# Uptake, metabolism, and cytotoxicity of isomeric cholesterol-5,6-epoxides in rabbit aortic endothelial cells

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**Abstract** The isomeric cholesterol-5,6-epoxides represent two common cholesterol autoxidation products and along with their principal metabolic product, 3β,5α,6β-cholestane triol, are purportedly angiotoxic. The uptake and cytotoxic action of these compounds was examined in cultured rabbit aortic endothelial cells emphasizing mechanisms of uptake and metabolic fate. The isomeric cholesterol epoxides are incorporated with equal facility and in a dose-dependent manner. The pattern of uptake, which is markedly influenced by media serum concentration and by temperature, suggests that these compounds are partly incorporated through association with serum lipoproteins. After incorporation, both epoxide isomers are rapidly converted to cholestane triol which readily exits the cells. Cholestane triol is further metabolized to an ester-type product representing up to 10% of the added cholesterol epoxides by 24 h of incubation. The order of cytotoxic potency of these cholesterol oxides is: cholestane triol > cholesterol-β-epoxide > cholesterol-α-epoxide, with LD50 concentrations ranging from 23 to > 150 μM in confluent cells. Cholestane triol and cholesterol-β-epoxide are twice as cytotoxic to subconfluent cells as compared to confluent cells, whereas cholesterol-α-epoxide is essentially equitoxic to confluent and subconfluent cells. Cholesterol epoxide cytotoxicity is significantly reduced by treatments in the absence of serum in accord with substantial reduction in uptake when incubations are performed in serum-free media. Our findings show that these cytotoxic cholesterol oxides are incorporated by endothelial cells through a combination of receptor-mediated and nonspecific or passive mechanisms; however, the efficacy of uptake and resulting toxicity is substantially influenced by serum lipoproteins. —**Sevanian, A., J. Berliner, and H. Peterson.** Uptake, metabolism, and cytotoxicity of isomeric cholesterol-5,6-epoxides in rabbit aortic endothelial cells. *J. Lipid Res.* 1991. **32:** 147–155.

**Supplementary key words** low density lipoproteins • cholesterol epoxide • cholestane triol • cholesterol • serum lipids

Hypercholesterolemia has long been considered a major risk factor for the development of atherosclerosis (1, 2). More recent studies suggest that progression of atherosclerosis may be mediated via free radical-induced modification of serum lipoproteins (3–5) involving, in part, peroxidative damage to lipid components. Peroxidation of serum lipoproteins is thought to facilitate their rapid uptake and accumulation in resident macrophages and other cells of the vessel wall, and may also account for toxicity to the endothelium and underlying tissues (5–7). Several investigators have turned their attention to cholesterol oxidation as a contributing factor to atherosclerosis based on studies that showed that cholesterol oxides were angiotoxic and diets containing cholesterol oxidation products were highly atherogenic while similar diets free of these products were relatively nonatherogenic (8, 9).

Cholesterol oxides are formed during free radical-induced lipid peroxidation wherein cholesterol is a major component (10–13). Many of these cholesterol oxidation products are detected in human serum (14), are significantly elevated in humans with hypercholesterolemia (15), and can be isolated from foods (16, 17), tissues (18, 19), serum lipoproteins (20), and in human atheromatous plaques (21). However, it is unclear as to whether these cholesterol oxides are merely passive products of lipoprotein lipid peroxidation (in which case they may serve as markers for oxidatively modified lipoproteins) or whether they contribute to the toxicity of oxidized lipoproteins.

Cholesterol oxidation in biological systems gives rise to numerous products, some of which predominate when biomembranes are subjected to lipid peroxidation (10, 13). Common cholesterol oxidation products include: cholesterol-5α,6α-epoxide (αCE), cholesterol-5β,6β-

Abbreviations: LDL, low density lipoproteins; CE, cholesterol epoxide; αCE, cholesterol-5α,6α-epoxide; βCE, cholesterol-5β,6β-epoxide; CT, 3β,5α,6β-cholestane triol; FBS, fetal bovine serum; REC, rabbit aortic endothelial cells; TCA, trichloroacetic acid; VLDL, very low density lipoproteins; HDL, high density lipoproteins; PBS, phosphate-buffered saline.

epoxide ($\beta$CE), and 3$\beta$,5$\alpha$,6$\beta$-cholestane triol (CT). The toxicities of these oxides, as well as other cholesterol oxidation products, have been examined in vascular explants (22), aortic smooth muscle cells (23), endothelial cells (24), and fibroblasts (25). Although some understanding of their general toxicity is at hand, relatively little is known about the influence of cholesterol oxide uptake and metabolism on endothelial cell toxicity. Endothelial cells represent a first line of exposure to cholesterol oxides as presented in serum lipoproteins. We describe in this report the relationship between uptake, metabolism, and cytotoxicity of three major cholesterol oxides, i.e., $\alpha$CE, $\beta$CE, and CT, using rabbit aortic endothelial cells.

