Coexistence of Oxidized Lipids and α-Tocopherol in All Lipoprotein Density Fractions Isolated From Advanced Human Atherosclerotic Plaques

Xianwa Niu, Vivienne Zammit, Joanne M. Upston, Roger T. Dean, Roland Stocker

Abstract—After investigation of the contents and redox status of antioxidants and lipids in homogenates of both normal artery and atherosclerotic plaque, we now investigated them in the density fractions (very low, low, high, and protein fractions) of atherosclerotic plaque freshly obtained from carotid endarterectomy. By using the optimum extraction method (homogenization in carbonate buffer) and after density gradient ultracentrifugation, we isolated and characterized density fractions of plaque for apolipoproteins, size and contents of α-tocopherol (α-TOH), unesterified cholesterol, cholesteryl linoleate (Ch18:2), and hydroxides and hydroperoxides of Ch18:2, ie, Ch18:2-O(O)H. The distribution of apolipoproteins was more heterogeneous than that in the corresponding lipoproteins isolated from blood, and the majority of material in all plaque density fractions was present in large particles eluting in the void volume of gel-filtration columns. The content of unesterified cholesterol per unit of protein in low- and high-density fractions was 10-fold that in corresponding plasma lipoproteins. Low- and very-low-density fractions contained most of the lesion lipids and α-TOH. Two to five percent of lesion Ch18:2 was present as Ch18:2-O(O)H and distributed more or less equally among all density fractions, yet the content of α-TOH per unit of Ch18:2 was higher than that in corresponding plasma lipoproteins. These results demonstrate that α-TOH and oxidized lipids coexist in all lesion density fractions, further supporting the notion that large proportions of lipids in lipoproteins of advanced stages of atherosclerosis are oxidized. However, although not ruling it out, our results do not support the suggestion that advanced stages of atherosclerosis are associated with gross deficiencies in the lipoproteins’ vitamin E content. (Arterioscler Thromb Vasc Biol. 1999;19:1708-1718.)

Key Words: antioxidants ▪ atherosclerosis ▪ oxidative stress ▪ vitamin E ▪ lipid peroxidation

Substantial evidence suggests that the accumulation and modification of plasma LDL in vessel intima contribute to the development of atherosclerosis.1 This includes the oxidative modification of the protein and lipid moieties of LDL, as indicated by an increased particle electrophoretic mobility,2-3 LDL aggregation,4 oxidatively modified apolipoprotein B100 (apoB100) and fragments derived from it,5-6 and the presence of oxidized free and esterified fatty acids (see Reference 7 and references therein). Plasma contains numerous water and lipid-soluble antioxidants,8 and there is little direct evidence of LDL (lipid or protein) oxidation in the circulation, even in patients suffering from severe atherosclerosis.9 The former has contributed to the generally held view that oxidative modification of LDL takes place in the intima rather than circulation.1

As extracellular fluids are poor in enzymic antioxidant defenses,8 we have investigated previously the contents and redox status of aqueous and lipid-soluble antioxidants in homogenates of normal human arteries and advanced lesions. Surprisingly, although fully consistent with corresponding plasma values, we observed7 significant concentrations of both ascorbate (the first line of aqueous defense8,10) and α-tocopherol (α-TOH, the most abundant fat-soluble antioxidant11). In fact, the concentrations of ascorbate and urate (per unit of protein) and α-TOH (per unit of bisallylic hydrogen-containing lipid) in lesion intima were at least comparable with those in normal artery intima and plasma. Despite the presence of these antioxidants, which in combination completely prevent radical-induced lipid peroxidation in plasma12 and other extravascular fluids,13,14 homogenates of human plaque contained very large amounts of oxidized lipids, with eg, up to 15% of the fatty acid moiety of cholesteryl linoleate (Ch18:2) being present in oxidized forms.7,15

There are several possible explanations for these apparently paradoxical data. For example, it might be that lipid oxidation occurred at a time when antioxidants were depleted. If so, then antioxidant replenishment might follow, whereas the oxidized lipids remained in the lesion. Unfortunately, this possibility is virtually impossible to assess in relation to advanced human lesions, because of lack of availability of suitable materials. A second possibility is that there might be physical separation between antioxidants and oxidative...
Characterization of Human Plaque Lipoproteins

Niu et al

Isolation of Plasma Lipoproteins

Ten milliliters of human blood from healthy subjects or patients undergoing carotid endarterectomy was collected into heparinized Vacutainers (Becton Dickinson) and plasma was separated from blood cells by centrifugation at 514g and at 4°C for 15 minutes. After adjustment of density to 1.21 g/mL with solid KBr, 2 mL of plasma was layered under 3.1 mL of ice-cold buffer A (phosphate buffered [10 mmol/L] saline, pH 7.3, containing 0.54 mmol/L EDTA and 10 μmol/L butylated hydroxytoluene). Before use, buffer A was supplemented with Chexel-100 (0.3 g/100 mL), stirred overnight at room temperature, and filtered through a 0.22-μm nitrocellulose filter (Millipore, Bedford, MA). This treatment removed contaminating transition metals, as verified by the ascorbate autoxidation method; the buffer was then flushed with argon. LDL and HDL were isolated by density gradient ultracentrifugation at 417 000g and at 15°C for 4 hours as described previously.18

