Cholesterol Autoxidation in Phospholipid Membrane Bilayers

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Lipid peroxidation in unilamellar liposomes of known cholesterol-phospholipid composition was monitored under conditions of autoxidation or as induced by a superoxide radical generating system, γ-irradiation or cumene hydroperoxide. Formation of cholesterol oxidation products was indexed to the level of lipid peroxidation. The major cholesterol oxidation products identified were 7-keto-cholesterol, isomeric cholesterol 5,6-epoxides, isomeric 7-hydroperoxides and isomeric 3,7-cholestane diols. Other commonly encountered products included 3,5-cholestadiene-7-one and cholestane-3β,5α,6β-triol. Superoxide-dependent peroxidation required iron and produced a gradual increase in 7-keto-cholesterol and cholesterol epoxides. Cholesterol oxidation was greatest in liposomes containing high proportions of unsaturated phospholipid to cholesterol (4:1 molar ratio), intermediate with low phospholipid to cholesterol ratios (2:1) and least in liposomes prepared with dipalmitoylphosphatidylcholine and cholesterol. This relationship held regardless of the oxidizing conditions used. Cumene hydroperoxide-dependent lipid peroxidation and/or more prolonged oxidations with other oxidizing systems yielded a variety of products where cholesterol-5β,6β-epoxide, 7-ketocholesterol and the 7-hydroperoxides were most consistently elevated. Oxyradical initiation of lipid peroxidation produced a pattern of cholesterol oxidation products distinguishable from the pattern derived by cumene hydroperoxide-dependent peroxidation. Our findings indicate that cholesterol autoxidation in biological membranes is modeled by the peroxide-induced oxidation of liposomes bearing unsaturated fatty acids and suggest that a number of cholesterol oxidation products are derived from peroxide-dependent propagation reactions occurring in biomembranes.
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The oxidation of cholesterol in biological systems has received considerable attention for many years. There is general agreement that cholesterol oxidation in cells can result from enzymatic processes, as well as from free radical-mediated membrane lipid peroxidation (1,2). The 7α-hydroxylation of cholesterol during bile acid synthesis (3) is an example of microsomal cytochrome P-450 catalyzed oxidation. In the presence of its cofactors NADPH and O_2, cytochrome P-450 monooxygenase catalyzes peroxidation of microsomal lipids (4–6). This lipid peroxidation is accompanied by cholesterol autoxidation with the formation of several products, including the isomeric cholest-5-ene-3β,7β- and cholest-5-ene-3β,7α-diols (3,7-diols) (4,5), with the latter contributing to the enzymatically derived cholest-5-ene-3β,7α-diol (3).

Free radical oxidation of cholesterol yields several products that are commonly encountered in tissues and microsomal preparations. The radical species responsible for cholesterol oxidation appear to derive from activated oxygen. Accordingly, specific radical generating systems, or defined dioxygen species, have been employed in cholesterol oxidation studies using membraneous and nonmembraneous preparations (7–12). Moreover, activated oxygen appears to be generated by cytochrome P-450 monoxygenase (13,14) with the induction of lipid peroxidation (14) and cholesterol oxidation (15).

A qualitative similarity is apparent among cholesterol oxidation products formed in several artificial systems as well as those isolated from peroxidized biomembranes. In many cases, the nature of oxidizing species remains ill defined, or in those instances where defined species were utilized, the matrix within which cholesterol oxidation took place differed from the organized state of lipids in biomembranes. A thorough review of cholesterol oxidation products formed in aqueous colloidal solutions vs products isolated from natural sources is presented by Smith (16).

Oxidation of cholesterol in model membranes has recently been described for iron-catalyzed (10,11) and hydrogen peroxide- or ultraviolet light-induced autoxidations (17). These studies employed long periods of imposed oxidation (up to 24 hr) and utilized phospholipids containing varying degrees of unsaturation. The model membranes were in the form of large multilamellar vesicles in two reports (10,11) and small unilamellar vesicles in the other (17). In the former studies the membrane arrangement likely permitted direct access of oxidants generated in the aqueous phase to only a small proportion of lipids. The later study was concerned primarily with the stability of cholesterol and the effect of antioxidants, whereas little attention was given to product formation.

In this report the identities of the major detectable oxidation products of cholesterol in unilamellar vesicles (liposomes) are described. In addition, specific free radical generating systems have been used to induce lipid peroxidation with subsequent examination of cholesterol oxidation products. These liposomes afford a reasonably exact formulation of lipid composition permitting a measure of control over the mode and extent of peroxidation, conditions that are not readily achieved using membranes from cells.

MATERIALS AND METHODS

Chemicals and reagents. Bovine liver phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were purchased from Sigma (St. Louis, MO), and the solvents in which they were delivered were evaporated under a stream of argon. These lipids were then stored in methylene chloride at a concentration 5 mg/ml under argon at −80 C.

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; TBAR, thiobarbituric acid-reacting products; CuOOH, cumene hydroperoxide; HX, hypoxanthine; ADP, adenosine diphosphate; XO, xanthine oxidase; SOD, superoxide dismutase; TLC, thin layer chromatography; 7-keto, cholest-5-ene-3β-ol-7-one; 3,5-diene, 3,5-cholestadiene-7-one; α-CE, cholestan-5α,6α-epoxy-3β-ol; CT, 5α-6β-triol; 7-OOH, 3β-hydroxycholest-5-ene-7α-hydroperoxide; β-CE, cholestan-5β,6β-epoxy-3β-ol; HPLC, high pressure liquid chromatography; DPPC, dipalmitoylphosphatidylcholine; PI, peroxidizability index.

