

# Disease Stage-Dependent Accumulation of Lipid and Protein Oxidation Products in Human Atherosclerosis

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**Oxidative modification of low-density lipoprotein is thought to promote arterial lipid accumulation and atherogenesis. Previous studies reported on the presence of certain lipid or protein oxidation products in lesions, although a systematic investigation measuring several oxidation parameters and the accumulation of nonoxidized lipids and antioxidants at various stages of atherosclerosis has not been performed in the same tissue. Using the intimal lipoprotein-containing fraction of human aortic lesions, we demonstrate here that cholesterol accumulated with lesion development and that this increase was already significant at the fatty streak stage. By comparison, cholesterylestes increased significantly only in fibrofatty and more complex lesions that also contained significantly increased amounts of cholesterylester hydro(pero)xides and 27-hydroxycholesterol. Cholesterylester hydroxides were the major lipid oxidation product detected. Despite accumulation of oxidized lipid,  $\alpha$ -tocopherol was also present and maintained at a comparable level over the disease process. Of the oxidized protein moieties measured only *o,o*-dityrosine increased with disease, although chlorotyrosines were present at relatively high levels in all lesions compared to healthy vessels. Our data show that accumulation of nonoxidized lipid precedes that of oxidized lipid in human aortic lesions. (*Am J Pathol* 2002, 160:701–710)**

The development of atherosclerosis to clinical manifestations is a protracted process that spans several decades. It is well established that lipids, specifically cholesterol, accumulate as the disease progresses<sup>1</sup> and that these lipids are derived from plasma low-density lipoprotein (LDL). Aberrant uptake of LDL and its associated lipids

by intimal monocytes that have infiltrated from the blood is believed to generate foam cells and thereby initiate atherosclerosis.<sup>2</sup> Foam cells are the hallmark of the fatty streak/early lesion from which the more advanced and complicated lesions derive. In advanced lesions, a fibrous cap is formed from collagen and a lipid-rich necrotic core. Vessel lumen narrowing because of the enlarged fibrous cap and plaque rupture lead to clinical manifestations.

The definitive mechanisms leading to the initiation and development of atherosclerosis are unknown. However, retention of LDL in the vessel wall because of interaction and complex formation with extracellular matrix components is well-documented<sup>3</sup> and is thought to be important. Further, a bulk of evidence suggests that oxidative modifications to LDL contribute to its retention.<sup>4–6</sup> Both arterial wall retention and oxidative modification of LDL are thought to contribute to foam cell formation.

*In vitro* studies with plasma LDL have shown that oxidative modifications proceed in different stages and involve changes to LDL's antioxidants, lipids, and protein components. The nature and extent of these alterations vary depending on the type of oxidant involved, the oxidant to lipoprotein ratio used, and the extent to which oxidation is allowed to proceed. Originally<sup>4</sup> the pathway was described by the sequential depletion of endogenous antioxidants including  $\alpha$ -tocopherol ( $\alpha$ -TOH), followed by the formation of lipid hydroperoxides and their fragmentation to aldehydes and other reactive secondary products of lipid oxidation. The latter can modify apolipoprotein B-100 so that the altered particle becomes a ligand for the scavenger receptor,<sup>7</sup> and *in vitro* exposure of macrophages to such oxidized LDL can lead to foam cell formation.<sup>8</sup> More recently, it has become apparent that not all oxidants convert the lipoprotein along the above pathway. Thus, hypochlorite (a two-electron oxidant produced by myeloperoxidase) generates a high-uptake LDL by primarily oxidizing the protein component, although some consumption of  $\alpha$ -TOH and formation of lipid hydroperoxides is always observed.<sup>9</sup> The fact that

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**Table 1.** Comparison of Macroscopic and Histology Classification of Aortic Lesions

Stary classification group	Intima, <i>n</i>	Age range, years	Macroscopic assessment	Histology versus macroscopic assessment (% agreement)
I-II	24	20-64	No/initial lesions	88
II-III	24	20-66	Fatty streak lesions	79
IV-V	18	35-66	Fibro-fatty lesions	83
V-VI	11	35-66	Ulcerated/complex lesions	91

oxidized high-uptake LDL can be formed via different pathways raises questions of their relative importance and, hence, the extent of lipid *versus* protein modification during the oxidative modification of LDL *in vivo*.

Whether *in vivo* LDL lipid peroxidation proceeds beyond the depletion of  $\alpha$ -TOH, LDL's major antioxidant, is unclear. This is an important issue, as secondary lipid oxidation products including aldehydes are not formed to any substantial extent in the presence of the vitamin.<sup>10</sup> Low  $\alpha$ -TOH to cholesterol content has been reported in advanced lesions,<sup>11</sup> although our earlier studies with advanced human lesions showed normal levels of  $\alpha$ -TOH per cholesteryl ester (CE).<sup>12,13</sup> Expressing  $\alpha$ -TOH per CE is more appropriate as the vitamin scavenges peroxy radicals produced during the peroxidation of fatty acids containing bisallylic hydrogen;<sup>14</sup> CE represent the major source of such fatty acids in lipoproteins and lesions, whereas cholesterol does not contain bisallylic hydrogen.

The mechanisms responsible for oxidation of LDL within the vessel wall remain undefined. Several oxidants have been proposed to be involved, including macrophages, 15-lipoxygenase, myeloperoxidase and oxidants derived from its action, reactive nitrogen metabolites, NAD(P)H oxidases, and transition metals.<sup>15</sup> Unlike the situation with atherosclerosis in animals in which a gene knock-out approach can be used, much of the evidence supporting the involvement of the different oxidants in human atherosclerosis is derived from the detection of generic or specific oxidation products in different types of lesion materials. A problem with this approach has been that to date, the disease stage-dependent accumulation of several different lipid and protein oxidation products has not been studied systematically in the same tissue samples and together with the accumulation of nonoxidized lipids. We therefore examined various parameters that reflect the oxidation status of both lipid (CE, cholesterol) and protein residues (tyrosine (Tyr), phenylalanine (Phe), and the content of lipophilic antioxidants ( $\alpha$ -TOH, ubiquinol-10) in the intimal lipoprotein-containing fraction of human aortic lesions spanning atherosclerotic disease development.