## MATERIALS AND METHODS

### Preparation of cholesterol oxides

Cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO) and was found to be > 99% pure as determined by gas chromatography using chromatographic conditions described by Maerker and Unruh (26). Cholesterol epoxides were prepared from cholesterol by reaction with m-chloroperoxybenzoic acid as described previously (27). This procedure was also used for the preparation of radiolabeled cholesterol epoxides. Accordingly, 1$\alpha$,2$\alpha$-[$^3$H]cholesterol, obtained from Amersham, Inc. (Arlington Heights, IL), was mixed with nonradioactive cholesterol to give a stock solution with a specific activity of 100 $\mu$Ci/mol. The reaction yielded a nearly complete conversion to cholesterol epoxides (CE) producing $\alpha$CE and $\beta$CE at a ratio of 75:25, respectively. The isomeric mixture was separated by high performance liquid chromatography (27) resulting in isolation of each isomer at > 98% purity. The mobile phase (composed of hexane–isopropanol) was removed by rotary evaporation, leaving the purified cholesterol epoxide crystals which were dissolved in absolute ethanol. CT was prepared from radioactive or nonradioactive $\alpha$CE by mild hydrolysis using perchloric acid, as described previously (25), and dissolved in absolute ethanol. The specific activities of $\alpha$CE, $\beta$CE, and CT were adjusted with corresponding unlabeled compounds immediately prior to their addition to cultured cells. Final specific activities of these compounds are presented in the figure legends. All compounds were added in ethanol (0.5% by volume) to the culture medium which was prepared at least 30 min prior to addition to cells.

### Cell culture

Rabbit aortic endothelial cells (REC), obtained from New Zealand Albino rabbits, were used between passages 9 and 13. Their phenotypic characteristics have been described previously (28) and were checked routinely via positive factor VIII surface antigen, angiotensin converting enzyme activity, and morphological appearance. These phenotypic markers were expressed at consistent levels throughout the study. Cells were maintained in 80/20 DMEM/M199 with 15% heat-inactivated fetal bovine serum (FBS) (Gibco, NY). Conditioned medium was also added at the time of medium change at a 1:4 ratio with fresh medium. Cells were passaged using a 1:3 split ratio, were allowed to grow to confluence, and transferred by mechanical disruption using a rubber policeman with dispersion by vigorous pipetting. Cultures were maintained by weekly media changes and had a doubling time of approximately 28 h. Fresh complete media was added to the cells 24 h prior to each experiment.

### Analysis of cholesterol epoxide distribution in serum components

To analyze the distribution of cholesterol epoxide among the serum components of the cell culture medium, 0.50 $\mu$Ci of [$^3$H]$\alpha$CE (100 $\mu$Ci/$\mu$mol) was added to 22 ml of heat-inactivated FBS. The serum was then incubated in a shaker bath for 30 min at 37°C. Immediately after incubation the serum was subjected to ultracentrifugal fractionation of lipoproteins (29). Briefly, this involved layering the medium over 12.5% sucrose and centrifugation for 21 h at 50,000 *g*. Fractions corresponding to densities ranging from 1.020 to 1.050 g/ml were collected and layered onto a 0–15% sucrose gradient and recentrifuged as above. Fourteen fractions were then collected, their densities were determined and aliquots were taken for measurement of radioactivity and protein content (Bio-Rad protein assay kit).

### Uptake and metabolism of cholesterol epoxide

Studies on the uptake and metabolism of the cholesterol oxides involved addition of the radiolabeled compounds to complete media (medium containing 15% FBS) at specified concentrations in the presence of 10 $\mu$M [$^3$H]$\alpha$CE or $\beta$CE. The dishes were harvested at 1–2 h intervals up to 8 h and at selected intervals from 8 to 24 h. In experiments designed to test the effects of serum on uptake into cellular and extracellular components, cells were incubated in media prepared with FBS ranging from 15 to 0.5%, in the presence of $\alpha$CE at 100 $\mu$Ci/$\mu$mol. After 4 h incubation, cells were divided into two identical groups and washed three times with serum-free medium. To one group 0.1% trypsin was added for 10 min at 37°C, the cells were pelleted, and the supernatant was analyzed for trypsin-releasable counts. The cells from the second group were recovered by centrifugation, washed three times with serum-free medium, and aliquots were collected for measurement of radioactivity. This represents

total cellular radioactivity while the difference between total and trypsin releasable counts represents intracellular radioactivity.

Cholesterol oxide metabolites were analyzed by extraction of the culture medium or cell pellet. Extraction of lipids was performed as described by Weithmann, Peterson and Sevanian (30). Aliquots of the total lipid extracts were applied to silica gel-coated plates which were developed in toluene–ethyl acetate 3:2. Measurements of radioactivity associated with zones correpsonding to authentic αCE, βCE, and CT standards were carried out as described previously (31). In those instances where uptake and/or metabolism of CT was of interest, plates were developed in a solvent system consisting of toluene–ethyl acetate–methanol 1:1:0.2. Plates were scanned using a Berthold radiochromatogram scanner (Weibald, Germany), and radioactive zones were recovered for more precise measurements using a liquid scintillation counter.