Various batches of these phospholipids were assayed for their content of thiobarbituric acid-reacting products (TBAR), and only samples containing no TBAR were used to prepare liposomes. Cumene hydroperoxide (CuOOH) and hypoxanthine (HX) were obtained from Aldrich (Milwaukee, WI); and cholesterol, adenosine diphosphate (ADP), xanthine oxidase (XO), bovine erythrocyte superoxide dismutase (SOD) and hematin were purchased from Sigma. 4[^{14}C]-Cholesterol was obtained from Amersham (Arlington Heights, IL) and repurified on a monthly basis by thin layer chromatography (TLC) as previously reported (18) to remove cholesterol oxidation products. A similar procedure was used to purify unlabeled cholesterol obtained from Sigma. The absence of cholesterol oxidation products was confirmed by gas and thin layer chromatography (19). The levels of cholesterol oxidation products were typically below our limits of detection.

Authentic cholesterol oxidation products: 3,7-diols, cholest-5-ene-3β-ol-7-one (7-keto), 3,5-cholestadiene-7-one (3,5-diene), cholestan-5α,6α-epoxy-3β-ol (α-CE) and 25-hydroxycholesterol were obtained from Sterloids (Wilson, NH). α-CE was found to be contaminated with 1% and 3% cholestan-3β,5α,6β-triol (CT) and cholesterol, respectively, and was further purified as described previously (20). CT was purchased from Sigma and was chromatographically pure. 3β-hydroxy-5α-cholest-6-ene-5α-hydroperoxide and 3β-hydroxycholest-5-ene-7α-hydroperoxide (7-OOH) were provided by Dr. Leland Smith. Cholestan-5β,6β-epoxy-3β-ol (β-CE) was prepared by reaction of cholesterol with metachloroperoxybenzoic acid in ether, as described previously (20). This reaction produces ca. 75% and 25% of the α- and β-CE diastereomers, respectively, requiring purification of β-CE by high pressure liquid chromatography (HPLC) (20).

Liposome preparation. Three different phospholipid formulations were used for preparing liposomes. These formulations were based on the type or proportions of phospholipids and consisted of either pure dipalmitoylphosphatidylcholine (DPPC), bovine liver PC or a mixture of PC and bovine liver PE in a molar ratio of 4:1. The phospholipids were mixed with cholesterol as methylene chloride solutions in quantities sufficient to achieve final desired molar ratios of phospholipid to cholesterol of either 2:1 or 4:1. At this point, a total of 8 μCi (at 56 μCi/μmol) of radiolabeled cholesterol was added to the sample. For a typical experiment, a total of 35 mg of lipid was mixed, and the solvent was evaporated under a stream of argon. The resulting film was suspended by vigorous mixing for 1 min in 2.4 ml of cold argon-saturated 10 mM Tris buffer containing 150 mM KCl at pH 7.4. The suspension was then transferred under an atmosphere of argon to a screw-cap polycarbonate tube. The lipid suspension was sonicated for 10–15 min in a cup-horn using a Heat Systems-Ultrasonics W-225R sonifier at a power setting of 20. The temperature throughout this period was maintained at 30 C (with the exception of DPPC liposomes, which were maintained at 38 C) using a circulator bath. The sample was then centrifuged for 30 min at 65,000 $\times$ g at 4 C. The supernatant was recovered for further use. All procedures were carried out under subdued light. The amount of phospholipid in each preparation was assayed by phosphorus determination (21). Confirmation of the ratio of PC and PE was accomplished by extraction and resolution of the phospholipids via TLC (22), recovery of phospholipids and measurement by phosphorus analysis (21). Cholesterol content was verified by HPLC as detailed.

Peroxidation of liposomes. The three types of liposomal preparations described were incubated in the presence of one of the four oxidizing systems described here. Incubations under these oxidizing conditions were carried out using 2.5 mg of lipid per sample suspended in a total volume of 1.0 ml Tris buffer.

(i) Autoxidation of the liposomes involved dispersing aliquots into aerated Tris buffer followed by incubation in a shaking water bath (80 oscillations/min) for intervals up to 4 hr at 37 C.

(ii) A standard enzymatic O_2^--generating system was used. This involved additions of ADP-Fe^{+3} (prepared by premixing ADP and $FeSO_4$ in water at a ratio of 12:1), where the final concentrations were 120 and 10 μM, respectively. The ADP-Fe^{+2} complex is known to autoxidize rapidly in water yielding ADP-Fe^{+3} (23,24). Incubations were begun by successive additions of 0.50 units XO and 500 μM HX (final concentration). Incubations of these and all other samples were as described for the autoxidation experiments noted above. Under these conditions, O_2^- generation, as measured via cytochrome C reduction (25), remained linear for 15 min at a rate of 1.9 μmol/min and subsided by 20 min. Prolonged incubations (i.e., intervals as long as 3 hr) are presumed to represent oxidations other than those induced by enzymatically generated oxyradicals. The dependence of lipid peroxidation on O_2^- formation was confirmed in preliminary experiments in which >85% of lipid peroxidation was prevented by the addition of 50 μg of SOD. Inhibition of lipid peroxidation by SOD indicates that O_2^- is fulfilling the dual role of maintaining iron in its reduced and catalytically active state and yielding secondary oxidizing species (26).

(iii) Cumene hydroperoxide (200 μM) and 50 μM hematin, prepared as described previously (27), were added to the liposome suspensions. Incubations were initiated by addition of a 100 μl sonicated solution of CuOOH in Tris buffer.

(iv) Irradiation using a ^{60}Co source was carried out by adding 200 μl samples of liposomes to 800 μl of distilled water (pH 7.0) in borosilicate screw-cap test tubes. The tubes were sealed under air or N_2O (20-min purging of samples in a glove bag) prior to subjecting the samples to γ-irradiation. The dose rate of our radiation source was 4.87 Gy/min, and samples were irradiated for set intervals up to 120 min.

Analysis of cholesterol oxidation products. At fixed time intervals, the incubations were terminated by removing 100 μl aliquots for determination of TBAR (28) and immediately thereafter adding 7 vol of chloroform/methanol (2:1, v/v) to the remaining sample. The mixture was briefly centrifuged to separate the organic phase, which was collected and saved. The upper aqueous phase was reextracted as above, and the organic phases were pooled and evaporated under nitrogen. Using this method, >98% of the radioactivity in all samples was extracted.