Isolation of Human Atherosclerotic Plaque Density Fractions

Human plaques were obtained from patients undergoing carotid endarterectomy at the Royal Prince Alfred Hospital, New South Wales, with approval from the local Human Ethics Review Committee and with the informed consent of all patients. Patients were asked to fill in a questionnaire regarding supplementation with antioxidants; none of the patients was taking vitamin E or other antioxidants supplements. All plaques represented advanced lesions with complicating factors (Table 1). Immediately after surgical removal, plaques were placed in saline and brought to the laboratory on ice. Within 60 minutes of surgical removal, plaque samples were rinsed in buffer A and a small piece removed for histology (Table 1). After removal of surrounding adventitia and media, plaques were rinsed again in buffer A, blotted dry, and weighed. Approximately 0.16 g of blotted intima was added per milliliter of ice-cold, previously Chexel-treated, and argon-flushed buffer B (100 mmol/L sodium carbonate, pH 11, containing 2.7 mmol/L EDTA, 10 μmol/L butylated hydroxytoluene, 0.01% aprotinin, 1 mmol/L PMSF, 0.002% elastatinal, 2 mmol/L benzamidine, 1 μmol/L D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone, 0.008% gentamicin, and 0.008% chloramphenicol). The plaque was then minced (referred to as “minced plaque”) and homogenized for 5 minutes at 4°C, using a 10-ML polytetrafluoroethylene-lined glass homogenizer (Wheaton) with the piston rotating at 200 to 500 rpm. These conditions have been previously established to maximize overall recovery of protein, oxidized lipids (FC and Ch18:2), oxidized lipids (hydroperoxides [Ch18:2-OOH] and hydroxides [Ch18:2-OH] of Ch18:2), and antioxidants (α-T0H, ascorbate, and urate).7 The resulting “raw homogenate” was centrifuged at 2000g and at 4°C for 10 minutes, the density of the resulting supernatant (referred to as homogenate) adjusted to 1.21 g/mL, and the sample subjected to density ultracentrifugation as described above. Where possible, a sample of venous blood was collected from the patient the night before the operation, the plasma prepared, supplemented with 0.6% sucrose, and stored overnight at −20°C. After plaque collection, the patient’s plasma sample was thawed and also subjected to density ultracentrifugation together with the corresponding plaque sample. Where a patient plasma sample was not available, a freshly prepared “control plasma” obtained from a healthy subject was prepared and subjected to density ultracentrifugation together with the plaque sample.

In an attempt to further optimize the extraction recovery while maintaining gross structural integrity of the lipid/protein particles present in the plaque samples, we compared homogenization in buffer A or B versus the mild buffer extraction method described by Ylä-Herttuala et al16,19 (Figure 1). For the latter, plaque finely minced with a pair of scissors on ice in either buffer A or B was extracted at 4°C for 18 to 24 hours on an orbital shaker. The resulting plaque suspension was centrifuged at 2000g and at 4°C for 10 minutes, and the resulting pellet resuspended in buffer A or B and homogenized as described above for plaque (resulting in homogenized pellet). The supernatant of the centrifuged plaque suspension, referred to as extract was retained for further analyses (see Figure 1 and below). For recovery experiments, raw homogenates, homogenates, extracts, and homogenized pellets were extracted and analyzed.
for protein, lipids, and \( \alpha \)-TOH as described below. Recoveries were calculated as percentages of the material present in either homogenate versus raw homogenate (homogenization method) or extract versus extract plus homogenized pellet (buffer extraction method). For the recovery of \( \alpha \)-TOH, \( \alpha \)-tocotrienol was used as external standard.

After density gradient ultracentrifugation (417,000 g, 15°C, 4 hours) 4 density fractions were collected by direct aspiration using a syringe with a 25-gauge needle. They were designated as very low density (VLDF, 400 to 700 mg/L), LDF ('700 mg/L), high density (HDF, >720 mg/L), and protein fraction (PF, 700 to 1000 mg/L). LDF and HDF gave no consistent discrete bands and therefore were collected as a constant volume at positions corresponding to those of plasma LDL and HDL. No significant differences in the position of plasma LDL and HDL were observed between healthy and patient plasma samples. The fractions corresponding to the uppermost 400 to 700 mg/L and the bottom 700 to 1000 mg/L were designated as VLDF and PF, respectively. The material between the above density fractions, ie, all remaining material in the centrifuge tube, was also collected and pooled for analysis and designated as pooled remainder materials. This limited the possibility of omission of significant amounts of materials from the various analyses.

### Analyses of Lipids, Vitamin E, and Total Thiols and Protein

Plasma lipoproteins and plaque density fractions were extracted with acidified methanol and hexane\(^1\) and the organic extract analyzed by HPLC for FC and Ch18:2, \( \Delta 18 \) Ch18:2-O HH and Ch18:2-OH \( \Delta 20 \) (representing hydroperoxides and hydroxides of CEs, respectively),\(^{15} \) and vitamin E (\( \alpha \)-TOH, \( \gamma \)-tocopherol, and \( \alpha \)-tocopheryl quinone).\(^{21} \) To determine the recovery of reduced glutathione, a modified version of total thiol determination by Ellman\(^2\) was used. In brief, an aliquot (100 mg/L) of homogenate or extract was incubated with 900 mg/L of 100 mmol/L potassium phosphate, pH 7.4, containing 400 mmol/L NaCl, 1 mmol/L EDTA, and 10 mmol/L dithionitrobenzoate at 37°C for 1 to 2 hours in the dark. Subsequently, the absorbance at 412 nm was determined, using the incubation buffer devoid of dithionitrobenzoate as a blank. Total protein was determined by using the bicinchoninic assay (as per instructions of the manufacturer); BSA was used as a standard.