## Materials and Methods

### Materials

Cholesteryl linoleate (C18:2), cholesteryl arachidonate (together referred to as CE), and coenzyme Q<sub>9</sub> (ubiquinone-9) were purchased from Sigma Chemical Co. (St. Louis, MO). D,L- $\alpha$ -TOH was purchased from Eastman-

Kodak (Rochester, NY). [<sup>3</sup>H]C18:2 [cholesteryl-1,2,6,7-<sup>3</sup>H(N)], 91.5 Ci/mmol, was from Dupont-NEN (Boston, MA). (25R)-Cholest-5-en-3 $\beta$ -26-diol (27HC) was from Research Plus Inc. (Bayonne, NJ) and cholest-5-en-3 $\beta$ -ol-7-one (7KC) was from Steraloids Inc. (Wilton, NH). Organic solvents of HPLC quality were obtained from Mallinckrodt (Clayton, Australia) and Merck (EM Science, Gibbstown, NJ). Before use, all aqueous solutions were stored over Chelex-100 (Bio-Rad Laboratories, Richmond, CA) to remove contaminating transition metals. All other chemicals were of the highest available purity and nanopure water (Millipore Systems, Australia) was used throughout.

### Specimens

Postmortem materials were obtained from 20- to 66-year-old patients from the NSW Institute of Forensic Medicine, Sydney, Australia, with the approval of the local Human Ethics Review Committee. Aorta were resected within 24 hours of death and washed in ice-cold 50 mmol/L phosphate buffer containing 3-aminotriazole (10 mmol/L), diethylenetriamine pentaacetic acid (0.1 mmol/L), and butylated hydroxytoluene (20  $\mu$ mol/L), pH 7.4 (buffer A). Aortas were stored in buffer A at -80°C.

### Lesion Classification

Lesion stages were initially classified macroscopically according to Stary and colleague's<sup>16</sup> nomenclature and definition. The descending thoracic and abdominal aorta from a total of 35 patients was resected and designated into four distinct groups; initial lesions (Stary classes I-II), fatty streak lesions (II-III), fibro-fatty lesions (IV-V), and complex/ulcerated lesions (V-VI), representing the spectrum of atherosclerosis. A tissue section from each specimen was removed and fixed in 10% formalin in phosphate-buffered saline and transferred to 70% (v/v) ethanol for histology, using hematoxylin and eosin staining (Table 1). Remaining aortas were then stored in buffer A at -80°C until used.

### Sample Preparation

Sample preparation was essentially as described.<sup>17</sup> Briefly separated intima was weighed and tissue samples from the same group pooled to yield ~10 g. Each 10-g pool represented a single sample (*n* = 1) for analysis. Intimas were frozen in liquid N<sub>2</sub>, pulverized, and recon-

stituted in 50 mL of buffer B [10 mmol/L phosphate/0.15 mol/L NaCl, pH 7.4, containing 0.3 mmol/L ethylenediaminetetraacetic acid, 0.1 mmol/L diethylenetriamine pentaacetic acid, 10 mmol/L 3-aminotriazole, 20  $\mu$ mol/L butylated hydroxytoluene, 1 mmol/L phenylmethyl sulfonyl fluoride, 0.002% (w/v) elastatinal, 2 mmol/L benzamidine, 1  $\mu$ mol/L D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone, 0.008% (w/v) gentamicin, 0.008% chloramphenicol, and 10  $\mu$ mol/L reduced glutathione]. Ubiquinol-9 (5  $\mu$ mol/L) prepared from ubiquinone-9,<sup>18</sup> and  $\alpha$ -tocotrienol (1  $\mu$ mol/L<sup>12</sup>) were also added as internal standards for estimation of recoveries during the processing steps. The samples were gently agitated end-over-end overnight in the dark at 4°C and then centrifuged at 2750  $\times$  *g* for 30 minutes at 4°C in a swing-bucket centrifuge. The resulting tissue supernate (SN-1) was further ultracentrifuged at 100,000  $\times$  *g* for 30 minutes at 4°C, the pellet and the top lipemic layer discarded, and a tissue supernate (SN-2) collected for subsequent analyses. SN-2 contains all lesion lipoproteins as it represents the starting material for sequential floatation ultracentrifugation.<sup>17</sup>

Aortic lesion SN-2 samples (<500  $\mu$ L) were added to hexane:methanol (5:1, v/v) for extraction of CE hydro(pero)xides (CE-O(O)H), cholesterol, CE, and  $\alpha$ -TOH and the hexane phase reconstituted in propan-2-ol and subjected to HPLC as described previously.<sup>19,20</sup> For oxysterol analyses aliquots (500  $\mu$ L of SN-2) were extracted with chloroform:methanol (2:1, v/v) with added 19-hydroxycholesterol as an internal standard (5  $\mu$ g/tube). The chloroform phase was evaporated and transferred with diethyl ether (2.5 mL) to a screw-cap tube before cold alkaline saponification. Total cholesterol was also determined in separate aliquots (50  $\mu$ L) after saponification initiated by a methanolic KOH solution (20% w/v, 2.0 mL). Cholesteryl propyl ether was used as an internal standard (5  $\mu$ g/tube). The tubes were flushed with argon and the contents mixed overnight at 4°C before water (2.0 mL) and hexane (2.5 mL) were added. The tubes were then mixed for 30 seconds and centrifuged at 1600  $\times$  *g* for 5 minutes at 10°C. Oxysterol and cholesterol in the ether/hexane phase were then determined.

