of log and electronic flow cytometer compensation was performed to exclude two overlapping emission spectra. For each sample, 10 000 cells were acquired and the data were analyzed with the FlowMax software (Partec) in order to determine the percentage of Annexin V-positive cells which were not simultaneously stained with propidium iodide.

Flow cytometric measurement of the mitochondrial transmembrane potential with the cationic lipophilic dye $DiOC_6(3)$

The mitochondrial transmembrane potential ($\Delta\Psi_m$) was measured with 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$: λ_{Ex} max: 484 nm, λ_{Em} max: 501 nm) (Molecular Probes Inc., Eugene, OR, USA) used at a 40 nmol/L final concentration in untreated or treated U937 cells, as previously described (Miguet et al., 2001). The $DiOC_6(3)$ mitochondrial transmembrane potential related fluorescence was recorded by flow cytometry with a GALAXY flow cytometer (Partec) equipped with an argon laser emitting at 488 nm. The green fluorescence was collected through a 520/10 nm band pass filter and the fluorescent signals were measured on a logarithmic scale. For each sample, 10 000 cells were acquired and the data were analyzed with FlowMax software (Partec).

Determination of cell permeability with propidium iodide

Cell permeability was determined after staining with the phenanthrene dye propidium iodide (λ_{Ex} max 540 nm, λ_{Em} max 625 nm) (Sigma), which only enters into dead cells (Yeh et al., 1981). A stock solution of propidium iodide was prepared in phosphate-buffered saline (PBS) at a concentration of 1 mg/ml. Propidium iodide was used at a final concentration of 5 μg/ml in a cell suspension adjusted to 10^6 cells/ml. Fluorescence was immediately quantified by flow cytometry of 10 000 cells analyzed on a logarithmic scale consisting of 4 decades of log on a GALAXY flow cytometer (Partec) at excitation and emission wavelengths of 488 nm and 590/10 nm, respectively.

Characterization of nuclear morphology by staining with Hoechst 33342

The nuclear morphology of untreated and treated cells was studied by staining with Hoechst 33342 (Sigma), as previously described (Lizard et al., 1995). In these conditions, dead cells are characterized by condensed and/or fragmented nuclei (considered as apoptotic cells) as well as by swollen nuclei, whereas living cells have round and regular nuclei (Lizard et al., 1995; Monier

et al., 2003). The morphological aspect of the nuclei was observed with an inverted Laborlux IX70 microscope (Olympus, Tokyo, Japan) or with an Axioskop right microscope (Zeiss, Jena, Germany) by using UV light excitation. 300 cells were examined per sample.

Staining with MDC

Staining with MDC was performed as previously described (Miguet-Alfonsi et al., 2002; Monier et al., 2003). MDC (λ_{Ex} max 340 nm, λ_{Em} max 530 nm) (Sigma) was prepared at a concentration of 0.1 mol/L in DMSO and added to the culture medium at a final concentration of 0.05 mmol/L. After 30 min of incubation at 37°C, cells were collected by centrifugation, resuspended in culture medium (2×10^6 cells/ml) and 50 µl of the cell suspension were applied to glass slides, coverslipped and immediately examined under an Axioskop right microscope (Zeiss) using UV light excitation. 300 cells were examined for each sample.

AO staining and lysosomal integrity measurements

Acridine orange (AO) is a weak base which accumulates in its charged form within lysosomes of living cells because of the low lysosomal pH, and which produces a red fluorescence when excited by a blue light (Olsson et al., 1987, 1989). During prolonged exposure to cytotoxic agents, including oxysterols, the red fluorescence of AO decreases markedly (Yuan et al., 2000). The shift in AO fluorescence from granular red to diffuse green reflects leakage and redistribution of AO from the lysosomes, indicating impairment of the lysosomal membranes or the ability of the lysosomes to maintain low pH. According to these considerations, AO is widely used to perform lysosomal integrity measurements and the percentage of AO-negative cells (which do not emit a red fluorescence) determined by flow or image cytometry permits quantification of the percentage of cells with destabilized lysosomes (Yuan et al., 2000). In the present investigation, a 1 mg/ml stock solution of AO (Sigma) was prepared by dissolving the dye in distilled water (Olsson et al., 1987). After staining with AO (1 volume of AO at 10 µg/ml mixed with 1 volume of cell suspension, incubation for 15 min at 37°C), cells were washed twice in culture medium, resuspended in culture medium, and immediately analyzed by flow cytometry. The fluorescent signals were measured on a GALAXY flow cytometer (Partec) equipped with an argon laser emitting at 488 nm and the red fluorescence of AO was collected through a 630 nm long pass filter. For each sample, fluorescence was quantified in 10 000 cells on a logarithmic scale and data were analyzed with FlowMax software (Partec).

Flow cytometric measurement of the production of superoxide anions with HE

The production of superoxide anions ($O_2^{\cdot -}$) was studied by using HE on untreated or treated U937 cells. HE is a nonfluorescent compound which can diffuse through cell membranes and which is rapidly oxidized in ethidium under the action of $O_2^{\cdot -}$ (Rothe and Valet, 1990). HE (Molecular Probes Inc.) was initially prepared at a concentration of 10 mmol/L in DMSO and was used at a 2 µmol/L final concentration on cell samples of 10^6 cells per ml of culture medium. After 15 min of incubation at 37°C, cells were analyzed by flow cytometry with a GALAXY flow cytometer (Partec) equipped with a laser emitting at 488 nm. The red fluorescence of ethidium was collected through a 590/10 nm band pass filter and the fluorescent signals were measured on a logarithmic scale. For each sample, 10 000 cells were acquired and data were analyzed with the FlowMax software (Partec).

Measurement of HMG-CoA reductase activity by [^3H]acetate incorporation into sterols

Approximately 5×10^6 U937 cells were incubated with 20 µg/ml of oxysterols or cholesterol or with 100 µmol/L of lovastatin for an hour. At the end

of this time, 5 μCi of [^{3}H]sodium acetate were added and cells were incubated for an additional 24 h. Cells were then collected and rinsed twice with PBS, and lipids were extracted by the method described by Guijarro et al. (1998).

Measurement of interleukin-8 secretion by ELISA

To measure interleukin-8 (IL-8) secretion, U937 cells were incubated for 24 h in the presence or absence of oxysterols (or cholesterol) at 10 and 20 μg/ml, or with lovastatin at 100 μmol/L. At the end of the incubation time, the culture medium was collected and used to assay levels of IL-8 by ELISA according to manufacturer's procedure (Working protocol for IL-8 module set, Bender MedSystems^{TM}, Vienna, Austria).

