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Oxidation of Lipids in Low Density Lipoprotein Particles

NORIKO NOGUCHI*, RIKA NUMANO, HAJIME KANEDA and ETSUO NIKI

Research Center for Advanced Science and Technology, The University of Tokyo, 4-6-1 Komaba, Meguro, Tokyo 153, Japan

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This study was undertaken to understand further the mechanisms and dynamics of the oxidation of lipids in low density lipoprotein (LDL) particles, aiming specifically at elucidating the material balance between oxygen uptake and products found and also the relative susceptibilities to oxidation of cholesteryl ester in the core and phosphatidylcholine in the outer monolayer in the LDL particles. It was found that considerable amount of oxygen uptake could not be accounted for by conjugated diene or total peroxides. Total peroxide was measured from the phosphine oxide formed from triphenylphosphine or diphenylpyrenylphosphine by reduction of peroxides. Cholesteryl ester hydroperoxides and phosphatidylcholine hydroperoxides were the major peroxides formed in LDL oxidation, but they accounted for about 60% of total peroxide. Cholesterol was also oxidized, but its oxidation was significant only at the later stages of the reaction. It was also found that the oxidizability of cholesteryl ester relative to phosphatidylcholine was larger within the LDL particle than in homogeneous solution and this was interpreted in the context of the physical properties of LDL particle.

Keywords: Lipid peroxidation, cholesteryl ester, phosphatidylcholine, low density lipoprotein, total peroxide, phosphine oxide

Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); BHT, 2,6-di-*tert*-butyl-4-methylphenol; CE, cholesteryl ester; CEOH, CE hydroxide; CEOOH, CE hydroperoxide; DPPP, diphenyl-1-pyrenylphosphine; EDTA, ethylenediaminetetraacetic acid; LDL, low density lipoprotein; PBS, phosphate buffered saline; PC, phosphatidylcholine; PCOOH, PC hydroperoxide; PUFAs, polyunsaturated fatty acids; TBARS, thiobarbituric acid reactive substances; TPP, triphenylphosphine

INTRODUCTION

The oxidation of lipids in low density lipoprotein (LDL) has received much attention in connection with the oxidative modification of LDL which is accepted to play a key role in the pathogenesis of atherosclerosis.[1] Lipid hydroperoxides, primary products of lipid peroxidation, and their decomposition products such as malondialdehyde, 4-hydroxynonenal and acrolein modify specific amino acid residues in the apolipoprotein B-100 (apo B) protein moiety of LDL.[2] The lipid-derived radicals may attack apo B directly and

*Corresponding author. Fax: +81-33481-4574. E-mail: nonoriko@oxygen.rcast.u-tokyo.ac.jp

modify protein. The protein modification results in the loss of recognition of the oxidatively modified LDL by the native LDL receptor and the enhanced recognition by the macrophage scavenger receptors.[3] Oxidized LDL exerts additional atherogenic properties;[4] for example, it is thought to play role in recruitment and retention of monocytes and macrophages and have the potential to damage endothelial cells and release various cytokines.

One of the characteristics of lipid peroxidation in LDL is related to the structural property of the particle. Human LDL is a spherical particle with a diameter of about 22 nm and an average molecular weight of 2.5 million. The neutral lipids such as cholesteryl esters and triglycerides form a hydrophobic core, while amphipathic phospholipids and free cholesterol form a surface monolayer and surround the core. The approximate number of molecules of phospholipids, free cholesterol, cholesteryl esters and triglycerides are 700, 600, 1600, and 100 per LDL particle.[5] The total number of fatty acid molecules bound in the different classes is 2700 on average, of which about half are polyunsaturated fatty acids (PUFAs), the major PUFAs being linoleic acid with smaller amount of arachidonic acid. They are distributed unequally in the different lipid classes, for example, linoleic acid is predominantly bound in cholesteryl esters, whereas more arachidonic acid is found in phospholipids than in cholesteryl esters. It has been found that more cholesteryl ester hydroperoxides are formed than phospholipid hydroperoxides independent of the site of initial radical production,[6–8] that is, outside or within LDL particle. The oxidation of LDL induced by copper also gave more cholesteryl ester hydroperoxides than phospholipid hydroperoxides.[8] This may be simply due to a different concentrations of PUFA in cholesteryl esters and phospholipids as suggested by Bowry and Stocker,[9] or other effects such as physical properties of LDL particles. Another interesting point is that the amount of hydroperoxides formed from CE and PC does not account for oxygen uptake observed during oxidation of LDL.[8] The present study was carried out to obtain deeper insight into the mechanisms and dynamics of lipid peroxidation in LDL particles.