To account for inadvertent oxidation occurring during aortic lesion work-up, [<sup>3</sup>H]C18:2 (50  $\mu$ Ci) was added to four separate aortic (~1 g wet weight) lesions. The lesions were subjected to the tissue and analyte extraction processes described above in buffer B (5 mL). Radiolabeled C18:2 and/or C18:2-O(O)H fractions were identified by RP HPLC coupled with on-line radiometric detection.

The hydroxylation product of Tyr (3,4-dihydroxyphenylalanine; DOPA) and those of Phe (*o*-Tyr, *m*-Tyr) and *o,o*-dityrosine (diTyr) in aortic tissue were determined as described previously.<sup>21</sup> Briefly SN-2 aliquots (900  $\mu$ L) were mixed with sodium deoxycholate (100  $\mu$ L, 0.3% w/v) and trichloroacetic acid (100  $\mu$ L, 50% v/v). The delipidated protein samples were then freeze-dried and subjected to gas phase, acid-catalyzed hydrolysis using 6 N HCl containing mercaptoacetic acid (5% v/v) and phenol (1% v/v) under anaerobic conditions. Freeze-dried hydrolysates were reconstituted in water (100  $\mu$ L) and filtered (0.22  $\mu$ m) before HPLC.<sup>21</sup>

## HPLC Analyses

CE-O(O)H, cholesterol, CE, and  $\alpha$ -TOH in lesion supernates were quantified using RP HPLC coupled with UV, electrochemical, and postcolumn chemiluminescence detection as described.<sup>19,20</sup> For detection of radiolabeled compounds the above HPLC conditions were combined with on-line radiometric detection and a scintillant-to-flow ratio of 1.

Oxysterols were separated by normal phase HPLC using an Alltima Silica column (0.46  $\times$  15 cm; 100 Å, 5  $\mu$ m) with a 3-cm guard column (Ultremex, 3- $\mu$ m particle size; Phenomenex, Thornleigh, Australia) and eluted with hexane/propan-2-ol/acetonitrile (94.8/4.6/0.6, v/v/v) at 1 mL/min. The mobile phase was sparged with helium during analysis and lipids detected at 210 and 234 nm. Total cholesterol was determined by RP HPLC using propan-2-ol:acetonitrile: water (54:44:2, v/v/v). All chromatography assignment and quantification of individual peaks was based on co-elution and area comparison with authentic standards.

DOPA, *m*-Tyr, *o*-Tyr, and diTyr in hydrolysates were isolated on a Zorbax ODS column at 1 mL/min elution and a gradient of solvent A (100 mmol/L sodium perchlorate in 10 mmol/L sodium phosphate buffer, pH 2.5) and solvent B (80% methanol in water) as described.<sup>22</sup> The eluant was monitored by in-series UV (280 nm) and fluorescence detection (280 nm excitation for all components and emission wavelength of 320 nm for DOPA, *m*-Tyr and *o*-Tyr and 410 nm for diTyr). The identity and quantity of representative samples were checked by electrochemical detection<sup>23</sup> and mass spectroscopy.<sup>21,22</sup> Artifacts formation of *m*-Tyr, *o*-Tyr, DOPA, and diTyr during protein hydrolysis of atherosclerotic tissue using the methodology described herein has been investigated in detail.<sup>21,23,24</sup> The maximum artifact formation of the analytes determined by this method consistently ranges from 20  $\mu$ mol/mol of *m*-Tyr, *o*-Tyr, and diTyr to 200  $\mu$ mol/mol of DOPA per parent amino acid.

## Gas Chromatography/Mass Spectroscopy (GC/MS) Determination of Chlorinated Tyrosine

3-Chlorotyrosine (ClTyr) and 3,5-dichlorotyrosine (diClTyr) were determined by stable isotopic dilution GC/MS as described.<sup>25</sup> Briefly, samples were dried and proteins hydrolyzed using methanesulfonic acid (4 mol/L) containing 1% (v/v) phenol (100  $\mu$ L). The hydrolysate was diluted in 0.1% trifluoroacetic anhydride (200  $\mu$ L) and purified using solid phase extraction. Tyr, ClTyr, and diClTyr were eluted using 80% methanol containing 0.1% trifluoroacetic anhydride (1.1 mL) and dried. Amino acids were derivatized with *n*-propanol/HBr (3.5 mol/L) (300  $\mu$ L) and then with trifluoroacetic anhydride/ethyl acetate (1:4, v/v) (100  $\mu$ L). Derivatized samples were finally diluted in ethyl acetate (300  $\mu$ L) before GC/MS analysis. The initial column temperature of 65°C was maintained for 2 minutes and then increased to 150°C (30°C/minute), 170°C (4°C/minute), 200°C (5°C/minute), and to 260°C (30°C/minute). The injector, auxiliary channel, and ion source

**Table 2.** Protein-Standardized Unmodified and Oxidized Lipid Contents in Aortic Lesions

Lesion group, <i>n</i>	Protein	Chol	CE	CE-OOH	CE-O(O)H	27HC	7KC
I-II (4)	1.51 (0.16)	10.6 (1.2)	13.1 (3.4)	ND	0.10 (0.03)	0.02 (0.02)	0.03 (0.01)
II-III (8)	1.43 (0.31)	28.6* (7.1)	34.2 (12.6)	ND	0.18 (0.05)	0.29 (0.12)	0.05 (0.01)
IV-V (9)	1.44 (0.13)	79.9* (19.7)	69.7* (18.6)	20.5 (5.1)	0.82* (0.30)	0.64* (0.08)	0.09 (0.02)
V-VI (7)	1.49 (0.62)	92.5* (29.3)	43.6 (14.9)	14.0 (5.9)	1.00* (0.31)	0.96* (0.13)	0.14* (0.03)

The protein, lipid, and oxidized lipid content in the lipoprotein-containing tissue supernate (SN-2) derived from the intima of aortic lesions were determined as described in Materials and Methods. Each *n* value represented a tissue pool (10 g) derived from up to six pieces of intima obtained from different subjects and overall a total of 35 human aortas were used. The data represent mean values (SEM) expressed as nmol/mg protein for all analytes except protein (mg/ml) and CE-OOH (pmol/mg). ND, none detected.