Measurement of IL-8 mRNA level by RT-PCR

Ten × 10^6 U937 cells were incubated in the presence or absence of oxysterols (10–20 μg/ml), cholesterol (10–20 μg/ml), or lovastatin (100 μmol/L) for 18 h. For RT-PCR, total RNA was purified with the RNeasy mini kit (Qiagen, Courtaboeuf, France) according to manufacturer's recommendations. Total RNAs were reverse-transcribed with the Omniscript Reverse Transcriptase kit (Qiagen) and cDNAs were amplified by polymerase chain reaction (PCR) with HotStar Taq polymerase (Qiagen) using the following primers for IL-8: 5′-ACAGC-AGAGCACACAAGCTT-3′ (forward) and 5′-CT-GGCAACCCTACAACAGAC-3′ (reverse). With these IL-8 specific primers, PCR was performed using 25 consecutive cycles (denaturation: 94°C/1 min; annealing: 56°C/1 min; extension: 72°C/1 min). β-Actin gene transcription was used as an internal standard with the following actin specific primers: 5′-TGCTATCC-AGGCTGTGCTAT-3′ (forward) and 5′-GATGGAGTTGAAGGTAGTTT-3′ (reverse). With these actin primers, PCR was carried out for 25 consecutive cycles (denaturation: 94°C/1 min; annealing: 50°C/1 min; extension: 72°C/1 min). Amplification products were run on a 0.8% agarose gel, stained with ethidium bromide, visualized by UV illumination, and digitized with a Biocom analyzing system.

Multiplexed flow cytometric analyses of inflammatory cytokines

The production of inflammatory cytokines was investigated in the culture medium of untreated U937 cells or cells treated for 24 h with different oxysterols (20 μg/ml) by using a multiplexed particle-based flow cytometric assay (Vignali, 2000; Kellar and Iannone, 2002). At the end of the incubation time, the culture medium was collected and used to assay the levels of IL-8, IL-1β, IL-6, IL-10, TNF-α, and IL-12 by two-color flow cytometric analysis performed on a GALAXY flow cytometer (Partec) using the BDTM Cytokine Bead Array (BD Biosciences Pharmingen, San Diego, CA, USA) according to the manufacturer's procedure. To this end, fluorescent polystyrene beads (diameter: 7.5 μm; excitation and emission wavelengths of 488 and above 600 nm, respectively) are coupled via a covalent linkage to antibodies raised against one of six cytokines (IL-8, IL-1β, IL-6, IL-10, TNF-α, IL-12) and represent a discrete population unique in their FL-3 intensity. Thus, the fluorescent beads coupled to antibodies serve as capture for a given cytokine, which can be detected simultaneously in a mixture. The captured cytokines are detected via a direct immunoassay using six different antibodies coupled to phycoerythrin (PE), which emits at a wavelength of 585 nm. Samples were run on a GALAXY flow cytometer equipped with a laser emitting at 488 nm; data were acquired on FlowMax software (Partec) and further analyzed using Becton Dickinson Cytometric Bead Array software. Forward vs. side scatter gating was employed to exclude any sample particles other than polystyrene beads. Data were displayed as two-color dot plots (FL2 (PE): band pass 580 ± 10 nm vs. FL3 (beads): long pass 665 nm) so that the six discrete FL-3 microparticle

dye intensities were distributed along the Y-axis. Standard curves were plotted (cytokine calibrator concentration vs. FL-2 mean fluorescence intensity) using a four-parameter logistic curve fitting model. Cytokine concentrations were determined from these standard curves.

Statistical analyses

Statistical analyses were performed with StatView software (Cary, NC, USA) using a one-way analysis of variance (ANOVA) followed by a Dunnett *t*-test.

Results

Characterization of oxysterol-induced cytotoxic activity

The characterization of cytotoxic activity induced by 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, cholesterol-5α,6α-epoxide, cholesterol-5β,6β-epoxide, 19-hydroxycholesterol, 22*R*-hydroxycholesterol, 22*S*-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol used at 10 and 20 μg/ml against U937 cells was performed at 24 h of treatment using various assay methods: evaluation of the externalization of phosphatidylserines determined with Annexin V FITC; measurement of the loss of mitochondrial transmembrane potential and cellular permeability with $DiOC_6(3)$ and propidium iodide, respectively; and characterization of nuclear morphology by staining with Hoechst 33342 which distinguishes between necrotic and apoptotic forms of cell death (Lizard et al., 1995). As shown in Figure 1, only 7β-hydroxycholesterol, 7-ketocholesterol, and cholesterol-5β,6β-epoxide were found to be significantly toxic ($p < 0.001$) to the cells. This cytotoxicity was characterized by a high percentage of cells with externalized phosphatidylserines (Figure 1A), a loss of mitochondrial transmembrane potential indicated by an increased percentage of cells with depolarized mitochondria (Figure 1B), an enhancement of cells permeable to propidium iodide (Figure 1C), and the occurrence of cells with swollen, fragmented, and/or condensed nuclei (Figure 1D). The latter characteristics are in agreement with a complex mode of cell death including apoptosis (Monier et al., 2003; Berthier et al., 2004). To further understand the mechanisms underlying oxysterol-mediated cytotoxicity, AO uptake was used to study lysosomal integrity (Olsson et al., 1989). In these conditions and in comparison with control cells, significant differences between the percentage of cells with destabilized lysosomes were only observed with the cytotoxic oxysterols (7β-hydroxycholesterol, 7-ketocholesterol, and cholesterol-5β,6β-epoxide) (Figure 2A). Moreover, as the presence of MDC-positive structures was recently reported in the cytoplasm of 7β-hydroxycholesterol- and 7-ketocholesterol-treated U937 cells (Miguet-Alfonsi et al., 2002; Monier et al., 2003), these structures were also analyzed following treatment with the different oxysterols included in the present investigation. Of note, large MDC-positive structures were only identified with the cytotoxic oxysterols oxidized at C7 (7β-hydroxycholesterol and 7-ketocholesterol) (Figure 2B). Moreover, as the inhibition of HMG-CoA reductase activity was often suggested to contribute to oxysterol-induced cytotoxicity (Bansal et al., 1989), the effects of oxysterols on this enzyme were determined, with lovastatin (100 μmol/L) used as a positive control. It is noteworthy to mention that no relationship was found between oxysterol-induced cytotoxic activity and HMG-CoA reductase activity (Figure 3): 7-ketocholesterol and 7β-hydroxycholesterol, which are potent inducers of cell death, did not affect HMG-CoA reductase activity; cholesterol-5β,6β-epoxide, which is less toxic than 7β-hydroxycholesterol and 7-ketocholesterol, dramatically increased HMG-CoA reductase activity; 25-hydroxycholesterol and 27-hydroxycholesterol, which are not cytotoxic, reduced HMG-CoA reductase activity;