#### Exchange of Oxidized Ch18:2 and \( \alpha \)-TOH Between Lipoprotein Density Fractions

LDL and HDL containing radiolabeled \( \alpha \)-TOH and Ch18:2-OH were prepared by incubating 6.5 mL of fresh human plasma and 15 \( \mu \)L of \( ^{13} \)C-\( \alpha \)-TOH (1.2 Ci/L in DMSO) plus 15 \( \mu \)L of \( ^{3} \)H-Ch18:2-OH (0.9 Ci/L in ethanol) for 6 hours at 37°C. Radiolabeled LDL and HDL (\( ^{13} \)C/[\( ^{3} \)H]LDL and \( ^{13} \)C/[\( ^{3} \)H]HDL, respectively) were isolated by density gradient ultracentrifugation and stored under argon at 4°C. To determine whether compounds of the radiolabeled lipoproteins exchanged into lesion lipoprotein density fractions during sample workup, individual carotid plaques were minced, divided into 2 equal portions, and 150 \( \mu \)L of \( ^{13} \)C/[\( ^{3} \)H]LDL or \( ^{13} \)C/[\( ^{3} \)H]HDL was added to 1 portion. The plaque suspensions were then homogenized as described above. In addition, \( ^{13} \)C/[\( ^{3} \)H]LDL and \( ^{13} \)C/[\( ^{3} \)H]HDL were homogenized in buffer in the absence of plaque. The homogenates were then subjected to density gradient ultracentrifugation as described above and the density fractions isolated sequentially from the least to the most dense. The fractions isolated between VLDF and LDF, LDF and HDF, and HDF and PF were also

### Table 1. Description of Subjects and Human Atherosclerotic Plaque Histology

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (y)</th>
<th>Sex</th>
<th>Risk Factors</th>
<th>Lesion Stages</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>M</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>80</td>
<td>M</td>
<td>Ex-smoker, hypertension</td>
<td>Vb*</td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>M</td>
<td>Ex-smoker, hypercholesterolemia</td>
<td>Vb</td>
</tr>
<tr>
<td>4</td>
<td>75</td>
<td>M</td>
<td>Ex-smoker, hypertension</td>
<td>Vb</td>
</tr>
<tr>
<td>5</td>
<td>67</td>
<td>F</td>
<td>Hypertension, hypercholesterolemia</td>
<td>Vb†</td>
</tr>
<tr>
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<td>61</td>
<td>M</td>
<td>Ex-smoker, hypertension</td>
<td>Vb</td>
</tr>
<tr>
<td>7</td>
<td>72</td>
<td>M</td>
<td>Ex-smoker, hypertension</td>
<td>Vb</td>
</tr>
<tr>
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<td>60</td>
<td>M</td>
<td>Diabetes mellitus, hypertension</td>
<td>Vlc</td>
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<td>M</td>
<td>Ex-smoker, diabetes mellitus</td>
<td>Va</td>
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<tr>
<td>10</td>
<td>76</td>
<td>M</td>
<td>Ex-smoker</td>
<td>Vb</td>
</tr>
</tbody>
</table>

All plaques used in this study were obtained from carotid endarterectomy and represented advanced lesions. See Stary et al\(^{23} \) for the classification of lesions.

ND indicates not done; Vb, fibroatheroma + calcification; Va, fibroatheroma; Vlc, complicated lesion + thrombus.

*Hemorrhage into plaque; †ulceration + hemorrhage into plaque.

Figure 1. Schematic outline of approach used to validate method for extracting lipoprotein density fractions from advanced human atherosclerotic plaques.
collected and analyzed. An aliquot of each density fraction (30 μL) was added to 10 mL of scintillant (Ultima-Gold, Packard Instruments) and counted by using a TRI-CARB 2100 TR Liquid Scintillation Analyzer (Packard Instruments) with [14 C]/[3 H] dual-labelments) and counted by using a TRI-CARB 2100 TR Liquid Scintillation Analyzer (Packard Instruments) with [14 C]/[3 H] dual-label program.

**SDS-PAGE and Western Blot Analysis of Apolipoproteins**

Proteins were separated under reducing conditions by SDS-PAGE using mini gradient gels (4% to 15%). The separated proteins were either visualized by silver staining or transferred onto nitrocellulose, reacted with the appropriate apolipoprotein antibody, and the complexes detected by enhanced chemiluminescence (ECL, Amersham).

**Histology**

A sample of each plaque was fixed in 10% phosphate-buffered (50 mmol/L) formaldehyde for up to a week and then transferred to 70% ethanol and stored at 4°C until processed at the School of Pathology, University of New South Wales, using hematoxylin and eosin staining. For classification of the severity of atherosclerotic lesions, the definition and nomenclature of Stary et al was used.

**Gel-Filtration Chromatography**

The particle sizes of plasma lipoproteins and plaque density fractions were assessed by gel-filtration fast protein liquid chromatography (FPLC). The sample (VLDF, 8 to 17 μg; LDF, 3 to 25 μg; HDF, 34 to 105 μg; LDL, 78 to 232 μg; and HDL, 989 μg) was injected onto a Superose 6 column (30 x 1.5 cm, inside diameter; Pharmacia) eluted at 0.25 mL/min and at 4°C until processed at the School of Pathology, University of New South Wales, using hematoxylin and eosin staining. For classification of the severity of atherosclerotic lesions, the definition and nomenclature of Stary et al was used.

**Statistics**

Student’s paired t test was used to compare data, with P ≤ 0.05 considered significant.

**Results**

**Optimization of Plaque Extraction Efficiency**

Homogenization is a fast method to extract lipids and antioxidants with high recoveries from control arteries and advanced human plaque, although it may cause inadvertent structural alteration or damage to lipoproteins. Buffer extraction is a more gentle method, although it is comparatively slow and incomplete and hence may recover lipids and antioxidants to a lower and varying extent. We therefore first compared the recovery for protein, selected lipids, and vitamin E from plaque by homogenization versus buffer extraction. We also examined the effect of PBS (buffer A) versus alkaline carbonate buffer (buffer B) (see Reference 25). In 3 separate experiments using 3 different plaques, the recoveries of FC, Ch18:2, cholesteryl arachidonate (Ch20:4), and α-TOH were higher in homogenates than the corresponding buffer extracts (Figure 2). Also, the mean recovery of protein was significantly higher in homogenates prepared with buffer B than buffer A (Figure 2).