\**P* < 0.05 compared with group I-II.

temperatures were set to 250, 280, and 150°C, respectively. Pulsed splitless injection was used with selective ion monitoring. The background levels of diCITyr and diTyr in bovine serum albumin determined by this method range from 15 to 40 μmol/mol.<sup>25</sup>

### Statistical Analysis

Kruskall-Wallis one-way analysis of variance on ranks and Mann-Whitney *t*-tests were used to evaluate significant differences at a *P* value 0.05 (two-tailed).

### Results

We have determined previously the extent of lipid accumulation, lipid oxidation, and antioxidant status in advanced human atherosclerotic lesions, and have shown that α-TOH is not extensively depleted despite the coexistence of oxidized CE.<sup>12,13</sup> Herein we report on the characterization of antioxidants, lipid, oxidized lipid, and proteins from the intimal lipoprotein fraction of human aorta representing the earlier spectrum of the disease process.

Aortic intimal tissue was designated into four groups based on disease status according to Sary and colleagues:<sup>16</sup> I-II, initial lesions; II-III, fatty streaks; IV-V, fibro-fatty lesions; and V-VI, ulcerated/complex lesions (Table 1). A total of 35 aortas were used, and all materials were obtained from patients ≥20 years old. Thereby the material in group I-II contained intima with diffuse thickening and initial lesions and is distinct from fatty streak lesions and normal artery tissue.<sup>16</sup> Consequently a large number (*n* = 24, Table 1) of individual tissue samples representing stage I-II were required to generate four separate pools of tissue for analysis. Macroscopic assessment enabled processing of the specimens before histology thereby averting a freeze/thaw cycle and potential generation of artifacts. Although visual assessment does not reflect intimal thickness we obtained >80% agreement (Table 1), demonstrating that visual classification was useful and appropriate.

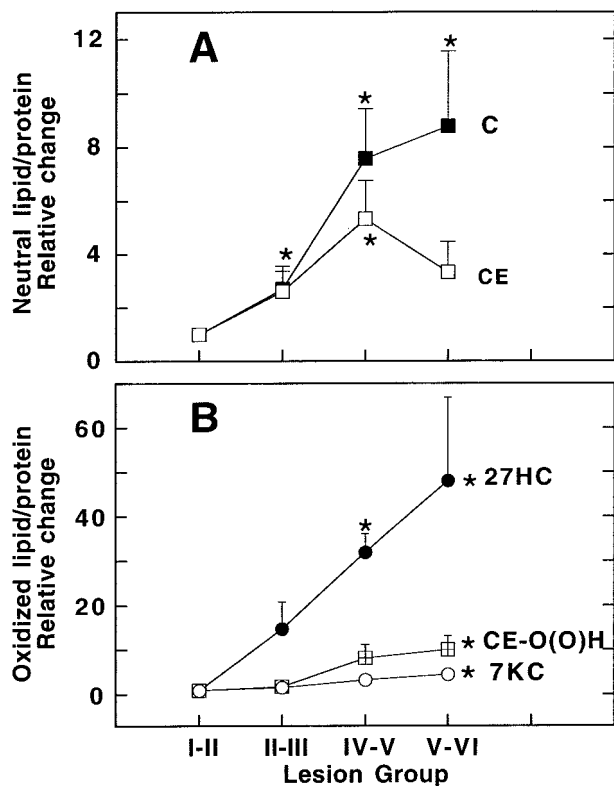
After low-temperature pulverization of the intima, the reconstituted tissue (SN-1) was subjected to centrifugation and removal of a lipemic layer to yield a tissue supernate designated SN-2. This supernate contains all tissue lipoproteins and thereby all analytes reported below reflect lipoprotein-associated parameters. Next we investigated the potential autoxidation of lipid (C18:2)

and recovery of the internal standard, ubiquinol-9 to assess artifactual oxidation during sample work-up. Overall, tests with [<sup>3</sup>H]C18:2 (see Materials and Methods) indicated that the radiolabel eluted as unmodified C18:2 (*n* = 4), whereas on average, 60% (mean, *n* = 4) of added ubiquinol-9 was recovered as total CoQ<sub>9</sub> in SN-2 (data not shown). The contents of lipid, both unmodified and oxidized, lipid-soluble antioxidants, and protein oxidation markers in SN-2 from the various lesions were then determined to compare relative changes in lipid, protein, and oxidation parameters in the disease process more systematically and comprehensively than reported previously.

An increase in cholesterol is a prominent early disease marker.<sup>1</sup> In agreement, the content of cholesterol per protein increased with increasing lesion severity, with significant changes noted in the early disease stages (Table 2). Lipoprotein-associated CE (the sum of cholesteryl arachidonate and C18:2) also accumulated (Table 2), although significant changes were not obtained until fibro-fatty lesions were present. The level of CE per cholesterol in lesion lipoproteins decreased in the most advanced lesions (V-VI).