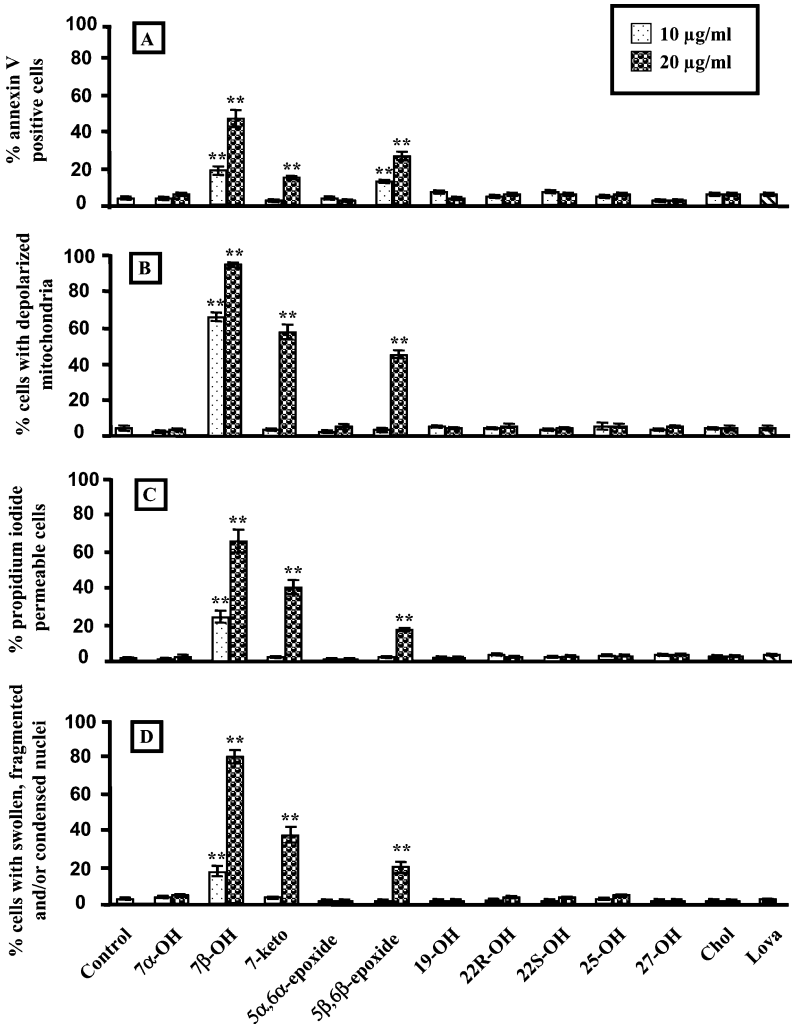


Figure 1. Effects of oxysterols, cholesterol, and lovastatin on phosphatidylserine externalization, mitochondrial transmembrane potential, cellular permeability to propidium iodide, and morphological nuclear changes in U937 cells. U937 cells were cultured for 24 h in the absence (control) or presence of oxysterols or cholesterol (10 and 20 µg/ml, corresponding to 25 and 50 µmol/L, respectively) or lovastatin (100 µmol/L). The detection of phosphatidylserine externalization (A) was made with the Annexin V-fluorescein isothiocyanate (FITC) reagent in the presence of propidium iodide and the percentage of Annexin V-positive cells, which were not simultaneously stained with propidium iodide, was quantified by flow cytometry; the loss of the mitochondrial transmembrane potential (B) was measured with $DiOC_6(3)$ to determine the percentage of cells with depolarized mitochondria by flow cytometry; cellular permeability (C) was evaluated by staining with propidium iodide and the percentage of propidium iodide-positive cells was quantified by flow cytometry; morphological nuclear changes occurring during cell death (D) were defined by the percentage of cells with swollen, fragmented and/or condensed nuclei identified by fluorescence microcopy after staining with Hoechst 33342. Data are the mean ± SEM of three independent experiments performed in triplicate. **Indicates statistically significant differences ($p < 0.001$) between untreated (control) and oxysterol-, cholesterol- or lovastatin-treated cells. 7α-Hydroxycholesterol: 7α-OH; 7β-hydroxycholesterol: 7β-OH; 7-ketocholesterol: 7-keto; cholesterol-5α,6α-epoxide: 5α,6α-epoxide; cholesterol-5β,6β-epoxide: 5β, 6β-epoxide; 19-Hydroxycholesterol: 19-OH; 22*R*-hydroxycholesterol: 22R-OH; 22*S*-hydroxycholesterol: 22S-OH; 25-hydroxycholesterol: 25-OH; 27-hydroxycholesterol: 27-OH; cholesterol: Chol; lovastatin: Lova.

