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Influence of Cholesterol Oxidation Derivatives on Membrane Bound Enzymes in Cultured Aortic Smooth Muscle Cells[1] (42153)

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Abstract. Cholestane-3β,5α,6β-triol and 25-hydroxycholesterol are two of the most cytotoxic and also relatively abundant of the autoxidation derivatives of cholesterol. Cultured aortic smooth muscle cells which were incubated with 10 μg/ml of either sterol for 24 to 48 hr showed a marked decrease of 5'-nucleotidase activity in isolated crude membranes. It was further demonstrated that 5'-nucleotidase activity was also markedly decreased in plasma membrane-enriched fractions when cells were incubated with cholestane-3β,5α,6β-triol. Na^+,K^+-ATPase activity in crude membranes showed a significant decrease (32%) only in cells incubated with cholestane-3β,5α,6β-triol for 48 hr. There was no effect on Na^+,K^+-ATPase activity in cells incubated for 24 hr with either sterol.

Certain autoxidation derivatives of cholesterol have been known to cause cytotoxicity to a variety of cells in culture, including vascular smooth muscle cells (1, 2). These compounds may, therefore, be atherogenic, particularly in view of the probable important role of vascular smooth muscle cells in initiation of atherogenesis (3–5). They have been shown to be potent inhibitors of cholesterol biosynthesis in cultured cells (6, 7), which may result in depletion of the cholesterol from cell membranes. The latter could affect a number of membrane functions, including permeability to metabolites, endocytosis and the activities of membrane-bound enzymes. The viability of cells often depends on the integrity of their cell membranes; it is conceivable, therefore, that the cell injury induced by autoxidation derivatives of cholesterol is attributable to their effects on membrane functions. In the present experiments we studied the effects of the two most cytotoxic autoxidation derivatives of cholesterol; cholestane-3β,5α,6β-triol and 25-hydroxycholesterol, on the membrane-bound enzymes of cultured aortic smooth muscle cells. Although there are several reports on cholesterol and cell membrane functions (9, 10), the effect of cholesterol oxidation derivatives on membrane bound enzymes has not completely been explored.

Materials and Methods. *Preparation of aortic smooth muscle cell cultures.* Cultured aortic smooth muscle cells were grown from explants of New Zealand white rabbit aortas as described previously (1). The explants were nourished with Eagle's basal medium (BME), which was supplemented with 10% fetal calf serum, L-glutamine (1.29 mg/ml), penicillin (20 units/ml), streptomycin (20 mcg/ml), and fungizone (0.05 mcg/ml). The flasks were incubated in a tissue culture incubator at 37°C under an atmosphere of 5% CO_2 and 95% air. In approximately 2 weeks the smooth muscle cells had reached confluency. The cells were detached by trypsin, counted, and aliquots of cells seeded into 75-ml flasks. After several days of incubation, the subcultures were confluent, and these cells were used for the membrane studies.

The sterols were first solubilized in an ethanol vehicle and appropriate volumes were added to the culture media. In all cases, ethanol was less than 0.4% of the final volume of the medium. The monolayer cultures treated with sterols or vehicle alone were incubated 24 to 48 hr at 37°C. Viability tests based on trypan blue exclusion were done before and after incubation with the test sterols or vehicle. Viability was based on the

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ability of the cells to exclude the dye. For harvesting, the cells were washed four times with 0.15 *M* NaCl (pH 7.4). The cells from each flask were scraped into 2.0 ml of 0.15 *M* NaCl (pH 7.4) with a rubber policeman and collected by centrifugation for 5 min at 1100*g* at 4°C. The cells were resuspended in 0.15 *M* NaCl with a Vortex mixer and collected again by centrifugation for 5 min at 1100*g*. This washing step was repeated two times. The washed cells were resuspended in 1.0 m*M* Tris–HCl (pH 7.4) and incubated for 20 min to allow for cell swelling. This and all subsequent steps were carried out at 0–4°C. The cell suspensions were transferred to a Tenbroeck homogenizer and the cells were disrupted by 25–40 strokes using a tight fitting pestle. The extent of cell breakage was monitored by phase contrast microscopy. The crude homogenate was pelleted by centrifugation at 33,000*g* at 4°C for 1 hr. The crude particulate fraction was resuspended in 0.25 *M* sucrose containing 1.0 m*M* Tris–HCl (pH 7.4). Aliquots were taken for the determination of protein and cholesterol and for assay of enzyme activity.

