# Cellular Sterol Accumulation Stimulated by Cholesterol 5β,6β-Epoxide in J774 Macrophages (43896)

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*Abstract.* **A significant accumulation of cellular free cholesterol and steryl esters is observed in J774 macrophages when cells are exposed to low-density lipoproteins (LDL) containing cholesterol 5β,6β-epoxide. This cellular sterol accumulation is mainly due to the formation of esterified cholesterol and desmosterol. Cellular steryl esters increased to 39.4 and 22.4 μg/mg cell protein with 0.8 μ*M* of cholesterol 5β,6β-epoxide and 3,5-cholestadien-7-one, respectively, whereas hardly detectable levels were observed with the absence of oxysterols. The total cellular sterols increased 45% above the value of control with cholesterol 5β,6β-epoxide. The uptake of [$^3$H] cholesteryl oleate-LDL was also enhanced by cholesterol 5β,6β-epoxide. The rapid displacement of desmosterol with cholesterol was observed when cells were treated with cholesterol 5β,6β-epoxide or 3,5-cholestadien-7-one in the presence of LDL. Cholesterol 5β,6β-epoxide became associated with LDL in the culture conditions, and its uptake into J774 cells and the cytotoxicity were reduced significantly by the association with LDL. The comparison of selected oxysterols for their ability to stimulate cellular sterol accumulation indicated that cholesterol 5β,6β-epoxide is the most potent. Cholesterol esterification was enhanced significantly by cholesterol 5β,6β-epoxide whereas cholesterol 5α,6α-epoxide and 3,5-cholestadien-7-one produced a modest response. In contrast, although cholestantriol, the metabolic hydrolysis product of cholesterol epoxides, also associated with LDL, it showed no stimulating effect on both cellular sterol content and sterol esterification. These results indicate that some oxysterols, such as cholesterol 5β,6β-epoxide and possibly 3,5-cholestadien-7-one, stimulate cellular sterol accumulation in J774 macrophages and may play an important role in atherogenesis.** [P.S.E.B.M. 1995, Vol 209]

A striking characteristic of atherosclerosis is the accumulation of lipids, mainly cholesteryl esters, in the arterial wall. Recent studies suggest that the oxidative modification of low-density lipoprotein (LDL) may play an important role in the development of atherosclerosis (1). Oxidized LDL has cytotoxic effects and enhances macrophage lipid accumulation (2). Growing evidence shows that LDL may be oxidized *in vitro* by endothelial cells (3), smooth muscle cells (4), and macrophages (5, 6), as well as *in vivo* in atherosclerotic lesions (7–9). However, it remains unclear what is the specific etiologic agent. High levels of serum cholesterol have been shown to increase risk of atherosclerosis. Cholesterol undergoes spontaneous autoxidation to produce oxidized derivatives, or oxysterols, which may be involved in atherogenesis (10). Cholesterol oxidation products are found in foods (11, 12), human serum (13), human breast fluid (14), rat lung and liver (15), human prostatic secretions and tissues (16), and in atheromatous plaques (17). Although there is some understanding of their toxicity to cells (18–20) and as potent inhibitors of cholesterol biosynthesis (21, 22),

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little is known about the effect of oxysterols on the cholesterol ester accumulation, which results in foam cell formation in macrophages. The acyl-CoA:cholesterol acyltransferase (ACAT) reaction is a critical step in this atherosclerotic process where the reaction's sterol substrate specificity is largely undefined.

Several studies (23, 24) have demonstrated that most foam cells loaded with cholesteryl esters arise from circulating monocyte-macrophage cell origin. Monocytes have few binding sites for native LDL but take up large amounts of acetylated or oxidized LDL via scavenger receptors (26, 27) which are not down-regulated in the presence of excess cholesterol (25). Mouse peritoneal macrophages in culture can accumulate esterified cholesterol when incubated with modified lipoproteins such as acetylated LDL (25) and have been considered an appropriate model in which to study the metabolism of cholesterol that may be atherogenic.

Therefore, the present study investigated effects of certain oxysterols on the accumulation of cholesterol and cholesteryl esters in J774 macrophages. We observed significant cholesteryl ester accumulation caused by two oxysterols, namely cholesterol 5β,6β-epoxide and 3,5-cholestadien-7-one. The possible atherogenic role of these oxysterols is discussed.

## Materials and Methods

**Oxysterols.** Cholesterol 5α,6α-epoxide, cholesterol 5β,6β-epoxide, cholestan 3β,5α,6β-triol (cholestantriol), 3,5-cholestadien-7-one and 25-hydroxycholesterol were obtained from Steraloids, Inc. (Wilton, NH). The purity was confirmed as one compound by TLC and HPLC analyses. The chromatographic standards, cholesterol, cholesteryl arachidonate, cholesteryl linoleate, cholesteryl oleate, cholesteryl palmitate, cholesteryl heptadecanoate, cholesteryl stearate, and desmosterol were from Sigma Chemical Co. (St. Louis, MO). [1,2,6,7-$^3$H] Cholesterol (75.3 Ci/mmole), [Cholesteryl-1,2,6,7-$^3$H(N)] cholesteryl oleate (71.0 Ci/mmole), and [9,10-$^3$H] oleic acid (7.40 Ci/mmole) were obtained from New England Nuclear (Boston, MA). [$^3$H] Cholesterol 5β,6β-epoxide and [$^3$H] cholestantriol were synthesized from [1,2,6,7-$^3$H] cholesterol by reaction with *m*-chloroperoxybenzoic acid (28). In brief, [1,2,6,7-$^3$H] cholesterol was diluted with nonradioactive cholesterol in $CH_2Cl_2$ to yield a specific activity of 0.5 mCi/μmole. While stirring, *m*-chloroperoxybenzoic acid in $CH_2Cl_2$ was gradually added over a 10-min period to the cholesterol. The reaction continued for 30 min at room temperature and was terminated by adding 10% sodium sulfite. The organic phase was washed with 5% sodium bicarbonate twice, then washed with water and saturated NaCl. The products of cholesterol epoxides (80% yield) and cholestantriol (5% yield) were isolated by TLC (silica gel $F_{254}$; E. Merck) with a mobile phase of chloroform:acetone (9:1, v/v). [$^3$H] Cholesterol 5β,6β-epoxide was further isolated from [$^3$H] cholesterol 5α,6α-epoxide by reverse phase TLC (RP-18, Whatman) with a mobile phase of methanol. The yield ratio of cholesterol 5β,6β-epoxide to cholesterol 5α,6α-epoxide was 1:4. [$^3$H] Cholestantriol was also purified by reverse phase TLC (RP-18, Whatman) with a mobile phase of methanol.