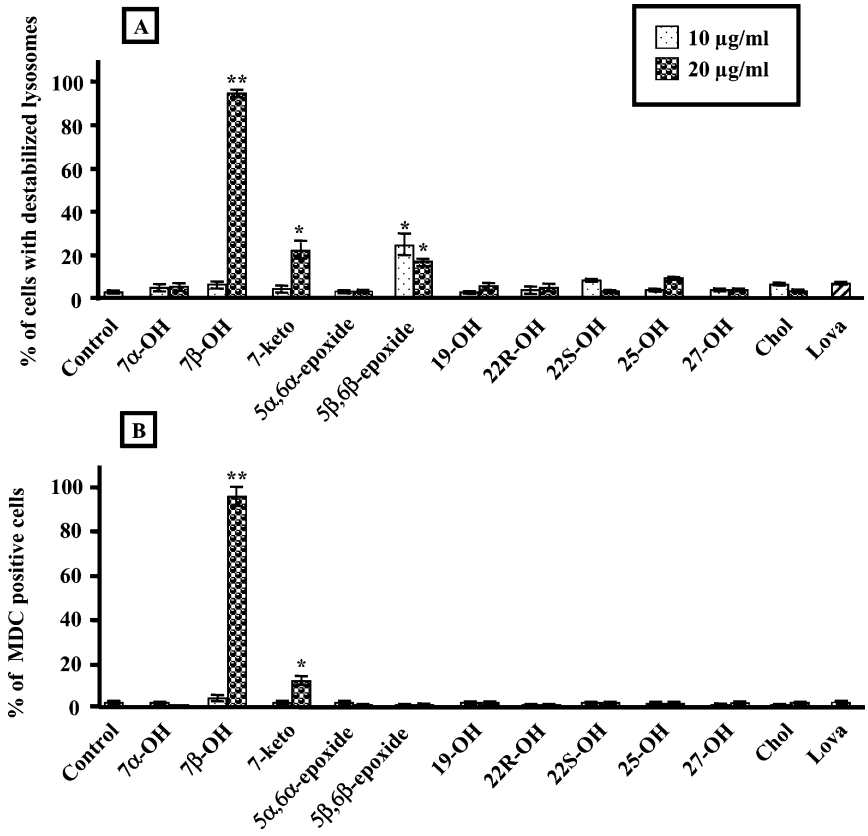


Figure 2. Effects of oxysterols, cholesterol, and lovastatin on lysosomal destabilization and the formation of MDC-positive cytoplasmic structures in U937 cells. U937 cells were cultured for 24 h in the absence (control) or presence of oxysterols or cholesterol (10 and 20 µg/ml, corresponding to 25 and 50 µmol/L, respectively) or lovastatin (100 µmol/L). The percentage of cells with destabilized lysosomes (A) was defined by flow cytometry after staining with acridine orange (AO) (cells with destabilized lysosomes do not emit red fluorescence), and the percentage of MDC positive cells (B) containing MDC positive cytoplasmic structures was determined by fluorescence microscopy. Data are the mean ± SEM of three independent experiments performed in triplicate. *, ** Indicate statistically significant differences, $p < 0.05$ and $p < 0.001$, respectively, between untreated (control) and oxysterol-, cholesterol-, or lovastatin-treated cells. 7α-Hydroxycholesterol: 7α-OH; 7β-hydroxycholesterol: 7β-OH; 7-ketocholesterol: 7-keto; cholesterol-5α,6α-epoxide: 5α,6α-epoxide; cholesterol-5β,6β-epoxide; 5β,6β-epoxide: 19-hydroxycholesterol: 19-OH; 22*R*-hydroxycholesterol: 22R-OH; 22*S*-hydroxycholesterol: 22S-OH; 25-hydroxycholesterol: 25-OH; 27-hydroxycholesterol: 27-OH; cholesterol: Chol; lovastatin: Lova.

7α-hydroxycholesterol, 19-hydroxycholesterol, 22*R*-hydroxycholesterol, 22*S*-hydroxycholesterol, and cholesterol-5α,6α-epoxide, which are also not cytotoxic, had no effect on HMG-CoA reductase activity. In addition, the highest inhibition of HMG-CoA reductase activity was observed with lovastatin, which has no cytotoxic activity, and none of the effects induced by the oxysterols were observed in the presence of cholesterol (Figures 1 and 2).

Effect of oxysterols on superoxide anion production

The production of superoxide anions ($O_2^{\cdot -}$) was quantified by flow cytometry with HE on U937 cells cultured for 24 h in the absence or in the presence of oxysterols (7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, cholesterol-5α,6α-epoxide, cholesterol-5β,6β-epoxide, 19-hydroxycholesterol, 22*R*-hydroxycho-

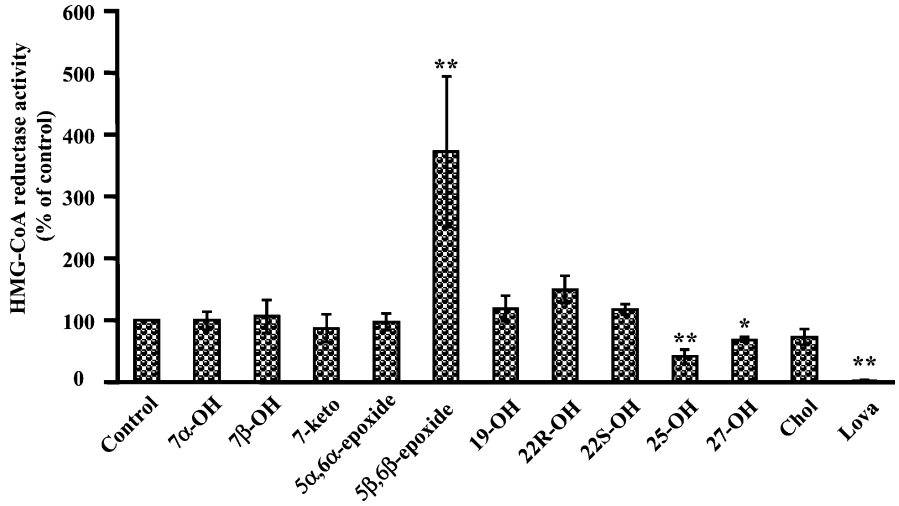


Figure 3. Effects of oxysterols, cholesterol, and lovastatin on HMG-CoA reductase activity. U937 cells were cultured for 24 h in the absence (control) or presence of oxysterols and cholesterol at 20 μg/ml (50 μmol/L) and lovastatin at 100 μmol/L and incorporation of [^{3}H]acetate was measured (in order to evaluate HMG-CoA reductase activity) as described in the Methods (control = 100%). *,** Indicate statistically significant differences, $p < 0.05$ and $p < 0.001$, respectively, between untreated (control) and oxysterol-, cholesterol-, or lovastatin-treated cells. Values are representative of three independent experiments. 7α-Hydroxycholesterol: 7α-OH; 7β-hydroxycholesterol: 7β-OH; 7-ketocholesterol: 7-keto; cholesterol-5α,6α-epoxide: 5α,6α-epoxide; cholesterol-5β,6β-epoxide: 5β,6β-epoxide; 19-hydroxycholesterol: 19-OH; 22*R*-hydroxycholesterol: 22R-OH; 22*S*-hydroxycholesterol: 22S-OH; 25-hydroxycholesterol: 25-OH; 27-hydroxycholesterol: 27-OH; cholesterol: Chol; lovastatin: Lova.

lesterol, 22S-hydroxycholesterol, 25-hydroxycholesterol and 27-hydroxycholesterol) (10 and 20 μg/ml), cholesterol (10 and 20 μg/ml) or lovastatin (100 μmol/L). As shown in Figure 4A, an enhancement of $O_2^{\cdot-}$ production was detected in all treated cells. However, this increase was particularly striking in cells treated with the cytotoxic oxysterols 7β-hydroxycholesterol and 7-ketocholesterol, as well as with 25-hydroxycholesterol and cholesterol-5α,6α-epoxide, which are not cytotoxic. This stimulation of $O_2^{\cdot-}$ production in 25-hydroxycholesterol- and cholesterol-5α,6α-epoxide-treated cells underlines the fact that oxysterol-induced cytotoxicity is not strictly related to $O_2^{\cdot-}$ overproduction.