The dried lipids were suspended in 1.0 ml argon saturated toluene/ethyl acetate (3:2, v/v) and applied to 3-ml solid phase "Diol" extraction columns (Analytichem Corp., Harbor City, CA) conditioned with the same solvent. Eluents were collected under mild vacuum suction,

and the columns were washed with another 2 ml of toluene/ethyl acetate. The combined eluents were immediately evaporated under nitrogen and saved in hexane/benzene (95:5, v/v) under argon at −80 C. Using this technique, >99% of the radioactivity associated with cholesterol or its oxidation products was recovered. The columns were subsequently eluted with methanol to collect the phospholipids. No radioactivity was present in this fraction.

In some experiments, an aliquot of the toluene/ethyl acetate eluent (cholesterol fraction) was removed for TLC as described previously (18). This was done to isolate cholesterol and CT, the latter not being suitably resolved by the HPLC method described below. The identity of CT was confirmed using an authentic nonradiolabeled standard applied along with each sample. The details for recovering and measuring CT by TLC are presented elsewhere (18). The radioactive zones corresponding to cholesterol and CT were scraped from the plates, and the level of radioactivity in each sample was determined by liquid scintillation spectrometry.

The remainder of each cholesterol fraction was reduced to a 100-μl volume, of which 20 μl was subjected to HPLC. HPLC of cholesterol and its oxidation products was performed with a Perkin Elmer Series 4 liquid chromatograph fitted with two 3-μ particle size 100 × 4.6 mm columns (Chromanetics, Inc., Jessup, MD) in series that were eluted isocratically with hexane/isopropanol (95.8:4.2, v/v) at a flow rate of 1.5 ml/min. Detection of peaks was accomplished with an Erma Instruments differential refractometer. The eluent was subsequently delivered to a fraction collector set at 15-sec sampling intervals. The collected samples were then measured for radioactivity by liquid scintillation spectrometry. Using this approach, a radioactivity chromatogram was superimposed on the refractive index chromatogram of authentic cholesterol oxidation standards added to the samples prior to injection. A representative refractive index chromatogram of cholesterol and several oxidation products is shown in Figure 1.

The cholesterol content in each sample was determined following the chromatographic run using an external standard method. Corrections for variations in recovery were based on the radioactive counts in the original sample and the total radioactivity measured after HPLC (i.e., the sum of the radioactivity in all collected fractions). Recoveries ranged from 86 to 96%. The extent of cholesterol oxidation and the amounts of each oxidation product were estimated from the level of radioactivity associated with each peak. Calculations of the amount of each oxidation product were based on the radioactive counts associated with that product expressed as a percentage of the original cholesterol radioactivity. The cholesterol content in each sample was then multipled by the calculated percent for each oxidation product identified yielding a value, in micrograms, for each product formed. This method of calculation assumes that the specific activities of cholesterol and its oxidation products are equal. Verification of each oxidation product identified during the chromatographic run was made by "spiking" radioactive peaks with known amounts of authentic cholesterol oxide standards. The detection limit for most oxidation products by refractometry was 0.5 μg, whereas the limit for peak resolution measured via radioactivity was 3 times background (i.e., 36 dpm).

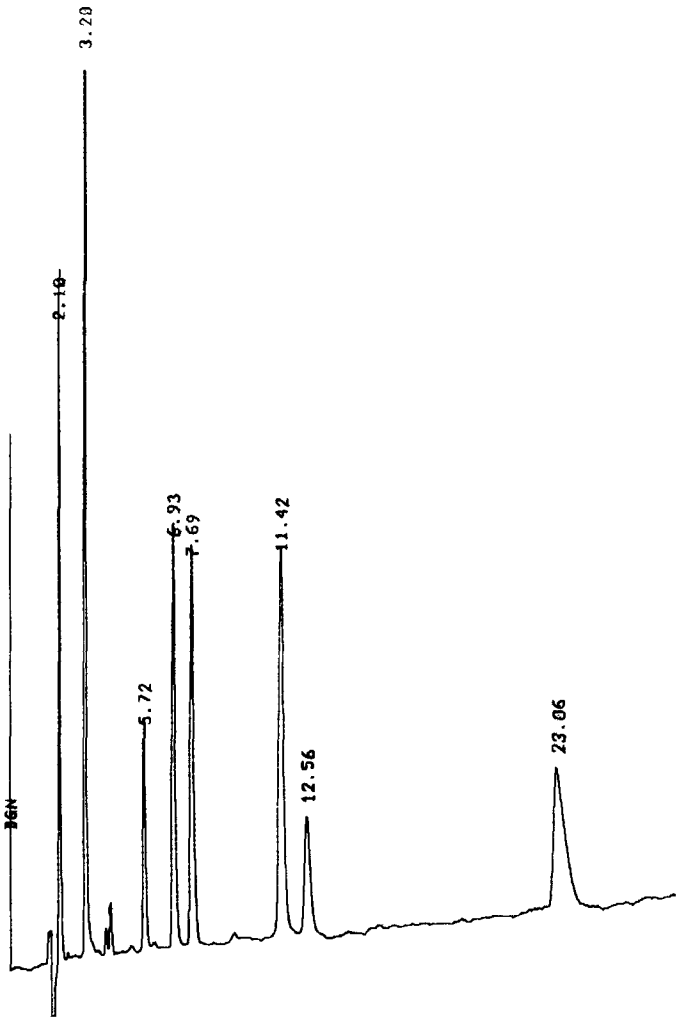

FIG. 1. A high pressure liquid chromatograph of cholesterol oxidation standards. The chromatographic conditions are described in the text. The eluent was monitored by differential refractometry and the integrator response was set at A = 128 for the tracing shown. The oxidation products are indicated by their retention times and are as follows: 2.10, 3,5 diene; 3.20, cholesterol; 5.72, 25-hydroxycholesterol; 6.93, α-CE; 7.69, β-CE; 11.42, 7-keto; 12.56, 7α-OOH; 23.06, 7α-diol.