**Lipoprotein-Deficient Serum and LDL.** Lipoprotein-deficient serum (LPDS) (density >1.21 g/ml) was prepared from fetal bovine serum (FBS) (GIBCO, Grand Islands, NY) by ultracentrifugation at 59,000 rpm at 4°C for 36 hr in a Ti.70 Beckman rotor. The protein concentration was adjusted to 50 mg/ml by dilution with 150 m*M* NaCl. LDL was isolated by sequential ultracentrifugation in the density range 1.019–1.063 g/ml according to the method of Goldstein *et al.* (29). [$^3$H] Cholesteryl oleate-LDL was prepared by incorporation of [cholesteryl-1,2,6,7-$^3$H(N)] cholesteryl oleate into LDL using a procedure of Faust *et al.* (30) with the difference that [$^3$H] cholesteryl oleate was used, instead of cholesteryl linoleate.

**Cell Culture.** The murine cell line J774A.1 (BALB/c macrophage) obtained from the American Type Culture Collection (ATCC) was maintained at 37°C in RPMI-1640 (GIBCO) containing 10% heat-inactivated FBS, 1.0% PS (penicillin G 5000 units/ml and streptomycin 5000 μg/ml, GIBCO) under a humidified atmosphere of 5% $CO_2$ and 95% air.

Cells were plated in six-well (35 mm) culture plates (Corning) at a density of $3.0 \times 10^5$ cells/well containing RPMI-1640 plus 10% LPDS media and grown for 2 days. The media were then removed and fresh media (RPMI-1640 plus 10% LPDS) containing lipoprotein (LDL, 300 μg/ml), or without LDL, and an appropriate oxysterol (0.8 μ*M*) dissolved in DMSO (final concentration was 0.5%) were added. DMSO without oxysterol served as a control. To determine LDL derived cellular sterols, [$^3$H] cholesteryl oleate-LDL was used to measure uptake of radiolabeled LDL. In this experiment, the final DMSO concentration was 0.2%. After incubation for the indicated period of time, cells were washed twice with phosphate-buffered saline (PBS), pH 7.4. To each well, 1 ml 0.1% SDS (sodium dodecyl sulfate) was added, and the cells were kept at room temperature for 1 hr. An aliquot of the sample (0.8 ml) was transferred to a test tube and the lipids were extracted as described below. The rest of the sample was used for the protein content measurement by the Bio-Rad protein assay.

**Lipid Extraction and High-Performance Liquid Chromatography.** To each aliquot of cell lysate, 15 μg cholesteryl heptadecanoate was added as an internal standard (31). Lipid extracts of cells were prepared

by extracting three times with 2 ml hexane-isopropanol (3:2, v/v). After evaporation, the extract residue was dissolved in acetonitrile-isopropanol (55:45, v/v) solvent and an aliquot was injected for high-performance liquid chromatography (HPLC) analysis. HPLC analysis was performed on a WATERS 600 liquid chromatography system equipped with a WATERS 990 photodiode array detector. The separation was carried out on a 5 μm LiChrosorb RP-18 column (4.0 mm i.d. × 250 mm; E. Merck) with HPLC-grade acetonitrile-isopropanol (55:45, v/v) at a flow-rate of 1.0 ml/min at 40°C. Cholesterol and cholesteryl esters were detected at 210 nm. The retention times of reference standards are respectively 7.0 min for cholesterol, 11.7 min for cholesteryl arachidonate, 13.7 min for cholesteryl linoleate, 17.1 min for cholesteryl oleate, 18.5 min for cholesteryl palmitate, 20.5 min for cholesteryl heptadecanoate, and 22.7 min for cholesteryl stearate. The sterol mass was quantitated by the peak areas and normalized by the peak areas of the internal standard, cholesteryl heptadecanoate.

To analyze the unsaponifiable sterols of the ester fraction, cell lysate was extracted three times with hexane-isopropanol (3:2, v/v) and separated on TLC plates (silica gel $F_{254}$; E. Merck) using a mobile phase of petroleum ether:diethyl ether:acetic acid (90:10:1, v/v/v). The sterol ester fraction from TLC was saponified using alcoholic KOH with a final concentration of 10% KOH in 50% ethanol at 70°C for 75 min. The unsaponifiable sterols were extracted three times with hexane and dried. The unsaponifiable fraction was resuspended in acetonitrile-isopropanol (50:50, v/v) and analyzed by HPLC as described above, except that the mobile phase was acetonitrile-isopropanol (50:50, v/v).

**Assay for Incorporation of [$^3$H]-Oleate into Cholesteryl Ester.** Cells were plated and grown in RPMI-1640 plus 10% LPDS media for 2 days as described before. Fresh media containing LDL (300 μg/ml), a test oxysterol (0.8 μ*M*) in DMSO (final concentration was 0.5%), and [$^3$H] oleate (final concentration, 0.1 m*M*, 6.25 μCi/μmole) were then added. After incubation in the humidified atmosphere of 5% $CO_2$ for 12 hr, cells were washed with PBS and lipids were extracted by the method of Bligh and Dyer (32). Lipids charged on TLC plates (silica gel $F_{254}$; E. Merck) were separated using petroleum ether:diethyl ether:acetic acid (90:10:1, v/v/v) and the corresponding cholesteryl ester spot was scraped and quantified by liquid scintillation counting.

