Oxysterols, Cholesterol Biosynthesis, and Vascular Endothelial Cell Monolayer Barrier Function (43198)

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Abstract. **A spectrum of cholesterol oxidation derivatives (oxysterols) is generated in food products exposed to heat or radiation in the presence of oxygen. One of these derivatives (cholestan-3β,5α,6β-triol) was shown to compromise the selective barrier function of cultured vascular endothelial cell monolayers, an action that may initiate atherosclerotic lesion formation. This study sought to investigate the relationship of cholesterol synthesis inhibition by several naturally occurring oxysterols to depression of vascular endothelial cell monolayer barrier function, determined as an increase in albumin transfer across cultured endothelial monolayers. All oxysterols tested caused a variable time- and dose-dependent elevation in *trans*-endothelial albumin transfer, and they were also able to inhibit cholesterol biosynthesis to varying degrees. Pure cholesterol was without effect on both counts. The correlation between the increase in albumin transfer related to oxysterol exposure and the ability of oxysterols to suppress cholesterol biosynthesis was, however, poor. Moreover, mevinolin, a water-soluble competitive inhibitor of cholesterol synthesis, reduced the rate of cholesterol synthesis to 0.9% of control but did not significantly increase albumin transfer. Cholestan-3β,5α,6β-triol caused a 660% elevation in albumin transfer while cholesterol synthesis remained at 11% of control. We conclude that changes in endothelial barrier function caused by exposure to the oxysterols examined, but not pure cholesterol, are probably related to factors other than the well-known action of cholesterol biosynthesis inhibition. These findings may have implications in the development of atherosclerosis.**
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In 1913, dietary cholesterol was first reported to increase the risk for development of atherosclerosis in animals (1). Since that time, high levels of serum cholesterol have been shown to increase risk of atherosclerotic lesion formation in both animals (2–5) and humans (6–8). However, it remains unclear whether the etiologic agent is cholesterol itself, or perhaps some other associated factor. Some of these associated factors that have been implicated are oxidation products of cholesterol, or oxysterols (9–12).

It has been suggested that damage to the vascular endothelium may be involved in the etiology of atherosclerosis (7, 13, 14) by compromising one of the major functions of the endothelium, i.e., to act as a selectively permeable barrier to blood components such as cholesterol-rich lipoprotein remnants. Many oxysterols have been shown to be toxic to cells including vascular endothelial cells (9, 11, 12, 15), smooth muscle cells (16, 17), fibroblasts (18–21), and P815 mastocytoma cells (22, 23).

Oxysterols are known to be potent inhibitors of cholesterol biosynthesis (24, 25), whereas cholesterol itself is without effect (26, 27). In this regard, the cytotoxic effect of the oxysterol 25-hydroxycholesterol was suggested to be related to its inhibitory effects on cholesterol biosynthesis (28). However, the correlation between the ability of oxysterols to inhibit cholesterol biosynthesis and their cytotoxicity is inconsistent (29). Oxysterols have also been shown to insert into biologic membranes, resulting in changes of cell shape (30). Insertion of oxysterols into cell membranes may also compromise their ability to maintain various functions necessary for continued cell viability, such as transmembrane ion gradients (22, 23, 28, 31).

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Therefore, the present study investigated the hypothesis that certain oxysterols might be primary initiators of atherosclerosis by injuring vascular endothelial cells and subsequently by decreasing barrier function of the endothelium. The movement of bovine serum albumin across confluent monolayers of cultured vascular endothelial cells was used as a model of the movement of high-molecular-weight substances such as cholesterol-rich lipoprotein remnants across the *in situ* endothelium. The effects of five oxysterols, each of which has been reported to be found in food products (32–36), on *trans*-endothelial albumin transfer were determined in this model system. The role of oxysterol-induced depression of cholesterol biosynthesis in decreasing endothelial monolayer barrier function was also studied.

