Oxidized lipid accumulates in the presence of α -tocopherol in atherosclerosis

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Oxidative modification of low-density lipoproteins in the arterial wall is a key feature of atherogenesis and widely believed to cause and/or accelerate lesion development. Linked to this is the expectation that vascular antioxidants are depleted during oxidation *in vivo*. However, whether α -tocopherol (vitamin E), an important lipid-soluble antioxidant, is depleted early in atherogenesis and can prevent lipid peroxidation *in vivo* is unresolved. To address this we examined the content of specific configurational isomers (*cis/trans*) of lipid hydro(pero)xides in lesions, which represent the major non-enzymic oxidation products, as formation and accumulation of *cis/trans* isomers is influenced by α -tocopherol in studies *in vitro*. Concordant with our previous findings that large amounts of oxidized lipid co-

exist with relatively normal α -tocopherol levels in human lesions, we now show that *cis/trans* isomers predominate over other products in human carotid and aortic lesions and in lesion lipoproteins. Further, dietary vitamin E supplementation of rabbits after arterial injury significantly increases both the aortic levels of α -tocopherol and the overall content of *cis/trans* isomers. These data are fully consistent with α -tocopherol acting as a hydrogen donor during lipid oxidation *in vivo* and suggest that α -tocopherol does not prevent lipoprotein lipid oxidation in the diseased vessel wall.

Key words: hydroxylinoleate, lipid oxidation, lipoprotein, stereo isomer, vitamin E.

INTRODUCTION

A large body of work implicates oxidative modification of low-density lipoprotein (LDL) in the cause and/or progression of human atherogenesis (see [1–5] for reviews). Thus LDL oxidation occurs early in the disease and is thought to precede foam-cell formation [1,2]. Whether α -tocopherol (α -TOH; biologically the most important lipid-soluble antioxidant and the active form of vitamin E [6]) is depleted early in atherogenesis and can prevent lipid peroxidation *in vivo* are unresolved issues. Low vitamin E content in atherosclerotic lesions has been reported when α -TOH is standardized to cholesterol [7]. However, we have observed that advanced human lesions contain normal levels of α -TOH per cholesteryl ester (CE; the major class of oxidizable lipid in LDL) despite the co-existence of large amounts of oxidized CE [8,9]. The latter suggests that intimal lipid oxidation occurs in the presence of α -TOH. However, the possibility remains that α -TOH is temporarily depleted and subsequently replenished in response to oxidative stress.

We propose that to resolve whether lipid oxidation *in vivo* occurs in the presence of α -TOH, the positional and configurational isomers of cholesteryl linoleate hydro(pero)xide [C18:2-O(O)H] should be assessed, as C18:2-O(O)H represents the major non-enzymic lipid-oxidation product found in human lesions [8–10]. Enzymic oxidation of polyunsaturated fatty acid yields highly characteristic positional and stereo-specific products. In contrast, free-radical-induced lipid oxidation generates a complex, though not random, array of products and configurational isomer specificity is influenced by the presence of hydrogen donors such as α -TOH [11,12]. We [13] and others [14] have shown that the presence of α -TOH in lipoproteins selectively generates *cis/trans*-C18:2-O(O)H isomers during lipid peroxidation *in vitro*.

We show herein that *cis/trans* isomers of C18:2-O(O)H are formed predominantly during plasma and lesion lipoprotein oxidation in the presence of endogenous α -TOH. Further, esterified *cis/trans*-C18:2-O(O)Hs are the primary products found in human lesions and in a rabbit model of arterial injury, suggesting that lipid peroxidation *in vivo* occurs in the presence rather than the absence of α -TOH.

EXPERIMENTAL

Materials

D,L- α -TOH was from Kodak (Rochester, NY, U.S.A.) and 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH) and 2,2'-azobis-(2,4-dimethylvaleronitrile) were from Polysciences (Warrington, PA, U.S.A.). Soya bean 15-lipoxygenase (15-LO; 630000 units/mg), heparin from porcine intestinal mucosa and its de-N-sulphated form, desulphoheparin, were from Sigma (St Louis, MO, U.S.A.). Carotid lesion material was obtained post-operation [9] and aortic lesion material post-mortem from subjects of 20–66 years old from the NSW Institute of Forensic Medicine, Sydney, Australia, with the approval of the local ethics committee. Human plasma was obtained from healthy volunteers [13], again with the approval of the local ethics committee. Rat chondrosarcoma proteoglycan (PG) and human arterial PG were generous gifts from Dr Wayne Comper, Monash University, Melbourne, Australia, and Dr Germán Camejo, AstraZeneca, Mölndal, Sweden, respectively. Glycosaminoglycans (GAGs; heparin and de-N-sulphated heparin; 1 mg/ml) were prepared in 5 mM Hepes, pH 7.2, containing 20 mM NaCl, and PG (1 mg/ml) was prepared in 5 mM Hepes, pH 7.2, containing 10 mM NaCl.