To evaluate the recovery of α-TOH under the different workup conditions, α-tocotrienol was added as external standard to the plaque samples before homogenization or buffer extraction, as this form of vitamin E has physical and redox properties similar to those of α-TOH. The recovery of α-tocotrienol was 74.0 ± 3.3% and 52.3 ± 13.3% (mean ± SD; n = 4; buffer A and B data combined) for homogenization and buffer extraction, respectively; there was no difference between buffers A and B. The recovery of reduced glutathione, added to aliquots of a minced plaque, was used to estimate both the extent of artifactual oxidation of thiols during sample processing as well as recovery of an aqueous antioxidant. The recovery for exogenous GSH after homogenization or buffer extraction was 74.0 ± 12.7% and 79.3 ± 2.2% (mean ± SD, n = 3), respectively, for buffer A and 64.8 ± 11.2% and 61.1 ± 12.5% (mean ± SD, n = 3), respectively, for buffer B.

Figure 3 shows that independent of the buffer used, both homogenization and buffer extraction resulted in the recovery of a very wide range of proteins, likely to be representative of the plaque population. To examine the possibility of lipoprotein aggregation during sample processing, each of 4 plaque samples derived from homogenization or buffer extraction with buffer A or B, and after centrifugation, was serially diluted (4 to 5 dilutions), using the corresponding buffer. The protein concentration was then determined, before the absorbance was measured at 600 nm as an index of turbidity. The turbidity-to-protein ratio was about twice as high in homogenate than extract for 3 separate lesion samples, independent of the buffer used (data not shown), indicating that homog-
enization caused somewhat more aggregation than buffer extraction.

We also subjected the plaque density fractions to gel-filtration FPLC. All fractions contained large particles eluting as the major peak with the void volume from the column (at ≈30 minutes). This was observed independent of whether plaque samples were homogenized or buffer extracted (Figure 4; data for VLDF and HDF not shown), or whether buffer A or B was used (data not shown). By contrast, plasma LDL and HDL eluted at 47 and 62 minutes, respectively. When LDF (derived from homogenate or extract) was spiked with plasma LDL immediately before FPLC, 2 separate peaks eluting with the void volume and native LDL, respectively, were observed (data not shown). This suggested that either aggregation occurred during the plaque workup and/or LDL exists as aggregates in plaque. To discriminate between these 2 possibilities, we homogenized or buffer-extracted plasma LDL and subsequently reisolated the lipoprotein by density ultracentrifugation. This resulted in some LDL aggregation and loss of apoB, although a substantial proportion of the material remained in a form that coeluted with native LDL (Figure 4D through 4F). The extent of aggregation and apoB loss (assessed by the total area of peaks eluting from the FPLC column) was somewhat larger for homogenization than buffer extraction (compare Figure 4E and 4F), consistent with the turbidity results described above.

Together, these results indicate that some lipoprotein aggregation occurred during the sample processing, the extent of which was larger in density fractions derived from homogenates than buffer extracts. Despite this, however, all of the endogenous lipid/protein particles recovered eluted as high molecular weight complexes, even with the comparatively milder buffer extraction. This indicated that at least a portion of plaque LDL was present as large particles/aggregates before sample workup. Therefore, and because higher overall recoveries were obtained with homogenization than buffer extraction, all plaque lipoprotein density fractions subsequently used for further characterization were prepared by homogenization, using buffer B.

Further Characterization of Lipoprotein Density Fractions Derived From Advanced Human Plaque

Table 2 and Figure 5 summarize the densities and contents of the different apolipoproteins in the various density fractions prepared from homogenates of advanced human plaque. As can be seen, the densities of plaque LDL and HDF were similar to those of plasma LDL and HDL, whereas the density of VLDF was higher than that of plasma VLDL (ie, ρ < 1.006 g/mL). As expected, the more buoyant VLDF and LDF contained most of the monomeric apoB and fragments derived from it (Figure 5B). It is well recognized that human atherosclerotic lesions contain fragments of apoB (see References 25 and 27). VLDF and LDF also contained most of the apoE detected, although significant amounts were also present in HDF (Figure 5C). Traces of high molecular weight material reacting with anti-apoE antibody were observed in VLDL and LDF (Figure 5C). HDF was the major contributor to plaque apoAI, although substantial amounts of this apolipoprotein were also present in LDF (Figure 5D). Only traces of apoAII could be detected in all density fractions (Table 2).

The concentration of FC (per protein) was ≈10-fold higher in plaque LDL and HDF than plasma LDL and HDL (Table 3), with 60% of the fractionated FC being present in LDL. By contrast, the ratio of readily oxidizable CEs (Ch18:2 plus Ch20:4) to protein was similar for LDL and normal or patient LDL (data not shown). Overall, this resulted in a lower ratio of CEs to FC in plaque LDL and HDF than plasma LDL and HDL (Table 3).

In human lipoproteins, Ch18:2 is the most abundant CE containing bisallylic hydrogens, which are most prone to oxidation. A substantial proportion (2% to 5%) of Ch18:2

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**Figure 3.** SDS-PAGE of plaque homogenate and buffer extract by using buffer A or B. Proteins (4 μg per lane) present in homogenate (H), extract (BE), and plasma LDL (nLDL) were separated by gradient (4% to 15%) PAGE and stained with silver nitrate. The results shown are typical of 3 separate experiments, using 3 different plaque samples.

