Oxidation of cholesterol in low density and high density lipoproteins by cholesterol oxidase

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Abstract The cholesterol oxidase-catalyzed oxidation of cholesterol in native low density (LDL) and high density lipoproteins (HDL_3), as well as in monolayers prepared from surface lipids of these particles, has been examined. The objective of the study was to compare the oxidizability of cholesterol, and to examine the effects of lipid packing on oxidation rates. When [^{3}H]cholesterol-labeled lipoproteins were exposed to cholesterol oxidase (*Streptomyces* sp.), it was observed that LDL [^{3}H]cholesterol was oxidized much faster than HDL_3 [^{3}H]cholesterol. This was true both at equal cholesterol concentration per enzyme unit, and at equal amounts of lipoprotein particles per enzyme unit. About 95% of lipoprotein [^{3}H]cholesterol was available for oxidation. The complete degradation of lipoprotein sphingomyelin by sphingomyelinase (*Staphylococcus aureus*) resulted in a 10-fold increase in the rate of LDL [^{3}H]cholesterol oxidation, whereas the effects on rates of HDL_3 [^{3}H]cholesterol oxidation were less dramatic. A monolayer study with LDL surface lipids indicated that degradation of sphingomyelin loosened the lipid packing, because the ceramide formed occupied a smaller surface area than the parent sphingomyelin, and since the condensing effect of cholesterol on sphingomyelin packing was lost. The effects of sphingomyelin degradation on lipid packing in monolayers of HDL_3-derived surface lipids were difficult to determine from monolayer experiments. Based on the finding that cholesterol oxidases are surface pressure-sensitive with regard to their catalytic activity, these were used to estimate the surface pressure of intact LDL and HDL_3. The cut-off surface pressure of a *Brevibacterium* enzyme was 25 mN/m and 20 mN/m in monolayers of LDL and HDL_3-derived surface lipids, respectively. Since this enzyme was able to oxidize [^{3}H]cholesterol in native LDL and HDL_3, it was estimated that the surface pressure in these lipoproteins was probably less than 25 mN/m and 20 mN/m, respectively. —**Slotte, J. P., and L. Grönberg.** Oxidation of cholesterol in low density and high density lipoproteins by cholesterol oxidase. *J. Lipid Res.* 1990. **31:** 2235–2242.

Supplementary key words sphingomyelin • sphingomyelinase • surface pressure

The enzyme cholesterol oxidase (EC 1.1.3.6) has the ability to convert 3-β-hydroxy-sterols to their respective keto derivatives. The conversion of cholesterol (5-cholesten-3-β-ol) to cholestenone (4-cholesten-3-one) by cholesterol oxidases has been used to probe the localization and distribution of cholesterol in different biological structures (1–8).

We have recently provided evidence indicating that the interaction of cholesterol with sphingomyelins significantly retards the enzyme-catalyzed oxidation of cholesterol in biological as well as in monolayer membranes (8, 9). It was further observed that the catalytic activity of cholesterol oxidases at the water/lipid interphase was markedly affected by the surface pressure (i.e., the lipid packing) of the substrate membranes (9) (L. Grönberg and J. P. Slotte, unpublished results).

In studies on the surface pressure-dependency of the catalytic activities of cholesterol oxidases, we have further observed that the "cut off" surface pressure in 1-palmitoyl-2-oleoyl-phosphatidylcholine/N-oleoyl-sphingomyelin/cholesterol monolayer membranes was markedly different for *Streptomyces* and *Brevibacterium* sp. enzymes. Whereas oxidation of cholesterol by the *Streptomyces* enzyme was abolished above a surface pressure of 30 mN/m, the *Brevibacterium* enzyme lost catalytic activity around 25 mN/m (L. Grönberg and J. P. Slotte, unpublished results). This different surface pressure-dependency of various cholesterol oxidases can be used to get information about lipid packing and surface pressures of intact cell membranes and lipoproteins.

The present studies were performed to investigate the interactions between cholesterol oxidases and cholesterol in native lipoproteins as well as in monolayers prepared from lipoprotein surface lipids. We report the unesterified cholesterol in LDL and HDL_3 displayed markedly different susceptibilities to oxidation by cholesterol oxidase. Our results further suggest that the surface pressure of native LDL and HDL_3 was less than 25 mN/m and 20 mN/m, respectively.

Abbreviations: LDL, low density lipoprotein; HDL, high density lipoprotein.

EXPERIMENTAL PROCEDURES

Isolation of lipoproteins

Low density lipoproteins (LDL, d 1.019–1.063 g/ml) and high density lipoproteins (HDL_3, d 1.12-1.21 g/ml) were prepared from fresh human plasma (EDTA, 4 mM) by sequential ultracentrifugation (10, 11). The stock solutions of LDL (3–6 mg protein/ml) and HDL_3 (14–20 mg protein/ml) were dialyzed extensively against Tris-HCl (20 mM), NaCl (140 mM), and EDTA (1 mM; pH 7.5), and were passed through 0.22-μm filter and stored sterile in the dark at 4°C. The lipoproteins were used within 2–4 weeks of preparation. The purity of the lipoprotein batches was routinely verified by agarose electrophoresis followed by lipid (Oil Red O) and protein (Coomassie Brilliant Blue) staining.

Preparation of [^{3}H]cholesterol-labeled lipoproteins

To label the lipoproteins with unesterified [^{3}H]cholesterol, cholesterol in LDL and HDL_3 was allowed to equilibrate (24–48 h at 4°C) with [^{3}H]cholesterol (60 Ci/mmol; New England Nuclear Du Pont) dried onto the wall of a sterile, plastic disposable tube. About 20 μCi of [^{3}H]cholesterol was added per 5 and 10 mg protein of LDL and HDL_3, respectively.