### Cytotoxicity assays

Cholesterol oxides were added to either confluent (i.e., stationary) or subconfluent (logarithmically growing) cell cultures at various concentrations in order to establish cytotoxic dose ranges. Cytotoxic potencies were estimated on subconfluent and confluent cells from 72-h growth curves in the presence of the test compound (see Table 4), or by 24-h treatments of confluent cells with the test compound, replating, and measurement of plating efficiency at 24 h and cell growth for the following 72 h. Cell densities were typically $10^5$ cells/ml for confluent cells and $5 \times 10^4$ cells/ml for subconfluent cells.

Duplicate dishes were used for each experimental condition and the data represent results from three to five independent experiments. Results are expressed as the mean and standard error for each condition presented.

## RESULTS

### Uptake and metabolism

The uptake of αCE and βCE by confluent REC was measured over a 24-h period as shown in **Fig. 1A**. Incorporation of both cholesterol epoxide isomers was similar with rapid uptake during the first 10–16 h, and no net uptake thereafter. At 24 h approximately 40% of the added epoxides were incorporated into cells. The conversion of the cholesterol epoxides to CT is also shown in Fig. 1A. Both αCE and βCE were converted to CT at similar rates over the 24-h incubation interval. It should be noted that CT formation represents the amounts recovered from isolated and washed REC (i.e., intracellular CT). Approximately 17% of each epoxide was metabolized to CT. Less than 0.2% of these epoxides were converted to CT in the absence of REC. These results resemble the pattern of uptake and metabolism of cholesterol epoxides seen in V79 cells (25). The extent of cholesterol epoxide uptake was concentration dependent as shown in Fig. 1B.

Since cholesterol epoxide solubility is enhanced in the presence of serum, the extent of uptake could be affected by media serum concentration and nonspecific binding to cells could be confused for uptake if one simply measures radiolabel associated with cells. **Table 1** shows that only 12% of added [$^3$H]CE is trypsin-releasable after incubation for 4 h in medium containing 15% FBS. When serum concentrations were reduced, a progressive increase in the proportion of trypsin-releasable counts was obtained, and in the absence of serum, approximately 40% of the total cellular cholesterol epoxide was trypsin-releasable. After

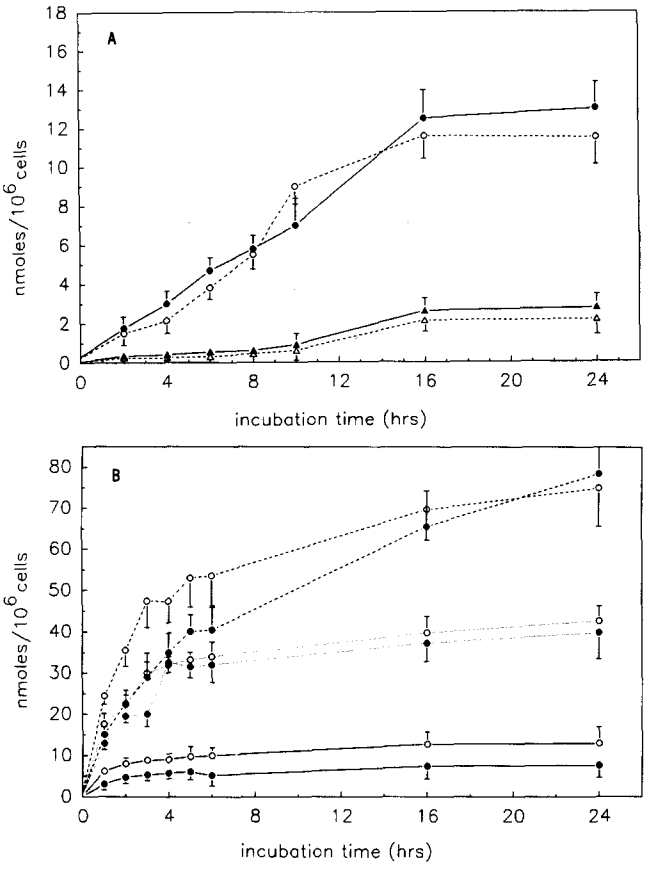


**Fig. 1.** A: The uptake of αCE (●) and βCE (○) by confluent REC is shown. Cells were incubated with $3 \times 10^{-2}$ μCi (100 μCi/μmol) of each epoxide at 10 μM final concentration in 25-mm multiwell dishes containing 2 ml complete medium. After the specific incubation period, the medium was removed, the cells were washed and scraped from the dish, and lipids were extracted from the cell pellet. The lipid extract was applied to thin-layer plates to isolate cholesterol oxides and CT as described in Methods. The isolated zones corresponding to αCE, βCE, and CT were scraped into scintillation vials and radioactivity was measured. The total CT formed from αCE (▲) and βCE (△) is also shown. B: The uptake of αCE (●) and βCE (○) by REC is shown as a function of epoxide concentration. Cells were incubated with 10 (———), 30 (·····) and 50 μM (-------) of each epoxide and the uptake of radioactivity was determined as described in Fig. 1A.