Abbreviations used: 18:2, linoleic acid; 18:2-OH, linoleate hydroxide; AAPH, 2,2'-azobis-(2-amidinopropane) dihydrochloride; C18:2, cholesteryl linoleate; C18:2-O(O)H, cholesteryl linoleate hydro(pero)xide; CE, cholesteryl ester; CE-O(O)H, cholesteryl ester hydro(pero)xide; GAG, glycosaminoglycan; 15-LO, 15-lipoxygenase; LDL, low-density lipoprotein; α -TOH, α -tocopherol; LDL_{1/2}, a mixture of α -TOH-containing- and α -TOH-depleted-LDL particles; NP, normal phase; PG, proteoglycan; RP, reversed phase.

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13-(*S*)- and 9-(*S*)-C18:2-OH were from Cayman Chemicals (Ann Arbor, MI, U.S.A.) and all positional, configurational and stereo-isomers of C18:2-OH were prepared by vitamin E-controlled autoxidation of cholesteryl linoleate (C18:2) as described in [15]. [^3H]C18:2 {[cholesteryl-1,2,6,7- ^3H (N)]C18:2; 91.5 Ci/mmol} was from Dupont/NEN (Boston, MA, U.S.A.) and [^{14}C]C18:2 (55 mCi/mmol) from Amersham Bioscience (Little Chalfont, Bucks, U.K.). Aqueous solutions were stored over Chelex-100 (Bio-Rad, Richmond, CA, U.S.A.) to remove contaminating transition metals, except GAG or PG solutions (see above) or buffers containing added Ca^{2+} or Mg^{2+} ions. Other chemicals were of the highest available purity and nanopure water (Millipore Systems, Sydney, Australia) was used throughout.

Preparation of plasma, normal LDL and α -TOH-depleted LDL

Human plasma was obtained from heparin-treated whole blood and LDL (approx. 1 μM in apolipoprotein B-100) was isolated from plasma by density-gradient ultracentrifugation [16]. Low-molecular-mass, water-soluble antioxidants were removed from plasma and LDL preparations by gel filtration before use. α -TOH-depleted LDL was prepared by aqueous peroxyl radical (AAPH) oxidation as described in detail previously [17]. Native and α -TOH-depleted LDL were mixed (1:1) to generate a mixture of α -TOH-containing- and α -TOH-depleted-LDL particles ($\text{LDL}_{1/2}$). LDL concentration was determined from the content of non-esterified cholesterol assuming 550 mol of cholesterol per mol of apolipoprotein B-100 [18].

Oxidation of plasma, LDL and lesion homogenate

Plasma and lesion supernate oxidation was initiated by AAPH and LDL lipid oxidation by addition of AAPH or Cu^{2+} at 37 °C. Aliquots (50–200 μl) were removed at the times indicated and lipid hydroperoxides were converted stoichiometrically into hydroxides with NaBH_4 (50 mM) before extraction. Chemical reduction did not result in isomerization of C18:2-OOH and approx. 85 % of the total cholesteryl ester hydro(pero)xide [CE-O(O)H] was recovered as C18:2-OH, as judged by reversed-phase (RP) and normal-phase (NP) HPLC (results not shown).

Preparation and oxidation of GAG- and PG-LDL

LDL aggregates were formed in 5 mM Hepes, pH 7.2, containing NaCl (20 mM), MgCl_2 (2 mM) and CaCl_2 (4 mM) and GAG at a LDL/GAG ratio of 1:0.06. Turbid solutions were obtained when heparin was used, whereas desulphoheparin and buffer controls did not cause apparent aggregation. Samples were centrifuged at 5000 g for 5 min at 4 °C and the pellet formed in the heparin-exposed samples was re-constituted in 5 mM Hepes, pH 7.2, containing NaCl (140 mM), using a volume equal to that of the supernate. The native and desulphoheparin controls remained clear solutions and were used without further treatment. PG-LDL aggregates were formed by addition of a 10 mM Ca^{2+} solution to LDL in 5 mM Hepes containing NaCl (10 mM), pH 7.2, followed by the addition of rat chondrosarcoma (50 $\mu\text{g}/\text{ml}$) or human arterial PG (150 $\mu\text{g}/\text{ml}$). Each of the GAG-exposed LDL samples was diluted in 5 mM Hepes containing 140 mM NaCl, pH 7.2. PG-LDL aggregates were supplemented with EDTA (5 μM). Oxidations were initiated by addition of Cu^{2+} , AAPH or 15-LO to GAG-LDL and of 15-LO to PG-LDL, in aerated solutions at 37 °C.

Preparation of lesion supernates

Carotid endarterectomy lesions (approx. 1 g wet weight), typical of advanced and complicated lesions [9], were washed in cold

deoxygenated PBS (50 mM, pH 7.4) containing 0.54 mM EDTA and 10 μM butylated hydroxytoluene. Intima was resected, finely minced and homogenized in cold, deoxygenated sodium carbonate (100 mM) buffer, pH 11 (4–5 ml) [9]. The homogenates were centrifuged at 2000 g for 10 min at 5 °C and supernates were used either directly or for the isolation of lesion lipoproteins by density-gradient ultracentrifugation as described in [9]. Protein concentration was determined using the bicinchoninic acid assay according to the manufacturer's instructions (Pierce, Rockford, IL, U.S.A.).