The extent of cholesterol oxidation was indexed to the level of lipid peroxidation, as measured by TBAR. The level of lipid peroxidation and cholesterol oxidation was determined in all liposome preparations prior to and at various time intervals after the imposed oxidizing conditions already described. All samples were analyzed in duplicate, and values are expressed as the mean and standard deviation calculated from three to four independent experiments for each oxidizing condition described.

RESULTS

The incorporation of cholesterol into liposomes yielded small unilamellar vesicles with characteristics that have been described previously (29,30). The ratio of cholesterol to phospholipid varied no more than 15% from the prescribed ratios at the time of preparation. Furthermore,

TABLE 1

Effect of ^{60}Co Irradiation under N_2O on Cholesterol Oxidation in 1:4 Cholesterol-Phospholipid Liposomes

Duration (min)	Products (in μg)							μmol TBAR[a]
	α-CE	β-CE	7-OOH	7-keto	3,7-diol	3,5-diene	CT	
0	0.072 ±0.009	0.125 ±0.015	0.022 ±0.002	0.147 ±0.025	0.069 ±0.012	0.117 ±0.030	0.102[b]	0.30 ±0.09
30	0.074 ±0.008	0.152 ±0.025	0.029 ±0.007	0.153 ±0.003	0.069 ±0.008	0.081 ±0.014	ND	0.40 ±0.10
60	0.085 ±0.005	0.126 ±0.014	0.024 ±0.007	0.148 ±0.010	0.065 ±0.005	0.084 ±0.015	0.166[b]	0.50 ±0.11
90	0.110 ±0.018	0.160 ±0.025	0.028 ±0.005	0.137 ±0.025	0.064 ±0.010	0.108 ±0.055	0.198[b]	0.49 ±0.15

ND, Not determined. Inadequate chromatography or insufficient numbers of samples prevented accurate determinations of products and quantitation.

[a]The levels of TBAR are expressed as equivalents of malonaldehyde and are calculated from the optical densities measured at 532 nm using a molar extinction coefficient of 1.56×10^4.

[b]Standard deviations not calculated as value represents the average of two measurements.

the type of phospholipid used had no effect on cholesterol assimilation, and the resulting liposomes were stable for the duration of the experiments described. In these experiments, we used either a 1:2 or 1:4 ratio of cholesterol to phospholipid, such that the liposomes differed in terms of the mole percent of cholesterol and degree of unsaturation. The fatty acyl composition of these PC/PE liposomes is described elsewhere (30).

Table 1 shows the major cholesterol oxidation products detected following γ-irradiation of liposomes consisting of a 1:4 molar ratio of cholesterol/phospholipid under an atmosphere of N_2O. In the absence of oxygen, the predominant radical species derived by γ-irradiation of water is ·OH (31). Several oxidation products were measured following irradiation periods as long as 90 min, however, most of these products were present in the original samples (0 min control) and were presumably formed during liposome preparation. This appeared to be the case for all liposome preparations in this report. Cholesterol oxidation was unavoidable during the preparative sonication of liposomes. The levels of oxidation products were low, however, there was some variability in content and proportions of products among different liposome batches. The total amount of cholesterol oxides at zero time were generally less than 0.1% of the total cholesterol content. Accordingly, these products would be undetectable by conventional HPLC methods, and the samples could mistakenly be assumed to be free of oxidized lipids. The only oxidation products to accumulate during γ-irradiation were α-CE and CT. Other cholesterol oxidation products either did not accumulate, or in some cases, decreased below the original levels during the course of irradiation. It should be noted that no TBAR were formed, and levels of 7-OOH remained unchanged over the course of irradiation.

A comparison of lipid peroxidation and cholesterol oxidation in the three liposomal formulations is presented in Table 2 for samples subjected to γ-irradiation under aerobic conditions. Under these conditions, the major oxidizing species formed are O_2^- via the e^-aq, ·OH and H_2O_2 derived from O_2^-. All the cholesterol oxides were found to accumulate in each of the liposomal systems examined. The extent of lipid peroxidation was greatest in the most unsaturated samples (1:4, cholesterol/phospholipid) where the levels of most cholesterol oxidation products and TBAR were as high at 20 min as those formed in 1:2 cholesterol/phospholipid after 60 min of irradiation. Cholesterol oxidation in the cholesterol:DPPC liposomes was least among the three systems studied; however, there was a distinct accumulation of 7-OOH that was not found in the more unsaturated systems. The principal products in all cases were α-CE, β-CE, 7-keto and 3,7-diol. Both α-CE and β-CE were produced in approximately equal proportions during oxidation of cholesterol/DPPC liposomes, whereas the formation of β-CE was favored in liposomes bearing a higher degree of unsaturation. Based on the limited number of lipid peroxidation products analyzed in this study, it appears that product complexity increases during oxidations in the presence of air and, to a lesser extent, as the lipid system becomes more unsaturated.

Subsequent experiments were then conducted using the chemical oxidizing systems. The O_2^- generating system, XO + HX + ADP-Fe^{+3}, is thought to produce a variety of oxidizing species capable of initiating lipid peroxidation (26). The exact species responsible for membrane lipid peroxidation has yet to be determined, although ·OH and perferryl radical are widely viewed as the likely agents. Use of CuOOH plus hematin is representative of lipid peroxide-induced lipid peroxidation. These reactions comprise the propagation stage of lipid peroxidation (14).