Effects of oxysterols on IL-8 secretion and IL-8 mRNA synthesis

To investigate whether or not oxysterols influence IL-8 production, U937 cells were incubated for 24 h in culture medium alone or supplemented with 10 or 20 μg/ml of various oxysterols (7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, cholesterol-5α,6α-epoxide, cholesterol-5β,6β-epoxide, 19-hydroxycholesterol, 22*R*-hydroxycholesterol, 22*S*-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol), 10 or 20 μg/ml of cholesterol or 100 μmol/L of lovastatin. 25-Hydroxycholesterol and especially 7β-hydroxycholesterol were the only oxysterols able to significantly enhance IL-8 secretion (Figure 4B). The levels of IL-8, as measured by ELISA, were 20- to 80-fold higher, respectively, in 25-hydroxycholesterol- and 7β-hydroxycholesterol-treated cells than in untreated (control), cholesterol- or lovastatin-treated cells (Figure 4B). Interestingly, in the presence of 7β-hydroxycholesterol and 25-hydroxycholesterol, no induction or secretion of other pro-inflammatory cytokines (IL-1β, IL-6,

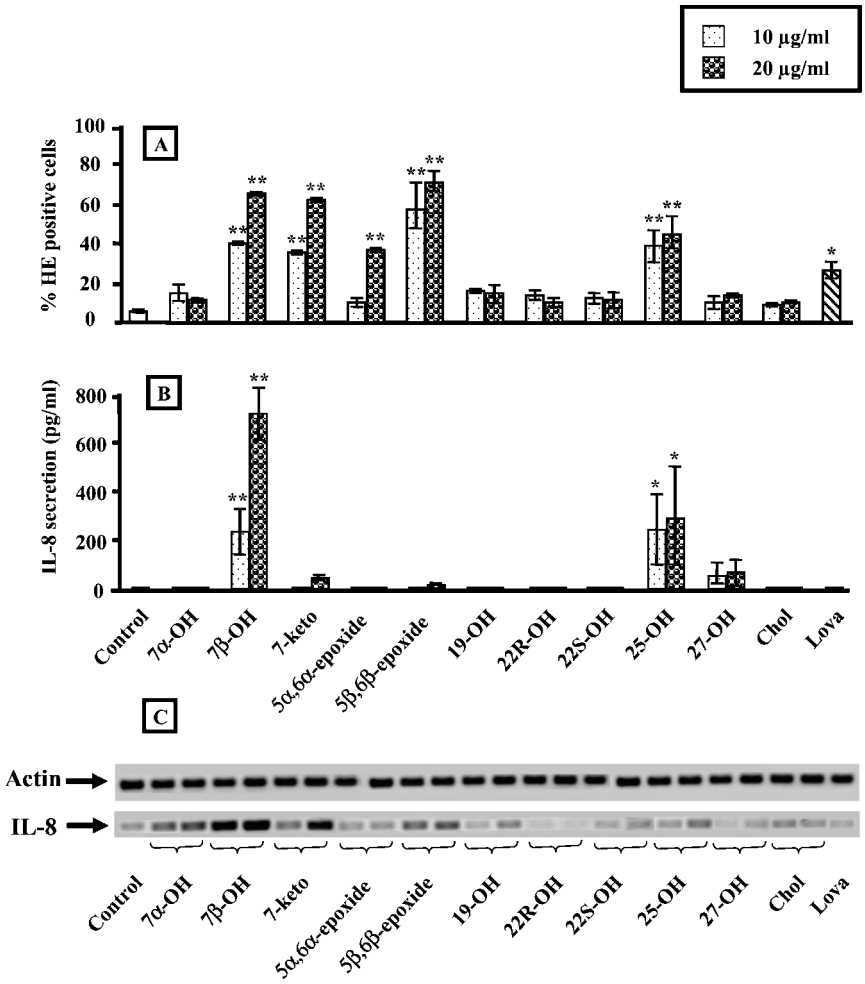
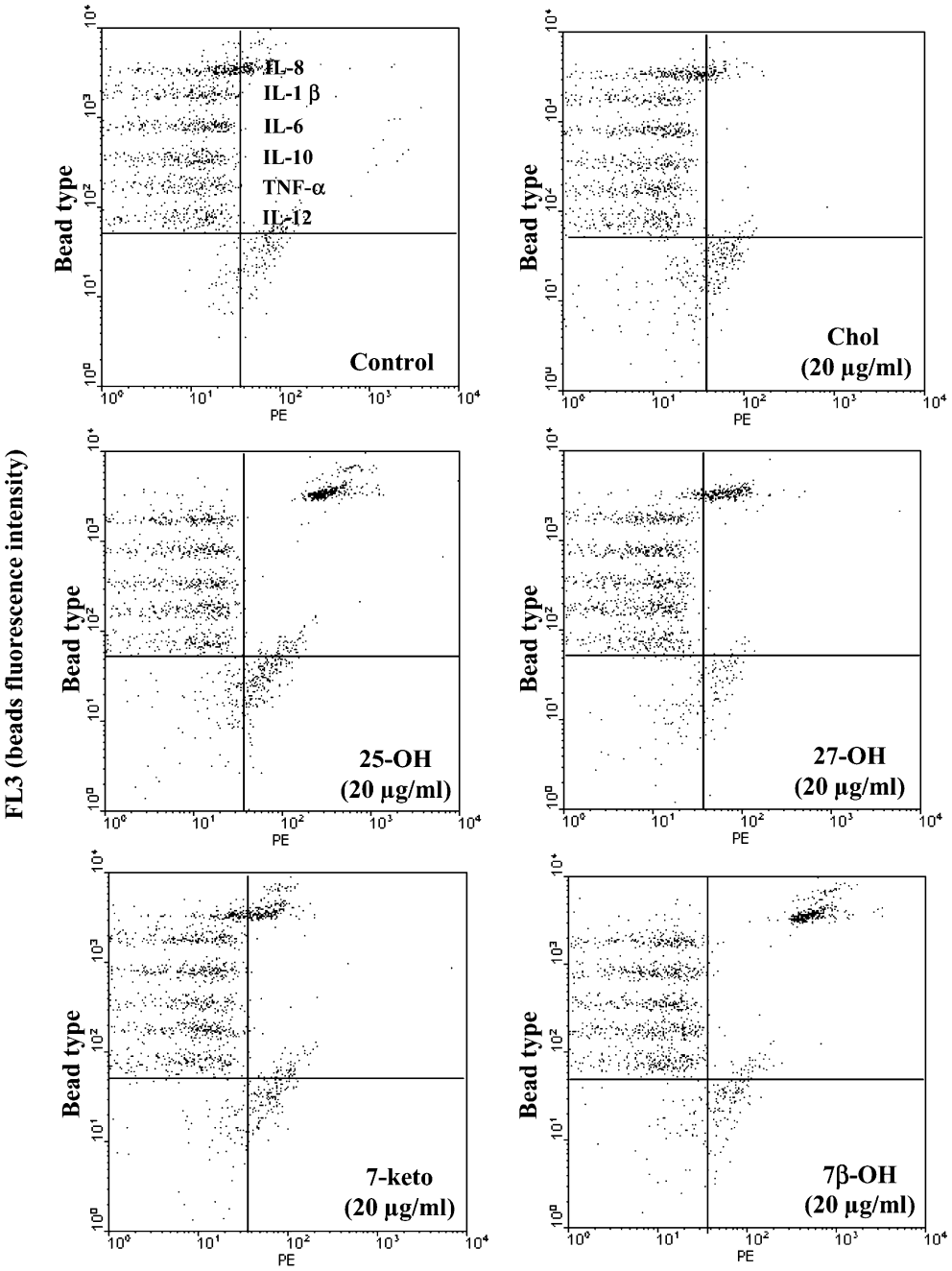