MATERIALS AND METHODS

Materials

A hydrophilic azo compound, 2,2′-azobis(amidinopropane) dihydrochloride (AAPH), and a lipophilic azo compound, 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), used as a peroxyl radical source[10] were purchased from Wako Pure Chemical Ind. (Osaka, Japan) and used as received. Soybean phosphatidylcholine (PC) was purchased from Sigma Chemical Co. (St. Louis, MO) and purified as previously described.[8] Synthetic dimyristoyl PC and linoleoyl-palmitoyl PC, cholesteryl esters and cholesterol were from Sigma and used without further purification. Diphenyl-1-pyrenylphosphine (DPPP) was a kind gift of Dr. Cynshi at Chugai Pharmaceutical Company. Triphenylphosphine and triphenylphosphine oxide were from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Other chemicals were those of the highest grade available commercially.

Oxidation of Soybean PC Liposome

The oxidation of soybean PC multilamellar vesicles was performed as reported previously.[11] Briefly, appropriate amounts of soybean PC (final concentration 5.1 mM) and AMVN were dissolved in methanol and the solution was taken into a round bottom flask. Methanol was removed under reduced pressure to obtain a thin film on the flask wall, which was slowly peeled off by shaking with 0.1 M NaCl aqueous solution containing 0.1 mM EDTA to obtain white milky aqueous suspensions of multilamellar liposomal membranes. The small unilamellar vesicles were prepared by sonicating the above multilamellar membranes (Branson Sonifier 250). When hydrophilic AAPH was used instead of AMVN as a

radical initiator, it was added to the liposomal suspensions as an aqueous solution. The oxidation of soybean PC membranes was performed at 37°C in air and the accumulation of PC hydroperoxides (PCOOH) was measured with an HPLC using a UV detector at 234 nm. Straight phase LC-Si column (particle size 5 μm, 4.6 mm × 250 mm, Supelco, Tokyo) was used and the eluent was methanol/40 mM phosphate buffer (90/10, v/v), flow rate being 1.0 ml/min. The amount of PCOOH was also measured from phosphine oxide formed from phosphine by the reaction with peroxides.

Measurement of Total Peroxide

When one molecule of triphenylphosphine (TPP) or diphenyl-1-pyrenylphosphine (DPPP) reduces one molecule of peroxide, one molecule of triphenylphosphine oxide (TPP=O) or diphenyl-1-pyrenylphosphine oxide (DPPP=O), respectively, is formed. Thus the amount of peroxide can be measured quantitatively by measuring the amount of phosphine oxide. TPP and TPP oxide were separated with an HPLC and measured by an absorption at 220 nm with a UV detector (JASCO, UV970). DPPP and DPPP oxide were also analyzed with an HPLC equipped with two detectors in series, UV and fluorescence (JASCO, FP920) detectors. They were monitored by UV absorption at 350 nm and fluorescence intensity at 380 nm with excitation at 352 nm. Analytical normal phase LC-Si column (4.6 × 250 mm; 5 μm particle size) was used (Supelco, Tokyo). Hexane/2-propanol (88/12, v/v) and hexane/*tert*-butyl alcohol (70/30, v/v) at a flow rate of 1.0 ml/min were used for analyses of TPP and DPPP, respectively. To measure the amount of PCOOH in the oxidation of soybean PC liposome, 200 μl of DPPP (300 μM) solution in 2-propanol was added to 100 μl of liposomal suspension and the mixture was incubated for 5 min at 37°C after vortex mixing for 1 min. An aliquot of the reaction mixture was injected onto HPLC. The same volume of buffer without PC was treated by the same way and the value was abstracted from that of liposomal suspension. DPPP oxide was prepared by a reaction of DPPP with excess linoleic acid hydroperoxide in benzene solution at 37°C. A complete depletion of DPPP was confirmed.