In addition to increasing content of unmodified lipid, an accumulation of lipoprotein lipid oxidation products was also found with increasing disease severity. CE-containing polyunsaturated lipids are the primary targets for one-electron mediated oxidation, and oxidized CEs are found in lesions<sup>12,13,26,27</sup> but are essentially absent in plasma LDL.<sup>13</sup> CE hydroperoxides (CE-OOH) were not detected in early lesions, ie, stages I-III (Table 2). However, they were detected in fibro-fatty and advanced lesions, albeit at much lower amounts (<2.5%) than the sum of CE hydroperoxides and hydroxides (CE-O(O)H) (Table 2) indicating that CE hydroxides (ie, CE-OH) were the major product. Similar to hydroperoxides, CE-O(O)H were significantly increased only in fibro-fatty and more complex lesions (≥stage IV) (Table 2). We also measured 7KC as a marker of one-electron-mediated cholesterol oxidation and 27HC, an enzymatic product of the mitochondrial cytochrome P450 enzyme, sterol 27-hydroxylase. Of these, 27HC was present at higher levels than 7KC (Table 2). Whereas 7KC content increased significantly only in the most complex lesions (group V-VI), disease stage-dependent increases in 27HC were significant in fibro-fatty lesions (Table 2).

To assess the dependency of the changes in nonoxidized and oxidized lipids on lesion development, we

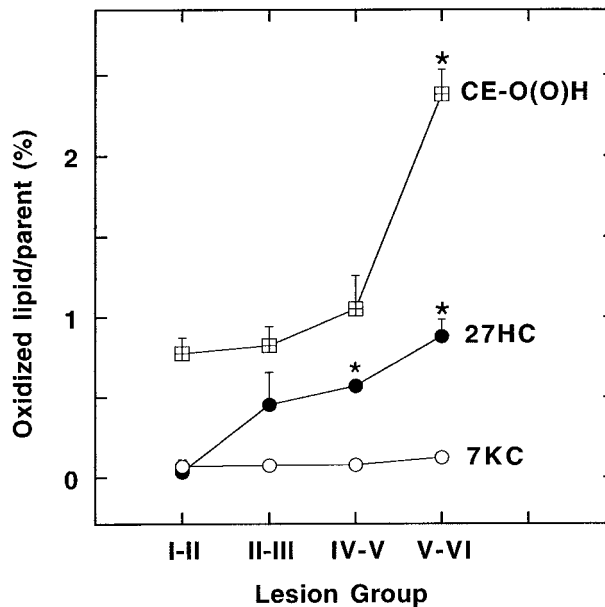


**Figure 1.** Disease-stage-dependent changes in protein-standardized lipid and oxidized lipid in aortic lesions. Intimas were processed to yield a tissue supernate (SN-2) that contains all lesion lipoproteins as described in Materials and Methods. The content of both unmodified (A) and oxidized lipid (B) in SN-2 per protein (expressed as nmol/mg) was then determined by HPLC (Table 2). Relative changes in protein-standardized cholesterol (C, ■) and CE (□) CE-O(O)H (●), 27HC (○), and 7KC (μ) were expressed as a fraction of those observed in tissue containing no/initial lesions (group I-II). The data represent mean values ± SEM with asterisks indicating  $P < 0.05$  compared with group I-II.

expressed the content of each type of lipid relative to the corresponding parent molecule in the intima showing the least disease, ie, stage I-II (Figure 1). Thus, cholesterol increased up to eightfold with lesion development (Figure 1A). Also, the relative changes in CE were similar to cholesterol except in the most complex lesions where CE markedly decreased. Similar to unmodified lipid, lipoprotein-associated CE-O(O)H, 27HC, and 7KC increased significantly with lesion severity (Figure 1B).

To distinguish the above changes in lipid load from the proportion of lipids oxidized, we next expressed the different oxidation products per respective parent molecule in a disease stage-dependent manner (Figure 2). The amount of CE-O(O)H per CE increased significantly in advanced lesion stages (V-VI) and comprised 2.4% of the parent molecule (Figure 2). Compared to CE-O(O)H however, the percentage of 27HC per parent molecule comprised 0.9% even at the most advances stages of the disease. Furthermore, the intimal content of 7K per total cholesterol did not increase significantly (Figure 2).

Despite the relatively large content of oxidized lipid, the lipid-soluble antioxidants  $\alpha$ -TOH and ubiquinol-10 (measured as CoQ<sub>10</sub>) remained detectable at each disease stage (Table 3). In fact, the intimal content of  $\alpha$ -TOH expressed per protein was similar for most lesions except



**Figure 2.** Content of parent molecule-standardized oxidized lipids in lesions. The extent to which individual lipids in lesion lipoproteins were oxidized was examined by measuring the content of CE-O(O)H per CE (□) and 27HC (●) and 7KC per total cholesterol (○) (expressed as percent of parent) in the various lesion groups. The data represent mean values ± SEM with asterisks indicating  $P < 0.05$  compared with group I-II.

for a significantly elevated level in stages IV-V (Table 3). In general, the  $\alpha$ -TOH/CE ratio remained constant independent of lesion severity (Table 3). CoQ<sub>10</sub>, the reduced form of which is an effective endogenous antioxidant for LDL lipid,<sup>28</sup> was present at 2 to 5 times lower concentrations than  $\alpha$ -TOH and the levels of this antioxidant did not seem to alter with disease progression (Table 3).

In addition to lipid, protein amino acid moieties may be susceptible to oxidative attack in the intima. Reaction of Tyr and Phe with hydroxyl radicals yields DOPA and *o*-Tyr, *m*-Tyr, and Tyr radical formation can generate diTyr. In addition, myeloperoxidase generates ClTyr, diClTyr, or diTyr and there is substantial evidence to suggest participation of myeloperoxidase during atherogenesis.<sup>15</sup> In contrast to nonoxidized and oxidized lipid few of the protein oxidation markers determined in this study varied significantly between lesion groups when standardized to total protein (Table 4) or to parent molecule (Figure 3). A notable exception was diTyr that increased significantly in the most advanced lesions (Table 4 and Figure 3).