Table 3 presents a compilation of data found in Tables 4, 5 and 6, but for the sake of brevity, two oxidizing conditions are selected for comparison. The extent of cholesterol oxidation was found to be influenced by its content in liposomes and by the peroxidizability index (PI) of the component lipids (32). In DPPC/cholesterol liposomes, the only double bonds present are those of

TABLE 2

Effect of Aerobic ^{60}Co Irradiation on Cholesterol Oxidation in Various Cholesterol-Phospholipid Liposomes

Lipid ratio/ duration	Products (in μg)							μmol TBAR[a]
	α-CE	β-CE	7-OOH	7-keto	3,7-diol	3,5-diene	CT	
Chol/PL (1:2)								
0 min	0.10 ±0.04	0.10 ±0.03	0.06 ±0.01	0.05 ±0.01	0.18 ±0.03	0.08 ±0.01	0.04[b]	0.51 ±0.50
2 min	0.26 ±0.08	0.33 ±0.06	0.13 ±0.04	0.10 ±0.03	0.45 ±0.11	0.23 ±0.04	ND	7.96 ±1.29
20 min	0.28 ±0.04	0.38 ±0.07	0.24 ±0.03	0.22 ±0.02	0.50 ±0.12	0.27 ±0.02	ND	10.72 ±2.16
60 min	0.30 ±0.05	0.56 ±0.15	nil	0.37 ±0.02	ND	ND	0.10 ±0.02	32.32 ±2.87
120 min	0.44 ±0.06	0.65 ±0.09	nil	0.55 ±0.10	0.58 ±0.09	0.29 ±0.03	ND	29.11 ±4.97
Chol/PL (1:4)								
0 min	0.22 ±0.02	0.25 ±0.09	nil	0.04 —	0.23 ±0.06	nil	0.06[b]	1.46 ±0.36
2 min	0.32 ±0.02	0.59 ±0.08	ND	0.12 ±0.01	0.50 ±0.10	0.04 ±0.02	ND	22.97 ±4.99
20 min	0.40 ±0.04	0.76 ±0.02	0.08 ±0.02	0.24 0.04	0.51 ±0.08	0.12 ±0.02	0.09[b]	33.22 ±8.05
60 min	0.40 ±0.06	0.79 ±0.04	0.17 ±0.02	0.60 ±0.07	0.88[b]	0.30 ±0.07	ND	46.66 ±2.96
Chol/DPPC (1:4)								
0 min	0.13 ±0.02	0.08 ±0.03	nil	0.04 —	0.21 ±0.05	0.01 —		
60 min	0.36 0.06	0.34 ±0.06	0.20 ±0.05	0.39 ±0.14	0.45 ±0.13	0.12 ±0.01		
120 min	0.50 ±0.11	0.50 ±0.06	0.25 ±0.07	0.54 ±0.10	0.55 ±0.08	0.23 ±0.05		

ND, not determined (see Table 1 for explanation).

[a]The levels of TBAR are expressed as equivalents of malonaldehyde and are calculated from the optical densities measured at 532 nm using a molar extinction coefficient of 1.56×10^4.

[b]Standard deviations not calculated as value represents the average of time measurements.

TABLE 3

Relationship of Cholesterol Oxidation to Lipid Peroxidation in Liposomes of Differing Unsaturated Fatty Acid Content

	Chol/DPPC (1:4)	Chol/PC:PE (1:2)		Chol/PC:PE (1:4)	
μmol Conditions	Total chol. oxidized[b]	Total chol. oxidized[b]	μmol TBAR[a]	Total chol. oxidized[b]	TBAR[a]
Atuox.					
60 min	0.13	0.20	12.82	0.41	15.30
90[c]/120[d] min	0.27	0.24	14.74	0.59	23.00
ADP-Fe^{+3} + XO					
60 min	0.43	ND	ND	0.73	42.94
90[e]/120[f] min	0.62	0.53	46.75	1.02	49.09

ND, not determined. Inadequate chromatography prevented confirmation of species or accurate measurement and quantitation.

[a]The levels of TBAR are expressed as molar equivalents of malonaldehyde and are calculated from the optical densities measured at 532 nm using a molar extinction coefficient of 1.56×10^4. The values shown are the net formation of TBAR obtained by subtracting the levels measured in the starting (unincubated) liposomal preparations from the levels measured at the time intervals indicated.

[b]Values are expressed as percent of cholesterol in the original sample and are the sum of all oxidation products detected.

[c]90-Min incubations were used instead of 120 min for (1:4) Chol/PC:PE liposomes.

[d]120-Min incubations were used for (1:2) Chol/PC:PE and Chol/DPPC liposomes.

[e]90-Min incubations were used for (1:2) and (1:4) Chol/PC:PE liposomes.

[f]120-Min incubations were used for Chol/DPPC liposomes.

TABLE 4

Major Oxidation Products of Cholesterol in Phospholipid Liposomes (1:2 Molar Ratio of Cholesterol to Phospholipid)

Conditions (min)	Products (in μg)						μmol TBAR*
	α-CE	β-CE	7-OOH	7-keto	3,7-diol	3,5-diene	
Control							
0	0.08 ±0.03	0.09 ±0.02	nil	0.05 ±0.02	0.04 ±0.01	0.01 —	0.45 ±0.11
Autox.							
10	0.25 ±0.09	0.25 ±0.05	nil	0.14 ±0.03	0.24 ±0.07	nil	1.05 ±0.57
30	0.23 ±0.07	0.31 ±0.10	ND	0.14 ±0.03	0.24 ±0.06	0.02 ±0.006	4.86 ±1.40
60	0.26 ±0.05	0.26 ±0.06	0.01 —	0.23 ±0.04	ND	ND	13.25 ±4.80
90	0.18 ±0.05	0.23 ±0.05	0.05 ±0.01	0.31 ±0.11	0.24 ±0.05	0.06 ±0.02	11.97 ±6.90
120	0.29 ±0.04	0.32 ±0.07	ND	0.35 ±0.05	0.23 ±0.06	ND	15.15 ±5.99
ADP-Fe^{+3} + XO							
10	0.38 ±0.07	0.31 ±0.03	0.26 ±0.07	nil	0.24 ±0.03	nil	15.15 ±0.90
30	0.44 ±0.12	0.49 ±0.12	0.76 ±0.25	0.14 ±0.03	0.21 ±0.06	nil	20.23 ±8.90
90	0.44 ±0.10	0.43 ±0.17	0.35 ±0.20	0.41 ±0.11	0.33 ±0.06	0.03 ±0.009	47.20 ±15.01
CuOOH + hematin							
10	0.20 ±0.02	0.32 ±0.07	0.08 ±0.02	0.13 ±0.04	0.24 ±0.06	nil	6.86 ±0.66
30	0.18 ±0.05	0.36 ±0.09	0.24 ±0.17	0.30 ±0.05	0.33 ±0.06	0.05 ±0.005	22.24 ±4.56
60	0.16 ±0.05	0.29 ±0.07	0.30 ±0.12	0.54 ±0.17	0.36 ±0.13	0.11 ±0.02	28.84 ±3.97
90	0.20 ±0.04	0.37 ±0.09	0.30 ±0.20	0.59 ±0.09	0.37 ±0.11	0.15 ±0.03	33.78 ±12.97
180	0.28 ±0.04	0.44 ±0.11	ND	0.60 ±0.09	ND	ND	35.70 ±14.66