TABLE 1. Levels of intracellular versus extracellular αCE as influenced by media serum concentrations

| Percent Serum in Medium | Percent Total Extracellular[a] | pmoles αCE/$10^6$ Cells Intracellular[b] | n[c] |
|---|---|---|---|
| 0 | 39 ± 7 | | 3 |
| 0.1 | 23 ± 4 | 1.0 ± 0.40 | 3 |
| 1.0 | 21 ± 3 | 2.9 ± 0.50 | 3 |
| 5.0 | 16 ± 5 | 5.0 ± 1.23 | 3 |
| 10.0 | 15 ± 3 | 9.1 ± 2.72 | 5 |
| 15.0 | 12 ± 3 | 8.6 ± 3.04 | 3 |
| 10% LDL[d] | 13 ± 3 | 2.3 ± 1.10 | 5 |
| 10.0 Incubated at 4°C | 32 ± 9 | 0.82 ± 0.30 | 5 |

[a]Trypsin-releasable counts after 4 h incubation with REC at 37°C.
[b]Non-trypsin-releasable counts after 4 h incubation with REC at 37°C. Total amount of labeled αCE added was 100 pmol per $10^6$ cells.
[c]n = Number of experiments involving duplicate cultures per experiment.
[d]Isolated LDL was pretreated with labeled αCE for 30 min followed by ultracentrifugal re-isolation as described in the text. The content of radioactivity in the LDL was determined and an amount equal to 10% serum was added on the basis of protein content.

the 30-min preincubation in medium, a considerable amount of precipitable material was apparent, particularly when high concentrations of cholesterol epoxides were added. The amount of material remaining in suspension was evaluated by adding 0.01 μCi of labeled αCE or βCE along with the corresponding unlabeled compounds and then centrifuging the medium at 3000 rpm for 15 min. Aliquots of the supernatant were then counted to determine the amount of remaining suspended material. For αCE or βCE, 62 ± 1.2% of the added radioactivity remained in suspension. This was the case regardless of the percent serum content of the medium. However, in serum-free medium only 52 ± 1.4% of the added compound remained in solution when 25–100 μM was initially added. The remaining "insoluble" material was recoverable as a fine crystalline precipitate. When medium containing cholesterol epoxides was added to cells, the precipitate was seen by microscopic examination to settle on the monolayer whereas the suspended compound had a cloudy micellar appearance. This partial "insolubility" of cholesterol epoxides could affect their intra- (vs. extra-) cellular distribution and the actual concentration of material available to cells. Table 1 also shows that the intracellular cholesterol epoxide was not as high when using 10% LDL versus 10% serum, suggesting that uptake may be facilitated by serum proteins other than LDL. The proportions of trypsin-releasable counts for LDL prelabeled with αCE were similar to those obtained after treatments in 10% serum-containing medium. Treatments at 4°C in serum-containing medium produced similar levels of trypsin-releasable counts as treatments at 37°C in the absence of serum, suggesting that incorporation by cells is a metabolically active process.

In order to further explore the fate of cholesterol epoxide upon addition to cultured REC, distribution of labeled αCE in complete medium was examined. Since most of the radiolabel added to media was TCA-precipitable, a more detailed examination of αCE distribution among serum proteins was undertaken. Ultracentrifugal fractionation of αCE prelabeled serum revealed a rather complex distribution of radioactivity as shown in **Fig. 2.** More than 80% of the total counts added to complete medium, under conditions similar to standard cell culture, were recovered in fractions with densities similar to that of LDL. Addition of labeled αCE to serum-free media yielded an ultracentrifugal distribution of radioactivity largely among fractions with densities less than those found when αCE was added to complete medium; however, a considerable amount of overlap is evident for the two density profiles. Approximately 25% of the total cholesterol epoxide added to serum-containing medium appears to be associated with protein having a density distribution corresponding to LDL. Less than 3% of the added CE was associated with fractions corresponding to the densities for VLDL and HDL (data not shown).

The possibility of LDL-mediated uptake of cholesterol epoxide was examined by comparing αCE incorporation by REC grown under conditions of decreased LDL recep-