### Discussion

We have investigated biochemical changes in human aortas associated with atherogenesis by analyzing lipid, protein, and antioxidant contents and by measuring the accumulation of specifically oxidized moieties in the lipoprotein fraction prepared from aortic intimal lesions from early- to end-stage disease. Consistent with the notion that lipid accumulation increases with lesion severity we observed cholesterol to accumulate with advancing disease and this increase was already signifi-

**Table 3.** Disease Stage-Associated Changes in Lipid-Soluble Antioxidant Content in Aortic Lesions

Lesion stage	$\alpha$ -TOH	CoQ <sub>10</sub>	$\alpha$ -TOH/CE	$\alpha$ -TOH/cholesterol
I-II	53 (31)	33 (15)	4.1 (1.5)	4.8 (2.2)
II-III	142 (79)	45 (19)	5.3 (0.9)	4.9 (0.8)
IV-V	347 (86)*	75 (21)	7.3 (2.5)	5.4 (1.6)
V-VI	148 (81)	78 (33)	3.5 (1.5)	2.0 (0.9)

$\alpha$ -TOH and CoQ<sub>10</sub> content in the lipoprotein-containing tissue supernate (SN-2) derived from aortic lesions were determined by HPLC as described in Materials and Methods. The data represent mean values  $\pm$  (SEM) and are expressed as pmol/mg protein or mmol/mol CE or cholesterol.

\**P* < 0.05 compared with group I-II.

cant in the earliest stages, ie, fatty streaks. By comparison, CEs were significantly increased only in fibro-fatty and more complex lesions that also contained higher concentrations of protein-standardized CE-O(O)H and the enzymatic oxysterol, 27HC. The accumulation of cholesterol and CE preceded the increases in the corresponding oxidation products 27HC and CE-O(O)H, with the latter representing the major lipid peroxidation product detected. Together, these results suggest that in human atherosclerosis significant accumulation in primary lipoprotein lipid (per)oxidation products does not precede the accumulation of nonoxidized lipid.

According to the LDL oxidation theory of atherosclerosis,<sup>4</sup> oxidation of LDL occurs early in the disease process and precedes foam cell accumulation and fatty streak formation. Despite the popularity of the theory, the accumulation of native and oxidized lipid and protein in relation to disease stage is rarely quantified and compared. Rather, single oxidation parameters are commonly determined at only a single disease stage or, alternatively, lesion parameters are compared to the concentration of total plasma or LDL cholesterol. We therefore compared the relative changes in protein and selected lipids that represent the major oxidizable components of plasma lipoproteins, and oxidation parameters of both in the intima of human aortic tissue displaying lesions of increasing severity. Importantly, tissue was resected within 24 hours of death. Within this time frame there are no significant changes to the biochemical composition of arterial tissue and the contents of free and esterified cholesterol, and other lipids and protein remain stable.<sup>29</sup> In addition, the levels of CE-O(O)H per parent detected in the most severe aortic lesions (2.4%) were less than that observed for carotid lesions (3.5%) processed freshly within 2 hours of resection.<sup>13</sup> Further, we compared analytes from various grades of lesion derived from the same aortic tissue sample and verified that artifactual oxidation of CE did not occur during sample processing. As CEs are more susceptible to oxidation than cholesterol, Tyr

and Phe, the results obtained therefore likely represent the situation *in vivo*.

LDL lipid accumulation is a prominent and early marker for atherosclerosis. Consistent with this, the content of cholesterol significantly and linearly increased with disease severity (Table 2). As expected, the CE content also increased with increasing lesion severity, although the CE load per cholesterol in lesion lipoproteins tended to decrease in the most advanced lesions. This is consistent with an earlier report showing a decrease in C18:2 in fibrous plaques,<sup>30</sup> as CE measured herein represents the sum of C18:2 and cholesteryl arachidonate. This suggests increased hydrolysis of lipoprotein CE in advanced lesions and/or preferential loss of CE rather than cholesterol from lipoproteins.

We selected CE-O(O)H and 7KC as markers of non-enzymatic, free radical-mediated lipid (per)oxidation. The former are the major lipid oxidation products formed when lipoproteins undergo *in vitro* oxidation and contribute to the *in vitro* formation of high-uptake LDL.<sup>7</sup> In addition, CE-O(O)H are the major lipid oxidation products in human lesions<sup>1,12,26,31</sup> and hydroxylated fatty acids are associated with plaque instability.<sup>31</sup> Notably, CE-O(O)H and oxysterols are not found in normal iliac arteries<sup>12,32</sup> or in circulating plasma lipoproteins.<sup>13</sup> Our data show a disease stage-dependent increase in intimal CE-O(O)H (Table 2) suggesting that overall CE-O(O)H is a suitable parameter to monitor the extent of lipoprotein lipid oxidation in the vessel wall.

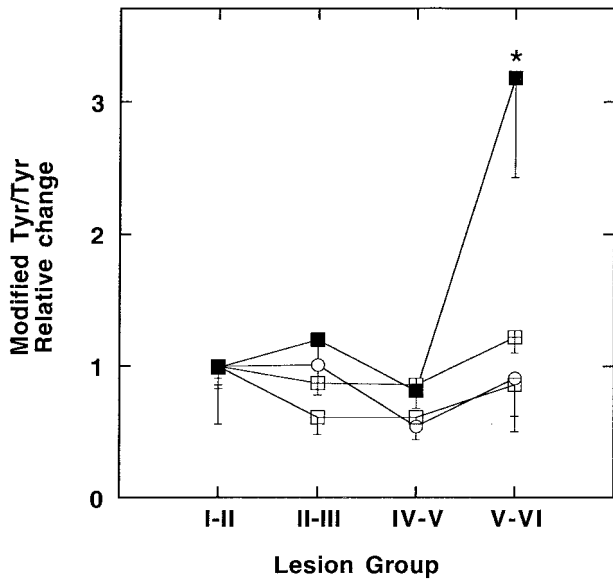
As lesion severity increased, the percentage of lipoprotein-associated CE present as CE-O(O)H increased to a maximum of 2.3%. Although significantly greater than any other oxidation parameter determined here, this value is at the low end of the range of values reported previously for carotid plaque samples.<sup>12,13</sup> The configurational isomer distribution of C18:2-OH (JM Upston, AC Terentis, R Stocker, unpublished results) indicate that particularly in the early stages of atherosclerosis, the majority of CE-O(O)H detected were formed in the