**Figure 4.** Gel-filtration chromatography of human atherosclerotic plaque LDF Methods. As a control sample, pooled plasma obtained from apoE gene knock-out mice (A) was used as described by Neuzil et al.**5**2**5**. Representative chromatograms of LDF from homogenized (B) or buffer extracted lesion material (C) are also shown. D through F, Isolated native LDL was subjected to no treatment (D), homogenization (E), or buffer extraction (F), as performed for plaque samples, followed by reisolation by density ultracentrifugation as described in Methods. Overall protein recovery was 55.4%, 37.8%, and 53.2% for D, E, and F, respectively, as assessed by area comparison with that of freshly isolated LDL. For simplicity, only the section of the chromatogram up to, and including, the corresponding plasma LDL peak is shown.
was present as either Ch18:2-OOH or Ch18:2-OH [referred to as Ch18:2-O(O)H] in all plaque density fractions (Table 4). Although most of the fractionated Ch18:2-O(O)H was present in VLDF and LDF, the degree of oxidized [expressed as percentage of Ch18:2-O(O)H per total Ch18:2] was at least comparable in the more dense (HDF and PF) and buoyant (VLDF and LDF) fractions (Table 4). Ch18:2-O(O)H were not detected in plasma lipoproteins isolated from the blood of normal subjects or the patients undergoing endarterectomy (Table 4), consistent with a previous report.

Despite the observed substantial extent of oxidation of Ch18:2, the concentration of α-TOH was similar to or higher in LDF and HDF than that in the corresponding lipoproteins isolated from normal or patient plasma, when the results were expressed per protein or Ch18:2, respectively (Table 5). Similar to the distribution of CEs, the majority of α-TOH was localized within VLDF and LDF. Consistent with the large increase in FC-to-protein ratio, the content of α-TOH per FC was lower in LDF and HDF than the corresponding normal or patient LDL and HDL samples (Table 5). All lipoprotein density fractions contained small amounts of γ-tocopherol. The ratio of γ- to α-isomer of vitamin E in LDF and HDF was not different than that in LDL and HDL isolated from normal and patient plasma samples (not shown). In addition, all lipoprotein density fractions prepared from advanced human plaque contained ≈3% to 11% of α-TOH as α-tocopherol quinone (Table 5), whereas this 2-electron oxidation product of vitamin E was barely detectable in normal or patient LDL and HDL.

To rule out that oxidized lipid and/or antioxidants exchanged between lesion lipoproteins during sample workup, we added LDL or HDL containing [14C]/[3H]-LDL and [14C]/[1H]HDL into any lesion lipoprotein fraction during the homogenization procedure. Figure 6 shows that no significant exchange of either [1H]-Ch18:2:0H or [14C]α-TOH occurred from either LDL or HDL into any lesion lipoprotein fraction not subjected to any treatment (data not shown), lipoproteins homogenized in buffer only (Figure 6, insets), and lipoproteins homogenized in the presence of plaque (Figure 6).

### Discussion

We previously described the coexistence of relatively large amounts of α-TOH and oxidized CEs in homogenates prepared from whole intimas of endarterectomy samples containing advanced fibrofatty lesions. Consistent with this, we report here that all lipoprotein density fractions derived from
such homogenates contain substantial and comparable amounts of both oxidized Ch18:2 and ω-TOH. As Ch18:2 represents the single major and most readily oxidizable lipid in lipoproteins, the results obtained show, for the first time, that lipids in all types of lipoproteins present in advanced lesions are oxidized to a similar extent, and that, on average, ω-TOH is not deficient in these lipoprotein density fractions at this late stage of the disease.

The lipoprotein density fractions used for the analysis of lipids and antioxidants were prepared by using conditions optimized for recovery of protein, lipids, and α-TOH (Figure 2), as we aimed at maximizing the proportion of the initial plaque material analyzed, and intimal lipoproteins must be released from the various components present in vessels. This formed part of the reason for choosing homogenization over buffer extraction, although homogenization caused some lipoprotein aggregation (Figure 4). The latter is consistent with the fact that native LDL isolated from healthy subjects28 and aortic LDL19,29 are prone to aggregation when exposed to physical stress. We note, however, that even when applying the milder16 buffer extraction method, the vast majority of all lipoprotein density fractions eluted with the void volume from the gel-filtration column, indicating that most of the material was present as aggregates. Our observation that HDF contained large particles/aggregates is fully consistent with a previous report of Heideman and Hoff.30 In apparent contrast to our results with LDF, several groups reported a proportion of human lesion LDL with a gel-filtration profile similar to that of plasma LDL,3,6,31,32 although lipoproteins like LDL are known to aggregate in the intima.33 The reason(s) for the discrepancy is unclear, although we used plaque samples with advanced and complicated lesions (Table 1), whereas the above studies of others used either grossly normal intima or rejected complicated lesions where fatty-fibrous material was present. Hollander et al34 reported the isolation of arterial LDL with size comparable with that of native LDL from complicated plaques, using high-salt buffers that are expected to reduce aggregation. However, these authors noted no

| TABLE 3. Contents of Lipids in Human Atherosclerotic Plaque Density Fractions and Plasma Lipoproteins |
|-----------------------------------------------|------------------|------------------|------------------|------------------|
| Free Cholesterol | Cholesteryl Linoleate | Cholesteryl Arachidonate |
|                  | (Mean±SD) | % in Each Fraction | (Mean±SD) | % in Each Fraction | (Mean±SD) | % in Each Fraction |
|                  |          |                   |          |                   |          |                   |
| Raw homogenate  | 8        | 0.5±0.3           | 0.5±0.2 | 0.07±0.02         |          |                   |
| VLDF            | 10       | 4.1±1.1           | 8.0±3.4 | 14.8              |          |                   |
| LDF             | 10       | 5.9±1.9           | 32.3±9.9| 59.8              |          |                   |
| HDF             | 10       | 0.8±0.3           | 6.2±1.6 | 11.5              |          |                   |
| PF              | 10       | 0.1±0.1           | 7.6±6.7 | 14.1              |          |                   |
| PRM             | 10       | 46.0±13.6         |          |                   |          |                   |
| nLDL†           | 4        | 0.6±0.2           | 1.5±0.5 | 0.15±0.03         |          |                   |
| nHDL†           | 4        | 0.1±0.0           | 1.8±0.7 | 0.17±0.07         |          |                   |
| pLDL‡           | 8        | 0.5±0.2           | 1.8±0.6 | 0.20±0.07         |          |                   |
| pHDL‡           | 8        | 0.1±0.0           | 2.0±0.5 | 0.27±0.12         |          |                   |

Plaque density fractions/plasma lipoproteins were extracted with hexane:methanol (5:1, vol/vol) and the organic phase analyzed for the lipids, using HPLC (UV at 210 nm). PRM indicates pooled remainder materials comprising all remaining material left after removal of VLDF, LDF, HDF, and PF.