**Lipid Identification.** An identification of desmosterol was obtained by examining the GC-MS of fraction C-1 collected from the DMSO control (Fig. 1B). An LKB-2091 mass spectrometer was used, operating at 100 eV ionization energy and 250°C source temperature connected to a Shimadzu model GC-9A gas chromatograph using a 10m DB-5 (J&W Scientific, Inc., Ranch Cordova, CA) methyl phenyl siloxane coated capillary column programmed at 10°C/min. The steroid eluted at 385°C, its mass spectrum clearly indicating a cholesterol derivative with an unsaturated side chain by virtue of its molecular ion at m/z 384(8) and fragment ions at m/z 369 (M-$CH_3$, 13), 366 (M-$H_2O$, 7), 351 (M-$C_3H_7$, 11), 300 (M-$C_6H_{12}$, 7), and 271 (33). However, several other spectra (ergosta-7,22-diene-3-ol; cholesta-7,24-dien-3-ol; cholesta-8,14-dien-3-ol; cholesta-7,14-dien-3-ol) had similar spectra (NIST/EPA/NIH Mass Spectral Data Base Version 4.0, Gaithersburg, MD) and the double bond location was confirmed by examining a deuteriochloroform solution of the compound with $^1$H NMR. A 300 MHz Varian spectrometer was used with a 32 degree pulse width and 1024 repititions to achieve satisfactory signal-to-noise. Angular and terminal methyls were observed at Δ0.67, 1.00, 1.59, and 1.67, along with a doublet for the 21-methyl at Δ0.98 (J6-7Hz). Corresponding literature values are Δ0.69, 1.02, 1.59, 1.68, and 0.95 (J6-7Hz) (33).

**Determination of Cholesterol 5β,6β-Epoxide Uptake, Conversion and Cytotoxicity.** [1,2,6,7-$^3$H]-cholesterol 5β,6β-epoxide (specific activity, 75.3 Ci/mmole) was used to determine the uptake and conversion to cholestantriol in J774 cells according to Sevanian *et al.* (20) with some modification. In brief, J774 cells were incubated with 0.8 μ*M* cholesterol 5β,6β-epoxide (0.5mCi/μmole) in RPMI1640 medium containing 10% LPDS with, or without 300 μg/ml LDL for indicated time intervals. At the indicated time points, the medium was removed, and the cells were washed once with 0.1% trypsin and two times with PBS. 0.1% (w/v) SDS-PBS was added to lyse cells, and the cells were kept at room temperature for 1 hr. Aliquots of the cell lysate samples were applied to silica gel 60$F_{254}$ thin-layer chromatography plates (0.5 mm), and the lipids were separated using toluene:ethyl acetate (3:2, v/v). The sterol bands were visualized by iodine vapor and the bands corresponding to cholesterol 5β,6β-epoxide and cholestantriol were scraped into scintillation vials and quantified by liquid scintillation counting. The protein content was measured by the Bio-Rad protein assay. For the cytotoxicity determination, trypan blue exclusion was used to count survival cells.

**Analysis of the Distributions of Cholesterol 5β,6β-Epoxide and Cholestantriol in Culture Medium.** An ultracentrifugal fractionation analysis was used to determine the distributions of cholesterol 5β,6β-epoxide and cholestantriol in the culture medium. [$^3$H] cholesterol 5β,6β-epoxide or cholestantriol (0.8 μ*M*, 2 μCi/μmole) was added to RPMI-1640 plus 10% LPDS medium with or without 300 μg/ml LDL, then the media were incubated in 37°C for 60 min. After the incubation, the medium was adjusted to a

density 1.21 g/ml by adding dry KBr and subjected to ultracentrifugal fractionation according to the method of Kulkarni *et al.* (34) with some modification. In brief, a discontinuous NaCl/KBr density gradient was formed by laying 9.5 ml 0.15 *M* NaCl containing 0.01% EDTA over 4.0 ml medium sample in a 13.5-ml Polyallomer Quick Seal ultracentrifuge tube (Beckman). Loaded tubes were sealed and centrifuged at 250,000*g* for 60 min at 10°C. Fractions were collected, and the radioactivities and the densities were determined.

## Results

**Accumulation of Cellular Sterols.** The sterol components of the J774 macrophage cells were analyzed by HPLC. As shown in Figure 1A, cholesterol and the major steryl esters are well separated and can be quantitated by the peak areas and that of the internal standard. A significant accumulation of free cholesterol and steryl esters was observed when J774 macrophages were incubated with LDL (300 μg/ml) and 0.8 μ*M* of either cholesterol 5β,6β-epoxide or 3,5-cholestadien-7-one (Fig. 1 C and D). When cells were incubated with LDL alone for 24 hr, only traces of steryl esters were found (Fig. 1B). When cells were treated with LDL plus either cholesterol 5β,6β-epoxide or 3,5-cholestadien-7-one, this resulted in an increase in cellular cholesterol and the accumulation of several steryl esters, and a decrease of cellular desmosterol, as is demonstrated in Figure 1 C and D.

During the course of our HPLC analysis of the cellular lipids of the J774 macrophage, desmosterol was clearly identified as component C-1 in Figure 1B exhibiting a retention time of 5.8 min relative to that of cholesterol at 7.0 min. Tabas *et al.* (35) have previously reported that J774 cells synthesize desmosterol instead of cholesterol as determined by pulse labeling experiments employing [$^3$H] mevalonate as a precursor. We further purified the C-1 by reverse phase HPLC (RP-18 column) with a mobile phase of acetonitrile-isopropanol (50:50, v/v). The C-1 compound was identified to be desmosterol by mass spectrometry and, more convincingly, by 300 MHz NMR. Desmosterol is present in J774 cells at 28.1 μg/mg cell protein, when cholesterol accumulation was not observed in the medium containing only LDL (Fig. 1B). When cells were loaded with cholesterol by addition of LDL plus 0.8 μ*M* cholesterol 5β,6β-epoxide or 3,5-cholestadien-7-one, desmosterol was significantly reduced to 6.3 μg/mg cell protein or 5.6 μg/mg cell protein, respectively (Table I). In the absence of LDL, cholesterol 5β,6β-epoxide had no effect on cellular sterol content at 0.8 μ*M*, whereas desmosterol was the major cellular sterol (Table I).