**Table 4.** Protein-Standardized Oxidized Protein Moieties in Aortic Lesions

Lesion stage, <i>n</i>	Tyr	DOPA	<i>m</i> -Tyr	<i>o</i> -Tyr	diTyr	CI Tyr	diCI Tyr
I-II (4)	288 (39)	216 (58)	37 (21)	53 (11)	21 (1)	170 (30)	110 (73)
II-III (8)	426 (88)	261 (46)	37 (16)	74 (16)	34 (9)	178 (26)	56 (14)
IV-V (9)	231 (27)	150 (32)	26 (8)	45 (9)	15 (4)	114 (18)	34 (6)
V-VI (7)	204 (12)	174 (18)	25 (4)	53 (7)	42 (8)*	174 (45)	87 (53)

Proteins in SN-2 were delipidated, hydrolyzed, and derivatized as described in Materials and Methods. Oxidized Tyr and Phe moieties were determined using HPLC with electrochemical detection (DOPA, *o*-Tyr, *m*-Tyr, and diTyr) or GC/MS (CI Tyr and diCI Tyr). Phe:Tyr was 0.74 (0.05) and did not vary between lesion groups. The data represent mean values (SEM) in pmol/mg except Tyr (nmol/mg).

\**P* < 0.05 compared with group I-II.



**Figure 3.** Relative changes in modified tyrosine residues in aortic intima. The type and extent to which Tyr residues were modified in intimal tissue was examined by measuring DOPA (■), diTyr (○), ClTyr (□) and diClTyr (◇). The content of oxidized Tyr residues, calculated per parent molecule, was expressed as a fraction of those observed in lesion group I-II. The data represent mean values  $\pm$  SEM with asterisks indicating  $P < 0.05$  compared with group I-II.

presence of  $\alpha$ -TOH. Indeed, we show herein that  $\alpha$ -TOH did not decrease with advancing atherosclerosis, consistent with our previous data<sup>12,13</sup> and a separate detailed GC/MS-based study of  $\alpha$ -TOH oxidation products in human lesion homogenates.<sup>33</sup> The notion that oxidation of lipoprotein lipid in the vessel wall proceeds via tocopherol-mediated peroxidation<sup>34,35</sup> suggests a likely mechanism for the formation of most CE-O(O)H in the presence of  $\alpha$ -TOH in lesions.

Our observation that even advanced lesions contain normal  $\alpha$ -TOH and at most 2.3% of its CE oxidized to CE-O(O)H has potentially important implications for several reasons. First, the overall extent of lipid oxidation is substantially less than that needed for the *in vitro* conversion of LDL to a high-uptake form by Cu(II).<sup>10,36</sup> This conclusion is consistent with the finding that the extent of oxidative change to aortic LDL, as assessed by electrophoretic mobility or other physical parameters, is less than that required for scavenger receptor-mediated uptake.<sup>37</sup>

Second, our observation has potential implications for the formation of both minimally modified LDL and isoprostanes. Oxovaleroyl, glutaroyl, and epoxyisopropane (the three active moieties identified in minimally modified LDL generated *in vitro*<sup>38</sup>) and isoprostanes are secondary lipid oxidation products. The formation of isoprostanes is essentially prevented as long as  $\alpha$ -TOH is present.<sup>39</sup> Consistent with this, isopropane levels in atherosclerotic lesions are 20-fold to 300-fold lower per parent molecule than lipid hydroxides.<sup>31</sup> Like isoprostanes, oxovaleroyl and glutaroyl are generated from arachidonic acid-containing phospholipids, most likely via fragmentation of arachidonic acid hydroperoxides, a process that is inhibited strongly in the presence of  $\alpha$ -TOH.<sup>40</sup> Indeed, the

generation of fragments of polyunsaturated fatty acids is almost completely prevented during LDL oxidation as long as  $\alpha$ -TOH remains present.<sup>10</sup> This indicates that the bioactive phospholipids generated during *in vitro* oxidation of LDL and present in minimally modified LDL may not be formed in substantial amounts in lesion lipoproteins the  $\alpha$ -TOH content of which remains essentially intact. Arachidonic acid is also present in cellular phospholipids and detection of its secondary oxidation products in homogenates of lesion material does not, by itself, establish their presence in lipoproteins. In fact, immunohistochemical studies suggest that lesion isoprostanes are predominantly associated with foam cells.<sup>41</sup> Similarly, using EO6, a monoclonal antibody specific for oxovaleroyl-containing phospholipid,<sup>42</sup> Palinski and colleagues<sup>43</sup> showed predominant co-localization of this epitope with foam cells in lesions of cholesterol fed New Zealand White and LDL receptor-deficient rabbits. Additional studies are needed to fully establish the precise extent of oxidation of lesion LDL, whether the oxidized arachidonate moieties detected<sup>41,43</sup> represent cellular membrane lipids (eg, from apoptotic or necrotic cells) or lipids from lipoproteins taken up, and how these oxidation parameters relate to lesion CE-O(O)H.

In contrast to CE-O(O)H, the oxidation of cholesterol was primarily enzymatic. Of the analytes measured, 27HC showed the greatest and earliest relative changes with disease progression (Figure 1), and accounted for 0.9% of total cholesterol (Figure 2). The disease-associated increase in the cholesterol-standardized 27HC content is consistent with a previous report<sup>44</sup> and infers a disease-associated increase in the activity of sterol 27-hydroxylase that co-localizes with macrophages in lesions.<sup>45</sup> Interestingly, the 27-hydroxylase pathway has been suggested to be important in the removal of extrahepatic cholesterol.<sup>46</sup> As the accumulation of cholesterol preceded that of 27HC, the formation of the latter may be interpreted as a physiological (potentially anti-atherogenic) response to cholesterol load.