Oxidation of Synthetic Phosphatidylcholine and Cholesteryl Ester

Linoleoyl-palmitoyl phosphatidylcholine and cholesteryl linoleate were oxidized at several different ratios (total number of bisallylic hydrogen being kept constant) by AMVN in *tert*-butyl alcohol at 37°C in air. The PCOOH and cholesteryl ester hydroperoxide (CEOOH) were measured with HPLC using a UV detector at 234 nm as previously described.[12]

Isolation of LDL

LDL was separated from the plasma of healthy donors by ultracentrifugation as described in the literature[13] within a density cut-off of 1.019–1.063 g/ml, and then dialyzed with cellulose membranes in phosphate-buffered saline (PBS, pH 7.4) containing 100 μM EDTA. It was sterilized with Mille-GV filter (Millipore, Tokyo, Japan) after dialysis. The protein concentration of LDL was measured using the bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL).

Oxidation of LDL and Analysis of Oxidative Modification of LDL

The oxidation of LDL (0.25 mg protein/ml) by AAPH or copper was carried out at 37°C under air in PBS (pH 7.4) containing 100 μM EDTA. LDL was dialyzed in PBS (pH 7.4) to remove EDTA prior to the oxidation by copper ion. AAPH and $CuCl_2$ were added as solutions in PBS (pH 7.4 or distilled water) into the LDL suspension. Oxygen consumption was followed by using a Clark-type oxygen electrode, Biological Oxygen Monitor, Model YSI 53 (Yellow Springs Instrument Co., Yellow Spring, OH). Free cholesterol and cholesteryl ester were separated by HPLC on an LC-18

column (particle size 5 μm, 4.6 mm × 250 mm, Supelco, Tokyo, Japan) eluted with acetonitrile/2-propanol (30/70, v/v) at flow rate of 1.0 ml/min and detected by UV at 210 nm as reported in the literature.[14] The formation of PCOOH and CEOOH was followed as previously described.[12] α-Tocopherol was analyzed with an HPLC detected with an electrochemical detector (Kotaki, Tokyo, Japan) set at +800 mV. LC-18 column (particle size 5 μm, 4.6 mm × 250 mm, Supelco, Tokyo, Japan) was used and the eluent was methanol/*tert*-butyl alcohol (90/10, v/v) containing 50 mM $NaClO_4$, flow rate being 1.0 ml/min. DPPP was used for measurement of total hydroperoxide formed in the oxidation of LDL. LDL suspension (10 μl) was diluted 10 times with H_2O (100 μM EDTA) and incubated with 125 μM DPPP methanol solution (400 μl with 0.003% BHT) at 37°C for 20 min. DPPP oxide was extracted with 500 μl H_2O and 2500 μl benzene. Twenty μl benzene solution was injected onto HPLC. Thiobarbituric acid reactive substances (TBARS) were measured by a conventional method[15] from the absorption at 532 nm. Lyso PC was measured with an HPLC by absorption at 205 nm using LC-Si column (particle size 5 μm, 4.6 mm × 250 mm, Supelco, Tokyo, Japan) and 2-propanol/hexane/ethanol/2 mM $NH_4H_2PO_4$ (120/90/25/25, v/v) as an eluent at flow rate of 1.0 ml/min. Chloroform extract of the LDL suspension by 2 volume of chloroform/methanol (2/1, v/v) was solvent-exchanged by 1/10 volume of methanol and injected onto the HPLC.

Lipids which were extracted from the same LDL by 2 volume of chloroform/methanol (2/1, v/v) were oxidized by AMVN in *tert*-butyl alcohol or *tert*-butyl alcohol/methanol (4/1, v/v) at 37°C in air. The formation of PCOOH and CEOOH was analyzed in the same way.

The data presented in this paper are the representative examples of several separate experiments. Although the different LDL samples showed a different length of lag time and rate of oxidation, they showed the similar pattern of oxidative modification.