Normalization of the specific activity of different cholesterol oxidases

To determine the apparent specific activity of cholesterol oxidase from *Streptomyces* sp. (Calbiochem, San Diego, CA) or *Brevibacterium* sp. (Beckman, Carlsbad, CA), the following assay was used. Different amounts of the enzymes were added to a 0.1 M phosphate buffer (pH 7.0) that contained 0.05% Triton X-100. The assay mixture was equilibrated at 25°C for 5 min before cholesterol (Sigma Chemical Co.) in 2-propanol was added to give a final substrate concentration of 100 μM. The absorbance increase at 240 nm was followed as a function of time. The apparent specific activity of cholesterol oxidase is expressed as units or milliunits per mg solid weight. One unit is equivalent to the conversion of 1 μmol of cholesterol to cholestenone per min at 25°C. The specific activities were used to calculate the amount of enzyme to be added in subsequent experiments.

Oxidation of LDL and HDL cholesterol

Oxidation of lipoprotein [^{3}H]cholesterol was performed with cholesterol oxidases from either *Streptomyces* sp. or *Brevibacterium* sp. Oxidation was performed at 37°C in Dulbecco's phosphate-buffered saline with constant lipoprotein concentration and variable cholesterol oxidase concentrations, or with variable lipoprotein amounts and constant cholesterol oxidase concentration. The oxidation time was 30 min, unless otherwise stated. The oxidation reaction was started by the addition of [^{3}H]cholesterol-labeled lipoproteins to phosphate-buffered saline (0.25 or 1.0 ml) containing cholesterol oxidase. The reaction was stopped by addition of chloroform–methanol 2:1 (v/v) to the reaction tubes (disposable glass tubes). The amount of [^{3}H]cholestenone produced from [^{3}H]cholesterol was determined from the lipid extract by thin-layer chromatography or gas–liquid chromatography (8). It was consistently observed that [^{3}H]cholesterol in LDL and HDL_3 traced perfectly the behavior of unesterified cholesterol mass in the lipoproteins, with regard to the cholesterol oxidase susceptibility. Therefore, most assays of the oxidation of lipoprotein cholesterol by cholesterol oxidase were performed on [^{3}H]cholesterol-labeled lipoproteins by thin-layer chromatography. In some experiments, the lipoproteins were pretreated for 15 min at 37°C with 0.1 U/ml of sphingomyelinase (*Staphylococcus aureus*, Sigma) before addition of cholesterol oxidase. This treatment caused a complete degradation of lipoprotein sphingomyelin mass (data not shown).

Extraction and isolation of lipoprotein surface lipids

Total lipids from LDL or HDL_3 were extracted with chloroform–methanol 2:1 (v/v). The organic phase was washed twice with Dulbecco's phosphate-buffered saline, and the solvent was evaporated to dryness. The lipids from about 5 mg LDL or 10 mg HDL_3 were dissolved in 0.5 ml chloroform and were applied to hexane-equilibrated NH_2 Bond Elut solid phase extraction columns. Neutral lipids were eluted with 4 ml chloroform–2-propanol 2:1 (v/v), fatty acids with 4 ml of diethyl ether containing 2% acetic acid, and finally the total phospholipids with 4 ml methanol, according to procedures reported by Kaluzny et al. (12). The purity of the phospholipids was over 99% as judged by thin-layer chromatography.

Surface pressure–mean molecular area isotherms of LDL and HDL_3 surface lipids

The surface pressure versus mean molecular area isotherms of lipoprotein surface lipids (with cholesterol or cholestenone) were measured at 37°C with a computer-controlled KSV 5000 surface barostat (KSV Instruments, Helsinki). The lipids in hexane–ethanol 9:1 were spread on 20 mM Tris-HCl, 145 mM NaCl (pH 7.0) in a thermostated rectangular Teflon trough with a total area of 487.50 cm^2 (6.5 cm × 75.0 cm). The monolayer was allowed to stabilize for 3–5 min before it was compressed at a barrier speed of 20 mm^2/sec. Data were sampled every 3 sec and were collected and analyzed with proprietary software (KSV Instruments, Helsinki).

Effects of sphingomyelin degradation on lipid packing in monolayers of lipoprotein surface lipids

The effects of sphingomyelin degradation on the monolayer area (at constant surface pressure) of pure

lipoprotein phospholipids or of a phospholipid/cholesterol mixture at 37°C were determined with a zero-order trough (9). The monolayer was compressed to 20 mN/m (mN/m = dyne/cm) and after a 5-min stabilization period, 20 mU/ml sphingomyelinase was added to the reaction compartment. The effect of sphingomyelin degradation on monolayer lipid packing was observed as a barrier movement (forward for area decrease and backward for area increase at constant surface pressure).

Cholesterol oxidase activity in monolayers containing lipoprotein surface lipids

The computer-controlled surface barostat (KSV Instruments, Helsinki) used for the monolayer experiments has been described previously (9). The determinations of enzyme activities of the two cholesterol oxidases (from *Streptomyces* and *Brevibacterium* sp.) were performed in a zero-order trough with 20 mM Tris-HCl, 145 mM NaCl, pH 7, as buffer. The reaction compartment was magnetically stirred (150 rpm) and thermostatted to 37°C. One hundred and twenty five μl of a lipid solution containing either LDL or HDL_3 surface lipids (0.1 mg/ml in hexane–ethanol 9:1 (v/v) was spread onto the buffer surface. The monolayer was allowed to stabilize for 3–5 min, whereafter it was compressed to a pre-determined surface pressure (barrier speed 6 mm^2/ sec). Constant surface pressure was maintained by compensatory barrier movement (computer controlled) throughout the experiment. After the monolayer had stabilized at the chosen surface pressure for 5 min, cholesterol oxidase (5 mU/ml) was added to the reaction compartment. The rate of oxidation of cholesterol in the monolayer was registered (at constant surface pressure) as a backward movement of the barrier due to an oxidation-dependent increase in the monolayer area. Data were sampled every 10 sec. Three different kinetic measurements were made at each surface pressure.