Preparation of plasma membrane-enriched fraction. Fractionation of the crude membranes was done according to the method of Schimmel *et al.* (13). The crude homogenate was centrifuged at 1700*g* for 10 min and the supernatant decanted and saved. The pellet was resuspended in 0.25 *M* sucrose with 1.0 m*M* Tris–HCl buffer (pH 7.4) by several strokes in a Tenbroeck homogenizer and centrifuged again at 1700*g* for 10 min. This procedure was repeated until the supernatant was clear. The 1700*g* pellet contained nuclei and unbroken cells. The combined 1700*g* supernatants were centrifuged at 205,000*g* at 4°C for 1 hr. The resultant postnuclear pellet was resuspended in 1.0 m*M* Tris–HCl (pH 7.4) and layered on a discontinuous sucrose density gradient of 55, 32, 27, and 13 sucrose (w/v). The gradient was centrifuged in a SW 50L swinging bucket rotor at 206,000*g* at 4°C for 14 hr in a Beckman L-2 ultracentrifuge. The bands of turbid material in each sucrose fraction were removed and the fractions were designated I, II, III, and IV. The particulate material in each fraction was pelleted by centrifugation at 106,000*g* for 1 hr. The membrane fractions were washed by resuspension in 0.25 *M* sucrose, 1.0 m*M* Tris–HCl (pH 7.4), and centrifugation at 106,000*g* for 30 min. The washed membrane fractions were resuspended in 0.25 *M* sucrose, 1.0 m*M* Tris–HCl (pH 7.4) and aliquots were removed for assay of enzyme activities. Protein in each fraction was determined. Other aliquots were utilized for neutral lipid extraction with chloroform:methanol (2:1 v/v). The lipid extracts from each fraction were dried under nitrogen and resuspended in isopropyl alcohol. Appropriate volumes (2 μl) were injected into a Perkin–Elmer 3920B gas chromatograph along with α-cholestane as internal standard. Sterols were identified by comparing retention times of standards relative to α-cholestane. A planimeter was used to determine relative peak areas of sterols in each fraction.

Measurement of 5′-nucleotidase activity and Na^+K^+-ATPase. The activity of 5′-nucleotidase (EC 3.1.3.5) was determined according to the method of Ikehara *et al.* (14). The final reaction mixture of 1.3 ml contained 0.1 *M* Tris–HCl (pH 7.5), 5.0 m*M* $MgCl_2$, 1.0 m*M* EDTA, and 50–100 μg (protein) crude membranes or 10–30 μg (protein) plasma membrane fraction. The assay mixture was preincubated at 37°C for 10 min, following which the reaction was initiated by the addition of 0.1 ml of 5′-adenosine monophosphate (final concentration 5.0 m*M*). The reaction was terminated after 20 min by the addition of 0.2 ml of 50% trichloroacetic acid. The precipitated material was removed by centrifugation at 1000*g* for 10 min. An aliquot of the supernatant was removed for the determination of inorganic phosphate. Zero time activity was determined by addition of trichloroacetic acid prior to substrate.

Na^+K^+-ATPase (E.C. 3.6.1.3) activity was determined by the method of Kimelberg and Papahadjopoulos (16). The final reaction mixture of 1.5 ml contained 50 m*M* Tris–HCl (pH 7.2), 100 m*M* NaCl,10 m*M* KCl, 0.1 m*M* EDTA, and 3.0 m*M* $MgCl_2$ with or without 0.5 m*M* ouabain. After the addition of 50–100 μg crude membrane (protein) or 20–40 μg plasma membrane (protein), the reaction tubes were preincubated for 10 min at 37°C. The reaction was started by the addition of 0.1 ml of adenosine triphosphate (ATP; 3.0 m*M* final concentration). The

reaction proceeded for 10 min and was stopped by the addition of 0.2 ml of 50% trichloroacetic acid. The tubes were then kept on ice to prevent acid hydrolysis of remaining ATP. The precipitated material was removed by centrifugation at 1000*g* for 10 min and an aliquot was removed for determination of inorganic phosphate. Zero time enzyme activity was determined by addition of trichloroacetic acid prior to the assay. Ouabain-sensitive Na^+K^+-ATPase was determined by subtracting ouabain-insensitive ATPase from total ATPase activity.

Analytic methods. Protein was measured by the method of Lowry *et al.* (11) using bovine serum albumin as the standard. Cholesterol was measured by the method of Abell *et al.* (12). Inorganic phosphate was determined by the modified method of Fiske and Subarrow (15) which was scaled down for much smaller amounts.