Figure 4. Effects of oxysterols, cholesterol, and lovastatin on the production of superoxide anions ($O_2^{\cdot-}$) and the induction of IL-8 secretion. U937 cells were cultured for 24 h in the absence (control) or presence of oxysterols or cholesterol (10 and 20 µg/ml corresponding to 25 and 50 µmol/L, respectively) or lovastatin (100 µmol/L). The production of $O_2^{\cdot-}$ (A) was determined by flow cytometry after staining with hydroethidine (HE) and results are expressed as a percentage of HE-positive cells. Aliquots of the medium from the cell cultures were assayed for IL-8 by ELISA (B). To determine the effects of oxysterols, cholesterol, and lovastatin on IL-8 mRNA levels (C), U937 cells were cultured for 18 h in the absence or presence of oxysterols or cholesterol al 10 and 20 µg/ml (25 and 50 µmol/L, respectively) or lovastatin at 100 µmol/L. Expression of IL-8 and actin mRNA was determined by RT-PCR. Data shown in (A) and (B) represent the mean ± SEM of three independent experiments performed in triplicate. *,**Indicate statistically significant differences, $p < 0.05$ and $p < 0.001$, respectively, between untreated (control) and oxysterol-, cholesterol- or lovastatin-treated cells. Data shown in (C) are representative of three independent experiments. 7α-Hydroxycholesterol: 7α-OH; 7β-hydroxycholesterol: 7β-OH; 7-ketocholesterol: 7-keto; cholesterol-5α,6α-epoxide: 5α,6α-epoxide; cholesterol-5β,6β-epoxide: 5β,6β-epoxide; 19-hydroxycholesterol: 19-OH; 22*R*-hydroxycholesterol: 22R-OH; 22*S*-hydroxycholesterol: 22S-OH; 25-hydroxycholesterol: 25-OH; 27-hydroxycholesterol: 27-OH; cholesterol: Chol; lovastatin: Lova.

IL-10, TNF-α, and IL-12) was observed by using a multiplexed particle-based flow cytometric assay (BDTM Cytokine Bead Array) (Figure 5). Similarly, following treatment with 7-ketocholesterol, 27-hydroxycholesterol, and cholesterol, which enhanced the secretion of IL-8 only slightly or not at all as quantified by ELISA (Figure 4B), no IL-1β, IL-6, IL-10, TNF-α, and IL-12


IL-8
IL-1 β
IL-6
IL-10
TNF-α
IL-12
Control
Chol
(20 µg/ml)
25-OH
(20 µg/ml)
27-OH
(20 µg/ml)
7-keto
(20 µg/ml)
7β-OH
(20 µg/ml)
Bead type
PE
FL3 (beads fluorescence intensity)
FL2 (cytokine secretion related to PE fluorescence intensity)

secretion was detected with the BDTM Cytokine Bead Array (Figure 5). The other oxysterols investigated (7α-hydroxycholesterol, cholesterol-5α,6α-epoxide, cholesterol-5β,6β-epoxide, 19-hydroxycholesterol, 22*R*-hydroxycholesterol and 22*S*-hydroxycholesterol) did not stimulate IL-8 secretion, as quantified by ELISA (Figure 4B), and did not induce IL-1β, IL-6, IL-10, TNF-α, and IL-12 secretions measured with the BDTM Cytokine Bead Array (data not shown). Of note, IL-8 secretion levels measured by ELISA and the BDTM Cytokine Bead Array were similar (data not shown). To determine if the significant enhancement of IL-8 secretion found with 7β-hydroxycholesterol and 25-hydroxycholesterol was associated with an enhancement of IL-8 gene expression, a semi-quantitative analysis of IL-8 mRNA was performed by RT-PCR. In comparison with untreated cells (control), the RT-PCR analysis revealed a marked and a slight increase in the IL-8 mRNA content of the cells incubated with 7β-hydroxycholesterol (10 and 20 μg/ml) and 25-hydroxycholesterol (20 μg/ml), respectively (Figure 4C), which are consistent with the results of the IL-8 secretion assays. However, no relationship between IL-8 secretion and IL-8 mRNA level was observed with the other investigated oxysterols. Thus, 7-ketocholesterol and 27-hydroxycholesterol, which slightly enhanced IL-8 secretion, either increase IL-8 mRNA level strongly or not at all, respectively (especially at 20 μg/ml) (Figure 4C). Markedly enhanced IL-8 mRNA levels were also observed with 7α-hydroxycholesterol and cholesterol-5β,6β-epoxide (at 10 and 20 μg/ml), and a slight increase of IL-8 mRNA levels was found with 19-hydroxycholesterol and 22*S*-hydroxycholesterol (mainly at 10 μg/ml), whereas these oxysterols did not stimulate IL-8 secretion (Figure 4C). Therefore, according to the oxysterol considered, the ability to enhance IL-8 mRNA is not necessary associated with enhanced IL-8 secretion. In addition, in the presence of 22*R*-hydroxycholesterol, IL-8 mRNA levels were hardly detectable and lower than in untreated cells (control) (Figure 4C).

Discussion

It is now well accepted that oxysterols (also named cholesterol oxidation products), which are 27-carbon derivatives of cholesterol resulting from oxidation on either the sterol nucleus or the C_{17} acyl chain, play important roles in atherosclerosis (Colles et al., 2001). Indeed, some of these compounds were found to be abnormally elevated in the plasma and atherosclerotic plaques of hypercholesterolemic patients (Lemaire et al., 1998; Iuliano et al., 2003; Vaya et al., 2001) and increased plasma levels of 7β-hydroxycholesterol were recently associated with an increased risk of atherosclerosis in humans (Ziedén et al., 1999; Yasunobu et al., 2001). Moreover, the ability of some oxysterols to trigger cytotoxic, pro-oxidative and/or pro-inflammatory reactions,