Calculation of enzyme activity

The oxidation of cholesterol to cholestenone was observed as an increase of the monolayer area. The enzyme activity in this report is presented as the amount of cholesterol (taken as percent of the total cholesterol available in the total monolayer) that was converted to cholestenone per time unit (seconds). The rate of conversion was calculated from the linear part of the area expansion curve, and represented the maximal rate. Since the degree of cholesterol-induced condensation of the phospholipid monolayer varied with surface pressure and lipid composition (9), the measured enzyme activities at different surface pressures were normalized to the corresponding mean molecular area isotherms at each surface pressure. The difference in the areas of corresponding cholesterol- and cholestenone-containing isotherms was used as a measure for total oxidation of cholesterol. The area increase that resulted from the enzymatic oxidation was directly related to this measure for total oxidation.

Assays

The amount of [^{3}H]cholestenone produced in LDL or HDL_3 from [^{3}H]cholesterol by the action of cholesterol oxidase was determined from the total lipid extract by thin-layer chromatography on Kodak Silica Gel sheets, or by gas–liquid chromatography, as described previously (8).

The protein content of the lipoprotein batches was determined according to Lowry et al. (13), with bovine serum albumin as standard. The amount of unesterified cholesterol in LDL and HDL_3 was determined by a cholesterol oxidase assay (14) from a total lipid extract of the two lipoprotein classes. Total phospholipid mass was determined with a phospholipid assay kit (Boehringer Mannheim, FRG).

RESULTS

Oxidation of LDL and HDL cholesterol by cholesterol oxidase from *Streptomyces* sp.

To determine the susceptibility of cholesterol in different lipoprotein classes to oxidation by cholesterol oxidase, we labeled the unesterified cholesterol pool of either LDL or HDL_3 with [^{3}H]cholesterol and exposed the lipoproteins to cholesterol oxidase from *Streptomyces* sp. Our initial experiments showed that the oxidation susceptibility of cholesterol in these two lipoprotein classes was markedly different. Since we wanted to compare LDL oxidation with HDL_3 oxidation, conditions were chosen so that different numbers of lipoprotein particles were exposed to a constant amount of enzyme for a fixed time (30 min) at 37°C. As shown in **Fig. 1**, the susceptibility of HDL_3 [^{3}H]cholesterol to oxidation by the *Streptomyces* sp. enzyme was markedly lower than the corresponding susceptibility found with LDL [^{3}H]cholesterol. This was true both at equal particle number per unit of cholesterol oxidase, as well as at equal cholesterol concentration per volume and enzyme unit (e.g., 10 μg cholesterol per 1 ml phosphate-buffered saline and 40 mU cholesterol oxidase; marked by arrows in Fig. 1).

Effects of sphingomyelin degradation on cholesterol oxidation in native lipoproteins

Previous studies from this laboratory have shown that the degradation of sphingomyelin with sphingomyelinase in intact cells renders the cellular cholesterol susceptible to oxidation by cholesterol oxidase (8, 9). To further elucidate the effects of sphingomyelin degradation on cholesterol oxidation and lipid packing, we tested the effects of sphingomyelin on cholesterol oxidation with native lipoproteins.

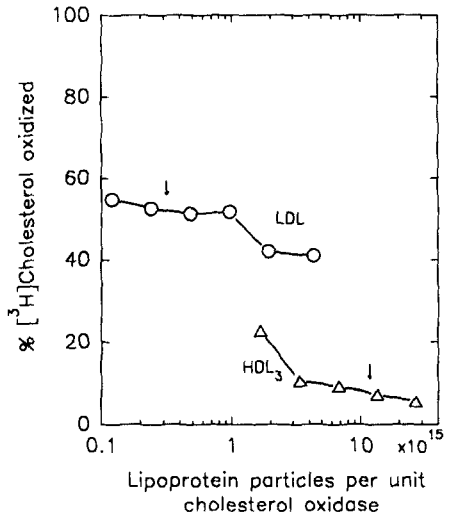

Fig. 1. Oxidation of LDL and HDL_3 cholesterol at varying amounts or lipoprotein particles per unit of cholesterol oxidase activity. Different amounts of either LDL (8.13–520 μg protein/ml) or HDL_3 (31–1000 μg protein/ml) were incubated in 1.0 ml phosphate-buffered saline together with 40 mU/ml of cholesterol oxidase from *Stretomyces* sp. After 30 min at 37°C the reaction was stopped and the samples were analyzed for [^{3}H]cholestenone and [^{3}H]cholesterol content. Values are defined as percent [^{3}H]cholestenone over the sum of [^{3}H]cholestenone and [^{3}H]cholesterol, and are averages from two or three representative experiments with each lipoprotein class (range ≤ 10%). The arrows indicate conditions where the unesterified cholesterol concentration was equal in the LDL and the HDL_3 systems.