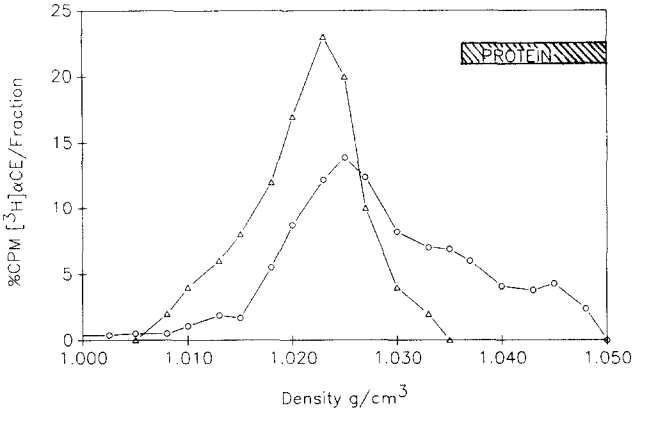


**Fig. 2.** Ultracentrifugal fractionation of fetal bovine serum lipoproteins containing [$^3$H]αCE. medium containing 15% fetal bovine serum was incubated for 30 min with 10 μM [$^3$H]αCE (sp act 6.8 μCi/μmol) at 37°C. Three ml of this medium was then subjected to ultracentrifugational fractionation as described in the text. After the second centrifugation step, 15 × 1.5 ml fractions were collected from the bottom of the centrifuge tube and analyzed for radioactivity, protein content, and density. The content of radioactivity, expressed as percent of total radioactivity added to the medium, is displayed in terms of the density for the specific fractions collected and measured. The distribution of radioactivity is shown for medium with (○) and without (△) serum. LDL was considered to sediment within the density range of 1.02–1.06 g/ml. Fractions containing protein are also indicated. Fractions containing VLDL and HDL were recovered after the first centrifugal step and are not shown (see text for details).

TABLE 2. Uptake of αCE[a] by REC and effect of temperature and inhibitors

| Condition | % [$^3$H]αCE Intracellular Uptake[b] | n |
|---|---|---|
| 37°C | 0.21 ± 0.05 | 5 |
| 4°C | 0.05 ± 0.02 | 3 |
| 25-OH cholesterol[c] | 0.13 ± 0.05 | 4 |

[a]A total of 10 pmol/ml of [$^3$H]αCE (100 μCi/μmol) was added to serum containing medium 30 min before addition to REC. Dishes were then incubated for the indicated periods.

[b]Non-trypsin-releasable counts incorporated in the presence of 2% serum-containing medium. Measurements were made using confluent endothelial cells. Values shown are the percent of total labeled αCE that is recovered with cells as non-trypsin-releasable counts.

[c]25-Hydroxycholesterol was added to confluent REC at a concentration of 3 μg/ml 3 days prior to analysis. Cells were then incubated in the presence of both αCE and 25-hydroxycholesterol.

tor function. The uptake of cholesterol epoxide was compared to the uptake of $^{125}$I-LDL (provided by Dr. Margaret Haberland, UCLA). As shown in **Table 2**, the uptake of cholesterol epoxide was reduced by 75% when cells were incubated at 4°C similarly to the reduction in LDL uptake. Treatment of cells with 25-hydroxycholesterol for 3 days to down-regulate the LDL receptor (32) caused a 29 ± 7.1% inhibition of LDL uptake and a similar, but not significant, decrease in cholesterol epoxide uptake. There was no difference in the number or viability of REC among the various treatment groups. Moreover, no differences were measured in the uptake of α-aminoisobutyric acid (an analog of alanine) (33) suggesting that general membrane transport functions were not markedly affected by the treatment protocol (data not shown). Incubation of REC with labeled αCE in the presence of 30 μM polyinosinic acid, an inhibitor of the LDL scavenger receptor (34), reduced the incorporation of label by 5–15% suggesting minimal involvement of scavenger receptors in the uptake process.

### Cytotoxicity of cholesterol oxides

The cytotoxicity of the cholesterol epoxides and CT have been examined with aortic smooth muscle cells (23, 35) and V79 and C3H/10T1/2 fibroblasts (36, 37), however, limited data exist on endothelial cell toxicity. The effects of serum on the cytotoxicity of cholesterol oxides was examined using αCE as a test compound. **Table 3** compares the effects of various doses of αCE on the survival of confluent REC for treatments in the presence and absence of 10% fetal bovine serum. The plating efficiency of REC was markedly affected by the absence of serum during the treatment period, therefore, all values for surviving fraction of cells were corrected on the basis of control incubations (involving addition of ethanol vehicle alone) which were assigned a surviving fraction value of 1.0. A significant decrease in survival was observed at αCE concentrations above 150 μM in serum-containing medium whereas no toxicity was seen with serum-free medium, even at the highest αCE concentrations tested (300 μM). Hence, the presence of serum components appears to facilitate cholesterol epoxide toxicity.

Cytotoxic potencies were also examined by means of 72-h growth curves in the presence of these compounds at various doses (**Table 4**). The data indicate that subconfluent cells are substantially more sensitive than confluent cells. The estimated LD50s in subconfluent cells for CT, βCE, and αCE are 15 μM, 25 μM, and 108 μM, respectively. These LD50 concentrations are similar to those reported previously for V79 cells (25) which are maintained as logarithmically growing cultures. By contrast, the cytotoxic potencies of these compounds were lower in confluent cells; the LD50s being 23 μM, 55 μM, and > 150 μM for CT, βCE, and αCE, respectively. Further examination of subconfluent cells treated with the cholesterol oxides revealed that about 20–50% of the cells that detached during the 24-h incubation period were not dead since after harvesting and washing these cells were able to reattach and grow. Cytotoxic analyses using the 24-h treatment protocol were also not, strictly speaking, measurements of cell death since about 80% of the treated cells remained attached to dishes, but did not divide, while control cells continued to divide. Thus, a major effect of these compounds may be inhibition of cell division which is subsequently manifested as cell death.