*Percentage of (total material recovered—PRM) in each of the density fractions.
†Isolated from the plasma of normal subjects.
‡Isolated from the plasma of patients undergoing carotid endarterectomy.

| TABLE 4. Contents of Oxidized CE in Human Atherosclerotic Plaque Density Fractions and Plasma Lipoproteins |
|-----------------------------------------------|------------------|------------------|------------------|------------------|
| Ch18:2-2-OH | Ch18:2-2-O(O)H, % of Total Ch18:2 (Mean±SD) |
| Cholesteryl Linoleate (Mean±SD) | % in Each Fraction | (Mean±SD) | Adjusted* | (Mean±SD) | % in Each Fraction | (Mean±SD) | Adjusted* |
|                  |                   |          |          |          |                   |          |          |
| Raw homogenate  | 9        | 27.3±20.9 | 17.3±10.4 | 35.6 | 5.7±4.3 | 17.9±10.8 | 36.5 | 2.1±1.6 |
| VLDF            | 10       | 15.9±12.6 | 19.6±6.5 | 40.8 | 5.7±4.3 | 21.8±7.0 | 44.4 | 2.6±2.0 |
| LDF             | 10       | 19.6±14.5 | 5.2±3.4 | 10.7 | 6.5±7.5 | 3.4±3.6 | 6.9 | 4.2±4.1 |
| HDF             | 10       | 38.9±44.5 | 6.3±4.5 | 12.9 | 15.2±24.6 | 6.0±5.3 | 12.2 | 4.8±5.8 |
| PF              | 9        | 39.1±49.2 | 51.4±10.4 | 51.5±12.3 | 0.0±0.0 |                   |          |          |
| PRM             | 10       | 0.5±0.0 |                   |          |          |          |          |          |
| nLDL†           | 8        | 0.5±0.0 |                   |          |          |          |          |          |
| nHDL†           | 12       | 0.0±0.0 |                   |          |          |          |          |          |

Plaque density fractions/plasma lipoproteins were extracted with hexane:methanol (5:1, vol/vol) and the organic phase analyzed for cholesteryl linoleate hydroxide (Ch18:2-2-OH) and hydroperoxide (Ch18:2-2-O(O)H), using HPLC (UV at 234 nm).

*Percentage of (total material recovered—PRM) in each of the density fractions.
†Isolated from the plasma of normal subjects and patients undergoing carotid endarterectomy.
TABLE 5. Contents of Vitamin E in Human Atherosclerotic Plaque Density Fractions and Plasma Lipoproteins

<table>
<thead>
<tr>
<th></th>
<th>nmol/mg of Protein (Mean±SD)</th>
<th>nmol/mol of Ch18:2 (Mean±SD)</th>
<th>mmol/mol of FC (Mean±SD)</th>
<th>% in Each Fraction</th>
<th>α-Tocopheryl Quinone, % Total α-TOH (Mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw homogenate</td>
<td>8</td>
<td>4.9±3.0</td>
<td>21.7±10.0</td>
<td>9.4±3.1</td>
<td>6.3±3.5</td>
</tr>
<tr>
<td>VLDL</td>
<td>10</td>
<td>77.5±24.5</td>
<td>24.1±8.7</td>
<td>20.5±9.8</td>
<td>21.0±10.7 39.6 3.4±2.4</td>
</tr>
<tr>
<td>LDF</td>
<td>10</td>
<td>32.1±17.6</td>
<td>28.5±12.4</td>
<td>5.7±2.9</td>
<td>21.4±8.5 40.3 3.4±3.1</td>
</tr>
<tr>
<td>HDF</td>
<td>10</td>
<td>4.5±3.0</td>
<td>58.9±65.3</td>
<td>6.9±5.5</td>
<td>4.2±2.2 7.9 10.5±12.2</td>
</tr>
<tr>
<td>PF</td>
<td>10</td>
<td>0.5±0.6</td>
<td>32.8±25.7</td>
<td>8.1±6.6</td>
<td>6.5±5.3 12.2 10.9±9.2</td>
</tr>
<tr>
<td>PRM</td>
<td>10</td>
<td></td>
<td></td>
<td>47.7±13.4</td>
<td></td>
</tr>
<tr>
<td>nLDL†</td>
<td>4</td>
<td>14.6±5.6</td>
<td>17.8±5.0</td>
<td>26.3±8.3</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>nHDL†</td>
<td>4</td>
<td>5.1±1.1</td>
<td>30.7±20.5</td>
<td>47.2±16.2</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>pLDL‡</td>
<td>8</td>
<td>10.1±3.4</td>
<td>12.4±6.1</td>
<td>19.4±4.5</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>pHDL‡</td>
<td>8</td>
<td>2.8±1.0</td>
<td>24.6±7.4</td>
<td>47.0±10.0</td>
<td>0.3±0.3</td>
</tr>
</tbody>
</table>

Plaque density fractions/plasma lipoproteins were extracted with hexane/methanol (5:1, vol/vol) and the organic phase analyzed for α-tocopherol and α-tocopheryl quinone by HPLC, as described in Methods.

†Isolated from the plasma of normal subjects.
‡Isolated from the plasma of patients undergoing carotid endarterectomy.