When [^{3}H]cholesterol-labeled LDL (at a fixed concentration) was exposed to increasing amounts of cholesterol oxidase (*Streptomyces* sp.) for different periods of time, it was shown that about 95% of the total [^{3}H]cholesterol-label (as well as the total cholesterol mass as verified by gas–liquid chromatography) was oxidizable (**Fig. 2**, left panel). Shorter exposure times and/or lower enzyme amounts obviously gave lower degrees of [^{3}H]cholesterol oxidation. When the sphingomyelin in LDL was degraded by exposure of the lipoproteins to sphingomyelinase, resulting in a total hydrolysis of LDL sphingomyelin (data not shown), the rate of [^{3}H]cholesterol oxidation increased markedly (about tenfold over untreated samples) at all enzyme concentrations tested (Fig. 2, right panel). As a result of the oxidation of LDL cholesterol with cholesterol oxidase, aggregation of LDL but not of HDL_3 was observed at the highest cholesterol oxidase concentration used (i.e., 400 mU/ml).

With the oxidation of HDL_3 [^{3}H]cholesterol, the highest enzyme concentration used (400 mU/ml) was not sufficient to oxidize more than 80% of the [^{3}H]cholesterol in HDL_3 after 60 min (**Fig. 3**, left panel). Lower enzyme amounts (40 or 4 mU/ml) failed to convert HDL_3 [^{3}H]cholesterol to [^{3}H]cholestenone during the 1-h oxidation period. Sphingomyelin degradation in HDL_3 (complete hydrolysis, data not shown) led only to a small increase in the extent of [^{3}H]cholesterol oxidation by the *Streptomyces* enzyme.

Effects of sphingomyelin degradation on molecular packing in monolayers of lipoprotein surface lipids

To obtain information about possible changes in molecular interactions in the polar shell of the lipoprotein particles as a result of sphingomyelin degradation, we simulated the reaction with monolayers containing lipoprotein surface lipids. One of the reaction products in the hydrolysis of sphingomyelin is ceramide, the mean molecular area of which is smaller than that of the parent molecule (15) (L. Grönberg and J. P. Slotte, unpublished results). When a monolayer containing LDL total phospholipids, but no cholesterol, was compressed to 20 mN/m and exposed to sphingomyelinase in the subphase, the formation of ceramide from sphingomyelin led to a monolayer area decrease, as demonstrated in **Fig. 4** (left panel). The presence of cholesterol (35:65 molar ratio to phospholipid) in the monolayer resulted, however, in a slight increase of the monolayer area when sphingomyelin was degraded (Fig. 4, left panel). The monolayer with total phospholipids from HDL_3 contained significantly less sphingomyelin (16),

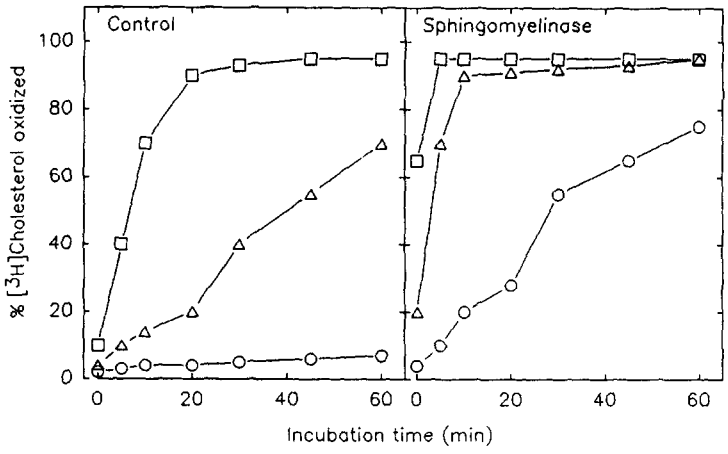

Fig. 2. Time-dependent oxidation of LDL [^{3}H]cholesterol at different cholesterol oxidase activities. LDL (143 μg/ml protein; about 60 μg/ml unesterified cholesterol) was incubated at 37°C for different periods of time in 1.0 ml phosphate-buffered saline together with 4 (○), 40 (△), or 400 (□) mU/ml of cholesterol oxidase from *Streptomyces* sp. The control panel shows oxidation of native LDL, whereas the sphingomyelinase panel shows oxidation of LDL that had been pre-exposed to 0.1 U/ml of sphingomyelinase for 15 min at 37°C prior to exposure to cholesterol oxidase. Values are averages from duplicate samples with each lipoprotein class at each cholesterol oxidase concentration (range ≤ 10%) of one representative experiment out of three.

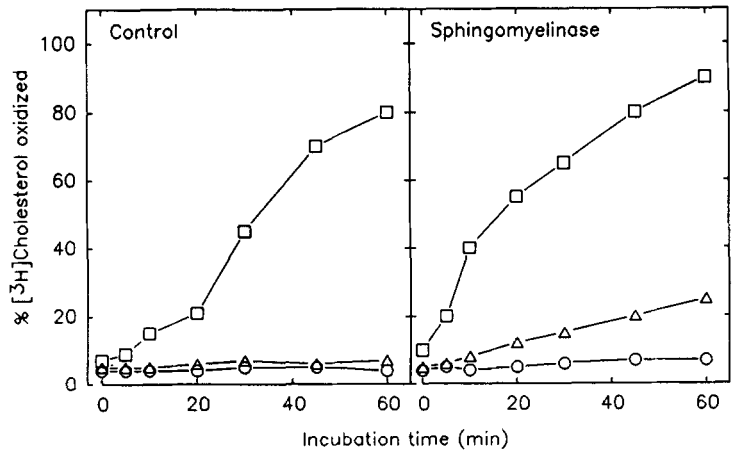

Fig. 3. Time-dependent oxidation of HDL_3 [^{3}H]cholesterol at different cholesterol oxidase activities. HDL_3 (1 mg/ml protein; about 50 μg/ml unesterified cholesterol) was incubated at 37°C for different periods of time in 1.0 ml phosphate-buffered saline together with 4 (○), 40 (△), or 400 (□) mU/ml of cholesterol oxidase from *Streptomyces* sp. The control panel shows oxidation of HDL_3 that had been pre-exposed to 0.1 U/ml of sphingomyelinase for 15 min at 37°C prior to exposure to cholesterol oxidase. Values are averages from duplicate samples with each lipoprotein class at each cholesterol oxidase concentration (range ≤ 10%) of one representative experiment out of three.