To test whether accumulated cellular cholesterol is derived from LDL, [$^3$H] cholesteryl oleate-LDL was

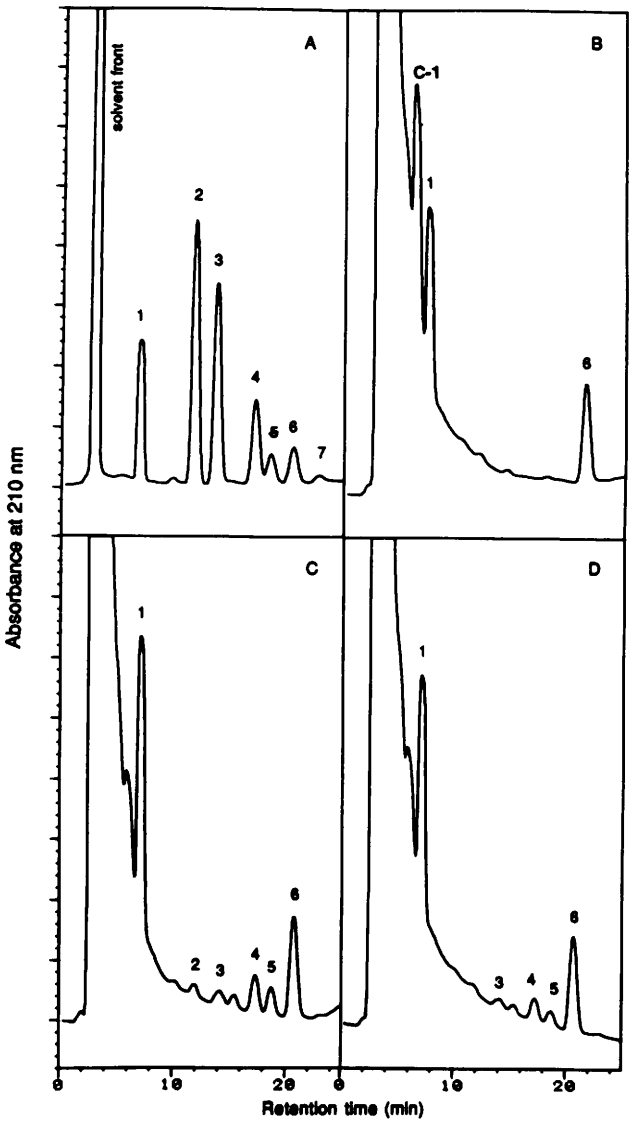


**Figure 1.** HPLC analysis of cellular sterol accumulation stimulated by cholesterol 5β,6β-epoxide and 3,5-cholestadien-7-one in the presence of 300 μg/ml LDL in J774 macrophages. (A) Separation of cholesterol and cholesteryl ester standards. (B) DMSO plus LDL control. (C) Cholesterol 5β,6β-epoxide (0.8 μM) plus LDL. (D) 3,5-cholestadien-7-one (0.8 μ*M*) plus LDL. 1 = cholesterol; 2 = cholesteryl arachidonate; 3 = cholesteryl linoleate; 4 = cholesteryl oleate; 5 = cholesteryl palmitate; 6 = cholesteryl heptadecanoate (internal standard); 7 = cholesteryl stearate; C-1 = identified to be desmosterol.

used to measure uptake of radiolabeled LDL. After incubation with [$^3$H] cholesteryl oleate-LDL in the absence and the presence of increasing amounts of cholesterol 5β,6β-epoxide, as shown in Figure 2, the cellular [$^3$H] cholesterol content was stimulated by cholesterol 5β,6β-epoxide in a dose-dependent manner. The increase of cellular [$^3$H] cholesterol is consistent with the increase of cellular sterols, except that only a 5% increase of [$^3$H] cholesteryl esters was observed when the concentration of cholesterol 5β,6β-epoxide increased from 0.4 to 0.8 μ*M* (Fig. 2). In this experi-

**Table I.** Effects of Oxysterols and Cholesterol on Cellular Cholesterol, Desmosterol and Their Esters in J774 Macrophages

| Sterols | Total sterols (μg/mg)[a] | Free cholesterol (μg/mg) | Steryl esters (μg/mg) | Distribution of steryl esters[b] | | | | Free desmosterol (μg/mg) |
|---|---|---|---|---|---|---|---|---|
| | | | | SA[c] | SL | SO | SP | |
| DMSO | 64.7 ± 2.3 | 33.4 ± 1.8 | 3.2 ± 0.7 | ND[d] | ND | 1.8 | 1.4 | 28.1 ± 1.4 |
| Cholesterol 5α,6α-epoxide | 70.8 ± 1.7 | 37.7 ± 1.1 | 8.4 ± 1.1 | ND | 0.6 | 4.0 | 3.8 | 24.7 ± 1.1 |
| Cholesterol 5β,6β-epoxide | 93.9 ± 2.1[e] | 48.2 ± 1.4[e] | 39.4 ± 2.2[e] | 0.2 | 0.9 | 14.5 | 23.8 | 6.3 ± 0.5[e] |
| 3,5-cholestadien-7-one | 68.5 ± 2.2 | 40.5 ± 1.1 | 22.4 ± 1.7[e] | ND | 0.6 | 9.8 | 12.0 | 5.6 ± 0.7[e] |
| Cholestantriol | 68.5 ± 1.5 | 36.6 ± 1.2 | 4.7 ± 0.5 | ND | ND | 2.0 | 2.7 | 27.2 ± 1.4 |
| 25-OH-cholesterol | 74.9 ± 1.1 | 37.5 ± 0.9 | 6.6 ± 0.4 | ND | ND | 2.8 | 3.8 | 30.8 ± 1.0 |
| Cholesterol | 70.6 ± 1.2 | 35.9 ± 1.3 | 3.8 ± 0.3 | ND | ND | 1.8 | 2.0 | 30.9 ± 1.4 |
| Non-LDL DMSO | 71.8 ± 3.6 | 7.9 ± 0.4 | ND | ND | ND | ND | ND | 63.9 ± 3.2 |
| Non-LDL Cholesterol 5β,6β-epoxide | 70.6 ± 3.3 | 8.1 ± 0.7 | ND | ND | ND | ND | ND | 62.6 ± 4.0 |

*Note.* Cells were incubated for 24 hr with or without LDL (300 μg/ml) containing DMSO or sterols (0.8 μ*M*).
[a] Values (μg/mg protein) represent mean ± SE ($n$ = 4) of two independent experiments, except for non-LDL treatments ($n$ = 2).
[b] Values of steryl ester contents are estimated based on cholesteryl ester standards.
[c] SA = steryl arachidonate; SL = steryl linoleate; SO = steryl oleate; SP = steryl palmitate.
[d] ND = Not detected.
[e] $P < 0.01$ compared with DMSO control in the same column by two-sample assuming unequal variances two-tailed $t$ test.