ND, not determined (see Table 1 for explanation).

*, See Table 1 for explanation.

cholesterol, and the PI is accordingly calculated as 0.50. The 1:2 cholesterol/phospholipid liposomes have a PI of 41.1, while the 1:4 cholesterol/phospholipid liposomes have a PI of 65.9. The values shown in Table 3 are expressed as the percentage of total cholesterol oxidized and are estimated from the sum of all cholesterol oxidation products measured. As in Tables 1 and 2, the extent of cholesterol oxidation was least in the DPPC and greatest in the 1:4 cholesterol/phospholipid liposomes. Cholesterol had a suppressive effect on lipid peroxidation, which could be predicted from the slow induction rates for autoxidation (33). Doubling the proportion of cholesterol (1:2 compared to 1:4 liposomes) reduced the level of lipid peroxidation (TBAR) by ca. 20%. Although a lower percentage of cholesterol was oxidized in the 1:2 liposomes, the actual amounts of cholesterol oxidation products were similar to that produced following oxidation of 1:4 liposomes. Thus, the dcrease in TBAR formation in the 1:2 cholesterol/phospholipid liposomes was accompanied by an increase in the amounts of cholesterol oxidized. It should be noted that a similar extent of cholesterol oxidation was achieved following 60-min treatments with ADP-Fe^{+3} + XO and 60 min of γ-irradiation in air.

The product profiles for the $O_2^{\cdot}$-dependent and CuOOH-induced peroxidations are shown in Tables 4 through 6. During the initial periods of oxidation (<30 min), these oxidizing systems yielded remarkably different proportions of cholesterol oxidation products, however, with prolonged incubations the profile of oxidation products began to resemble each other. α-CE and β-CE were formed in approximately equal amounts during the early intervals of oxidation. As the incubation period progressed, the relative amounts of β-CE increased, and it became a major product, particularly in unsaturated lipid systems. In liposomes bearing unsaturated fatty acids, 7-OOH was an early oxidation product that gradually accumulated.

TABLE 5

Major Oxidation Products of Cholesterol in Phospholipid Liposomes (1:4 Molar Ratio of Cholesterol to Phospholipid)

Conditions (min)	Products (in μg)							μmol TBAR*
	α-CE	β-CE	7-OOH	7-keto	3,7-diol	3,5-diene	CT	
Control								
0	0.08 ±0.01	0.10 ±0.03	0.03 —	0.04 ±0.01	0.08 ±0.02	0.02 —	0.06 ±0.01	1.09 ±0.47
Autox.								
10	0.25 ±0.05	0.25 ±0.03	0.05 ±0.01	0.05 ±0.01	0.28 ±0.09	ND	ND	8.50 ±1.38
30	0.26 ±0.07	0.27 ±0.06	0.05 ±0.01	0.15 ±0.05	0.33 ±0.05	0.04 ±0.01	ND	11.70 ±2.12
60	0.28 ±0.04	0.28 ±0.06	0.06 ±0.02	0.18 ±0.04	0.29 ±0.07	0.06 ±0.02	0.10 ±0.07	17.47 ±5.79
90	0.36 ±0.06	0.49 ±0.06	ND	0.18 ±0.02	0.35 ±0.06	0.09 ±0.03	0.15 ±0.05	25.16 ±11.00
ADP-Fe^{+3} + XO								
10	0.26 ±0.04	0.26 ±0.03	0.20 ±0.08	0.19 ±0.02	0.25 ±0.06	ND	ND	18.17 ±1.03
30	0.27 ±0.09	0.36 ±0.09	0.19 ±0.06	0.19 ±0.05	0.34 ±0.03	0.13 ±0.04	0.09 ±0.02	39.62 ±9.42
60	0.31 ±0.09	0.57 ±0.13	0.23 ±0.02	0.34 ±0.07	0.35 ±0.12	0.10 ±0.04	0.09 ±0.01	45.03 ±12.96
90	0.33 ±0.06	0.62 ±0.08	0.19 ±0.08	0.43 ±0.08	0.34 ±0.05	0.14 ±0.04	ND	51.18 ±14.27
CuOOH + hematin								
10	0.16 ±0.02	0.26 ±0.03	0.18 ±0.07	0.25 ±0.03	0.40 ±0.06	0.04 0.01	ND	25.16 ±6.01
30	0.27 ±0.08	0.59 ±0.14	0.23 ±0.05	0.72 ±0.16	0.73 ±0.15	0.08 ±0.02	0.24 ±0.06	59.69 ±8.02
60	0.44 ±0.11	0.79 ±0.19	0.22 ±0.05	0.94 ±0.26	0.88 ±0.16	0.16 ±0.02	0.46 ±0.04	76.96 ±16.97

ND, not determined (see Table 1 for explanation).

*, See Table 1 for explanation.