For aortic lesions the thoracic and abdominal aorta from a total of 31 subjects were resected and designated into three groups: fatty streaks, fibro-fatty and complex/ulcerated lesions [19]. Sample preparation was essentially as described previously [20]. Briefly, resected intima were weighed and tissue from the same lesion stage pooled to yield approx. 10 g. Each 10 g pool represented a single sample ($n = 1$) for analysis. Tissue was frozen in liquid N_2 , pulverized and reconstituted in 10 mM phosphate/0.15 M NaCl buffer, pH 7.4 (50 ml) containing various antioxidants, metal-ion chelators and protease inhibitors as specified in [20]. The samples were agitated overnight (4 °C) and centrifuged at 2750 g for 30 min at 4 °C. The resulting tissue supernate was ultracentrifuged at 100000 g for 30 min at 4 °C; the pellet and top layer were discarded and the supernate containing all lesion lipoproteins was collected.

Preparation and oxidation of lesion LDL

For isolation of lesion LDL, approx. 60 g of intima was resected from pools of four aortas, and the tissue was divided into approx. 10 g portions. Aortic tissue supernates were then prepared as described above. Lesion LDL was isolated from the supernates by sequential density ultracentrifugation ($d = 1.02$ – $1.07 \text{ g}/\text{ml}$) [20], dialysed extensively against PBS containing 100 μM EDTA at 4 °C under N_2 and concentrated before use. The final sample (5–7 ml) typically contained 250–600 μM non-esterified cholesterol and particle size was verified by FPLC and comparison with authentic plasma LDL. For oxidation experiments, AAPH was added (0.5 mM) and aliquots (200 μl) were taken at the indicated times for subsequent HPLC analyses as described below.

Extraction of neutral and oxidized lipid and antioxidants from plasma, LDL and tissue

Aliquots (50 μl) of plasma, LDL and GAG- and PG-exposed LDL were removed at times and added to hexane/methanol (5:1, v/v; 6 ml) for extraction of oxidized and neutral lipid and α -TOH into the hexane phase [16]. Hexane extracts were dried and reconstituted in propan-2-ol for RP HPLC. For isolation and quantification of configurational isomers of C18:2-OH formed during exposure of plasma or LDL to radical oxidants, separate aqueous aliquots (100 μl) were reduced with NaBH_4 prior to extraction, and lipids were reconstituted in hexane for NP HPLC. Carotid and aortic lesion supernates and samples of lesion lipoproteins (approx. 200 μl) were added to hexane/methanol (5:1, v/v; 12 ml). Endogenous CE-OHs in the hexane phase were isolated from CE-OOHs in lesion material by RP HPLC and collected for further analysis.

Rabbit lesion induction protocol

Groups of male New Zealand white rabbits were fed a α -TOH-deficient (10 i.u./kg of chow), -normal (50 i.u./kg of chow) or -supplemented (1000 i.u./kg of chow) chow as described in [21] that also contained 2 % cholesterol. All animal studies were

Figure 1 The accumulation of *cis/trans*-C18:2-O(O)H is controlled by α -TOH during oxidation of lipid in human plasma by aqueous peroxy radicals

Human plasma was oxidized by addition of 50 mM AAPH at 37 °C. At times, aliquots (20 μ l) were removed and the content of α -TOH (cross-hatched squares) and neutral lipids determined by RP HPLC. Further, 100 μ l aliquots were also removed and added to NaBH₄ (50 mM) to reduce hydroperoxy lipids to the hydroxide forms. Configurational isomers of C18:2-OH were isolated and quantified by NP HPLC (■, 13-*cis/trans*; ●, 9-*trans/cis*; □, 13-*trans/trans*; ○, 9-*trans/trans*) as described in the Experimental section. Data shown represent means $\pm$ S.D. of duplicate samples from three independent experiments utilizing separate plasma preparations.

approved by the Boston University Medical Center Institutional Animal Care and Use Committee. Chows and water were provided *ad libitum* and after 2 weeks animals were subjected to balloon injury of the aorta [21] followed by 8 weeks of further intervention. After this time animals were killed and aortic supernates were prepared as described in [22]. Briefly, aortas (0.15–0.84 g wet weight) were rinsed in cold, deoxygenated PBS (10 mM, pH 7.4) containing 2 mM EDTA and 20 μ M butylated hydroxytoluene. Intima was resected, frozen in liquid N₂ and pulverized. The tissue powder was collected into cold, deoxygenated buffer (2 ml) and homogenized on ice.