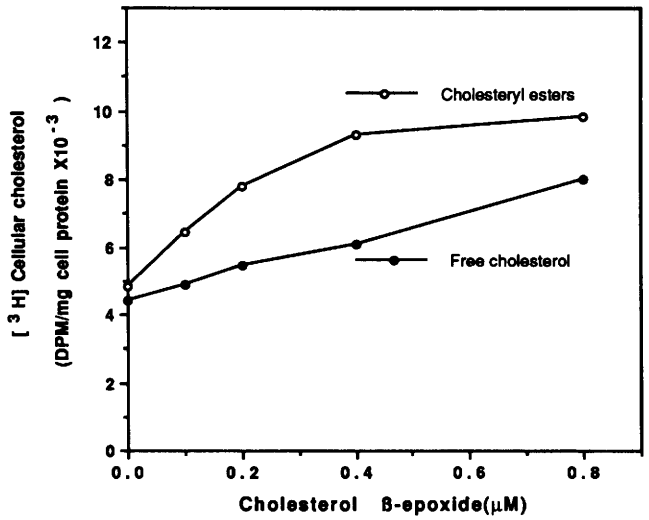


**Figure 2.** Dose-dependent uptake of [$^3$H] cholesteryl oleate-LDL in J774 macrophages stimulated by cholesterol 5β,6β-epoxide. Cells were treated with cholesterol 5β,6β-epoxide at indicated concentrations for 24 hr, and the radiolabeled cellular cholesterol and its esters were separated by HPLC. Values are means of duplicate experiments that did not differ more than 6%.

ment, since the [$^3$H] cholesterol was measured, the esterification of displaced desmosterol, as discussed below, and possibly the availability of ACAT substrates could result in the slower increase of cellular [$^3$H] cholesteryl esters.

In this study, we showed that endogenous desmosterol was rapidly displaced by exogenous cholesterol from LDL, the only cholesterol source, upon the 24 hr treatment of cholesterol 5β,6β-epoxide or 3,5-cholestadien-7-one. These results further support the finding that cholesterol 5β,6β-epoxide and 3,5-cholestadien-7-one stimulated LDL cholesterol uptake and accumulation in J774 cells. To ask whether the displaced desmosterol is esterified in J774 cells, we analyzed the unsaponifiable sterols of the cellular esters after 24-hr treatments of 0.8 μ*M* cholesterol 5β,6β-epoxide or 3,5-cholestadien-7-one in the presence of LDL. The cell culture and incubation conditions are the same as where we observed the rapid displacement of desmosterol by exogenous cholesterol. The unsaponifiable lipids were analyzed by HPLC, both cholesterol and desmosterol were detected at retention time of 7.0 min and 5.8 min, respectively in about equal proportions. The results suggest that the displaced desmosterol was esterified and the accumulated sterol esters contain both cholesteryl esters and desmosteryl esters.

The dose response effects of cholesterol 5β,6β-epoxide and 3,5-cholestadien-7-one were determined to gain further evidence. As shown in Figure 3A, in the absence of cholesterol 5β,6β-epoxide, cellular cholesterol was mainly free cholesterol at 34.3 μg/mg cell protein and free desmosterol at 30.3 μg/mg cell protein. However, with treatment of cholesterol 5β,6β-epoxide (0.8 μ*M*), the cellular free cholesterol increased to 47.2 μg/mg cell protein. More significantly, cholesteryl and desmosteryl esters increased to 38.2 μg/mg cell protein with 0.8 μ*M* cholesterol 5β,6β-epoxide from hardly detectable levels with the absence of this oxysterol. The cellular free desmosterol decreased substantially upon the treatment with choles-

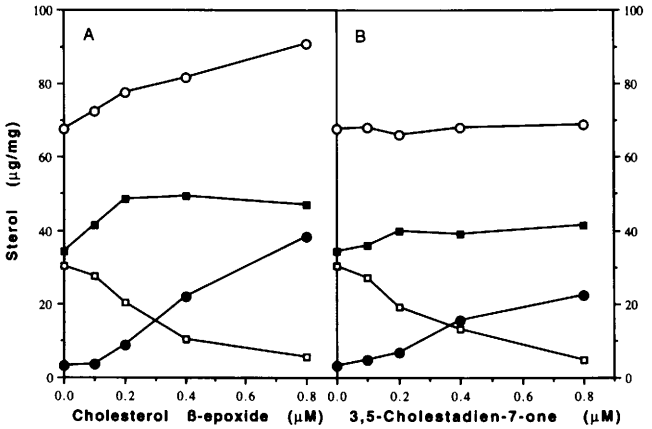


**Figure 3.** Dose-dependent effects on cellular cholesterol and desmosterol in J774 macrophages exposed to LDL (300 μg/ml) containing cholesterol 5β,6β-epoxide (A) or 3,5-cholestadien-7-one (B) at indicated concentrations for 24 hr. The cellular free cholesterol (■), free desmosterol (□), and their esters (●) are determined by HPLC. The total sterols (○) is the summary of three sterol components. Values are means of duplicate experiments that did not differ more than 7%.

terol 5β,6β-epoxide. The total cellular sterol content was significantly increased. A similar pattern of cholesterol accumulation was observed with treatment of 3,5-cholestadien-7-one over the same concentration range, however, no significant change in the total cellular sterol content was observed (Fig. 3B). Cellular cholesteryl and desmosteryl esters were increased significantly to 22.2 μg/mg cell protein with 0.8 μ*M* 3,5-cholestadien-7-one, but free cholesterol increased only slightly.

**Comparison of the Effect of Different Oxysterols.** The effects of a variety of oxysterols and cholesterol on the cellular cholesterol and desmosterol content of J774 cells were determined. As shown in Table I, total cellular sterol increased 45% above the value of control in J774 cells with cholesterol 5β,6β-epoxide, but was not significantly affected by other tested oxysterols, which include the steroisomeric cholesterol 5α,6α-epoxide, its hydrolysis product, cholestantriol, or its precursor, cholesterol. The total sterol accumulation induced by cholesterol 5β,6β-epoxide is mainly due to accumulation of esterified sterols. The total amount of sterols and distribution of esterified sterols are summarized in Table I. All of the oxysterols showed no cytotoxic effect at 1.0 μ*M* and lower concentrations determined by trypan blue exclusion. Under these conditions, cholesterol 5β,6β-epoxide showed the most potency to stimulate cellular cholesterol accumulation. The treatment with cholesterol 5β,6β-epoxide or 3,5-cholestadien-7-one resulted in significant accumulation of esterified sterols and a dramatic decrease in cellular free desmosterol content ($P < 0.01$), whereas the other oxysterols tested produced marginal or no effects. However, the total cellular sterols, cholesterol plus desmosterol, were not affected by 3,5-cholestadien-7-one. Both 25-hydroxycholesterol and cholesterol 5α,6α-epoxide produced slight increase in sterol esterification.