RESULTS

Analyses of Total Peroxides

Hydroperoxides are formed as the primary products in lipid peroxidation. One of the most quantitative methods to analyze hydroperoxides is to measure tertiary phosphine oxide formed by reaction of the hydroperoxides with tertiary phosphine. The analysis of lipid hydroperoxides was first performed with either TPP or DPPP in the oxidation of PC liposomal membranes. The unilamellar liposomal membranes prepared from soybean PC were oxidized with AAPH and DPPP oxide was measured after treatment of the products with DPPP. Furthermore, the oxidation products were analyzed with HPLC-UV for PC hydroperoxides. The amounts of PC hydroperoxides and DPPP oxide were in good agreement (Figure 1). Similar results were observed for the oxidation of multilamellar PC liposomal membranes induced by AMVN incorporated into the

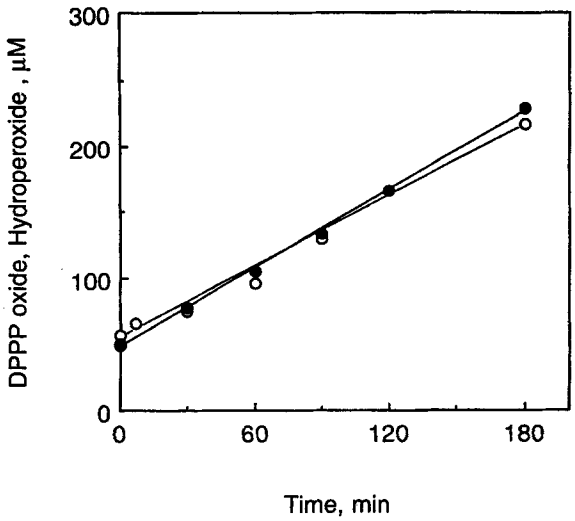

FIGURE 1 Formation of phosphatidylcholine hydroperoxide as measured by HPLC and diphenyl-1-pyrenylphosphine (DPPP). Soybean PC (5.1 mM) unilamellar vesicles were oxidized in the presence of AAPH (0.50 mM) at 37°C in air and PC hydroperoxide was analyzed with an HPLC using a UV detector at 234 nm (●). At the same time, the reaction mixture was treated with DPPP for 5 min and the DPPP oxide formed was analyzed with an HPLC equipped with a fluorescence detector (○) as described in Methods.

membranes (data not shown). Quantitative agreement was also observed with TPP oxide (data not shown). The amounts of hydroperoxides formed from the peroxyl radical derived from AMVN and AAPH were quite small compared with PCOOH under the present reaction conditions. These results show that DPPP oxide and TPP oxide are a good quantitative measure for lipid hydroperoxides.

LDL was oxidized with copper and oxygen uptake, formation of conjugated dienes, CE and PC hydroperoxides, TBARS, and consumption of α-tocopherol were followed. As observed previously,[8] CE and PC hydroperoxides increased some time after complete disappearance of α-tocopherol (Figure 2). Similar pattern was observed for DPPP oxide (data not shown). It should be noted that the amount of oxygen uptake was much greater than those of conjugated dienes and lipid hydroperoxides. Conjugated dienes reached a plateau after 200 min, while CE and PC hydroperoxides decreased probably due to their secondary oxidation or decomposition. The amount of TBARS was much smaller than oxygen uptake or even conjugated dienes and it increased significantly after lipid hydroperoxides started to decrease.

The copper-induced oxidation products of LDL were analyzed with DPPP in order to obtain more information on the fate of oxygen incorporated into LDL. The products were treated with DPPP and the resulting DPPP oxide was analyzed by HPLC equipped with a fluorescence detector. The amount of DPPP oxide was compared with that of PC and CE hydroperoxides which were measured with HPLC by UV at 234 nm (Figure 3). The amount of total peroxides measured as DPPP oxide was much greater than the sum of PCOOH and CEOOH observed. The missing oxygen may be involved in endoperoxides and peroxides of triglycerides, free cholesterol and protein and also in the secondary oxidation products such as aldehyde, ketone and alcohol of lipids. It was found that the amount of lyso PC was quite small, not more than a few percent of oxygen uptake.