Linoleate was found primarily in triglycerides in rabbit lesions and total lipids were extracted following hydrolysis as described in [23]. For this, NaBH₄ (50 mM) was added to lesion supernates to reduce lipid hydroperoxides that may degrade during hydrolysis. Lipids were extracted into hexane, dried and reconstituted in methanol/chloroform (8:1, v/v; 850 μ l). KOH (40%, w/v; 150 μ l) was then added and the samples were incubated at 60 °C for 30 min under argon before neutralizing with acetic acid. Finally, lipids were extracted into chloroform, dried and reconstituted in methanol. Recovery and oxidation of linoleic acid (18:2) during hydrolysis was assessed by addition of [¹⁴C]18:2 (2 μ M) to the supernates.

HPLC analyses

CE-O(O)H, cholesterol, CE and α -TOH in lesion, plasma and lipoprotein extracts were quantified using RP HPLC coupled with UV and electrochemical detection as described in [16,24]. Isolation of CE-OH from CE-OOH was achieved using a separate RP HPLC protocol [25]. Configurational and stereo-isomers of C18:2-OH were identified by NP and chiral-phase HPLC coupled with UV (234 nm) detection as described in [26]. Quantification

Figure 2 *cis/trans*-C18:2-O(O)H accumulation in LDL is influenced by the α -TOH content during oxidation by radical oxidants

LDL (approx. 0.7–1.2 μ M apolipoprotein B-100), comprising a mixture of α -TOH-containing- and α -TOH-depleted particles (LDL_{1/2}), was subjected to oxidation by 2 mM AAPH (A) or 5 μ M Cu²⁺ (B) at 37 °C. At times, aliquots (50–100 μ l) were removed and the content of α -TOH (cross-hatched squares) and C18:2-O(O)H isomers (■, 13-*cis/trans*; ●, 9-*trans/cis*; □, 13-*trans/trans*; ○, 9-*trans/trans*) quantified. The data shown are mean values with error bars showing maximum variation, and are derived from two independent experiments performed in triplicate.

and isolation of 18:2 and linoleate hydroxide (18:2-OH) isomers in hydrolysed lipid extracts was achieved using established RP, NP and chiral-phase HPLC conditions as described in [23]. For detection of radiolabelled compounds the above conditions were combined with online radiometric detection with a scintillant-to-flow ratio of 1. Assignment and quantification of individual peaks was based on co-elution and area comparison with authentic standards.

Determination of lipid autooxidation and product isomerization during processing

The method utilized to extract lipid from lesions largely preserves tissue antioxidants [8,9]; however, it is unknown whether tissue work-up induces oxidation and/or metabolism of isolated products. To account for inadvertent oxidation during processing, [³H]C18:2 (0.1 μ M; 915 Ci/mol) was added to two carotid and four aortic (approx. 1 g wet weight) lesions. The lesions were subjected to processing as described above; however, no significant oxidation of [³H]C18:2 was detected as judged by approx. 99% recovery of the radiolabel as unmodified C18:2 (results not shown).

Potential isomerization of lipid hydroperoxides was also determined as these may be unstable *ex vivo*. Three carotid lesions (0.6–0.9 g wet weight) were cut longitudinally and either

Figure 3 LDL exposed to GAG and PG oxidizes in the presence of α -TOH

(A–C) For GAG experiments, oxidation of native LDL (○, ●), desulphoheparin-exposed LDL (□, ■) and heparin-exposed LDL (△, ▲) was initiated by addition of various free-radical generators. Free-radical generators were utilized at molar oxidant/LDL ratios of 2 (Cu^{2+} ; A) and 1330 (AAPH; B) or 4840 (15-LO; C) units. Consumption of α -TOH (open symbols) and accumulation of CE-O(O)H (closed symbols) was determined by HPLC as described in the Experimental section. (D) Oxidation of rat PG- (cross-hatched squares) or human PG- (◆) LDL aggregates or native LDL (○) induced by 15-LO (3860 units:LDL molecule) was also followed. The results represent means $\pm$ S.D. of triplicate samples from three independent experiments.

$[^3\text{H}]13$ -*cis/trans*- or $[^3\text{H}]13$ -*trans/trans*-C18:2-OOH in 2 ml of sodium carbonate buffer was added to each piece. $[^3\text{H}]$ C18:2-OOH was prepared by incubating the lipid peroxyl radical-generating 2,2'-azobis-(2,4-dimethylvaleronitrile) (50 mM) with $[^3\text{H}]$ C18:2 (10 μM) for 60 min at 37 °C. $[^3\text{H}]$ C18:2-OOH (approx. 1 μM) was isolated by RP HPLC and isomers by NP HPLC, before addition to lesions. The lesions were processed and the supernate (2 ml) extracted and reconstituted in propan-2-ol. ^3H -labelled C18:2 and C18:2-O(O)H were identified by RP HPLC and the ^3H -labelled hydroperoxide and hydroxide forms individually collected, extracted and their content quantified. Isomerization of either hydroperoxide isomer was not detected, whereas reduction to the hydroxide form did occur and was equivalent for both isomers ($28 \pm 9\%$ for 13-*cis/trans*- and $21 \pm 3\%$ for 13-*trans/trans*-C18:2-OOH; mean $\pm$ S.D., $n = 3$). The nature of this reducing activity was not investigated further. $[^3\text{H}]$ C18:2-OH remained unmodified during processing (results not shown).