Since cholesteryl and desmosteryl esters are formed by the esterification of sterols, [$^3$H]-oleate incorporation into cholesteryl ester represents Acyl-CoA:cholesterol acyltransferase (ACAT) activity in the cell. To test the connection of the cellular cholesterol accumulation with ACAT activities, the [$^3$H]-oleate incorporation experiment was performed (Table II). The data show that cholesterol 5β,6β-epoxide at 0.8 μ*M* concentration stimulated [$^3$H]-oleate incorporation into cellular esters significantly (171% of control). In contrast, cholestantriol produced a weak inhibitory effect (86% of control) at the same concentration. Cholesterol 5α,6α-epoxide and 3,5-cholestadien-7-one produced a modest response, whereas 25-hydroxycholesterol produced a weak stimulation, with values of 139%, 130%, and 117% of control, respectively. Cholesterol had no significant effect. Without the addition of LDL to the cells which were preincubated with LPDS-containing medium, a low [$^3$H]-oleate incorporation (47% of control) was detected.

**The Metabolism and Cytotoxicity of Cholesterol 5β,6β-Epoxide in J774 Cells.** The uptake of [$^3$H]-labeled cholesterol 5β,6β-epoxide and the conversion to its metabolic product cholestantriol were measured in J774 cells. When J774 cells, cultured in lipoprotein-deficient medium, were incubated with 0.8 μ*M* [$^3$H] cholesterol 5β,6β-epoxide, a rapid uptake of cholesterol 5β,6β-epoxide and conversion to cholestantriol was observed (Fig. 4). However, when LDL (300 μg/ml) was added, the presence of LDL resulted in a significant decrease of uptake and conversion in J774 cells (Fig. 4). The total uptake of cholesterol 5β,6β-epoxide reduced about 10-fold in the presence

**Table II.** Effects of Oxysterols and Cholesterol on [$^3$H]-Oleate Incorporation into Steryl Esters in J774 Macrophages

| Sterols | Incorporation of [$^3$H]-oleate into steryl esters (DPM/100 μg cell protein)[a] |
|---|---|
| DMSO control | 3756 ± 104 (100)[b] |
| Cholesterol 5α,6α-epoxide | 5230 ± 109 (139) |
| Cholesterol 5β,6β-epoxide | 6418 ± 101 (171) |
| Cholestantriol | 3211 ± 101 (86) |
| 3,5-Cholestadien-7-one | 4875 ± 122 (130) |
| 25-Hydroxycholesterol | 4378 ± 125 (117) |
| Cholesterol | 4059 ± 103 (108) |
| Non-LDL DMSO | 1781 ± 96 (47) |

*Note.* Cells were incubated with 300 μg/ml LDL containing 0.8 μ*M* sterols for 12 hr.
[a] Values represent mean ± SE of triplicate experiments.
[b] The numbers in parentheses show percentage of control (DMSO).

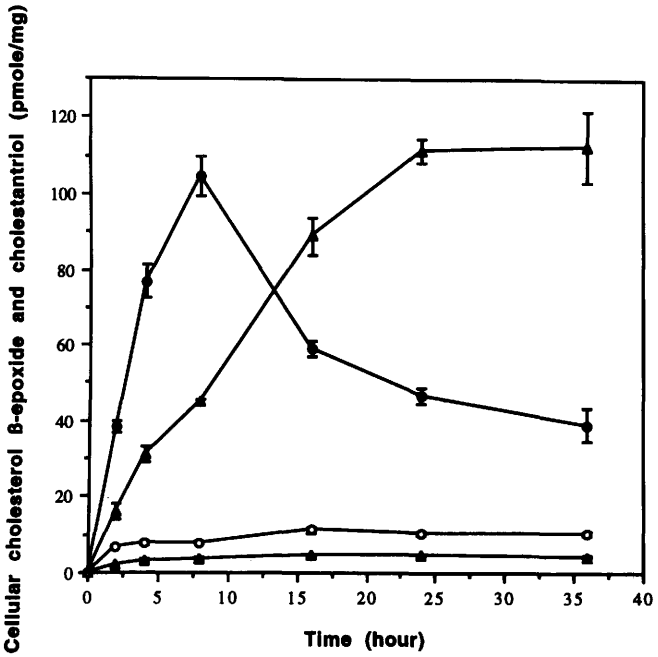


**Figure 4.** The uptake and conversion of [$^3$H] cholesterol 5β,6β-epoxide in the presence or absence of LDL in J774 macrophages. In the presence of LDL (10% LPDS + 300 μg/ml LDL), the cellular [$^3$H] cholesterol 5β,6β-epoxide (○) and [$^3$H] cholestantriol (△), and in the absence of LDL (10% LPDS alone), [$^3$H] cholesterol 5β,6β-epoxide (●), and [$^3$H] cholestantriol (▲) were determined at indicated time intervals. Values represent mean ± SE ($n$ = 4).

of LDL. At lower concentrations, a very similar uptake and conversion behavior of cholesterol 5β,6β-epoxide, in the absence or presence of LDL, were also observed (data not shown). Sevanian *et al.* reported that the uptake of isomeric cholesterol 5,6-epoxides in rabbit aortic endothelial cells may be facilitated by serum proteins other than LDL (20). Our results, seen in J774 cells, agree with that seen in rabbit aortic endothelial cells. In lipoprotein-deficient culture medium containing 10% LPDS, however, we observed a very rapid uptake and conversion of [$^3$H] cholesterol 5β,6β-epoxide to cholestantriol in J774 cells. After 24 hr, about 70% of cholesterol 5β,6β-epoxide was converted to cholestantriol. Although cholesterol 5β,6β-epoxide was effectively taken up by J774 cells, in the absence of LDL, there was no cholesterol accumulation observed. The result further indicates that cholesterol accumulation is stimulated only when both cholesterol 5β,6β-epoxide and LDL are present in the J774 culture.