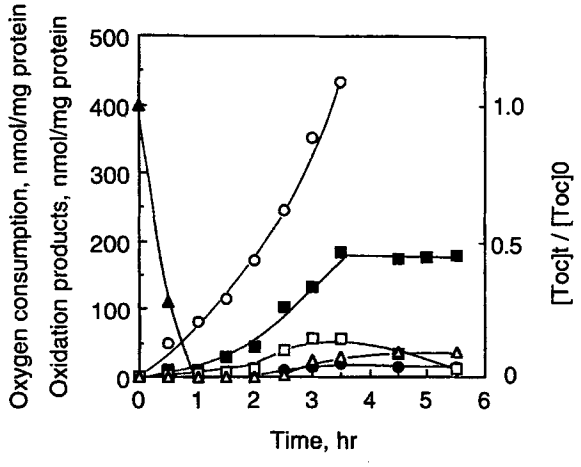

FIGURE 2 Oxidation of human LDL. Human LDL (0.25 mg protein/ml) was incubated with $CuCl_2$ (4 μM) at 37°C under air in PBS (pH 7.4) and oxygen uptake (○), vitamin E consumption (▲), and the formation of conjugated diene (■), hydroperoxides of phosphatidylcholine (PCOOH) (●) and cholesteryl ester (CEOOH) (□), and thiobarbituric acid reactive substances (△) were measured periodically. $[Toc]_t/[Toc]_0$ shows the fraction of α-tocopherol remaining.

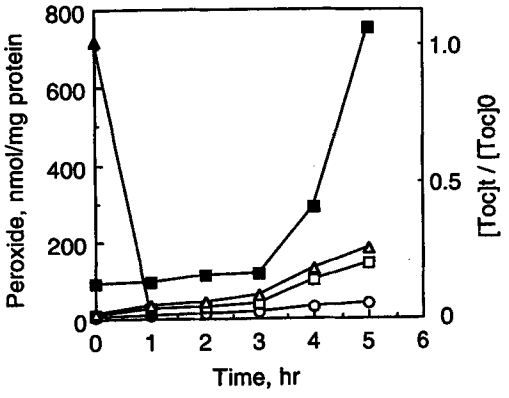

FIGURE 3 Peroxides formed in the oxidation of human LDL (0.25 mg protein/ml) by $CuCl_2$ (5 μM) at 37°C under air in PBS (pH 7.4). The formation of PCOOH (○) and CEOOH (□) was measured with HPLC using a UV detector at 234 nm. A sum of PCOOH and CEOOH was also plotted (△). The total peroxides (■) were measured with DPPP as described in Methods.

Oxidation of Cholesteryl Ester and Free Cholesterol

In order to estimate the oxidation of cholesterol, the consumption of free cholesterol and cholesteryl esters was followed during the oxidation of LDL. Free cholesterol and cholesteryl esters can be separated for different lipid classes by an HPLC detected by UV at 210 nm (Figure 4). The change in HPLC pattern with the extent of oxidation of LDL is illustrated in Figure 4. As observed by Esterbauer for total lipids,[2] the higher the extent of unsaturated fatty acids, the greater the rate of oxidation. At the initial stage, only the cholesteryl esters having polyunsaturated fatty acid moieties were oxidized, while cholesterol oleate, stearate and palmitate were not oxidized appreciably. Free cholesterol was not oxidized until later in the peroxidation reaction. These results suggest that the oxidation of cholesterol ring is not important in the respect to oxygen consumed when polyunsaturated fatty acid moieties are present.

Oxidation of Lipids in Outer Monolayer and Core of LDL Particle

The oxidation of LDL by copper, hydrophilic AAPH or lipophilic AMVN always results in greater yields of CE hydroperoxide than PC hydroperoxide. In order to understand the dynamics of lipid peroxidation in the LDL particle, the relative susceptibilities of PC and CE to oxidation were compared in the LDL particle and in *tert*-butyl alcohol or in a mixture of *tert*-butyl alcohol and methanol. The oxidation in alcohol was carried out after extraction of lipids from LDL with chloroform/methanol (2/1, v/v), followed by solvent exchange from chloroform to alcohol after removal of chloroform under nitrogen. Thus, it was possible to compare the rates of oxidation of CE and PC in LDL particle and in alcohol for the same substrates. A typical example of the results from these experiments is shown in Figure 5. As observed previously,[8] more CE hydroperoxide was formed than PC hydroperoxide, while they were formed in about same yield in alcohol solution. The formation of CE and PC hydroperoxides varied with LDL donors and the ratio of the rate of formation of CE hydroperoxide to that of PC hydroperoxide was measured for LDL samples from six different donors. The results are summarized in Table I. If the relative oxidizability of CE to that of PC is determined solely by the concentrations of active bisallylic hydrogens, the ratio should be the same in LDL particle and in