### Uptake and metabolism of cholestane triol

We reported previously that intracellularly formed CT was able to readily exit cells (37), indicating that the cell

TABLE 3. Effect of media serum on cytotoxicity of cholesterol epoxide

| Treatment Dose | Surviving Fraction[a] + Serum | − Serum |
|---|---|---|
| μM | | |
| 0 | 1.00 | 1.00 |
| 50 | 0.96 ± 0.03 | 0.89 ± 0.09 |
| 100 | 0.93 ± 0.09 | 0.80 ± 0.07 |
| 150 | 0.79 ± 0.12 | 1.20 ± 0.29 |
| 300 | 0.48 ± 0.06 | 1.03 ± 0.19 |

[a]The surviving fraction is obtained by replating all cells (i.e., detached and attached) at $10^4$ per dish after 24 h treatment with αCE at the treatment doses indicated. Subsequently, the cell number after 24 h culture in complete medium is divided by the number for untreated controls to obtain the value for surviving fraction. Control cultures are arbitrarily assigned a value of 1.00. Treatments were in the presence (+ serum) or absence (− serum) of 10% fetal bovine serum. The indicated treatment concentrations of αCE were added to media 30 min prior to adding the media to confluent REC. The plating efficiencies after 24 h treatment were 73 and 64% for (+ serum) and (− serum), respectively.

TABLE 4. Surviving fraction[a] of subconfluent and confluent endothelial cells following treatments with cholesterol oxides

| Compound | Concentration (μM) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 10 | 20 | 30 | 40 | 50 | 70 | 150 | 300 |
| αCE | | | | | | | | |
| Subconfluent | | 1.01 ± 0.05 | 0.98 ± 0.02 | | 0.77 ± 0.07 | 0.71 ± 0.09 | 0.61 ± 0.04 | 0.39 ± 0.10 |
| Confluent | | | 0.95 ± 0.04 | 0.94 ± 0.01 | 0.95 ± 0.02 | 0.90 ± 0.04 | 0.92 ± 0.13 | 0.54 ± 0.11 |
| βCE | | | | | | | | |
| Subconfluent | 0.89 ± 0.05 | 0.62 ± 0.07 | 0.45 ± 0.08 | 0.38 ± 0.10 | 0.33 ± 0.15 | 0.18 ± 0.13 | | |
| Confluent | 1.07 ± 0.03 | 0.97 ± 0.02 | 0.90 ± 0.05 | 0.85 ± 0.04 | 0.47 ± 0.20 | 0.20 ± 0.15 | | |
| Triol | | | | | | | | |
| Subconfluent | 0.59 ± 0.07 | 0.50 ± 0.04 | 0.23 ± 0.02 | 0.12 ± 0.10 | | | | |
| Confluent | 1.04 ± 0.09 | 0.88 ± 0.08 | 0.77 ± 0.09 | 0.57 ± 0.06 | 0.24 ± 0.11 | | | |

[a]The surviving fraction of cells represents the number of cells obtained after 72 h of growth in the presence of the test compounds, divided by the number of untreated (control) cells maintained under the same conditions. Control cells are assigned a value of 1.00.

membrane may be quite permeable to this compound. The uptake of radiolabeled CT by REC was, therefore, examined and the results are shown in **Fig. 3**. Incubation with 2 μM CT was accompanied by a rapid uptake for the first 4 h, amounting to nearly 30% of the added CT. Very little incorporation was seen beyond 4 h even though the cells showed no evidence of cytotoxicity beyond 24 h of treatment. It should be noted that the 2 μM treatment dose of CT is comparable to the amount of CT formed after 24 h of treatment with 10 μM αCE or βCE. Fig. 3 also indicates that the uptake of CT was dose-dependent over the concentration range of 1–5 μM and in each case the compound was largely incorporated during the first 4 h of incubation.

The facile cellular uptake of CT and apparent permeability of this compound promoted a more detailed examination of its formation and distribution after treating REC with αCE and βCE. **Fig. 4** describes the time course of CT formation and release into the culture medium after addition of 10 μM αCE or βCE to REC. The amount of CT recovered from the medium is plotted as a percent of total added cholesterol epoxide. The data show that CT release into the medium increased linearly with incubation time. Approximately 40% of the total CT produced was recovered from the medium and the extent of its formation and exit from cells was similar when either αCT and βCE were used as substrates. At 24 h an estimated 1.5 nmol CT/$10^6$ cells was found in the medium indicating that the extracellular CT concentration approached 1 μM (i.e., 10% of the cholesterol epoxide administered).