The rapid uptake and conversion of cholesterol 5β,6β-epoxide in the absence of lipoproteins resulted in a significant increase in the cytotoxicity produced by this oxysterol in J774 cells. Table III lists the $CD_{50}$ values (the concentration causing 50% cell death) determined by trypan blue exclusion. The $CD_{50}$ values of cholesterol 5β,6β-epoxide and cholestantriol increased as the culture medium lipoprotein content increases. When J774 cells are cultured in the medium with 10% FBS or 10% LPDS plus 300 μg/ml LDL, the cytotoxicity of cholesterol 5β,6β-epoxide decreased substantially, from the $CD_{50}$ value of 6 μ*M* to 14 μ*M* or 41 μ*M*, respectively (Table III). The LDL in the culture medium retained most of the cholesterol 5β,6β-epoxide resulting in significantly lower uptake and cytotoxicity. We can, thus, see a clear correlation between the uptake and the cytotoxicity of cholesterol 5β,6β-epoxide in J774 cells. These results suggest that LDL has a high affinity for cholesterol 5β,6β-epoxide and may significantly alter its entry into J774 cells. In the lipoprotein-deficient culture condition, the rapid conversion of cholesterol 5β,6β-epoxide to cholestantriol may also contribute to the increased cytotoxicity, since cholestantriol is well known to be more toxic than the cholesterol epoxides. The increased lipoprotein content in the culture medium also resulted in the decrease of cytotoxicity of cholestantriol. The $CD_{50}$ value increased from 3 μ*M*, in 10% LPDS medium, to 10 or 15 μ*M*, respectively, when J774 cells are cultured in the medium with 10% FBS or 10% LPDS plus 300 μg/ml LDL (Table III).

**Table III.** The $CD_{50}$ Values of Cholesterol 5β,6β-Epoxide and Cholestantriol for J774 Macrophages

| Oxysterols | $CD_{50}$ (μ*M*) | | |
|---|---|---|---|
| | LPDS | FBS | LPDS + LDL |
| Cholesterol 5β,6β-epoxide | 6 | 14 | 41 |
| Cholestantriol | 3 | 10 | 15 |

*Note.* Cells were cultured in RPMI 1640 medium containing 10% LPDS, 10% FBS, or 10% LPDS plus 300 μg/ml LDL for 24 hr.

To determine the association of cholesterol 5β,6β-epoxide and cholestantriol with LDL in the culture medium, the distribution of [$^3$H] cholesterol 5β,6β-epoxide and [$^3$H] cholestantriol among the fractions of culture medium was analyzed by ultracentrifugation. As shown in Figure 5, in the presence of 300 μg/ml LDL, both oxysterols are associated to the fractions with the density of LDL, whereas, in the absence of LDL, they are distributed among the bottom higher density fractions. The result demonstrated that cholesterol 5β,6β-epoxide is associated with LDL in the culture conditions where we observed cholesterol accumulation. Cholestantriol showed similar distribution among culture medium fractions, however, no cellular sterol accumulation was observed.

## Discussion

Accumulation of arterial wall macrophages loaded with cholesteryl esters (foam cells) is a characteristic of atherosclerotic lesions. The knowledge of what causes macrophages to take up large amounts of lipoprotein and accumulate cholesterol esters will lead

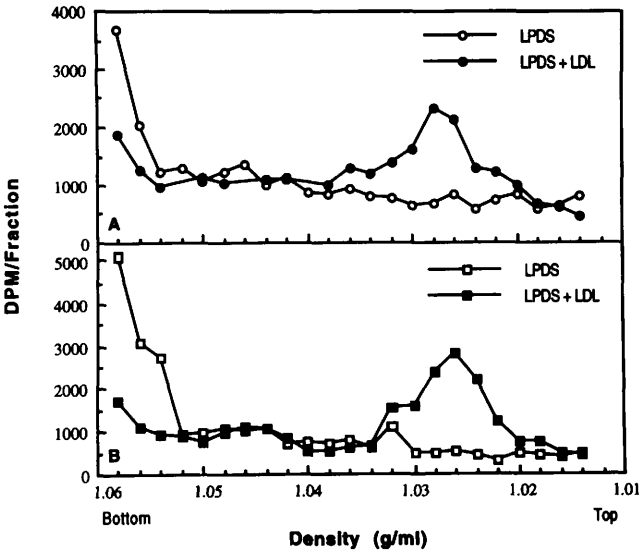


**Figure 5.** Ultracentrifugal analysis of the distributions of [$^3$H] cholesterol 5β,6β-epoxide (A) and [$^3$H] cholestantriol (B) among culture medium fractions, in the presence of LDL (10% LPDS + 300 μg/ml LDL) or the absence of LDL (10% LPDS alone).

to the understanding of the pathogenesis of the atherosclerotic lesion. In the present study, we have demonstrated that cellular cholesteryl and desmosteryl ester accumulation in J774 macrophages is stimulated markedly by cholesterol 5β,6β-epoxide and 3,5-cholestadien-7-one (Fig. 1). These two cholesterol oxidation products produced similar stimulating activities to increase cellular esters, but only cholesterol 5β,6β-epoxide produced a significant increase in total sterols which include cholesterol and desmosterol (Fig. 3). This effect was associated with the addition of LDL to the cell culture which had been preincubated in LPDS-containing medium where no stimulating effect was observed without the addition of LDL (Table I). We further tested the effect of cholesterol 5β,6β-epoxide on [$^3$H] cholesteryl oleate-LDL uptake, demonstrating that the cellular [$^3$H] cholesterol content was increased in the presence of cholesterol 5β,6β-epoxide (Fig. 2). These results suggest that the accumulated cellular cholesterol was derived from LDL, and cholesterol 5β,6β-epoxide and 3,5-cholestadien-7-one stimulated endocytosis of lipoproteins in J774 macrophages. In our results, J774 macrophages do not accumulate steryl esters when incubated with LDL alone for 24 hours (Fig. 1 and Table I). Tabas *et al.* reported that the incubation of J774 macrophages with LDL resulted in a marked accumulation of cholesteryl esters (36). This discrepancy may be due to the difference in the multiple variants of J774 cells, as previously reported by Khoo *et al.* (37). The HPLC method used in this study provides a single-step analytical procedure after lipid extraction.