Results. *Effect of cholestane-3β,5α,6β-triol and 25-hydroxycholesterol on 5′-nucleotidase activity.* The optimal substrate concentration for the biochemical determination of 5′-nucleotidase activity was found to be 5 m*M* adenosine monophosphate (AMP). An AMP concentration of 5 m*M* was selected because it was virtually saturating and because the reaction was linear at this concentration of AMP up to 30 min. An assay time of 20 min was used for all subsequent assays for 5′-nucleotidase activity. In all experiments, cell viability was determined by Trypan Blue exclusion. At least 90% of the cells were viable (based on cell counts of 300) after incubation with either sterol for the indicated time periods. Since cholestane-3β,5α,6β-triol or 25-hydroxycholesterol at final concentrations of 10 μg/ml were found to be minimally toxic to the cells (90% viability), this concentration was used for both sterols in all subsequent experiments.

Biochemical determination of 5′-nucleotidase activity in crude particulate fraction showed that both 25-hydroxycholesterol and cholestane-3β,5α,6β-triol caused significant decreases in enzyme activity at 24 hr, as well as at 48 hr (Table I). There was no significant difference in enzyme activity between 24 and 48 hr with either sterol. Cholestane-3β,5α,6β-triol was more potent than 25-hydroxycholesterol, as incubation of the cells with the former sterol for 48 hr resulted in a 48% decrease in 5′-nucleotidase activity compared to control values.

TABLE I. EFFECT OF CHOLESTANE-3β,5α,6β-TRIOL AND 25-HYDROXYCHOLESTEROL ON 5′-NUCLEOTIDASE ACTIVITY IN CULTURED AORTIC SMOOTH MUSCLE CELLS

Sterol	Time (hr)	5′-Nucleotidase activity (μM/min/mg $\times 10^{-2}$)	% Change compared to the control
Control (vehicle only)	—	30.76 ± 6.75	—
25-Hydroxy-cholesterol	24	18.11 ± 5.41*	−41.12
	48	22.60 ± 3.47*	−26.52
Cholestane-3β,5α,6β-triol	24	18.82 ± 5.89*	−38.81
	48	15.83 ± 3.09*	−48.53

Note. Control values represent the average values obtained at zero time, and after 24- and 48-hr preincubation with vehicle (ethanol). Values represent the means ± SEM of at least six experiments.

* $P < 0.01$ compared to control value.

Since 5′-nucleotidase and Na^+K^+-ATPase are localized on the plasma membrane in a variety of cell types, including aortic smooth muscle cells (17, 18), the various sucrose fractions were assayed to determine which of these contained the highest enzyme concentrations. Sucrose fractions III and IV were highest, and so these fractions were combined into one fraction and the remainder of the gradient combined (I and II) into another fraction. There were 5.2-fold and 4.3-fold enrichments in 5′-nucleotidase and Na^+K^+-ATPase, respectively, in this plasma membrane-enriched fraction (Table II). Analysis of the plasma membrane-enriched fraction for succinate cytochrome *c* reductase and NADPH cytochrome *c* reductase, marker enzymes for mitochondria and microsomes, respectively, showed that it contained no detectable mitochondrial contamination and only 15% of the total NADPH cytochrome *c* reductase activity (microsomal contamination). Ultrastructural examination of the plasma membrane-enriched fraction revealed numerous smooth vesicles ranging from 0.2 to 0.5 μm in diameter. There was no evidence of membranes with attached ribosomes, indicating that this fraction had no obvious microsomal contamination. The content of

TABLE II. ENZYME ACTIVITIES IN FRACTIONS OF CULTURED AORTIC SMOOTH MUSCLE CELLS

Fraction	5'-Nucleotidase (nmole/min/mg)	Na^+K^+-ATPase (nmole/min/mg)	Total protein (mg)
Crude homogenate	116.89	27.54	4.34
Nuclear pellet	57.11	34.86	2.08
Postnuclear pellet	194.59	—	1.82
Plasma membrane-enriched fraction	616.73	119.52	0.71
Combined mitochondrial and microsomal fractions	205.36	78.43	1.02

Note. Assay conditions were as described under Materials and Methods. Na^+K^+-ATPase represent ouabain-sensitive ATPase.

marker enzymes and ultrastructural appearance of this fraction were consistent with those reported by other investigators (19).

The inhibitory effect of cholestane-3β,5α,6β-triol on 5'-nucleotidase activity, determined with the plasma membrane-enriched fraction, was similar to that demonstrated with the crude membranes (Fig. 1). Incubation of the cells with cholestane-3β,5α,6β-triol for 48 hr resulted in a 55% decrease in 5'-nucleotidase activity as compared to control values.