Similar to 27HC, the presence of increased protein-standardized CE-O(O)H in fibro-fatty lesions may reflect the increased lipid load of that lesion stage and suggests that LDL lipid oxidation follows lipid accumulation in the temporal sequence of atherogenic events. However, it is possible that in the early disease stages lipid oxidation products are effectively metabolized or removed from the artery wall, but that their formation may still contribute to promotion of atherogenesis. Indeed, one study of human fetal fatty streak lesions reported the presence of malondialdehyde- and 4-hydroxynonenal-modified lysine (measured indirectly by immunological techniques) in the absence of monocytes/macrophages.<sup>47</sup> These authors suggested that intimal LDL accumulation and oxidation is an early event that contributes to monocyte recruitment into the vessel wall. To more precisely implicate a causal role of lipid (per)oxidation in atherogenesis the metabolism and/or removal of oxidation products is worthy of further study.

Consistent with our findings, the content of the free radical-derived, cholesterol-standardized 7KC, was similar in human fatty streak and advanced lesions,<sup>48</sup> yet

**Table 5.** Comparison of Modified Tyrosine Levels in Atherosclerosis and Normal Tissue

	DOPA	diTyr	CiTyr
Tissue/fluid			
Plasma LDL	630*	2*	10 <sup>†</sup>
Normal iliac artery	~500 <sup>‡</sup>	~30 <sup>‡</sup>	
Normal aorta		~30 <sup>§</sup>	80 <sup>†</sup>
Initial aortic lesions (I-II)	730 <sup>¶</sup>	70 <sup>¶</sup>	200 <sup>‡</sup>
Carotid and advanced aortic lesions	890 <sup>¶</sup> , ~1000 <sup>‡</sup>	~200 <sup>‡</sup> , 230 <sup>¶</sup>	178 <sup>¶</sup> , 420 <sup>†</sup>

The data represent mean values expressed as  $\mu\text{mol/mol}$  Tyr and are derived from \*, Fu and colleagues<sup>54</sup>; †, Hazen and colleagues<sup>49</sup>; ‡, Fu and colleagues<sup>21</sup>; §, Leeuwenburgh and colleagues<sup>50</sup>; and ¶, this study.

homogenate of endarterectomy plaque contains more 7KC than that of normal arteries.<sup>12,32</sup> A possible explanation for this difference is that we analyzed the lipoprotein fraction rather than the entire intima. Indeed, in pilot studies with the first tissue supernate (SN-1, containing SN-2 plus the lipemic layer; see Materials and Methods) we observed oxysterols and cholesterol-standardized 7KC to increase with increasing disease severity (data not shown). A lower 7KC and 27HC content in the SN-2 fraction compared to SN-1 is consistent with oxysterols being concentrated in the core of lesions, rather than in lipoproteins or the fibrous cap, as has been reported for 27HC (referred to as "26-OH-CHOL" by Garcia-Cruset and colleagues<sup>49</sup>).

Several groups have demonstrated the presence of oxidized protein moieties in atherosclerosis including CiTyr,<sup>50</sup> diTyr,<sup>21,51</sup> DOPA, *m*-Tyr, and *o*-Tyr.<sup>21</sup> However, of the protein oxidation products measured herein only diTyr increased significantly from early to late disease stage (Table 4, Figure 3). These data are consistent with increased diTyr levels found in aortic lesion-derived LDL compared to plasma LDL.<sup>51</sup>

Chlorinated Tyr was determined as both mono- and dichloro forms. CiTyr was present in higher amounts than diCiTyr (Table 4), with levels similar to those previously determined for lesion LDL ( $\sim 300 \mu\text{mol/mol}$  Tyr).<sup>50</sup> Interestingly, the diCiTyr to CiTyr ratio in lesion lipoproteins was high (Table 4) compared to that found for reactions of HOCl with free and peptide-bound Tyr.<sup>24</sup> In contrast, analysis of SN-2 after removal of all lipoproteins showed comparatively fewer products and lower ratios of diCiTyr to CiTyr (data not shown). A high ratio may be obtained under conditions of high local oxidant production (AJ Kettle, unpublished results), and our data supports the previous suggestion by Heinecke<sup>52</sup> that Tyr chlorination via myeloperoxidase activity is relatively specific for lesion lipoproteins. This is also consistent with the observation that myeloperoxidase binds to LDL.<sup>53</sup>

Excepting diTyr, no other Tyr modification showed a clear disease stage dependency and elevated levels of diTyr were detected only in the most advanced lesion stages. diTyr may be produced via myeloperoxidase activity and its increase in complex lesions is consistent with a recent study showing increased numbers of myeloperoxidase-expressing macrophages in advanced, but not early, atherosclerosis.<sup>54</sup> However an increase in the content of Tyr modification, particularly diTyr, appeared to be present in even early lesions compared to those values

derived for normal iliac tissue,<sup>21</sup> normal aorta,<sup>51</sup> and normal LDL<sup>55</sup> (Table 5). Thus, our results do not exclude the possibility that specific Tyr modifications may be early disease events and that such oxidation could contribute to the initiation of atherogenesis.

In conclusion, our data show that in human aortic lesions the accumulation of nonoxidized lipid precedes the accumulation of primary lipid (per)oxidation products that coexist with and are likely formed in the presence of  $\alpha$ -TOH. These data show that vitamin E does not generally become depleted and suggest, although do not prove, that appreciable lipoprotein lipid (per)oxidation represents a response to rather than a cause for lesion development.

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