Statistical analyses

Non-parametric tests (Mann-Whitney) were employed to evaluate significant differences at a P value of < 0.05 .

RESULTS

Previous mechanistic studies of LDL oxidation demonstrate a clear preference for *cis/trans* lipid hydro(pero)xide formation while α -TOH is present [13,14]. However, lesion LDL may be a heterogeneous mix of particles with varying α -TOH contents; some particles may be depleted of α -TOH considering that oxidation *in vivo* is proposed to be an early event [1] and that α -TOH is the most active redox component in LDL [27]. We therefore tested whether α -TOH acts as a hydrogen donor during oxidation of plasma, as this fluid contains various lipoproteins with different α -TOH contents and a significant proportion of high-density lipoproteins devoid of endogenous α -TOH [18]. Additionally, oxidation of $\text{LDL}_{1/2}$ was monitored to assess the effect of varying the α -TOH/LDL ratio on the profile of lipid peroxidation products.

During plasma lipid oxidation induced by aqueous peroxyl radicals (AAPH), distinct isomers of C18:2-O(O)H were generated depending on the α -TOH content, consistent with α -TOH exerting a hydrogen-donor effect (Figure 1). Gel-filtered plasma was used to eliminate ascorbate, which strongly inhibits AAPH-induced oxidation of lipids and tocopherol-mediated peroxidation of lipoprotein lipids [28], and the present study is

Figure 4 Human lesion *cis/trans*- and *trans/trans*-C18:2-OH configurational isomers

Representative NP HPLC chromatograms of C18:2-OH isomers in a preparation of oxidized authentic C18:2 (A) and in material isolated from human carotid lesion (B). The numbers above the peaks refer to the four positional and configurational isomers of C18:2-OH: 1, 13-*cis/trans*; 2, 13-*trans/trans*; 3, 9-*trans/cis*; 4, 9-*trans/trans*.

concerned with isomer formation during lipid oxidation of lipoproteins in the presence of α -TOH. Thus 9-*trans/cis*- and 13-*cis/trans*-C18:2-O(O)H isomers (hereafter referred to as *cis/trans* isomers) were formed exclusively during the α -TOH-containing phase of oxidation ($t < 240$ min). Detectable accumulation of the corresponding thermodynamically more stable *trans/trans* isomers only occurred once the level of α -TOH had decreased to $\leq 25\%$ (Figure 1). Following complete consumption of α -TOH, only *trans/trans*-C18:2-O(O)H isomers accumulated to a significant extent (Figure 1).

Similar to plasma, C18:2-O(O)H isomer accumulation during the oxidation of LDL_{1/2} initiated by AAPH (Figure 2A) or Cu²⁺ ions (Figure 2B) was influenced by α -TOH. Thus accumulation of *cis/trans*-C18:2-O(O)H isomers predominated during the α -TOH-containing phase, whereas, following α -TOH consumption, *trans/trans* isomers increased with time (Figure 2A). *trans/trans*-C18:2-O(O)H isomers were the predominant isomers generated in AAPH- and Cu²⁺-ion-mediated oxidation of α -TOH-depleted LDL (results not shown).

Intimal LDL is exposed to GAG and there is evidence that some of the lipoprotein is present as PG aggregates [29]. We therefore studied oxidation of *in vitro* GAG- or PG-treated LDL by radical oxidants to first establish whether lipid oxidation in these aggregates proceeded in the presence of α -TOH. LDL exposed to heparin (as a model GAG) was subjected to AAPH-, Cu²⁺- or 15-LO-induced oxidation (Figure 3). As shown previously [30,31], GAG-exposed LDL had increased suscep-

Table 1 Configurational isomers of endogenous C18:2-OH isolated from carotid lesions

Lesion lipoprotein fractions were prepared from supernates of carotid lesions as described in the Experimental section. α -TOH and C18:2-OH were quantified and isolated by RP HPLC and the positional and configurational isomers of C18:2-OH were determined by NP HPLC. The data shown represent means $\pm$ S.D. α -TOH data are derived from [9].

Lesion homogenate density fraction	<i>n</i>	<i>cis/trans</i> -C18:2-OH (%)	α -TOH (nmol/mg)
Very-low-density fraction	11	71 $\pm$ 8*	78 $\pm$ 25
Low-density fraction	11	64 $\pm$ 9	32 $\pm$ 18
High-density fraction	11	60 $\pm$ 9	5 $\pm$ 3

* $P < 0.05$ compared with the high-density fraction.

Table 2 *cis/trans*-Isomers dominate the product profile of C18:2-OH in aortic lesions

Endogenous C18:2-OH was isolated from supernates derived from aortic lesions as described in the Experimental section. The configurational and stereo-isomers of C18:2-OH were isolated further and identified by NP and chiral-phase HPLC. The total of C18:2-OH isolated from fatty streak lesions (nine separate tissue samples) was pooled to generate isomer data. For subsequent groups the data represent means $\pm$ S.D. expressed as a percentage of total C18:2-OH (*cis/trans* isomers) or 13-*cis/trans*-C18:2-OH (*S* isomers).