Materials and Methods

Cell Culture. Endothelial cells were obtained from porcine pulmonary arteries and cultured in tissue culture medium M-199 (GIBCO Laboratories, Grand Island, NY) containing 10% fetal bovine serum (lot 1111845, 3.4 mg of cholesterol/ml; HyClone Laboratories, Inc., Logan, UT) as described previously (12, 37). Cells from passage 5 to 15 were used in this study. Cultures were determined to be endothelial by uniform morphology and by quantitative determination of angiotensin-converting enzyme activity.

Materials. Sterols including 5-cholesten-3β-ol (cholesterol), cholestan-3β,5α,6β-triol (Triol), 5-cholesten-3β,25-diol (25OH), 5-cholesten-3β,7β-diol (7OH), 5-cholesten-3β-ol-7-one (7Keto), and cholestan-5α,6α-epoxy-3β-ol (Epoxy) were purchased from Steraloids of Wilton, NH. Mevinolin was purchased from Sigma Chemical Co., St. Louis, MO. Cholesterol was purified by recrystallizing from methanol three times. Sterols and mevinolin were first dissolved in absolute ethanol, and this solution added to M-199 containing 5% crystalline fatty acid-free bovine serum albumin (Sigma) and then diluted with culture medium yielding 0.2% ethanol and 0.5% bovine serum albumin in cell cultures. Control cultures received vehicle without sterols or mevinolin.

Determination of Endothelial Barrier Function. The ability of vascular endothelial cells to prevent the movement of bovine serum albumin across intact cultured monolayers was determined as described previously (12, 37). Briefly, cells were plated on gelatin-impregnated polycarbonate filters (13-mm diameter and 0.8-μm pore size); Nucleopore Corp., Pleasanton, CA) glued to polystyrene chemotactic chambers (ADAPS, Inc., Dedham, MA) and cultured for 48 hr, until confluent. The ability of endothelial cell lines to form confluent monolayers (determined as uniform albumin transfer of $\leq$1%/h) was assessed on a regular basis in conjunction with experiments. After 48 hr, the chemotactic chambers with attached filters and confluent endothelial monolayers were washed free of serum by gentle immersion in M-199 and incubated for 24 hr in basal medium, or basal medium supplemented with oxysterols or mevinolin. The basal medium was composed of M-199 enriched with 1% BME vitamins (GIBCO), 1% BME amino acids (GIBCO), and 5% fetal bovine serum (HyClone). After incubation with oxysterols or mevinolin, chemotactic chambers with attached monolayers were washed three times with M-199 and placed into 24-well plates (Corning Glass Works, Corning, NY) containing 1.5 ml of serum-free M-199. Each inner well of the chamber was then filled with 0.5 ml of M-199 containing 200 μM crystalline bovine serum albumin. After a 1-hr incubation, the media were sampled and their respective albumin concentrations determined by measuring the change in A_{630} after the addition of a reagent solution of bromcresol green (Sigma).

Measurement of Cholesterol Synthesis. To measure the rate of cholesterol synthesis by endothelial cell monolayers, cells were seeded into 6-well plastic culture plates (Corning) in 2 ml of basal medium, and incubated at 37°C in 5% for 72 hr. At this time, the culture medium was replaced with 2 ml of medium containing test compounds, and 6 μCi of [^{14}C]acetate (40–60 mCi/mmol; New England Nuclear, Boston, MA) were added to each well. After 6 hrs, the incubation was terminated by the addition of 0.4 ml of 10N KOH. Sterols synthesized from [^{14}C]acetate were extracted and precipitated with digitonin, and β-radiation was counted as described by Boissonneault and Heiniger (23).

Statistical Analysis. Experimental data were expressed as mean $\pm$ SE and all group sizes were equal to 6. Comparisons between groups were made using one-way analysis of variance; all comparisons yielding $P <$ 0.05 were subjected to Scheffe's least significant difference. The correlation between data of Figures 1 and 3 was determined by linear regression analysis. All statistical comparisons were conducted using the statistical software package ABSTAT 6.0 (Anderson-Bell Corp., Parker, CO).