Effect of 25-hydroxycholesterol and cholestane-3β,5α,6β-triol on ATPase activity. With 3.0 m*M* adenosine triphosphate, ATPase activity was linear with time and protein concentration under the conditions described under Materials and Methods for the biochemical determination of ATPase activity. A 3.0 m*M* ATP concentration has been widely used in biochemical assays of ATPase activity in cultured cells (19, 20). When the cells were incubated with 25-hydroxycholesterol or cholestane-3β,5α,6β-triol, it was found that only after a 48-hr incubation with cholestane-3β,5α,6β-triol was there significant inhibition of ATPase activity (Table III).

Effect of cholestane-3β,5α,6β-triol and 25-hydroxycholesterol on cellular cholesterol levels and their distribution in subcellular fractions. When cholestane-3β,5α,6β-triol was incubated with the cells for 1 to 4 days, cellular cholesterol concentrations showed an early decline at 24 hr and continued to decrease to 28 μg/mg protein at 4 days (62% of control value as shown in Fig. 2). In contrast, 25-hydroxycholesterol did not significantly affect cellular cholesterol levels at 24 hr, but further incubation with this sterol resulted in a precipitous drop in cholesterol

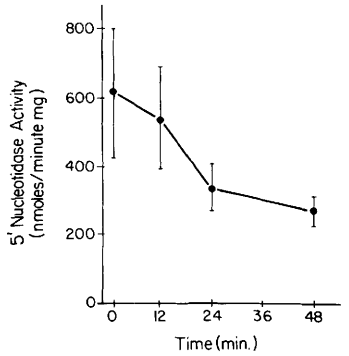

FIG. 1. Effect of cholestane-3β,5α,6β-triol on 5'-nucleotidase activity in the plasma membrane-enriched fraction. Cells were incubated with the sterol for time periods indicated prior to cell fractionation.

TABLE III. EFFECT OF 25-HYDROXYCHOLESTEROL AND CHOLESTANE-3β,5α,6β-TRIOL ON ATPase ACTIVITY IN CULTURED AORTIC SMOOTH MUSCLE CELLS

Sterol	Time (hr)	ATPase (μmole/min/mg × 10^{-2})	% Change compared to control
Control (vehicle only)	—	22.96 ± 4.18	—
25-Hydroxy-cholesterol	24	29.10 ± 8.44	+26.74
	48	21.09 ± 5.58	−8.14
Cholestane-3β,5α,6β-triol	24	23.22 ± 1.33	+1.13
	48	15.66 ± 3.39*	−31.79

Note. Control values represent the mean values obtained from zero time and from 24- and 48-hr incubation with vehicle (ethanol). ATPase represent ouabain-sensitive Na^+K^+-ATPase. Values represent means ± SEM of at least six experiments.

* $P < 0.01$ compared to control value.

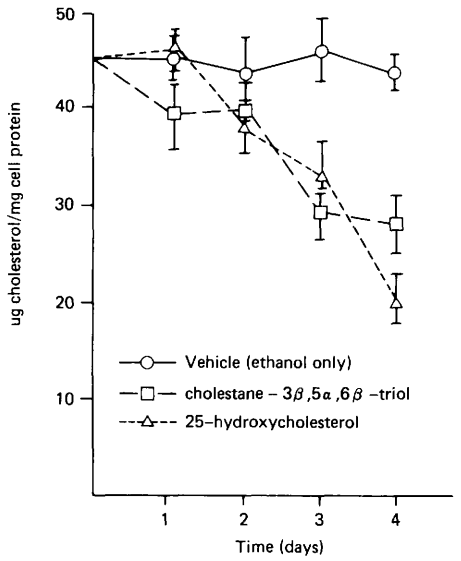

FIG. 2. Effect of cholestane-3β,5α,6β-triol and 25-hydroxycholesterol on cellular cholesterol levels. Cells were incubated with either sterol at a final concentration of 10 μg/ml for the time periods indicated.

levels to 21 μg/mg protein (47% of control value by Day 4 as seen in Fig. 2).

To determine whether cholestane-3β,5α, 6β-triol and 25-hydroxycholesterol specifically localize in particular membrane fractions, these sterols were incubated with the cells for 24 hr prior to lipid analysis of specific membrane fractions. The I and II membrane fractions were analyzed separately for sterol content. When the amounts of cholestane-3β,5α,6β-triol in each fraction were compared on the basis of unit protein, it was found that the plasma membrane-enriched fraction had the highest percentage of that sterol, 5.5 times higher than in the other subcellular fractions. In contrast, 25-hydroxycholesterol was more evenly distributed throughout the various subcellular fractions.