Lesion stage	<i>n</i>	<i>cis/trans</i> -C18:2-OH (%)	<i>cis/trans</i> -C18:2-OH 13- <i>S</i> isomer (%)
Fatty streak	9	87	50
Fibro-fatty	7	85 $\pm$ 2	50 $\pm$ 3
Ulcerated/complex	8	79 $\pm$ 6*	51 $\pm$ 1

* $P < 0.05$ compared with fibro-fatty lesions.

tibility to Cu²⁺-induced oxidation compared with native LDL or desulphoheparin-treated LDL, which was not aggregated (Figure 3A). In contrast, oxidation by AAPH (Figure 3B) or 15-LO (Figure 3C) was unaffected by GAG. Importantly, lipid oxidation in LDL aggregates proceeded in the presence of α -TOH for all oxidants tested (Figures 3A–3C). 15-LO-induced oxidation of solid LDL aggregates (heparin; results not shown) or rat or human PG-LDL also proceeded in the presence of α -TOH (results not shown) and was not affected by aggregation (Figure 3D).

The above studies performed *in vitro* show clearly that LDL lipid oxidation can proceed in the presence of α -TOH even in lipoprotein aggregates, and suggest that α -TOH may behave as a hydrogen donor *in vivo*. To test this further we determined the distribution of *cis/trans*- versus *trans/trans*-C18:2-OH in human carotid and aortic lesions representing the early stage through to the advanced disease. C18:2-OOH isomers were not investigated as they represent less than 10% of total CE-O(O)Hs [8]. In homogenates of carotid lesions, which represent advanced disease, a significant portion of endogenous C18:2-OH was present as *cis/trans* isomers. A representative chromatogram showing C18:2-OH positional (13- versus 9-) and configurational (*cis/trans* versus *trans/trans*) isomers is depicted in Figure 4.

cis/trans-C18:2-OH isomers were also the predominant isomers in carotid lesion lipoprotein fractions isolated by density-gradient ultracentrifugation corresponding in density to the plasma lipoproteins very-low-density lipoprotein, LDL and high-density lipoprotein (Table 1) as described in [9]. In these fractions the percentage of *cis/trans*-C18:2-OH decreased with increasing density and decreasing α -TOH content (Table 1). The relative amounts of the positional isomers (13- versus 9-)

Figure 5 *cis/trans*-C18:2-O(O)H accumulation in aortic-lesion LDL is influenced by α -TOH

LDL was isolated from aortic lesions and subsequently exposed to AAPH (0.5 mM) in PBS, pH 7.4, at 37 °C. At times, aliquots (200 μ l) were removed and the content of α -TOH (cross-hatched squares) and C18:2-O(O)H isomers (■, 13-*cis/trans*; ●, 9-*trans/cis*; □, 13-*trans/trans*; ○, 9-*trans/trans*) were isolated and quantified. The initial concentration of α -TOH was approx. 3 μ M. The data shown are representative of two separate experiments using different aortic-lesion LDL preparations, each prepared from a pool of four human aortas.

of C18:2-OH were equal in carotid supernates and lipoprotein fractions (results not shown), suggesting that oxidation *in vivo* was via a free-radical process.

Similar to carotid lesions, the predominant isomers of endogenous C18:2-OH in aortic lesions were *cis/trans* (Table 2). *cis/trans*-C18:2-OH constituted 79–87% of total C18:2-OH in supernates of aortic lesions (Table 2) and advanced lesions displayed a decreased proportion of *cis/trans*-C18:2-OH compared with earlier disease stages (fibro-fatty; Table 2). In addition, *cis/trans*-C18:2-O(O)H comprised 86.5 ± 3.4 (mean $\pm$ maximal deviation; $n = 2$ pools of four human aortas) of total isomers in lesion LDL isolated from human aortic lesions. Importantly, inadvertent oxidation of lesion lipids or isomerization of C18:2-OOH did not occur during processing (see the Experimental section). The stereo-isomer distribution (*S* versus *R*) of 13-*cis/trans*-C18:2-OH can indicate enzymic oxidation [32]. The aortic content of *S* and *R* isomers was equimolar independent of disease stage (Table 2), indicative of non-enzymic, free-radical-mediated oxidation.

To assess further whether α -TOH influences lipid peroxidation in lesion tissue we subjected human aortic lesion LDL to free-

radical-mediated oxidation. Oxidation of aortic lesion LDL by aqueous peroxy radicals (AAPH) proceeded in the presence of endogenous α -TOH (Figure 5). Similar to native LDL and plasma lipid oxidation, accumulation of *cis/trans*-C18:2-O(O)H predominated during the α -TOH-containing phase of oxidation ($t < 6$ h; Figure 5). The rate of accumulation of *trans/trans* isomers only surpassed that of the *cis/trans* isomers following consumption of α -TOH. Overall the data were concordant with α -TOH acting as a hydrogen donor during the oxidation of human lesion LDL.