Results

We previously reported that treatment of cultured endothelial cell monolayers with micromolar concentrations of Triol resulted in enhanced *trans*-monolayer movement of bovine serum albumin (12). The data expressed in Figure 1 confirm this finding, and extend it to include several other oxysterols. Of the oxysterols tested, Triol proved to be most potent at elevating albumin transfer following a 24-hr exposure and was maximally effective at a concentration of 25 μM. The oxysterol 7OH was the next most potent; it resulted in the same degree of disruption of monolayer barrier function as Triol but only at a 2-fold greater concentra-

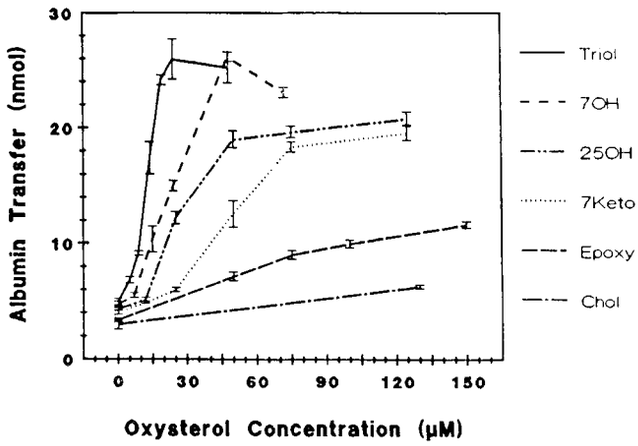

Figure 1. Differential impairment of endothelial barrier function by various oxysterols. Confluent endothelial monolayers were exposed to one of five oxysterols (Triol, 25OH, 7OH, 7Keto, and Epoxy) or cholesterol (Chol) at concentrations ranging from 0 to 140 μM for 24 hr. Albumin transfer was determined as described in Materials and Methods. Each point represents the mean ± SE of six cultures.

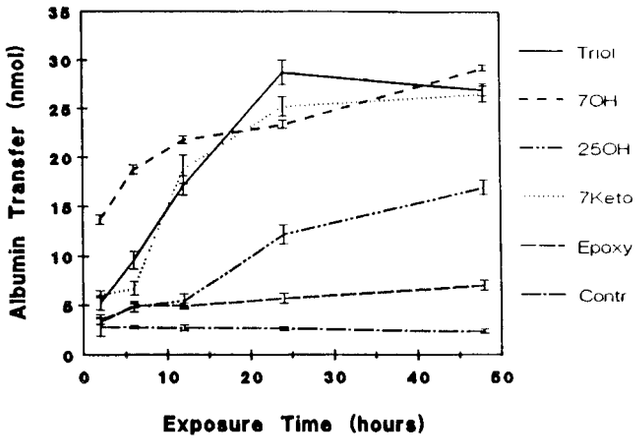

Figure 2. Endothelial barrier function as influenced by time of exposure to oxysterols. Endothelial cell monolayers were exposed to various concentrations of oxysterols (μM: Triol, 25; 7OH, 50; 25OH, 50; 7Keto, 75; Epoxy, 150) or without oxysterols (Contr) for 2, 6, 12, 24, or 48 hr, followed by determination of albumin transfer. Points represent group mean ± SE of six cultures.

tion (50 μM). The effects of 25OH also appeared to be greatest at 50 μM; however, maximum albumin transfer never reached that observed with Triol or 7OH. Albumin transfer in response to 7Keto did not reach a peak until exposure to 75 μM or greater. While albumin permeability increased as the concentration of Epoxy increased, a maximum albumin transfer was never achieved, even after exposure to 150 μM of this oxysterol. Throughout these experiments, pure cholesterol had no effect on albumin transfer at concentrations as high as 130 μM.