and the effects of sphingomyelin degradation were small (Fig. 4, right panel). For an unknown reason the degradation of sphingomyelin in a pure HDL_3 phospholipid monolayer led to a small but significant area increase, whereas no change in monolayer area was seen when cholesterol was present (at 0.13 mol fraction; Fig. 4, right panel).

Oxidation of cholesterol in mixed monolayers containing LDL and HDL surface lipids

We have previously reported that the activity of cholesterol oxidase is markedly dependent on lipid packing and surface pressure of the substrate containing membrane (9) (L. Grönberg and J. P. Slotte, unpublished observations). Further, since the surface pressure-dependency of cholesterol oxidases from different microorganisms varies considerably, these enzymes can potentially be used to estimate the apparent surface pressure of native membranes and lipoproteins.

The mean molecular area isotherms for LDL and HDL_3 surface lipids (cholesterol/phospholipid molar ratio 35:65 and 13:87 for LDL and HDL_3, respectively) are shown in **Fig. 5**. The substitution of cholesterol with cholestenone (the reaction product with cholesterol oxidase) gave larger mean molecular areas (Fig. 5) for LDL and HDL_3 surface lipids, consistent with the fact that cholesterol but not cholestenone gives a tighter packing of phospholipids (condensing effect; 9, 17). This change in mean molecular area can be registered in a zero-order trough and corresponds to the oxidation rate as catalyzed by cholesterol oxidase.

Fig. 6 shows the results of the oxidation of cholesterol in monolayers from LDL and HDL_3 surface lipids with cholesterol oxidases from either *Streptomyces* or *Brevibacterium* sp. It is evident that the *Streptomyces* enzyme was more potent at oxidizing monolayer cholesterol compared to the *Brevibacterium* enzyme (at equal enzyme concentrations). The surface pressure-dependency of the catalytic activity was also markedly different for the two enzymes. Whereas the cut-off surface pressure of the *Streptomyces* enzyme was somewhere above 30 mN/m (not currently determinable with the monolayer technique) with LDL

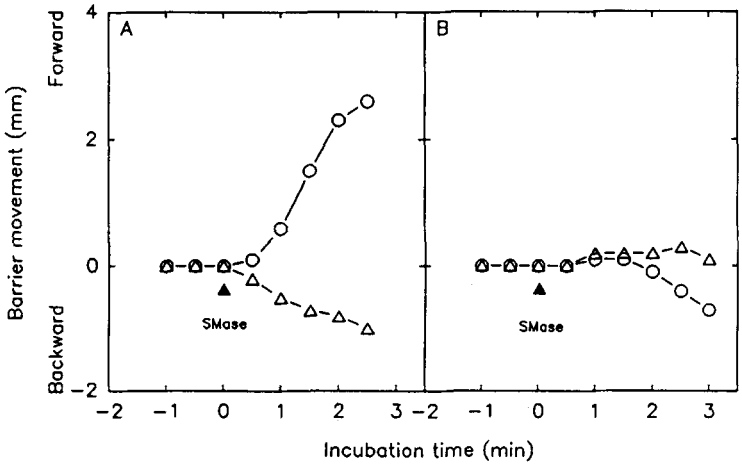

Fig. 4. Effects of sphingomyelin degradation on molecular packing in monolayers of lipoprotein surface lipids. Total phospholipids were isolated from either LDL or HDL_3, and the monolayers prepared were either pure total phospholipids (○), or phospholipids reconstituted with cholesterol (△). The cholesterol molar fraction was 0.35 in monolayers of LDL lipids (panel A) and 0.13 in monolayers of HDL_3-derived lipids (panel B). Twenty mU/ml of sphingomyelinase was added to the magnetically stirred reaction compartment (37°C) of the zero-order trough, and the effects of sphingomyelin degradation on monolayer area at constant surface pressure (20 mN/m) were recorded. Forward movement of the barrier represents a monolayer area decrease, and a backward movement a corresponding monolayer area increase. The values shown are derived from one representative experiment for each monolayer type out of at least three similar experiments (with two different batches of HDL_3).

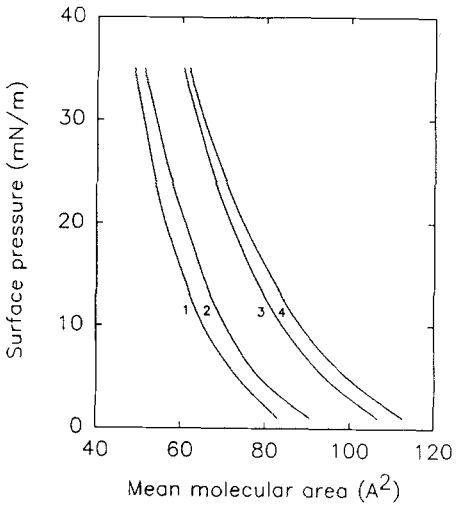

Fig. 5. Surface pressure versus mean molecular area isotherms for LDL and HDL surface lipids at 37°C on 20 mM Tris-HCl (pH 7.0) buffer containing 145 mM NaCl. Line 1 represents LDL phospholipids reconstituted with cholesterol (molar fraction 0.35 of cholesterol) whereas line 2 represents LDL phospholipids reconstituted with a similar amount of cholestenone. Lines 3 and 4 are for HDL_3 phospholipids with cholesterol and cholestenone, respectively (sterol molar fraction 0.13). Values represents average isotherms from three representative experiments (variability less than 2%).

surface lipids, the corresponding cut-off pressure of the *Brevibacterium* enzyme was about 25 mN/m.