Figure 6. Ch18:2-OH and α-TOH do not exchange between-lesion lipoprotein density fractions during homogenization of carotid plaque and subsequent preparative steps. [3H]-Ch18:2-OH and [14C]-α-TOH incorporated into LDL (A) or HDL (B) was homogenized in the presence or absence (insets) of human lesion material, as described in Methods and the legend to Figure 1. After ultracentrifugation, the density fractions were collected and 30 μL of each fraction was counted for [3H] (open columns) and [14C] (filled columns). The numbers on the x axis refer to VLDL/fraction (1); the material between fractions 1 and 3 (2); LDL/F (3); the material between fractions 3 and 5 (4); HDL/F (5); the material between fractions 5 and 7 (6); and PF (7). Two (presence of plaque) and 3 separate experiments (absence of plaque) were performed in duplicate. Data shown represent mean±range values. The recoveries of the added radiolabels ranged between 84% and 93%.

Qualitative difference between homogenization and buffer extraction, although the former method yielded significantly more lipid and protein,44 consistent with the results of the present study. Hoff and Gaubatz31 reported the bulk of lesion LDL from homogenized plaques to elute in the void volume fraction after gel filtration, with a very minor fraction eluting at a position similar to that of native LDL. Our results are not inconsistent with this, as we restricted the amount of material subjected to gel filtration so that we would not have detected such a minor fraction. Taken together, it thus seems possible that the reported differences in behavior of isolated plaque lipoprotein density fractions versus plasma lipoproteins reflect differences in property of the materials used rather than workup artifact. We are presently investigating this possibility further by comparing density fractions obtained by homogenization, buffer extraction, or powderization under liquid nitrogen,6 using lesions of different severity.

In addition to the structural differences indicated above, the lipoprotein density fractions isolated from advanced human lesions showed marked differences in composition when compared with those of plasma lipoproteins. Of these, the heterogeneity in apolipoprotein distribution, decreased CE-to-FC ratio, and increased content of oxidized lipids were most noteworthy. Differences in the relative distribution of apolipoproteins between lesion and plasma lipoproteins have been reported by others (see, eg, Reference 30, for apoAI), although the reason(s) for this is unknown. The CE-to-FC ratio may be decreased as a result of the action of lipase(s),35 consistent with the ~10-fold elevated FC/protein ratio in LDF versus native or patient LDL (Table 3). It is noteworthy that LDF, and LDF plus VLDL, comprise the bulk of recovered plaque FC and CE, respectively (Table 3), considering that linoleate comprises ~42% of all fatty acids in CE in these apoB-containing lipoproteins.31 The ratio of Ch18:2 to FC in VLDL and LDF observed in the present study (Table 3) closely resembled those reported previously by others for the apoB-containing, 1.006 to 1.063 g/mL density fraction,31 although in the case of LDF it was ~8-fold lower than that of plasma LDL.

α-TOH rapidly reacts with lipid peroxyl radicals and is generally regarded as the major lipid-soluble antioxidant in human tissues11 and lipoproteins.36 As such, it has received the greatest interest in both biochemical studies on the mechanism of LDL oxidation37,38 and as a supplementary
antioxidant for intervention studies.\textsuperscript{39} Considering this, surprisingly little is known about the concentration and distribution of $\alpha$-TOH in human atherosclerotic lesions. A major finding of the present work is that all lipoprotein density fractions isolated from advanced human plaque contain significant amounts of $\alpha$-TOH (Table 5). In fact, when expressed per protein or Ch18:2, the content of $\alpha$-TOH was increased in LDF (which carried most plaque lipid) versus plasma LDL, fully supporting the previously reported presence of relatively large amounts of this antioxidant in homogenates of human plaque.\textsuperscript{7} However, LDF’s content of $\alpha$-TOH was decreased when expressed per FC, in accord with a claim that $\alpha$-TOH is depleted in human atherosclerotic lesions,\textsuperscript{40} and raising the question of meaningfull expression of the vitamin concentrations.

Regarding the latter, linoleate and cholesterol can peroxidize in a free radical chain reaction; however, the chemical oxidizability of the former\textsuperscript{41} is $\approx 27$-times higher than that of the latter.\textsuperscript{32} [Chemical oxidizability is defined here as $k_p/(2k_{pe})$, where $k_w$ and $k_{pe}$ are the rate constants of radical chain propagation and termination, respectively.\textsuperscript{42}] The $\approx 20$- to 25-fold higher content of total peroxidized linoleate (assessed as hydroxy- plus o xo-octadecadienoate) than peroxidized cholesterol (assessed as $7\beta$-hydroxy- plus 7-keto-cholesterol) per parent moleule\textsuperscript{43,44} in advanced human plaque closely reflects this difference in chemical reactivity. This indicates that the peroxidation of fatty acids in the intima is mostly nonenzymatic and quantitatively more important than that of cholesterol. In addition, $\alpha$-TOH only moderately inhibits cholesterol peroxidation in model membranes, whereas it efficiently prevents that of linoleate in model membranes,\textsuperscript{42} suggesting that vitamin E primarily protects fatty acids rather than cholesterol from peroxidation. In this context we note that the mass distribution of $\alpha$-TOH in lesion lipoprotein density fractions reflects that of CE, not FC (compare Tables 3 and 5). We therefore conclude that expressing $\alpha$-TOH per Ch18:2 is meaningful, particularly as we have not been able to detect significant amounts of unesterified linoleate (J.M.U., R.S., unpublished data, 1997). Consequently, there is no good evidence for a depletion of $\alpha$-TOH in any of the lipoprotein density fractions isolated from advanced human plaque, corroborating our previous observation that plasma $\alpha$-TOH is also not depleted in patients suffering from severe atherosclerosis.\textsuperscript{9}