Figure 2 illustrates the time dependence of oxysterol-mediated elevation in albumin transfer across cultured endothelial monolayers. Oxysterols were tested at the lowest concentration able to maximally elevate albumin transfer after a 24-hr exposure, or at the highest concentration tested, as detailed in Figure 1 (concentration (μM): Triol, 25; 7OH, 50; 25OH, 50; 7Keto, 75; Epoxy, 150). In this experiment, albumin transfer in cultures exposed to Triol, 7OH, or 7Keto was not different by 24 hr. In contrast, albumin transfer in cultures exposed to 25OH or Epoxy never reached the extent of the other three oxysterols tested, even after a 48-hr exposure. Since high concentrations of cholesterol were not shown to increase *trans*-endothelial albumin transfer after 24 hr, it was not tested in this experiment. Barrier function was most acutely altered by 7OH, resulting in marked elevation in albumin transfer after just 2 hr exposure, followed by 7Keto and Triol, and finally by 25OH and Epoxy, which were not significantly different from control until 6 hr exposure.

Since many oxysterols inhibit the biosynthesis of cholesterol, and since a loss of membrane cholesterol may result in cell injury/dysfunction, we investigated this effect as a potential mechanism whereby oxysterols elevated albumin transfer across endothelial cell monolayers. To test this potential mechanism, the relative ability of the five oxysterols previously tested to inhibit the incorporation of [^{14}C]acetate into cholesterol was determined (Fig. 3). All of the oxysterols, but not pure cholesterol, were capable of depressing cholesterol biosynthesis. Under these conditions, the potency of the oxysterols to inhibit cholesterol biosynthesis was 25OH > 7OH > 7Keto > Triol > Epoxy. Treatment with 130 μM cholesterol had no significant effect on cholesterol biosynthesis. The relationship between the data contained in Figures 1 and 3, that is, the effect of inhibition of cholesterol biosynthesis on monolayer permeability to albumin, is poor at best, as evidenced by a correlation coefficient of 0.46.

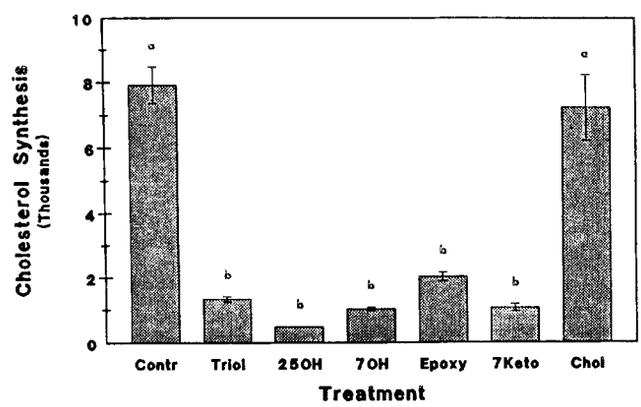

Figure 3. Effect of oxysterols on cholesterol synthesis from [^{14}C] acetate. Endothelial cell monolayers were exposed to various concentrations of oxysterols (μM: Triol, 25; 7OH, 50; 25OH, 50; 7Keto, 75; Epoxy, 150) or 130 μM cholesterol for 6 hr, followed by determination of cholesterol synthesis (cpm [^{14}C]acetate incorporation into digitonin-precipitable sterols/6-hr incubation/culture). Each point represents the mean ± SE of six cultures. Bars with different superscripts are significantly different at $P \leq 0.05$.

In order to further test a possible lack of relationship between cholesterol biosynthesis and oxysterol-induced disruption of endothelial barrier function, experiments were conducted comparing the effects of mevinolin, a water-soluble competitive inhibitor of hydroxymethylglutaryl-CoA reductase, and Triol on monolayer permeability and cholesterol synthetic rate. These data are present in Table I. Cholesterol synthesis was profoundly inhibited by both treatments, i.e., 12 μM mevinolin or 25 μM Triol. *Trans*-endothelial movement of albumin, however, was not altered by mevinolin, whereas exposure to Triol resulted in a marked elevation of albumin transfer across cultured endothelial monolayers. These data reject the hypothesis that the capability of oxysterols to decrease endothelial barrier function is related to their ability to inhibit cholesterol biosynthesis.