To test whether *cis/trans*-C18:2-O(O)H formation *in vivo* was dependent on arterial α -TOH, aortas from rabbits fed a high-cholesterol diet containing varying vitamin E content were balloon-injured to induce lesion formation. Previous studies have shown that in this model, vitamin E supplemented at 1000 i.u./kg of chow increased lesion size, as assessed by the intima-to-media ratio, but had no substantial effect on lesion morphology; vitamin E at 10 or 50 i.u./kg of chow had no effect on lesion size or morphology [21]. The lesion content of α -TOH in vitamin E-supplemented animals (1000 i.u.) significantly increased compared with the animals fed vitamin-deficient or normal chow (Table 3), as shown previously [21]. However, there was no difference in aortic α -TOH content in animals consuming 10 or 50 i.u. of vitamin E (results not shown) and therefore the data from these groups were combined. Concordant with an increased α -TOH content, the percentage of *cis/trans*-18:2-OH in lesions significantly increased in the vitamin E-supplemented animals (Table 3). However, the content of these isomers was already high in the normal/deficient groups (Table 3). Inadvertent oxidation of added [^{14} C]18:2 did not occur during sample processing (results not shown). Similar to the human lesions, 13-*cis/trans*-18:2-OH was racemic in all animals (Table 3), suggesting further that oxidation *in vivo* was mediated via free-radical rather than enzymic reactions.

DISCUSSION

The data presented herein show that the predominant configurational isomers of oxidized esterified 18:2 formed in the vessel wall during atherogenesis are *cis/trans*-18:2-OHs and that these products are generated predominantly in the presence of α -TOH. *cis/trans* Isomers predominate over the corresponding *trans/trans* isomers in human carotid and aortic lesions, in the lipoprotein fractions isolated from such tissue and in aortic lesions of rabbits following balloon injury and consumption of a cholesterol-fortified diet. Moreover, dietary supplementation of such rabbits with vitamin E significantly increased both the aortic content of vitamin E and the level of esterified *cis/trans*-18:2-OH. The latter is consistent with a hydrogen-donor effect on lipid oxidation *in vivo* by α -TOH. Together, the results

Table 3 18:2-OH configurational and stereo-isomers isolated from rabbit aortic lesions

Aortic lesion supernates were prepared from rabbits following arterial injury and feeding with a high-cholesterol (2%) and vitamin E-supplemented (1000 i.u. of α -TOH/kg of chow) or vitamin E-normal/-deficient (10 or 50 i.u. of α -TOH/kg of chow) diet, as described in the Experimental section. 18:2-OH was isolated from esterified lipid by hydrolysis as described in the Experimental section. RP HPLC was used to quantify 18:2 and α -TOH, and configurational and stereo isomers of 18:2-OH were determined by NP and chiral phase HPLC, respectively. The data shown represent means $\pm$ S.D.

Vitamin E (i.u./kg of chow)	<i>n</i>	α -TOH (nmol/mg)	<i>cis/trans</i> -18:2-OH (%)	<i>cis/trans</i> -18:2-OH 13- <i>S</i> isomer (%)
10 or 50	14	0.7 ± 0.5	62 ± 8	49 ± 5
1000	11	$27 \pm 16^*$	$72 \pm 7^*$	51 ± 3

* $P < 0.05$ compared with the 10 or 50 i.u. of vitamin E group.

suggest that lipid peroxidation *in vivo* occurred in the presence of α -TOH.

The mechanism(s) responsible for the accumulation of C18:2-O(O)H in the arterial intima is largely unknown, although lipid peroxidation is a distinctive hallmark of atherosclerosis [1]. Our previous studies show that antioxidants that may prevent oxidation, such as α -TOH and ascorbate, co-exist with extensively oxidized lipid in advanced carotid lesions [8,9]. This paradox may be explained by considering that such studies cannot determine whether endogenous antioxidants are deficient prior to, or are depleted during, lipid oxidation and are subsequently replenished. We have addressed these issues herein by determining the product profile of C18:2-O(O)H and not simply the extent of their accumulation. Our data show that specific lipid-oxidation products formed in the presence of α -TOH are found endogenously in atherosclerotic lesions.

The predominant lipid-oxidation product in human lesions is C18:2-O(O)H [8–10] and four positional and configurational isomers, 9-*trans/cis*-, 13-*cis/trans*-, 9-*trans/trans*- and 13-*trans/trans*-C18:2-O(O)H, may be formed. However, the configurational isomer specificity of C18:2-O(O)H in atherosclerotic lesions has not previously been studied extensively. In contrast, the stereo-specificity of one lesion isomer, 13-*cis/trans*-18:2-OH, has attracted interest and it has been suggested to be formed in part via enzymic reaction (15-LO) [32,33]. A small number of studies have documented, although not commented on, configurational isomers of C18:2-O(O)H in human lesions. Consistent with our results, one showed that 9-*trans/cis*-C18:2-OH was present in higher levels than the corresponding *trans/trans* isomer in human atheroma lipid [34]. More recently a high percentage of *cis/trans* isomers of oxidized C18:2 was isolated from human lesions [33,35], but the reason why these products should dominate the isomer profile remained unexplained. Here we provide a rational proposal for these observations; that *in vivo*-esterified lipid oxidation proceeds to a large extent in the presence of α -TOH rather than following its consumption.