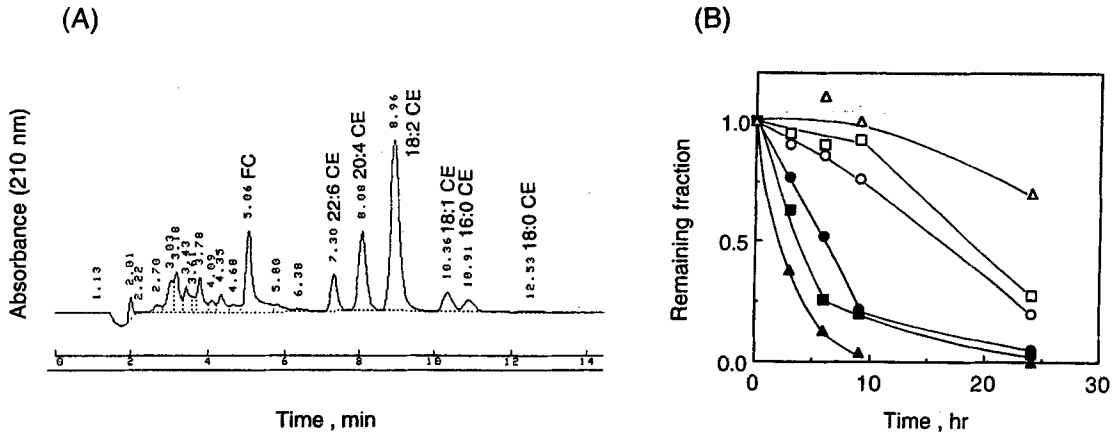

FIGURE 4 Oxidation of cholesteryl esters and free cholesterol in LDL. (A) Chromatogram profile of free cholesterol and cholesteryl esters extracted from human LDL. (B) Human LDL (0.25 mg protein/ml) was oxidized by AAPH (2 mM) and the decrease of each cholesteryl ester (▲: 22:6; ■: 20:4; ●: 18:2; ○: 18:1; □: 16:0) and free cholesterol (△) was followed with time.

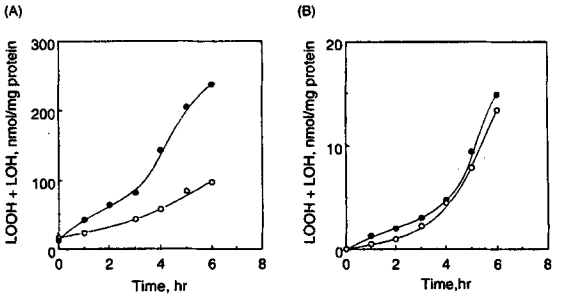

FIGURE 5 Oxidation of cholesteryl ester (CE) and phosphatidylcholine (PC) in LDL particles and in *tert*-butyl alcohol/methanol (4/1, v/v). (A) Human LDL (0.25 mg protein/ml, 0.5 μM) was incubated with AAPH (3 mM) in PBS (pH 7.4) at 37°C in air and the formation of CE (●) and PC hydroperoxides (○) was measured with an HPLC as described in Methods. (B) The lipids were extracted with twice as much chloroform/methanol (2/1, v/v) from the LDL (1.5 mg protein/ml) suspensions and the lipids were oxidized with AMVN (2 mM) in *tert*-butyl alcohol/methanol (4/1, v/v) at 37°C in air.

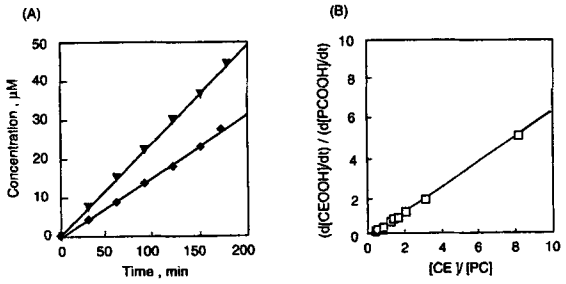

FIGURE 6 Oxidation of synthetic cholesteryl ester and phosphatidylcholine in *tert*-butyl alcohol. Cholesteryl linoleate and linoleoyl-palmitoyl phosphatidylcholine at several different ratios were oxidized with AMVN (6 mM) in *tert*-butyl alcohol at 37°C under air. (A) The formation of hydroperoxides of cholesteryl ester (CEOOH) (◆) and phosphatidylcholine (PCOOH) (▼) was followed with time. (B) The ratio of the rate of formation of CEOOH to that of PCOOH was plotted against the molar ratio of CE and PC.