We observed a prominent component (C-1) of cellular lipid extract in the LPDS or LPDS plus LDL-medium (Fig. 1B) during HPLC analysis. We identified the C-1 to be desmosterol by mass spectrometry. Desmosterol is an immediate precursor of cholesterol in the cholesterol biosynthesis pathway proposed by Bloch (38). The cellular desmosterol content decreased significantly when cells were incubated with LDL plus cholesterol 5β,6β-epoxide or 3,5-cholestadien-7-one (Fig. 1 C and D). Since J774 cells synthesize desmosterol instead of cholesterol (35), in lipoprotein deficient culture conditions, desmosterol is prominent. Tanimoto *et al.* (39) reported that desmosterol was significantly increased when the Bloch pathway was active, whereas another pathway via lathosterol to cholesterol was blocked by 3-methylcholanthrene. Our observation indicates that desmosterol accumulates in J774 macrophages when cells are not loaded with cholesterol and is consistent with the mevalonate pulse labeling results reported previously by Tabas *et al.* (35). When exogenous cholesterol uptake increased as induced by cholesterol 5β,6β-epoxide or 3,5-cholestadien-7-one, cellular desmosterol is reduced significantly, from 28.1 μg/mg cell protein to 6.3 or 5.6 μg/mg cell protein, respectively. We determined that desmosteryl esters also contribute to the cellular ester content based on HPLC analysis after saponification of steryl esters. The HPLC method used in Figure 1 may not be able to resolve cholesteryl esters from desmosteryl esters. This needs to be determined if the desmosteryl ester standards become available. Tabas *et al.* (35) reported that macrophage ACAT can effectively esterify desmosterol. These results suggest that upon the displacement of desmosterol by cholesterol, the free desmosterol is esterified by ACAT. The rapid displacement of desmosterol by exogenous cholesterol upon the treatment with cholesterol 5β,6β-epoxide or 3,5-cholestadien-7-one underscores that LDL endocytosis is enhanced. The results also suggest that cells prefer cholesterol rather than desmosterol as the functional cellular sterol. The stoichiometry of the displacement remains to be determined.

In examining the data of Table I, which summarizes the comparison of the effect of certain oxysterols, we find that only cholesterol 5β,6β-epoxide and 3,5-cholestadien-7-one significantly stimulated cellular steryl ester accumulation after 24 hr treatment. Concerning total cellular sterols, only cholesterol 5β,6β-epoxide showed significant stimulatory activity. The LDL-derived cholesterol is reesterified by ACAT (41), which is known to be related to the atherogenesis process. ACAT activity is normally low in macrophages cultured in the absence of exogenous cholesterol, but it is activated in response to the expansion of the cellular cholesterol pool (25). As shown in Table II, the absence of LDL in the medium resulted in low ACAT activity (47% of control). The addition of LDL (DMSO control) significantly en-

hanced ACAT activity, which was further increased by 71% above the value of the control by the addition of cholesterol 5β,6β-epoxide. Cholesterol 5α,6α-epoxide, 3,5-cholestadien-7-one, and 25-hydroxycholesterol enhanced ACAT activity by 39%, 30%, and 17%, respectively, above the value of control. The enhancement of ACAT activity produced by these oxysterols may be due to increased substrate availability corresponding to a stimulating effect on the endocytosis of lipoproteins. Cholestantriol slightly inhibited ACAT activity by a mechanism which is not clear.

Cholesterol 5α,6α-epoxide, cholesterol 5β,6β-epoxide and 3β,5α,6β-cholestantriol are commonly found as cholesterol oxidation products. In this study cholesterol 5β,6β-epoxide appears most effective in stimulating total cellular sterol accumulation. Sevanian *et al.* have determined that a significant amount of cholesterol epoxide is associated with the density fraction of LDL in standard cell culture (20). During the coincubation of LDL and cholesterol 5β,6β-epoxide or cholestantriol, we observed by ultracentrifugal analysis that they became associated with LDL fractions. Interestingly, the metabolic product of cholesterol epoxides, cholestantriol, showed no stimulating effect at the same concentration. Cholesterol 5β,6β-epoxide may oxidize the other moiety of lipoproteins or serve as a marker to be recognized directly by macrophages. In the absence of LDL, cholesterol 5β,6β-epoxide was effectively taken up by J774 cells and rapidly converted to cholestantriol (Fig. 4). The conversion of cholesterol epoxides to cholestantriol is catalyzed by cholesterol epoxide hydrolase (40). The observation indicates that J774 cells possess active cholesterol epoxide hydrolase. This finding suggests that the conversion of cholesterol epoxides to cholestantriol might be important for removing atherogenic cholesterol epoxide. Then cholestantriol may either be sequestered, further metabolized or excreted (20).

Bhadra *et al.* (42) reported that the major product of the oxidation of the cholesterol moiety of LDL in the presence of endothelial cells was cholesterol 5α,6α-epoxide. In the presence of $Cu^{+2}$ ion, 3,5-cholestadien-7-one predominated. Zhang *et al.* (43) also detected 5,6-epoxycholesterol in the $CuSO_4$ oxidized LDL and determined that 7-ketocholesterol was the major oxidation product. They concluded that the effects of oxysterols in the oxidized LDL on cholesterol esterification are largely dependent on the extent of oxidation, the relative proportions of oxysterols, and the availability of cholesterol for esterification. In their study, a mixture of oxysterols separated from oxidized LDL inhibited the cholesterol esterification in the presence of acetyl-LDL in mouse peritoneal macrophages, whereas individual 7-ketocholesterol, 5,6-epoxycholesterol, and 25-hydroxycholesterol showed no inhibitory effect or a modest stimulatory activity. In our experiments, we used native LDL instead of modified LDL and showed a significant stimulatory activity with cholesterol 5β,6β-epoxide and the modest or no activity with other tested oxysterols for both cellular sterol content and sterol esterification. This further suggests that the individual oxysterol may contribute to cellular sterol accumulation differently.

From the experimental data presented, we conclude that cholesterol and desmosterol accumulation, subsequently the possibility of foam cell formation, in J774 cells is stimulated by cholesterol 5β,6β-epoxide, and possibly 3,5-cholestadien-7-one as well. These oxysterols can induce a similar response as modified lipoproteins in J774 macrophages. Certain oxysterols are present in the oxidized LDL, either by endothelial cells or $CuSO_4$, as reported by Bhadra *et al.* (42) and Zhang *et al.* (43). Our current findings, together with these cited studies, suggest that the atherogenicity of oxidized LDL may in part be due to cholesterol oxidation products such as cholesterol 5β,6β-epoxide. Further studies are needed to assess further the potential of the atherogenicity of these oxysterols.

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