A similar difference in the apparent cut-off pressure was seen when HDL_3 surface lipids were used, although the cut-off pressure for the *Brevibacterium* enzyme was significantly lower, at about 20 N/m. The low amount of cholesterol present in the surface of HDL, and the correspondingly small condensing effect at higher surface pressures (cf Fig. 5) prevented us from analyzing the cut-off pressure of the *Streptomyces* enzyme. We tried to circumvent this problem by preparing a monolayer of HDL_3 surface lipids with a higher cholesterol saturation (35:65 molar ratio). The oxidation results with this monolayer indicated that the cut-off pressure of the *Brevibacterium* enzyme was still about 20 mN/m, but the cut-off pressure of the *Streptomyces* enzyme was shifted to higher surface pressures (not determinable).

Oxidation of LDL and HDL_3 by cholesterol oxidases from *Streptomyces* and *Brevibacterium* species

Since we knew the approximate cut-off surface pressures of the two cholesterol oxidases, we used both to study rates of [^{3}H]cholesterol oxidation in intact LDL and HDL_3. As with the monolayer system, it was apparent that also with native lipoprotein particles the *Streptomyces* enzyme was more potent at oxidizing [^{3}H]cholesterol compared with the *Brevibacterium* enzyme (**Fig. 7**). We also observed that HDL_3 [^{3}H]cholesterol was less susceptible for oxidation compared with LDL [^{3}H]cholesterol, irrespective of the source of the enzyme (Fig. 7). However, the main conclusion from the results in Fig. 7 relates to the possibility of estimating the apparent surface pressure in native LDL and HDL_3. Since both enzymes were capable of oxidizing [^{3}H]cholesterol in native LDL and HDL, it appears that the surface pressure in these particles must have been below 25 mN/m and 20 mN/m for LDL and HDL_3, respectively. This interpretation is based on the cut-off pressures observed with monolayer studies reported in Fig. 6.

DISCUSSION

Cholesterol oxidase was used in this study to examine the oxidation susceptibility of unesterified cholesterol in native lipoproteins as well as in monolayers prepared from lipoprotein surface lipids. Since the enzyme cholesterol oxidase has a poor membrane penetrating capacity (L. Grönberg and J. P. Slotte, unpublished observations), it has to interact with its membranous substrate at the water/lipid interphase. The physical properties of this water/lipid interphase have been shown to have marked effects on enzyme-catalyzed oxidation (9), and the process can therefore be used to obtain information on lipid-packing properties of native lipoprotein particles.

The finding that cholesterol in native LDL and HDL_3 was susceptible to oxidation by cholesterol oxidase contrasts quite dramatically with results obtained for cholesterol oxidation in native cell membranes (3, 5, 8). Cholesterol in native cell membranes is not a substrate of

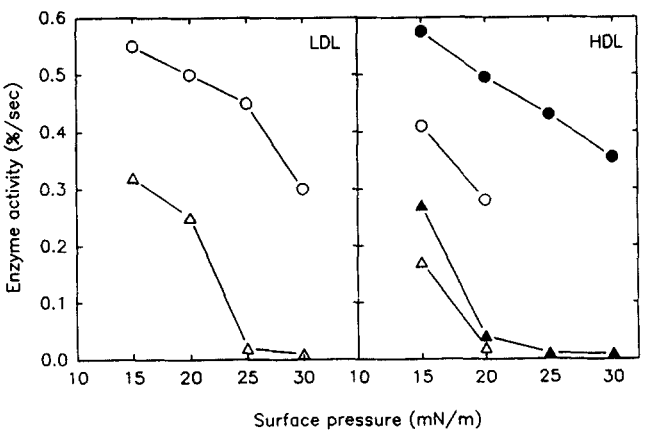

Fig. 6. Effects of surface pressure on the oxidizability of cholesterol in monolayers of lipoprotein surface lipids. Monolayers were prepared of either LDL phospholipids and cholesterol (cholesterol molar fraction 0.35; left panel) or HDL_3 phospholipids and cholesterol (cholesterol molar fraction 0.13 (open symbols) or 0.35 (filled symbols); right panel). The oxidation at different surface pressures was carried out at 37°C by cholesterol oxidase (5 mU/ml) from either *Streptomyces* sp. (○) or *Brevibacterium* sp. (△). Values are averages of three independent and representative experiments for each monolayer composition and enzyme type.