Figure 5. (opposite). Measurement of 7β-hydroxycholesterol-, 7-ketocholesterol-, 25-hydroxycholesterol-, 27-hydroxycholesterol-, and cholesterol-induced IL-8, IL-1β, IL-6, IL-10, TNF-α, and IL-12 secretion by using a multiplexed, particle-based, flow cytometric assay. To evaluate the ability of 7β-hydroxycholesterol, 7-ketocholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, and cholesterol to simultaneously induce the secretion of certain inflammatory cytokines, a multiplexed, particle-based assay was chosen. To this end, U937 cells were cultured in the absence (control) or presence of these oxysterols (20 μg/ml: 50 μmol/L) or cholesterol (20 μg/ml: 50 μmol/L) for 24 h. At the end of the incubation time, the culture medium was collected and used to assay the levels of IL-8, IL-1β, IL-6, IL-10, TNF-α, and IL-12 by a two-color flow cytometric assay performed with the BDTM Cytokine Bead Array. The X-axis indicates the quantity of cytokines secreted as a measure of the fluorescence intensity of capture antibodies coupled to PE. The Y-axis distinguishes six fluorescent polystyrene beads covalently coupled with antibodies directed against IL-8, IL-1β, IL-6, IL-10, TNF-α, or IL-12. Samples were run on a GALAXY flow cytometer equipped with a laser emitting at 488 nm. Data were acquired using FlowMax software and further analyzed using Becton Dickinson Cytometric Bead Array software. Forward vs. side scatter gating was employed to exclude any sample particles other than the polystyrene beads. Data shown are representative of three independent experiments.

which are major events involved in vascular dysfunction and atherogenesis (Salonen et al., 1997; Hansson, 2001), has also been frequently reported. Thus, cytotoxic effects and enhanced production of superoxide anions ($O_2^{\cdot -}$) were observed with oxysterols oxidized at C7 (7β-hydroxycholesterol and 7-Ketocholesterol) (Miguet-Alfonsi et al., 2002; O'Callaghan et al., 2001; Lizard et al., 1999). 7-ketocholesterol was also described to enhance IL-1β secretion in vascular endothelial cells, as well as the expression of adhesion molecules (ICAM-1, VCAM-1, and E-selectin), contributing to the recruitment of monocytes and T lymphocytes at the sub-endothelial level (Lemaire et al., 1998). 25-Hydroxycholesterol was reported to modulate the secretion of IL-8 (a pro-atherogenic cytokine involved in firm adhesion of monocytes to vascular endothelial cells) and inhibit the synthesis of TIMP-1 implied in the degradation of MMPs (Liu et al., 1997; Moreau et al., 1999). Taking these considerations into account and as oxysterols are suspected to favor the formation of atherosclerotic plaques, 10 commonly occuring oxysterols (7α-, 7β-hydroxycholesterol, 7-ketocholesterol, 19-hydroxycholesterol, cholesterol-5α,6α-epoxide, cholesterol-5β,6β-epoxide, 22*R*-, 22*S*-, 25-, and 27-hydroxycholesterol) were studied for both their cytotoxic activity and their capacity to induce superoxide anion production ($O_2^{\cdot -}$) and IL-8 secretion in U937 human promonocytic leukemia cells. We report here that these oxysterols may have cytotoxic, oxidative, and/or inflammatory effects, or none whatsoever.

Thus, among the 10 oxysterols studied, only 7β-hydroxycholesterol, 7-ketocholesterol and cholesterol-5β,6β-epoxide were cytotoxic (in the following order of toxicity: 7β-hydroxycholesterol > 7-ketocholesterol > cholesterol-5β,6β-epoxide) to human U937 promonocytic leukemia cells. These three oxysterols lead to phosphatidylserine externalization, loss of mitochondrial transmembrane potential, increased permeability to propidium iodide, morphological nuclear changes (swelling, condensation and/or fragmentation of the nuclei), and lysosomal destabilization associated or not with the formation of MDC-positive cytoplasmic structures (enriched in cholesterol, phosphatidylcholine, and sphingomyelin in 7-ketocholesterol-treated cells) (Kahn et al., 2004). Therefore, 7β-hydroxycholesterol-, 7-ketocholesterol-, and cholesterol-5β,6β-epoxide-induced cell death is a complex process, which includes typical features of apoptosis (presence of cells with fragmented and/or condensed nuclei) and which evokes the form of cell death triggered by nonesterified cholesterol (Tabas, 2002; Monier et al., 2003). In addition and in agreement with previous investigations (Yuan et al., 2000; Li et al., 2001), the present results favor the hypothesis that disruption of lysosomes induced by 7β-hydroxycholesterol, 7-ketocholesterol, and cholesterol-5β,6β-epoxide may be a critical event in oxysterol-induced apoptosis and that the subsequent leakage of hydrolytic enzymes, such as cathepsins B and L, into the cytosol might contribute to mitochondrial depolarization (Guicciardi et al., 2000) and caspase-cascade activation (Ishisaka et al., 1999). Moreover, these data are consistent with the notion that oxysterol-induced U937 cell death seems preferentially triggered by oxysterols resulting from oxidation of cholesterol on the sterol nucleus and that the α or β position of the hydroxyl and epoxide radicals plays a key role in the induction of the cell death process. Indeed, 7β-hydroxycholesterol and cholesterol-5β,6β-epoxide are cytotoxic, whereas the corresponding α isomers (7α-hydroxycholesterol and cholesterol-5α,6α-epoxide) are not. Moreover, as one of the best known properties of oxysterols is the ability of some of them to inhibit HMG-CoA reductase activity (Brown and Goldstein, 1974), and as some HMG-CoA reductase inhibitors have been reported to induce apoptosis of vascular smooth muscle cells in culture (Bansal et al., 1989), the relationship between oxysterol-induced inhibition of cholesterol synthesis and the induction of cell death has been defined. Of note, 25-hydroxycholesterol and 27-hydroxycholesterol,

which are two potent inhibitors of HMG-CoA reductase, had no cytotoxic effect, whereas 25-hydroxycholesterol was described to induce cell death against cell types, such as smooth muscle cells, involved in atheroma (Yin et al., 2000). Surprisingly, cholesterol-5β,6β-epoxide, which strongly enhances the activity of HMG-CoA reductase, was toxic. As for the other oxysterols investigated (7α-, 7β-hydroxycholesterol, 7-ketocholesterol, 19-hydroxycholesterol, cholesterol-5α,6α-epoxide, 22*R*-, 22*S*-, and 27-hydroxycholesterol), they did not exhibit a significant effect on HMG-CoA reductase activity. So far, the present data also strongly suggest the absence of a relationship between cell death and inhibition of HMG-CoA reductase activity, which is in agreement with previous work performed on bovine artery endothelial cells (Lizard et al., 1996).