Another major finding of the present study is that all lipoprotein density fractions of advanced lesions contain considerable yet comparable amounts of the oxidized forms of Ch18:2. Our results therefore provide a rationale for the examination of biological properties of oxidized lipoproteins other than LDL. We expect\textsuperscript{15} that cholesteryl o xo-linoleates, which we did not measure here, are present at comparable levels with Ch18:2-OH, so that overall some 4% to 9% of Ch18:2 in all density fractions of advanced lesions contain an oxidized fatty acyl chain. This value is lower than that observed in our previous study, probably because of the large variation in Ch18:2-OH content noted (1.7 to 48.7 mol%).\textsuperscript{7} However, the present value of oxidized linoleate to Ch18:2 is comparable with that in saponified lesion samples,\textsuperscript{45} indicating that most peroxidized fatty acids are esterified. Indeed, human lesions contain little unesterified fatty acids,\textsuperscript{46} and we have failed to detect substantial amounts of hydro(per-oxyoctadecanoate in unsaponified homogenates of advanced human lesions (J.M.U., R.S., unpublished data, 1997).

Although most interest has focused on oxidized LDL, we are unaware of evidence for intimal lipoprotein oxidation being specific for this or any other lipid/protein particle. In fact, from experiments with plasma\textsuperscript{46} and lipoprotein mixtures\textsuperscript{47} one would predict that lipids in intimal HDL and VLDL are just as “oxidizable” as those in intimal LDL, unless oxidant(s) specifically associates with the latter. Also, oxidized CE’s (and probably phospholipids) exchange between lipoproteins, and this is enhanced by CE transfer protein.\textsuperscript{48} As this exchange occurs within hours, yet lipoproteins reside in the intima for days,\textsuperscript{49} the simplest explanation for the observed comparable extent of Ch18:2 oxidation between all density fractions (Table 4) is that in advanced plaque, oxidized lipids are largely equilibrated, independent of where they are formed initially. Our results using radiolabeled LDL and HDL added to minced plaque exclude the possibility that such equilibration was the result of sample workup. Thus, several interesting questions are suggested, such as the effect of this on antioxidant strategy, proatherogenic activities of oxidized lipoproteins other than LDL, and the effect of oxidation on HDL’s ability to catalyze the elimination of (oxidized) intimal lipids.\textsuperscript{48}

Perhaps the most striking observation presented here is the coexistence of relatively large amounts of $\alpha$-TOH and oxidized lipids in all lesion density fractions (Table 5). This seems to contradict the commonly held view of action of vitamin E in LDL and other lipoproteins as chain-breaking antioxidants.\textsuperscript{37} However, it is becoming increasingly recognized that lipoprotein lipid peroxidation resembles emulsion polymerization,\textsuperscript{30} and that 1-electron oxidants can cause the peroxidation of a substantial proportion of lipids in isolated lipoproteins despite the presence of vitamin E (for review, see Reference 51). Although the present results are consistent with intimal lipid peroxidation proceeding via such tocopherol-mediated peroxidation,\textsuperscript{38} they do not provide direct evidence for its occurrence. As discussed previously in more detail,\textsuperscript{7} several issues must be considered. For example, we cannot exclude that the plaque density fractions analyzed contain a mixture of $\alpha$-TOH–depleted and $\alpha$-TOH–containing particles with and without oxidized lipids, or that formation of oxidized lipids occurred in the absence of $\alpha$-TOH and that the vitamin became replenished subsequently. However, in accordance with the above support for oxidized lipids, the comparatively more polar vitamin E can reasonably be expected to “equilibrate” between different intimal particles within the time frame of intimal residence time of lipoproteins. This, together with the high ratio of $\alpha$-TOH to CE’s and the large mean size of plaque lipoproteins, challenges the idea of the presence of $\alpha$-TOH–depleted particles, unless “micro-environments” are postulated where vitamin E becomes depleted. However, at present, there is no direct evidence for such microenvironments. Also, we are unaware of a precedent where vitamin E becomes replenished in vivo after its complete oxidation. In any case, this would probably be associated with undesirable additional lipid deposition, as the major carriers of vitamin E are lipoproteins.

Whether lipid peroxidation takes place in the presence or absence of $\alpha$-TOH may be assessed by the distribution of regioisomers of Ch18:2-O(OH). Thus, in humans, vitamin E
is likely the major H-donor capable of directly reacting with lipid peroxyl radicals, so that in its presence the kinetically preferred cis,trans Ch18:2-O(OH) isomers are formed, whereas in its absence, the thermodynamically favored trans, trans isomers accumulate.52,55 Preliminary results indicate that in advanced human lesions cis,trans predominate over trans,trans Ch18:2-O(OH) isomers,56 suggesting that most oxidized Ch18:2 detected were indeed formed in the presence of vitamin E.

In conclusion, the present results show that the lipids in all lipoprotein density fractions of advanced lesions are substantially oxidized yet contain normal amounts of α-TOH per readily oxidizable lipids. This, together with our previous mechanistic work on LDL lipid oxidation and the observation that also in the most advanced stages of atherosclerosis plasma vitamin E is not deficient and most intimal lipids seem to become oxidized in the presence of α-TOH, casts doubt on the rationale of dietary supplementation with vitamin E alone as a strategy to prevent intimal lipoprotein oxidation. Although our work confirms that lesion lipids are oxidized, it does not support the commonly presumed, although little supported, notion that the former is a consequence of a lack of vitamin E. Further work is needed to address whether intimal lipoprotein lipid peroxidation could be a result of a dysbalance between α-TOH and available antioxidants54 in favor of a prooxidant activity of the vitamin.

Acknowledgments

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References


Coexistence of Oxidized Lipids and $\alpha$-Tocopherol in All Lipoprotein Density Fractions Isolated From Advanced Human Atherosclerotic Plaques

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