Discussion

It has been suggested that the formation of atherosclerotic lesions may be initiated by damage to the endothelium of affected blood vessels (7, 13, 14), enhancing penetration of blood components, including cholesterol-rich lipoproteins, into the vessel wall. In this regard, oxysterols were shown to be toxic to the vascular endothelium, both *in vivo* (10, 11, 38) and *in vitro* (12), suggesting their direct involvement in atherosclerotic lesion formation. Pure cholesterol, on the other hand, is not of itself toxic to endothelial cells *in vitro* (12) and shows inconsistent toxicity *in vivo* (10, 38). Oxysterols can be formed by the oxidation of cholesterol during the cooking and processing of certain cholesterol-containing foods (32–36), and can be absorbed into the blood and carried by serum lipoproteins (39). Thus, it is important that the mode of toxicity of these compounds be understood.

We previously demonstrated that Triol depressed protein synthesis (40) and barrier function of cultured vascular endothelial cells (12). This effect may have been related in part to direct toxicity since release of cellular lactate dehydrogenase (LDH) into the culture medium was elevated in Triol-exposed cultures, although barrier function was altered as early as 2 hr after treatment initiation, whereas LDH release was not elevated until some time after 12-hr treatment. Thus, the temporal relationship between alteration of barrier function and LDH release suggests a mechanism other than direct toxicity resulting in cell death, i.e., cellular dysfunction. The present study extends these observations by testing other oxysterols, all of which (particularly Triol and 7OH) are found in foods (32–36), and it investigates the hypothesis that the effect of these compounds on endothelial barrier function is related to their ability to inhibit the synthesis of cholesterol.

Table I. Effect of Triol and Mevinolin on Endothelial Monolayer Permeability and Cholesterol Biosynthesis

Treatment	Permeability to albumin[a]	Cholesterol synthesis[b]
Control	2.97 ± 0.36[c,d]	22,776 ± 1,892[d]
Mevinolin (12 μM)	3.29 ± 0.44[d]	227 ± 40[c]
Triol (25 μM)	19.59 ± 0.89[c]	3,032 ± 496[e]

[a] Albumin permeability data measured as nmol of albumin transferred per 1-hr incubation.
[b] Cholesterol synthesis data measured as cpm [^{14}C]acetate incorporation into digitonin-precipitable sterols/6-hr incubation/culture.
[c] Mean ± SE, $n = 6$.
[d,e] Within a column, values with different superscripts are significantly different at $P \leq 0.05$.

The data shown in Figure 1 demonstrate that all of the oxysterols studied compromise the ability of cultured vascular endothelial cells to restrict the movement of macromolecular blood components, in this case bovine serum albumin, across the monolayer structure. Our results clearly show, however, that some oxysterols, e.g., Triol and 7OH, are more potent disrupters of endothelial barrier function than others, e.g., 25OH and Epoxy.

A time-response study of single concentrations of these oxysterols on albumin transfer was conducted using oxysterol doses that resulted in maximal albumin transfer following a 24-hr exposure period (Fig. 2). This study demonstrated a large range in the time necessary for different oxysterols to affect endothelial monolayer integrity. Thus, 7OH exposure resulted in an extremely rapid loss of endothelial monolayer integrity, followed by 7Keto, Triol, 25OH, and Epoxy. These data suggest that the chemical structure of the oxysterols in question is important to both short-term and long-term effects on endothelial barrier function, and may suggest different modes of action based upon physiochemical properties of individuals oxysterols.