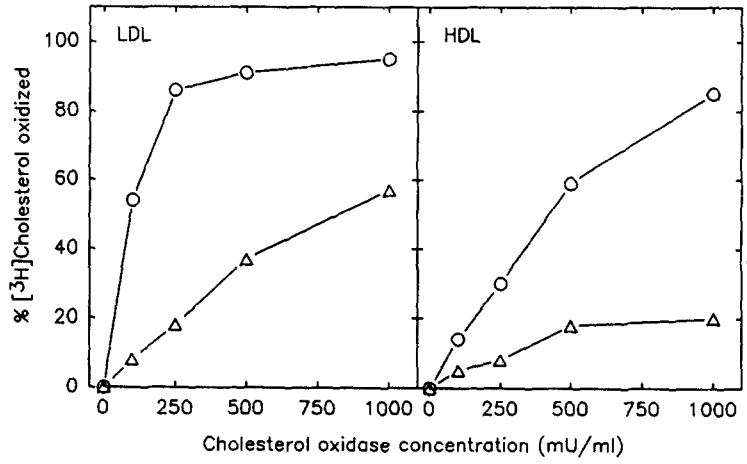

Fig. 7. Oxidation of [^{3}H]cholesterol in native LDL and HDL_3. [^{3}H]Cholesterol-labeled lipoproteins (0.52 mg/ml of LDL and 1.6 mg/ml of HDL_3, given as protein concentration) were exposed at 37°C for 30 min to various amounts of cholesterol oxidase from either *Streptomyces* (○) or *Brevibacterium* sp. (△) in phosphate-buffered saline (incubation volume 0.25 ml). Values for LDL (left panel) and HDL_3 (right panel) are averages from duplicate samples (range ≤ 10%) of one representative experiment for each enzyme type and lipoprotein class out of at least three similar experiments.

cholesterol oxidase, unless the membrane lipid packing is modulated by various treatments. Since cholesterol in LDL and HDL_3 was susceptible to oxidation, it indicates that the sterol molecules were available at the lipid/water interphase of the intact lipoprotein particles. This finding is in good agreement with the concept that the 3-beta hydroxyl group of sterol molecules is positioned proximal to the polar groupings of phospholipids and hydrated protein (18).

The difference in oxidizability of cholesterol in cultured cells versus intact lipoproteins further implies that the packing of lipids in the surface shell of lipoproteins is markedly looser than in cellular plasma membranes. The surface pressure dependency of the activity of cholesterol oxidase from *Brevibacterium* would suggest that the surface pressure in the polar shell of LDL and HDL_3 would be less than 25 and 20 mN/m, respectively (cf. Fig. 6). The corresponding surface pressure of intact cell membranes has been estimated to be about 31–35 mN/m for erythrocytes (19) and above 30 mN/m for BHK-21 cells and fibroblasts (L. Grönberg and J. P. Slotte, unpublished results). Our estimates of the surface pressure of native LDL and HDL_3 are fairly compatible with previously published estimates of the surface pressure in these lipoprotein classes. Based on studies where the adsorption of apolipoprotein A-I to monolayers prepared from either LDL or HDL_3 surface lipids was examined, Ibdah, Lund-Katz, and Phillips (17) suggested that the surface pressure of native LDL and HDL_3 would be less than or about 20 and 26 mN/m, respectively.

When rates of cholesterol oxidation were examined in LDL- or HDL_3-derived lipid monolayers, made to have an equal cholesterol surface concentration (35 mol %), the oxidation rates were found to be similar, whereas the cut-off (surface) pressure for enzyme activity was markedly different in the two monolayer types. The similarity in oxidation rates in LDL- and HDL_3-derived monolayers at equal cholesterol surface concentrations would suggest that oxidation rates were primarily determined by the surface concentration of cholesterol in membranes, rather than by overall cholesterol concentrations or by ratios of lipoprotein particles to enzyme concentration. The rate of cholesterol oxidation in intact lipoproteins could, however, be further enhanced by the degradation of sphingomyelin mass. This sphingomyelinase-induced increase in cholesterol oxidizability was much higher in LDL compared to HDL_3, in line with the fact that LDL contains more sphingomyelin than HDL_3 (14.5 vs 9.2 %, by weight respectively; 16).

The effects of sphingomyelin degradation on cholesterol oxidation rates in this study are analogous to experiments where phospholipase A_2 treatment of HDL was found to increase the interaction between filipin and unesterified cholesterol in the surface of HDL (20). The net result of sphingomyelin degradation in intact membranes or lipoprotein particles is apparently a more relaxed lipid packing. This can be explained by the fact that ceramide, produced by the sphingomyelinase reaction, occupies a smaller mean molecular area (at a given surface pressure) than its parent molecule (15) (L. Grönberg and J. P. Slotte, unpublished data). The hydrolysis of sphingomyelin also results in some loss of the cholesterol-induced condensation of phospholipid packing in the lipid layer, resulting in addition relaxation of the lipid packing (9; cf also Fig. 5).

The observation that only about 95–96% of LDL [^{3}H]cholesterol could be oxidized by cholesterol oxidase may suggest that a very small but significant fraction of LDL unesterified cholesterol was partitioned into a protected environment in the particle. The cholesterol oxidase could not reach this pool even after treatment with sphingomyelinase, indicating that the resistant [^{3}H]cholesterol was not associated with sphingomyelin. The oxidase-resistant cholesterol may have been associated with apolipoprotein B-100, since this apolipoprotein is known to be very hydrophobic and could well bind to itself cholesterol in addition to phospholipid.

To conclude, we have provided evidence suggesting that

unesterified cholesterol in LDL and HDL_3 is readily susceptible to oxidation by cholesterol oxidase. This finding alone implies that the molecular packing of lipids in the monolayer shell of LDL and HDL_3 is looser than the comparable packing in biological bilayer membranes. The looser surface packing may relate to the spherical nature of the particles and the curved membranous surface, and may have functional implications as well. It is well known that rates of surface transfer of unesterified cholesterol between lipoprotein particles are considerably faster than corresponding rates of cholesterol transfer from cell membranes to cholesterol acceptors (21, 22). This rate enhancement could, at least in part, result from the looser surface lipid packing in lipoproteins, although other factors such as the lipid composition also markedly determine exchange rates (23, 24).