Since there is strong evidence that oxidative stress has a deleterious effect on atherogenesis (Rosenblat et al., 2002), the capacity of oxysterols to stimulate superoxide anion ($O_2^{\cdot -}$) production was also evaluated. In agreement with our previous results (Miguet-Alfonsi et al., 2002; Monier et al., 2003), 7β-hydroxycholesterol and 7-ketocholesterol (but not 7α-hydroxycholesterol) were found to be capable of enhancing $O_2^{\cdot -}$ production and pro-oxidative activity was also found with cholesterol-5β,6β-epoxide, which triggered cell death, as well as with cholesterol-5α,6α-epoxide and 25-hydroxycholesterol, which were not cytotoxic. Therefore, among the 10 oxysterols studied, only five of them (7β-hydroxycholesterol, 7-ketocholesterol, cholesterol-5α,6α-epoxide, cholesterol-5β,6β-epoxide, and 25-hydroxycholesterol) demonstrated the ability to increase $O_2^{\cdot -}$ production and, depending on the oxysterol considered, overproduction of $O_2^{\cdot -}$ was not necessarily associated with cell death, suggesting that efficient defense processes directed against oxidative stress could be simultaneously induced (O'Callaghan et al., 2001). It is thus tempting to speculate that the diversity of oxysterol-related effects, including cytotoxic and/or oxidative activity, might depend on the physical and chemical properties of these molecules, which influence their distribution inside the cell, their interaction with receptors, and/or their ability to modify certain cellular structures, mainly those playing key roles in signal transduction such as lipid rafts (Sleer et al., 2001; Berthier et al., 2004).

As inflammatory reactions are widely involved in the development of atherosclerotic plaque (Hansson, 2001), a particular interest was also accorded to the ability of oxysterols to induce inflammatory cytokines, especially IL-8, which is a member of the CXC family of chemokines and which is a potent chemoattractant implicated in atherogenesis (Boiswert, 2004). Indeed, this cytokine is overproduced by macrophage foam cells in human atheroma (Wang et al., 1996) and stimulation of IL-8 production by oxidized low-density lipoproteins (oxLDL) has been described by other investigators (Terkeltaub et al., 1994). Of note, oxysterols generated during oxidation of oxLDL, including 7β-hydroxycholesterol and 25-hydroxycholesterol, have previously been shown to stimulate IL-8 secretion by human monocytes/macrophages and THP-1 cells (Liu et al., 1997). In agreement with these data (whereas the potency of 7β-hydroxycholesterol and 25-hydroxycholesterol to induce IL-8 secretion varies between U937 cells (present study) and THP-1 cells (Liu et al., 1997)), the high levels of IL-8 secretion and the potent enhancement of IL-8 mRNA observed in U937 cells treated with 7β-hydroxycholesterol and 25-hydroxycholesterol underline that these oxysterols significantly stimulate IL-8 at both the transcriptional and translational levels. In addition, the fact that 7β-hydroxycholesterol and 25-hydroxycholesterol, as well as 7-ketocholesterol and 27-hydroxycholesterol which only induce low levels of IL-8 secretion, are unable to induce other inflammatory cytokines (IL-1β, IL-6, IL-10, TNF-α, and IL-12), leads us to consider that oxysterol-induced IL-8 synthesis

and secretion involve highly specific mechanisms and receptors. Interestingly, as 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 7-ketocholesterol, which are a poor ligands for liver X receptors (LXRs) α and β (oxysterol nuclear receptors involved in the transactivation of numerous genes (Janowski et al., 1999)), stimulate IL-8 mRNA transcription associated or not with IL-8 secretion, conclusive evidence supports the hypothesis that these oxysterols do not transactivate IL-8 gene expression in a LXR-dependent manner. Conversely, as 22*R*-hydroxycholesterol and 25-hydroxycholesterol (in order of potency) are transcriptionnal activators of LXR_{α} and LXR_{β} (Janowski et al., 1999), and as 22*R*-hydroxycholesterol and 25-hydroxycholesterol reduced and enhanced IL-8 mRNA levels, respectively, these oxysterols might regulate IL-8 mRNA synthesis in a LXR-dependent manner. However, other putative receptors of oxysterols, including the anti-estrogen-binding site (AEBS) ubiquitously distributed in animal and human tissues (Lazier and Bapat, 1988), and the oxysterol-binding protein (OSBP) present in the cytosol of many cell types (Kandutsch et al., 1984), might contribute to the regulation of IL-8 mRNA levels. Thus, AEBS, which has mainly strong affinity for 7-oxygenated derivatives of cholesterol (7α-hydroxycholesterol, 7β-hydroxycholesterol, and 7-ketocholesterol) (Hwang, 1990), OSBP, which weakly and strongly recognizes 7-oxygenated derivatives of cholesterol and cholesterol derivatives hydroxylated on the side chain, such as 25-hydroxycholesterol, respectively (Beseme et al., 1987), as well as a subset of OSBP-related proteins (ORPs) (Olkkonen, 2004), which directly bind oxysterols, could act on the activation of transcription factors and/or on the synthesis of molecules contributing to the formation of the transcriptome involved in the regulation of IL-8 expression. In addition, the ability of some oxysterols (7α-hydroxycholesterol, 7-ketocholesterol, and 5β,6β-epoxide) to strongly enhance IL-8 mRNA levels, and to slightly or not increase IL-8 secretion, indicates either that these oxysterols are unable to activate post-transcriptional cellular events leading to IL-8 maturation and secretion, or that they repress these events. Consequently, depending on the complex oxysterol mixtures found in atherosclerotic lesions, the presence of 7α-hydroxycholesterol, 7-ketocholesterol, and/or 5β,6β-epoxide may either quench or promote IL-8 secretion. As for the absence of effect of cholesterol on IL-8 at the transcriptional and translational levels, it underlines that cholesterol can induce side effects only when its accumulation inside the cell occurs under its nonesterified or oxidized form (Wang et al., 1996; Tabas, 2002).

In conclusion, this present investigation clearly demonstrates that oxysterols have pleiotropic activities which may contribute to atherogenesis. Indeed, among the 10 oxysterols investigated, some of them were cytotoxic (7β-hydroxycholesterol > 7-ketocholesterol > cholesterol-5β,6β-epoxide), some induced $O_2^{\cdot-}$ overproduction (7β-hydroxycholesterol = 7-ketocholesterol = cholesterol-5β,6β-epoxide > 25-hydroxycholesterol > cholesterol-5α,6α-epoxide) and/or IL-8 secretion (7β-hydroxycholesterol > 25-hydroxycholesterol). Therefore, these oxysterols, which are frequently detected at enhanced levels in atherosclerotic plaques (Brown and Jessup, 1999), could be strongly involved in atherosclerosis, known as a complex slow degenerative process simultaneously involving the death of vascular and immunocompetent cells, oxidative stress, and enhanced cytokine secretion (Kockx and Knaapen, 2001; Hansson, 2001; De Nigris et al., 2003).

Acknowledgments

This work was supported by the Conseil Régional de Bourgogne, the Ligue Régionale Contre le Cancer de Côte d'Or, the Centre Hospitalier Universitaire de Dijon (PHRC 2002), and the Institut National de la Santé et de la Recherche Médicale (Inserm). The authors are indebted to Mr Jonathan Ewing for reviewing the English

version of the manuscript. We also thank Mr Serge Monier for his excellent technical assistance.

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