Since their cholesterol biosynthesis-inhibitory ability is known to be related to chemical structure (27) and binding affinity to a cytosolic receptor (41), it was of interest to determine if a relationship existed between the ability of different oxysterols to inhibit cholesterol biosynthesis and to increase endothelial monolayer permeability to albumin. All of the oxysterols tested were found to be potent inhibitors of [^{14}C]acetate incorporation into cholesterol under the conditions tested, and the relative potencies reported here do not differ from those reported previously (41, 42). However, the ability of the various oxysterols to increase *trans*-endothelial albumin transfer was poorly correlated with their ability to inhibit cholesterol synthesis.

We therefore further tested this relationship by utilizing the oxysterol Triol and a water-soluble competitive inhibitor of hydroxymethylglutaryl-CoA reductase, mevinolin, to inhibit cholesterol biosynthesis; the effects of these treatments on *trans*-endothelial movement of albumin were also studied. Mevinolin was able

to reduce incorporation of [^{14}C]acetate into cholesterol to a greater degree than Triol. Unlike Triol, however, it had no effect on endothelial monolayer permeability to albumin (Table I). These data, therefore, reject the hypothesis that inhibition of cholesterol synthesis per se results in an increase in endothelial monolayer permeability to albumin.

It has been suggested that oxysterols are capable of inserting into plasma membranes and of increasing their permeability to ions. Indeed, exposure of P815 cells or L cells to 25OH resulted in elevated cell permeability to calcium (23) and rubidium (28), while incorporation of 25OH into liposomes increased permeability to calcium (31). Exposure to certain oxysterols also resulted in changes in cell shape, including echinocyte formation in erythrocytes (30) and loss of microvilli in L cells (19). An influx of extracellular calcium and other ions would result in such morphologic changes and ultimately in cell death. We are currently testing the hypothesis that the ability of oxysterols to injure endothelial cells, and thereby to compromise *trans*-endothelial barrier function, is likewise related to a loss in membrane integrity followed by an elevation of endothelial cell plasma membrane permeability to extracellular ions, rather than solely to a depression in *de novo* synthesis of cholesterol.

Several oxysterols including 26-hydroxycholesterol (43), 7α and 7β-hydroxycholesterol, 7-ketocholesterol, and the α- and β-isomers of cholestan-5,6-epoxy-3β-ol (44) have also been detected in the nmol/liter range in normocholesterolemic human serum. Of interest, bile acid sequestrant therapy for reduction of serum cholesterol can influence the level of serum oxysterols. van Doormaal and co-workers (45) reported that hypercholesterolemic patients treated with colestipol experienced an increase in the level of serum 7α- and 7β-hydroxycholesterol (from 205 to 1225 nmol/liter and from 115 to 585 nmol/liter, respectively) without a change in serum 26-hydroxycholesterol levels. Likewise, Bascoul *et al.* (46) recently reported that hypercholesterolemic individuals consuming cholestyramine for its cholesterol-lowering effect experienced a 25-fold rise in serum 7α-hydroxycholesterol (from 100 to 2500 nmol/liter after 3–5 days treatment), over 75% of which was located in the low-density lipoprotein fraction. Similar effects were also noted with normocholesterolemic subjects. Several other studies have clearly demonstrated that oxysterols are carried in serum lipoproteins of animals (39) and humans (44–46). Therefore, cells that interact with lipoproteins can be expected to experience elevated levels of oxysterols relative to those levels found in serum. Thus, while the levels of oxysterols used in these experiments are somewhat greater than those found in whole serum from normal humans, it is conceivable that the level of oxysterols in the immediate vicinity of the endothelium are at least equal to if not greater than those used here.

Our findings support the hypothesis that depressed vascular endothelial cell monolayer barrier function by oxidation derivatives of cholesterol may be a primary determinant in the etiology of atherosclerosis. Inhibition of cholesterol biosynthesis was determined not to be a primary mechanism in altered monolayer barrier function, which may instead be related to the ability of cholesterol oxidation derivatives to partition into cellular lipid phases such as plasma and organelle-associated membranes.

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