Discussion. It has been suggested that slight perturbations in the microenvironment of intrinsic membrane bound enzymes can result in large changes in the activity of these enzymes (21). In this study, plasma membrane bound enzyme activity was used as an index of the functional status of the plasma membranes of cultured aortic smooth muscle cells which were incubated with either 25-hydroxycholesterol or cholestane-3β,5α,6β-triol, the two most cytotoxic of the autoxidation derivatives of cholesterol.

5′-Nucleotidase has been used extensively as a membrane marker for the plasma membrane in cell fractionation studies (22). The physiological significance of 5′-nucleotidase has not been established. Recent theories regarding its function include regulation of local blood flow by providing adenosine, a potent vasodilator (23), and salvaging extracellular AMP released by glycosyl transferase enzymes on the cell surface (24). In addition to 5′-nucleotidase, the plasma membrane of a variety of cell types contains enzymes with ATPase activity. The best characterized ATPase in plasma membranes is the Na^+K^+-ATPase, which has been shown to span the entire plasma membrane bilayer having extracellular sodium binding sites and intracellular potassium and adenosine triphosphate binding sites (25). There is wide agreement that the physiological role of this enzyme is the maintenance of intracellular ion concentrations.

In this study, the activity of 5′-nucleotidase was markedly decreased in crude membranes when the cells were exposed to 10 μg/ml of cholestane-3β,5α,6β-triol or 25-hydroxycholesterol. It was further demonstrated that 5′-nucleotidase activity was also markedly decreased in plasma membrane-enriched fractions when cells were incubated with cholestane-3β,5α,6β-triol. In contrast to 5′-nucleotidase, ATPase activity in crude homogenates from cells incubated for 24 hr with either sterol was not significantly different from control values. There was a significant decrease (32%) in ATPase activity in cells incubated with cholestane-3β,5α,6β-triol for 48 hr.

All of the cell cultures used in the present experiments showed more than 90% viability with the trypan blue exclusion test. The inhibitory effect on membrane bound enzyme activity by the oxidized sterols, therefore, is most likely the cause rather than the result of cell injury. There are at least two possible explanations for the effects of the oxidized sterols on cell membranes. Since many oxidized sterols have been found to be very potent inhibitors of cholesterol biosynthesis in cultured cells (6, 7), a consequence may

be a depletion of membrane cholesterol content. Another explanation is that the oxidation derivatives of cholesterol may be incorporated into cell membranes or replace cholesterol in cell membranes and thereby disrupt their hydrophobic interior (8). In the present study cholestane-3β,5α,6β-triol was found to increase in the plasma membrane fraction more than fivefold more than in the other cell fractions. Since phospholipid concentration is not altered significantly by the oxidized sterols (6), it is conceivable that the net effect would be a decrease in the cholesterol/phospholipid ratio. The fluidity of the plasma membrane is largely determined by its cholesterol content. A change in membrane fluidity may affect the positioning of enzymes and alter the potential space available to the enzyme and substrate (10). The demonstration of inhibitory effects on the activities of 5′-nucleotidase and Na^+K^+-activated ATPase by cholestane-3β,5α-6β-triol and 25-hydroxycholesterol in this study is consistent with the latter hypothesis. Only once previously has an oxidation derivative of cholesterol, 4-cholesten-3-one, been shown to inhibit Na^+K^+-ATPase activity (26). In the latter study up to 76% of the cholesterol in erythrocytic membranes was converted to 4-cholesten-3-one by cholesterol oxidase.

Inhibitory effects on vital membrane enzymes will result in alteration in the metabolic state of whole cells, initiating a complex sequence of events. Cells with impaired transport properties will undergo a loss of intracellular K^+ and inhibition of protein synthesis and other intracellular enzyme systems, followed by lysis due to osmotic imbalance, and eventually cell death. It has been suggested that arterial wall cells, particularly smooth muscle cells, are involved in the initiation and/or progression of atherosclerosis (27). If the effects observed in this *in vitro* study are found to occur *in vivo,* it is conceivable that these cholesterol oxidation derivatives might cause injury to endothelium and vascular smooth muscle cells, thus predisposing to the cellular proliferation and the lipid and connective tissue accumulations characteristic of atherosclerotic lesions. Experiments to test this hypothesis are currently in progress.

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