The identity of radiolabeled products was further examined by thin-layer chromatography of the lipid extracts from cells or culture medium. In addition to CT a third radiolabeled product migrating below CT was routinely detected. Isolation of this product by thin-layer chromatography followed by saponification resulted in more than 50% conversion back to CT. Treatment with rabbit liver esterase (Sigma, St. Louis, MO) resulted in conversion to CT, suggesting that this third metabolite

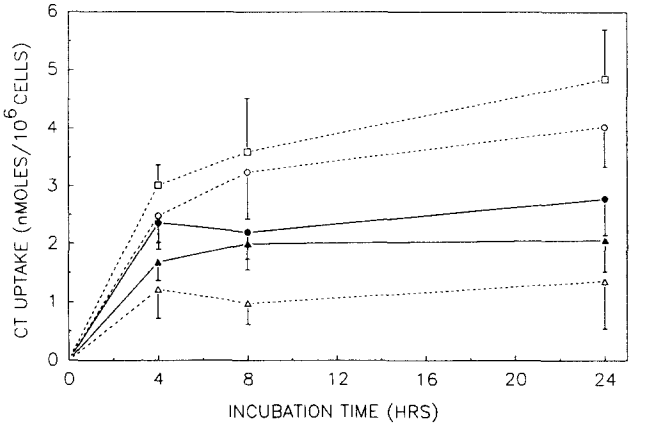


**Fig. 3.** Uptake of CT by confluent REC. Approximately 2 × $10^2$ cells in 25-mm multiwell dishes were incubated with 2 μM CT (●) (5 μCi/μmol) for the time intervals shown. In a similar manner cells were incubated with 1.0 μM (△) (10 μCi/μmol), 1.5 μM (▲), 2.5 μM (○), and 5 μM CT (□) (2 μCi/μmol). After incubation, the cells were washed twice with PBS containing 0.5% BSA and the monolayer was scraped and transferred with 1 ml PBS into scintillation vials to measure cellular radioactivity.

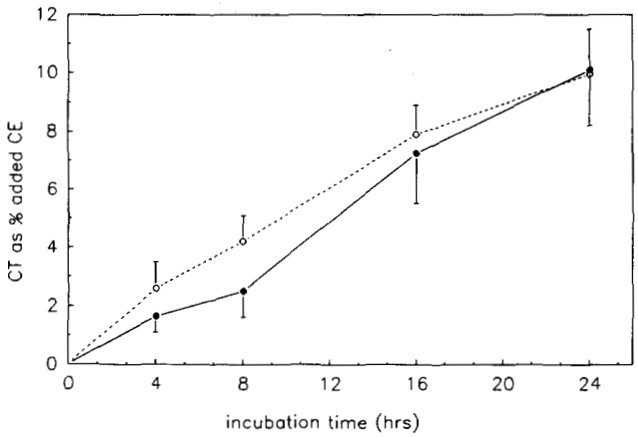


**Fig. 4.** The extent of CT recovered from media after incubating REC with 10 $\mu$M $\alpha$CE (●) or $\beta$CE (○). The results are expressed as the percent of added cholesterol epoxide recovered as CT from the incubation medium after the indicated incubation period. Cells were incubated with labeled cholesterol epoxides as described in Fig. 1. Thereafter, 1.0-ml aliquots of the medium were extracted and the recovered lipids were subjected to thin-layer chromatography to separate $\alpha$CE, $\beta$CE, and CT as described in the text. Zones corresponding to authentic CT standard (added to each sample as a carrier and visualized with iodine vapor) were recovered for assay of radioactivity. The level of CT recovered from the medium represents approximately 50% of total CT formed as described in Fig. 1.

was an esterified form of CT. Formation of a CT-glucuronide is considered unlikely since earlier studies with rat liver microsomes revealed no measurable glucuronyl-transferase activity towards CT (37) under conditions where considerable glucuronidation of *o*-aminophenol (38) was seen. Although positive identification of this product is not yet at hand, its significance as a metabolic product is indicated by accumulation to levels approximating 5–10% of the added cholesterol epoxide after 24 h of incubation.

## DISCUSSION

Cholesterol oxides have been shown to be angiotoxic (9, 22) and are suspected to contribute to atherogenesis (8). The source of elevated cholesterol oxides in hypercholesterolemic patients (14, 15), and in other human tissues (19, 39) is not known since these compounds are present in food (16) and can thus be ingested and distributed to various tissues (40). They may also be generated endogenously via free radical reactions (13, 18); however, the relative contribution from these two recognized sources remains uncertain. Despite evidence that cholesterol oxide levels may not always be elevated during hypercholesterolemia (41), there is sufficient data to suggest that serum concentrations in humans may at times be quite high. Accordingly, we relied on existing literature (14, 15, 18–20, 39) to establish biologically relevant concentrations for studies of cholesterol oxide metabolism and toxicity. Our findings show that all the cholesterol oxides examined elicit toxicity, as manifested by decreased cell division and survival at concentrations comparable to those reported in human serum and tissues. Indeed $\alpha$CE concentrations have been reported to be as high as 500 $\mu$M in hyperlipidemic human serum (15). Since some of the experiments conducted involved adding high concentration of cholesterol oxides ($>100$ $\mu$M) to media with low or no serum present, it is likely that the actual concentrations in solution, and hence freely available to cells, were considerably less than those initially added to the medium. Our estimates of 50–60% added cholesterol epoxides suggests that the indicated concentration could be corrected by this solubility factor. However, the remaining insoluble material was found to settle on the cells and largely become associated with the cells as trypsin-releasable material. Since an unknown amount of this material may become incorporated during incubation, we have chosen to express all data on the basis of the concentrations of initially added cholesterol oxides.