TABLE I Relative oxidizabilities of cholesterol ester (CE) and phosphatidylcholine (PC) in LDL particle and *tert*-butyl alcohol[a]

LDL sample	(d[CEOOH]/dt)/(d[PCOOH]/dt) = *A*		*A*(LDL)/*A*(*tert*-BuOH)
	LDL	*tert*-BuOH	
1	3.75	0.91	4.12
2	5.02	1.80	2.79
3	7.23	1.70	4.25
4	4.46	1.35	3.30
5	3.37	1.42	2.63
6	3.12	1.01	3.09

[a]LDL was oxidized in PBS with 3.0 mM AAPH at 37°C and the formation of CE and PC hydroperoxides was followed with an HPLC and the ratio (*A*) was measured. The total lipids extracted from LDL with chloroform/methanol (2/1, v/v) were oxidized in *tert*-butyl alcohol (*tert*-BuOH) (entry 1–5) or in *tert*-butyl alcohol/methanol (4/1, v/v) (entry 6) in the presence of 2.0 mM AMVN and CE and PC hydroperoxides were measured with an HPLC.

alcohol. However, Table I shows that the ratio of the rate of CE hydroperoxide formation to that of PC hydroperoxide formation in LDL particle is 3.4 times larger than in alcohol solution.

The relative susceptibilities of CE and PC in *tert*-butyl alcohol were measured using authentic cholesteryl linoleate and linoleoyl-palmitoyl PC. The ratio of the rate of formation of CE hydroperoxide to that of PC hydroperoxide was directly proportional to the molar ratio of CE and PC (Figure 6), the slope of the straight line of the plot in Figure 6B being 0.64. The data in Figure 5 also give a clue to the understanding of the oxidizabilities of lipids in LDL particles. The rates of formation of CE and PC hydroperoxides in the oxidation of LDL under the present conditions were respectively 4.09 and 1.31 nM/s, while those in alcohol were 2.18 and 2.15 nM/s. The LDL suspensions contained 0.25 mg protein/ml, which is equivalent to 1.20 ml LDL particles/l, assuming the molecular weight of apo B and LDL as 5.0×10^5 and 2.5×10^6 respectively and average density of LDL as 1.04. This implies that the lipids are condensed into the lipophilic compartment by $10^3/1.20 = 833$ times within LDL particles under the reaction conditions employed. On the other hand, the concentration of lipids in alcohol was equivalent to 1.5 mg protein/ml LDL, 6 times higher than that of aqueous LDL suspensions. Therefore, the concentrations of lipids in LDL particles were 833/6 = 139 times higher than those in alcohol. These results show that if we assume the rate of chain initiation by 3 mM AAPH and 2 mM AMVN is the same, the relative oxidizability of CE in LDL particles to that in alcohol is calculated as (4.09/139)/2.18 = 0.031, while that for PC is obtained (1.31/139)/2.15 = 0.0044 by assuming that the oxidizability is directly proportional (first-order

dependence) to the substrate concentration. These results suggest that the apparent oxidizabilities of CE and PC are much smaller in LDL particles than in solution, the decrease being more significant for PC than CE. These results suggest that CE is relatively more oxidizable compared with PC in the LDL particle than in homogeneous alcohol solution and that some physical properties of LDL particle as well as active bisallylic hydrogen concentration are important in determining the susceptibilities of lipids to oxidation.