TABLE 6

Major Oxidation Products of Cholesterol in Phospholipid Liposomes (1:4 Molar Ratio of Cholesterol to Dipalmitoylphosphatidylcholine)

Conditions	Products (in μg)						
	α-CE	β-CE	7-OOH	7-keto	3,7-diol	3,5-diene	CT
Control							
0 min	0.03 —	nil	0.05 ±0.01	0.03 ±0.005	0.10 ±0.04	nil	nil
Autox.							
60 min	nil	nil	0.09 ±0.02	ND	0.31 ±0.13	nil	nil
120 min	0.08 ±0.04	0.10 ±0.04	0.09 ±0.02	0.12 ±0.03	0.32 ±0.07	ND	ND
ADP-Fe^{+3} + XO							
60 min	0.11 ±0.04	0.14 ±0.05	0.15 ±0.05	0.20 ±0.03	0.26 ±0.12	0.11 ±0.02	0.09 ±0.03
120 min	0.13 ±0.02	0.16 ±0.05	0.25 ±0.02	0.28 ±0.07	0.35 ±0.07	0.18 ±0.04	0.09 ±0.04

In saturated liposomes, the extent of 7-OOH accumulation was as great as any of the other products measured. Under virtually all conditions, 7-keto and, to a lesser extent, 3,7-diol were major products that formed with prolonged incubations. Small but consistently detectable amounts of 3,5-diene were found under these conditions, however, its content relative to other products was greatest in the DPPC/cholesterol liposomes.

The relative proportions of cholest-5-ene-3β,7β-diol and cholest-5-ene-3β,7α-diol (the 3,7-diols) and the corresponding 7α- and 7β-hydroperoxides (7-OOH) could not be established with certainty due to the limited resolution of these isomers by HPLC. Approximation of the levels of radioactivity associated with each peak indicated that equal amounts of each isomer were formed under all circumstances. These approximations are subject to error of 25 to 35% due to peak overlap. We also note the absence of 25-hydroxycholesterol formation under all the oxidizing conditions studied.

DISCUSSION

The characterization of cholesterol oxidation products following free radical-induced membrane lipid peroxidation was studied using unilamellar liposomes. These artificial membranes permitted the manipulation of lipid composition which in this study involved the formulation of discrete proportions of unsaturated phospholipids and cholesterol. Based upon total lipid content the amount of cholesterol added to the liposomes was similar to the proportions found in cell membranes. For example, the plasma membrane of hepatocytes contains ca. 12.9% cholesterol by weight (34,35), while this percentage is lower in endoplasmic reticulum (3.3%) (35) and mitochondria (1.5%) (36). In some cases, the proportions of cholesterol may be even higher than those cited above (37).

It is possible to subdivide the cholesterol oxidation products identified in this study into two categories based on whether lipid peroxidation was mediated by oxyradical-dependent initiation reactions or by lipid peroxide-dependent propagation reactions. Liposomes subjected to anaerobic γ-irradiation represented one extreme, where cholesterol is probably oxidized by $\cdot$OH attack and the likelihood for lipid peroxidation chain reactions is low. Under these conditions the only products to accumulate were α-CE and CT (Table 1). γ-Irradiation in the presence of air (or $O_2^-\cdot$ generation via xanthine oxidase) yields a more complex array of oxyradicals, however, the principal oxidants are likely to be $\cdot$OH (or a perferyl radical) and H_2O_2, since $O_2^-\cdot$ has been shown to be incapable of directly oxidizing cholesterol (38). The presence of oxygen also permits oxidations to proceed via propagation reactions. The degree of propagation reactions may be limited either by short incubation intervals or more effectively by minimizing the degree of lipid unsaturation. The variety and amounts of cholesterol oxides formed are also increased with either prolonged irradiation or increased lipid unsaturation. Under these conditions, propagation reactions largely contribute to the accumulation of lipid peroxidation products, measured as TBAR and a host of cholesterol oxides. A comparison of data from Tables 1 and 2 indicates that in the absence of lipid peroxidation the yields of cholesterol oxidation products are low, where net formation of only two products is seen. By contrast, provision of oxygen permits considerable lipid peroxidation and an increased number of cholesterol oxidation products.

The free radical reactions involved in lipid peroxidation appear to be reflected by the types of cholesterol oxidation products. For example, in the absence of 7-OOH most of the other oxidation products are not encountered. There is, however, one important exception to this, i.e., α-CE formation after anaerobic γ-irradiation occurs in the absence of other products (Table 1). This indicates that 7-OOH are not produced under anaerobic conditions via $\cdot$OH, and, accordingly, no derived products are encountered. The absence of peroxidative chain reactions is expected during anaerobic irradiation and should be suppressed in a largely saturated lipid matrix such as the DPPC/cholesterol liposomes. However, a variety of cholesterol oxidation products are found after aerobic oxidation of DPPC/cholesterol liposomes, indicating the involvement of oxyradical initiation reactions. We note that in saturated liposomes subjected to aerobic oxidations, 7-OOH accumulates as a significant product (Tables 2 and 6), in agreement with the findings of Muto et al. (11). It is plausible that 7-OOH is more stable in saturated lipids where its decomposition is determined largely by interactions with other cholesterol molecules. Products such as 7-keto, 3,7-diol and the isomeric cholesterol epoxides may arise by reactions of 7-OOH with itself (39) or with O_2^- where both 7-OOH and O_2^- are generated by the aerobic oxidations described.

Lipid peroxidation via CuOOH plus hematin can be contrasted from the initiation-type reactions discussed above. Peroxidation was rapidly induced following addition of CuOOH, and the profile of cholesterol oxidation products after short incubation intervals ($\leq$30 min) resembled those obtained after longer incubations ($\geq$60 min) with the $O_2^-\cdot$-generating system. Under otherwise similar conditions, peroxide-dependent (CuOOH) oxidations produce a more rapid accumulation of TBAR, 7-OOH and derived products 7-keto and 3,7-diol, than O_2^--dependent oxidations. During the first 10 min of O_2^--dependent oxidation (Table 4 and 5), less oxidation products form, and with the exception of the 3,5-diene, approximately equal amounts of each product are detected. Beyond 30 min (when O_2^- is no longer being generated), the pattern of oxidation changes considerably and the cholesterol epoxides, 7-keto and 3,7-diols predominate. The oxidation profile resembles that obtained by autoxidation (although the amounts of each product are greater under these conditions of imposed oxidation), suggesting that lipid peroxidation is proceeding via autoxidation at these later incubation periods.