As the most important lipid-soluble redox active component, α -TOH is likely to dominate the hydrogen-donating capacity of vessel tissue. In both homogeneous and heterogeneous lipid the accumulation of specific products, and the extent of oxidation, is influenced by hydrogen donors [12]. The association between *cis/trans* isomer formation and the presence of α -TOH has been shown for lipid oxidation in lipoproteins isolated from plasma and initiated by aqueous peroxy radicals and 15-LO [14,26]. Furthermore, we show that the isomer profile produced during oxidation of lipid relevant to atherosclerosis is affected by the α -TOH content (Figures 1, 2 and 5). Thus during oxidation of LDL particles containing various levels of α -TOH, of plasma containing various lipoproteins with varying amounts of α -TOH and of aortic-lesion LDL, accumulation of *cis/trans*-C18:2-O(O)H occurs primarily during the α -TOH-containing phase.

We have utilized several approaches to determine whether the vitamin E content remains intact in the vessel wall in atherogenesis. These include analysis of the total vitamin E and oxidized lipid content in several lesion types and investigation as to whether *in vivo* lipid oxidation occurs in the presence or absence of α -TOH. In addition, the extent of *in vivo* vitamin E oxidation can be assessed by analysis of α -TOH oxidation [36]. In separate studies of carotid and aortic lesions, we observed that < 20% of α -TOH is oxidized, which is also inconsistent with the notion that intimal lipid oxidation progresses beyond α -TOH depletion [37].

In its classical mode of action, significant lipid peroxidation does not occur so long as α -TOH is present [6]. However, we and others have invariably demonstrated that up to 5% of C18:2

may be oxidized in advanced lesions, yet the α -TOH content remains essentially intact. Such data are consistent with the model of tocopherol-mediated peroxidation [17,28,38,39] in which lipid oxidation is initiated and promoted by α -tocopheroxyl radical. A key feature of this model is that α -TOH acts as a phase- and chain-transfer agent promoting lipid peroxidation so that substantial amounts of lipid hydro(pero)xides accumulate with relatively little consumption of α -TOH [17,28,38,39]. Whereas the data shown herein cannot provide direct evidence that lipid oxidation *in vivo* proceeds via tocopherol-mediated peroxidation, the results are concordant with the possibility that at least some of the intimal lipid oxidation occurs via this mechanism.

The results in Table 2 show that while the *cis/trans* isomers of C18:2-OH are the predominant oxidation products in human aortic lesions, their percentage decreases somewhat as the severity of the lesions increases. This suggests that some lipoprotein oxidation occurred in the absence of α -TOH, perhaps in vitamin E-depleted regions, and that the contribution of this increases slightly with developing disease. However, this lipoprotein lipid oxidation in putatively vitamin E-depleted regions is a minor contributor to the overall extent of lipoprotein oxidation, and its pathological relevance, if any [40], is unclear.

There is evidence to suggest that at least part of the LDL in arterial intima is present as PG aggregates [29] and that exposure to GAGs increases the susceptibility of plasma LDL to oxidation [30,31] and its atherogenicity [41]. We demonstrate herein (Figure 3) that GAG- or PG-exposed LDL also undergoes oxidation by radical oxidants in the presence of α -TOH, most likely via tocopherol-mediated peroxidation. The latter is supported by the observation that ascorbate (vitamin C), a co-antioxidant for α -TOH in LDL [38], effectively inhibits oxidation of GAG- and PG-exposed LDL (results not shown).

It is important to discriminate whether *in vivo* lipid oxidation occurs in the presence of vitamin E, or during a temporary episode of depletion. The view that vitamin E is deficient in lesions is based on the premise that as the major lipid-soluble antioxidant in tissue, α -TOH must be depleted before extensive lipid oxidation occurs [6], and on epidemiological evidence suggesting that antioxidants are important in mitigating cardiovascular disease [42]. The fact that ambivalent results have been obtained from large human intervention trials with vitamin E and several animal studies (e.g. see [43] for a review) suggests that α -TOH depletion may not be relevant to lipid peroxidation and antioxidation *in vivo*. It must also be considered that vitamin E's action *in vivo* may be largely unrelated to its antioxidant activity. For example, vitamin E supplementation in rabbits is found to preserve vascular function and prevent endothelial dysfunction induced by oxidized LDL [21,44].

In conclusion, our data show that in developing lesions lipid oxidation occurs largely in the presence of vitamin E and yields *cis/trans* isomers of oxidized esterified 18:2. Our results indicate that vitamin E is not depleted at the onset or during the development of atherosclerosis and that its presence does not prevent lipoprotein lipid peroxidation in the vessel wall. This may explain why vitamin E supplementation alone does not represent an effective anti-atherogenic strategy.

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