DISCUSSION

A variety of methods have been proposed for the measurement of lipid peroxidation.[16] For example, conjugated diene, TBARS, and iodometric titration have been often employed. The iodometric titration method, where iodine liberated by a reduction of hydroperoxide with sodium or potassium iodide in the presence of acid is measured, is accurate, quantitative, and reproducible, and multiple analyses can be carried out by automated assay.[17,18] The spectrophotometric[19,20] or potentiometric[21] triiodide assay has been also reported. However, the sensitivity of iodometric method is not high enough for biological samples. The TBA method is sensitive, but the inherent drawback of this method is the lack of specificity and quantitation relative to lipid peroxides. For example, various hydroperoxides and aldehydes react with thiobarbituric acid with different sensitivities depending on their types and analytical conditions.[22]

The analysis of lipid hydroperoxides with HPLC has a major advantage since different classes of hydroperoxides can be measured separately. The HPLC-post column method using chemiluminescence[23,24] or electrochemical[25] detection is quite sensitive. However, only such hydroperoxides that are eluted under the conditions employed can be measured with an HPLC. Furthermore, the sensitivity of HPLC-chemiluminescence method depends on the type of hydorperoxides, metals, reaction conditions and apparatus.[26]

It has been known that tertiary phosphine such as TPP reduces organic hydroperoxides to give corresponding phosphine oxide and alcohols. This is a stoichiometric reaction and proceeds quantitatively. Akasaka *et al.*[27] found that DPPP reduces hydroperoxides to corresponding alcohols and DPPP oxide, which is fluorescent and can be measured with quite a high sensitivity. This suggests that DPPP may well be used as a tool for measuring peroxides *in vitro* and *in vivo* and in fact has already been used successfully.[28] The present study on the oxidation of phospholipid in liposomal membranes shows that DPPP and TPP can be used to measure the lipid peroxides quantitatively. An excess amount of TPP or DPPP is required over hydroperoxide to reduce it rapidly and quantitatively. In order to minimize the interference, TPP oxide and DPPP oxide were analyzed separately in the present study from corresponding phosphines with an HPLC. TPP oxide is not fluorescent but has a reasonable absorbance between 200 and 240 nm and the extinction coefficients were obtained as $\varepsilon = 2.13 \times 10^4$ (206.6 nm), 2.03×10^4 (222.2 nm) and 1.95×10^4 (225.0 nm) $M^{-1}\,cm^{-1}$, respectively, which suggests that 1 μM peroxide (that is, 20 pmol, when 20 μl of the sample is injected) can be analyzed with accuracy. It may be noteworthy that this extinction coefficient is similar to that for conjugated diene.[29] DPPP oxide can be analyzed by either fluorescence or absorption, the former being much more sensitive than the latter. The detection limit for DPPP oxide with HPLC-fluorescence analysis is 0.1 nM, that is, 2 fmol when 20 μl of the sample is injected.

The present results confirm that the CE and PC hydroperoxides are indeed the major peroxides but that they do not account for all the peroxides; nor do the total peroxides account for the oxygen uptake. It should be borne in mind that significant amounts of products are missing in the

oxidation of LDL. The possible missing products may be peroxides from triglyceride, apo B, cholesterol and free fatty acids. Their decomposition products and secondary oxidation products may also be formed. It may be noteworthy that the formation of free fatty acid hydroperoxides and hydroxides and lyso PC were too small to account for the missing peroxide or oxygen uptake.

It has been found that cholesterol is oxidized by free radical mechanism to give various oxidation products.[30] They received much attention because of their toxicity,[31] although the mechanism underlying for their toxic effect has not been well elucidated. However, the reactivity of cholesterol toward peroxyl radical is much smaller than polyunsaturated fatty acids and, as observed in Figure 4B, the oxidation of cholesterol ring should not be important as long as polyunsaturated fatty acid moieties are remaining.

However, cholesterol exerts both dilution effect and physical effect. Free cholesterol is present in outer monolayer of LDL in quite a high concentration. This on the one hand dilutes the concentration of reactive phospholipids, but on the other hand it increases the rigidity of the outer monolayer and reduces the fluidity of phospholipids and decreases the susceptibility of phospholipid to oxidation induced by aqueous radicals. It was in fact observed in the oxidation of liposomal membranes that the decrease in the oxidizability of PC caused by the addition of free cholesterol was much larger than that accounted for by a simple dilution effect, although free cholesterol did not exert any effect in the lipid peroxidation in organic solution (unpublished results). On the other hand, the core of LDL particle composed of cholesteryl ester and triglycerides is more fluid as measured from the electron spin resonance parameters of spin probes incorporated into LDL particle.[32] Such a difference in physical properties between outer monolayer and inner core may at least in part explain why the oxidizability of CE relative to PC is high in LDL particles.

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