The yields of the α- vs β-CE are indicative of the prevailing process of cholesterol oxidation. Both isomers form under limited oxyradical-induced peroxidation. It appears that γ-irradiation under aerobic conditions or peroxidation via $O_2^-\cdot$ give rise to similar levels of epoxide isomers. Extensive peroxidation, assessed by high TBAR levels, clearly favors β-CE formation. In this respect, peroxyl species have been shown to mediate cholesterol oxidation by lipoxygenase (40) with marked β-stereoselectivity toward the cholesterol hydroperoxide and epoxide products. Gumulka et al. (41) proposed that the proportions of α- and β-CE provide a clue to the processes involved in cholesterol oxidation. They observed a predominance of β-CE following autoxidation, lipid peroxidation and

ozonization, whereas α-CE was preferentially formed by one-atom oxidants such as epoxidases and peracids. This contention is verified by our observations, and although the ratios of α- to β-CE in this study are lower than those reported previously (8,39,41,42), the directions of change are similar.

The formation of the isomeric epoxides by oxidation of cholesterol with H_2O_2 and other organic peroxo-compounds has been reported (8). Cholesterol epoxidation by reaction with hydroperoxides has been documented in organic solvents, aqueous dispersions (11,39,43), and considerable epoxidation also takes place when cholesterol is added to dispersions of lipid hydroperoxides isolated from hepatic microsomes (44). In each of these cases β-CE formation was predominant. Membranes consisting of only saturated fatty acids (Tables 2 and 6) may support cholesterol oxidation via oxyradicals, but cannot effectively produce lipid peroxides and sustain propagation reactions. The oxidation reactions are, therefore, limited to direct attack by oxyradicals (as in O_2^--dependent initiation of lipid peroxidation) or to interactions between oxidized cholesterol radicals. This results in a much reduced level of cholesterol oxidation and is accompanied by the formation of equal amounts of α- and β-CE. γ-Irradiation under anaerobic conditions (Table 1), or irradiation of DPPC/cholesterol liposomes in the presence of oxygen (Table 2), also yield equal amounts of α- and β-CE.

Based on these findings, we submit that oxyradical-induced cholesterol oxidation resembles the one-atom oxidants previously noted. These "initiation" reactions likely proceed by radical attack of the α-surface of cholesterol, while attack by peroxo-compounds (e.g., fatty acid hydroperoxides of phospholipids) preferentially involves the β surface, as originally proposed by Teng and Smith (2). The isomeric selectivity of oxyradicals vs peroxyl-mediated epoxidations may be explained on the basis of cholesterol orientation in membranes. According to Vandenheuval (45), cholesterol is embedded into the membrane bilayer with its α surface facing and at a 5-6 Å distance from the glycerol-phosphate backbone of an adjacent phospholipid. This places the α surface of the 5,6 double bond in relative proximity to oxyradicals generated in the aqueous phase, however, the radical species responsible for epoxidation remains to be identified. The β surface, on the other hand, is oriented toward adjacent fatty acyl groups facing the membrane interior. Attack by oxyradicals would be hindered by the proximate C-19 methyl group of cholesterol and the hydrophobic environment surrounding the β surface of the 5,6 double bond. In this arrangement the double bond is estimated to be 4-5 Å from an ω-3 double bond of a neighboring polyunsaturated fatty acid. Lipid peroxidation involving these polyunsaturated fatty acids would thus favor reaction of the peroxides with the β surface of cholesterol.

In addition to cholesterol epoxide formation, considerable amounts of CT and 3,5-diene were detected. The only known source for CT is the isomeric cholesterol epoxides, and under the conditions used in this study, CT could only be formed by spontaneous hydrolysis of the epoxides. However, the previously reported rates for spontaneous hydrolysis of either epoxide isomer (18,46) appear to be too slow to produce the amounts of CT detected. Nevertheless, an accumulation of CT would be consistent with cholesterol oxidation in the wake of lipid peroxidation and cholesterol epoxide formation. Formation of 3,5-diene has been proposed to derive from 7-OOH via dehydration of 7-keto (47). It appears that accumulation of 3,5-diene, relative to other products, is favored under conditions where 7-OOH can undergo decomposition without participating in further chain reactions. These conditions prevail in a saturated lipid matrix (Table 6) or under anaerobic conditions (Table 1).

Our findings are in general accordance with those of Terao et al. (10) and Muto et al. (11). They concluded that cholesterol was cooxidized with unsaturated fatty acids and that peroxyl and alkoxyl radicals derived from unsaturated fatty acids were largely responsible for the generation of the cholesterol oxides. The 7-keto and 3,7-diol products, also found as major products in these earlier studies, were proposed to derive from thermolytic decomposition of 7-OOH. The other major products were the isomeric cholesterol epoxides, which were proposed to arise from alkoxyl and peroxyl-radical attack of cholesterol in a process showing marked β-stereoselectivity (11).

Lipid peroxidation in tissues is expected to be minimized by a plethora of antioxidants and scavengers that prevent both radical initiation and chain reactions. The detection of low levels of lipid peroxidation products in living systems may be considered proof for low-level free radical reactions. If this is indeed the case, then the cholesterol oxidation profile from biological systems should resemble the oxyradical profiles described in this report. Based on cholesterol epoxide content, in vivo lipid peroxidation should be accompanied by approximately equal β- vs α-CE ratios. Available evidence, though sparse (19,48,49), supports this hypothesis. It may be possible to examine the course of lipid peroxidation in more complex systems containing cholesterol by "finger-printing" the oxidation products in a manner similar to that described in this report.

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