The skillful assistance of Maria Backlund in some of the experiments, and of Jarmo Tenhunen in the preparation of lipoproteins is warmly acknowledged. We thank Dr. Jarl B. Rosenholm, at the Department of Physical Chemistry, for generously giving us access to the KSV Surface Barostat. This research was supported by grants from the Ella and Georg Ehrnrooth Foundation, the Borg Foundation, and the Council for Sciences, Academy of Finland.

Manuscript received 7 June 1990 and in revised form 10 September 1990.

REFERENCES

1. Gottlieb, M. H. 1977. The reactivity of human erythrocyte membrane cholesterol with a cholesterol oxidase. *Biochim. Biophys. Acta.* **466:** 422–428.
2. Moore, N. F., E. J. Patzer, Y. Barenholz, and R. R. Wagner. 1977. Effect of phospholipase C and cholesterol oxidase on membrane integrity, microviscosity, and infectivity of vesicular stomatitis virus. *Biochemistry.* **16:** 4708–4715.
3. Patzer, E. J., R. R. Wagner, and Y. Barenholz. 1978. Cholesterol oxidase as a probe for studying membrane organisation. *Nature.* **274:** 394–395.
4. Pal, R., Y. Barenholz, and R. R. Wagner. 1979. Effect of cholesterol concentration on organization of viral and vesicle membranes. Probed by accessibility to cholesterol oxidase. *J. Biol. Chem.* **255:** 5802–5806.
5. Lange, Y., and B. V. Ramos. 1983. Analysis of the distribution of cholesterol in the intact cell. *J. Biol. Chem.* **258:** 15130–15134.
6. Thurnhofer, H., N. Gains, B. Mutsch, and H. Hauser. 1986. Cholesterol oxidase as a structural probe of biological membranes: its application to brush-border membranes. *Biochim. Biophys. Acta.* **856:** 174–181.
7. Slotte, J. P., J. F. Oram, and E. L. Bierman. 1987. Binding of high density lipoproteins to cell receptors promotes translocation of cholesterol from intracellular membranes to the cell surface. *J. Biol. Chem.* **262:** 12904–12907.
8. Slotte, J. P., G. Hedström, S. Rannström, and S. Ekman. 1989. Effects of sphingomyelin degradation on cell cholesterol oxidizability and steady-state distribution between the cell surface and the cell interior. *Biochim. Biophys. Acta.* **985:** 90–96.
9. Grönberg, L., and J. P. Slotte. 1990. Cholesterol oxidase catalyzed oxidation of cholesterol in mixed lipid monolayers: effects of surface pressure and phospholipid composition on catalytic activity. *Biochemistry.* **29:** 3173–3178.
10. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1355.
11. Schumaker, V. N., and D. L. Puppione. 1986. Sequential flotation ultracentrifugation. *Methods Enzymol.* **128:** 155–170.
12. Kaluzny, M. A., L. A. Duncan, M. V. Merritt, and D. E. Epps. 1985. Rapid separation of lipid classes in high yield and purity using bonded phase columns. *J. Lipid Res.* **26:** 135–140.
13. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
14. Heider, J. G., and R. L. Boyett. 1978. The picomole determination of free and total cholesterol in cells in culture. *J. Lipid Res.* **19:** 514–518.
15. Yedgar, S., R. Cohen, S. Gatt, and Y. Barenholz. 1982. Hydrolysis of monomolecular layers of synthetic sphingomyelins by sphingomyelinase from *Staphylococcus aureus*. *Biochem. J.* **201:** 597–602.
16. Skipski, V. P., M. Barclay, R. K. Barclay, V. A. Fetzer, J. J. Good, and F. M. Archibald. 1967. Lipid composition of human serum lipoproteins. *Biochem. J.* **104:** 340–352.
17. Ibdah, J. A., S. Lund-Katz, and M. C. Phillips. 1989. Molecular packing of high density and low density lipoprotein surface lipids and apolipoprotein A-I binding. *Biochemistry.* **28:** 1126–1133.
18. Tardieu, A., L. Mateu, C. Sardet, B. Weiss, V. Luzzati, L. Aggerbeck, and A. M. Scanu. 1976. Structure of human serum lipoproteins in solution II. Small-angle X-ray scattering study of HDL_3 and LDL. *J. Mol. Biol.* **101:** 129–138.
19. Demel, R. A., W. S. M. G. van Kessel, R. F. A. Zwaal, B. Roelofsen, and L. L. M. Van Deenen. 1975. Relation between various phospholipase actions on human red cell membranes and the interfacial phospholipid pressure in monolayers. *Biochim. Biophys. Acta.* **406:** 97–107.
20. Blau, L., R. Bittman, P. Lagocki. R. Byrne, and A. M. Scanu. 1982. Organization of unesterified cholesterol in high density lipoprotein probed by filipin. *Biochim. Biophys. Acta.* **712:** 437–443.
21. Lund-Katz, S., B. Hammerschlag, and M. C. Phillips. 1982. Kinetics and mechanism of free cholesterol exchange between human serum high and low density lipoproteins. *Biochemistry.* **21:** 2964–2969.
22. Phillips, M. C., L. R. McLean, G. W. Stoudt, and G. H. Rothblat. 1980. Mechanism of cholesterol efflux from cells. *Atherosclerosis.* **36:** 409–422.
23. Clejan, S., and R. Bittman. 1984. Decreases in rates of lipid exchange between mycoplasma gallisepticum cells and unilamellar vesicles by incorporation of sphingomyelin. *J. Biol. Chem.* **259:** 10823–10826.
24. Phillips, M. C., W. J. Johnson, and G. H. Rothblat. 1987. Mechanisms and consequences of cellular cholesterol exchange and transfer. *Biochim. Biophys. Acta.* **906:** 223–276.