REC appear to effectively metabolize cholesterol epoxides and CT. These cholesterol oxides are rapidly incorporated and can be concentrated by cells; however, CT also appears to readily exit cells after formation from cholesterol epoxides. Accordingly, REC may be a source of extracellular CT when exposed to cholesterol epoxides; however, there is a virtual absence of CT in serum as reported in previous studies (14, 41, 42, and our own (A. Sevanian) unpublished results), suggesting that CT must either be sequestered or further metabolized. Since CT is substantially more toxic than cholesterol epoxides, the ongoing metabolism of cholesterol epoxides, regardless of their origins, may be an intoxicating rather than detoxifying reaction, as suggested from previous studies (37). REC contain cholesterol epoxide hydrolase activity at levels comparable to other cells and tissues (25, 43) and, therefore, may be particularly susceptible to the enhanced toxicity afforded by rapid conversion of cholesterol epoxides to CT. The subsequent metabolism of CT may be required as a critical detoxifying process. To our knowledge, this is the first time that a metabolic product of CT has been identified in endothelial cells. Further studies are needed to establish the identity of this compound and determine whether the formation of this product represents an important elimination or detoxifying process.

Examining data for toxicity together with the results for uptake and metabolism suggests that the levels of these cholesterol oxides in the medium determine the extent of their uptake by REC and, in turn, the degree of cytotoxicity. Although these compounds were added in free form to the medium, there is compelling evidence that they become, at least in part, associated with serum lipoproteins. Indeed, recent analysis of human and rabbit serum confirms earlier reports (20) that most of the cholesterol oxides are associated with LDL and VLDL, respectively

(A. Sevanian and H. Hodis, unpublished results). Four sets of observations suggest that cholesterol oxides may become associated with serum lipoproteins prior to their uptake by REC. *1*) Addition of labeled cholesterol epoxide to fetal bovine serum results in association of approximately 25% of the radioactivity with LDL fraction after ultracentrifugation. *2*) Incubation of αCE-prelabeled medium with serial dilutions of unlabeled serum-containing medium produces a progressive inhibition of αCE uptake (Table 1). Uptake of αCE by REC appears to be saturable (Fig. 1) and increasing cell numbers increases the amount of cholesterol oxide incorporation (data not shown). *3*) The presence of serum in the medium, or addition of LDL to serum-free medium, significantly enhances cholesterol epoxide toxicity as compared to treatments in serum-free medium (Table 3). Cox, Comai, and Goldstein (24) also noted that the cytotoxic action of 25-hydroxycholesterol was enhanced when endothelial cells were treated with fetal bovine serum vehicle as compared to an ethanolic vehicle. *4*) The uptake of αCE is markedly reduced when cells are incubated at 4°C. Based on these findings, we propose that transport via serum lipoproteins (and particularly LDL) may be an effective means by which cholesterol oxides are delivered to and incorporated by endothelial cells; however, considerable uptake may occur via nonspecific or passive (absorption) processes under standard cell culture conditions.

A genotoxic mechanism for cholesterol epoxide toxicity has been proposed from several studies (25, 37, 44) and the potential for disrupting DNA synthesis (45) could account for the greater toxicity seen with subconfluent cells. It should be noted that subconfluent smooth muscle cells are also more sensitive to 25-hydroxycholesterol (24). Since subconfluent cultures have a large proportion of cells in log phase, it is plausible that their sensitivity to cholesterol epoxides and CT may be due to interference of DNA synthesis and replication. There is no evidence that CT is directly genotoxic; however, the possibility remains that its toxicity is exerted by inhibition of cholesterol biosynthesis and/or metabolism (35, 46) or by membrane disruption. In any event, incorporation and cytotoxicity are markedly influenced by serum lipoproteins suggesting that assimilation into cellular compartments is an important criterion for the toxic action of these compounds. Injury to endothelial cells via DNA or membrane damage may provide a locus for lesion development on the vessel wall.

There is at present a paucity of evidence for oxidative modification of serum lipoproteins obtained directly from animals or humans (47). Nevertheless, compelling evidence is emerging to suggest that oxidative modification of lipoproteins (particularly LDL) renders toxicity to smooth muscle and endothelial cells and alters the mode of LDL uptake by endothelial cells and monocytes (48, 49). We propose that measurement of cholesterol oxides be considered as another means for identifying oxidatively modified lipoproteins in addition to providing evidence for lipid peroxidation in tissues. The active uptake of lipoproteins containing these cholesterol oxides may be a process through which these cytotoxic compounds contribute to atherosclerosis.

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