Vitamin E Oxidation in Human Atherosclerotic Lesions

Andrew C. Terentis, Shane R. Thomas, Jeanne A. Burr, Daniel C. Liebler, Roland Stocker

Abstract—Oxidation of low-density lipoproteins (LDL) is a key process in atherogenesis, and vitamin E ( \( \alpha \)-tocopherol, TOH) has received attention for its potential to attenuate the disease. Despite this, the type and extent of TOH oxidation and its relationship to lipid oxidation in the vessel wall where lesions develop remain unknown. Therefore, we measured oxidized lipids, TOH, and its oxidation products, \( \alpha \)-tocopherylquinone (TQ), 2,3- and 5,6-epoxy-\( \alpha \)-tocopherylquinones by gas chromatography–mass spectrometry analysis in human lesions representing different stages of atherosclerosis. We also oxidized LDL in vitro to establish “footprints” of TOH oxidation product for different oxidants. The in vitro studies demonstrated that tocopherylquinone epoxides are the major products when LDL is exposed to the one-electron (ie, radical) oxidants, peroxyl radicals, and copper ions, whereas TQ preferentially accumulates with the two-electron (nonradical) oxidants, hypochlorite, and peroxynitrite. In human lesions, the relative extent of TOH oxidation was maximal early in the disease where it exceeded lipid oxidation. Independent of the disease stage, TQ was always the major oxidation product with all products together representing <20% of the total TOH present, and the oxidation product profile mirroring that formed during LDL oxidation by activated monocytes in the presence of nitrite. In contrast, oxidized lipid increased with increasing disease severity. These results suggest that two-electron oxidants are primarily responsible for TOH oxidation in the artery wall, and that the extent of TOH oxidation is limited yet substantial lipid oxidation takes place. This study may have important implications regarding antioxidant supplements aimed at preventing LDL oxidation and hence atherogenesis. (Circ Res. 2002;90:333-339.)

Key Words: tocopherol | LDL oxidation | atherogenesis | hypochlorous acid

The oxidation of low-density lipoproteins (LDL) in the artery wall is considered to be a key contributor to atherogenesis.\(^1\) In support of this, a wide variety of markers of oxidized lipid and protein have been detected in human atherosclerotic lesions, including F\(_2\)-isoprostanes,\(^2\) cholesteryl ester hydro(pero)xides (CE-O(O)H),\(^3,\(^4\) and chloroand nitrotyrosine.\(^5,\(^6\) The exact identity of the oxidant(s) responsible for LDL oxidation in vivo remains undetermined although several oxidants have been implicated, including transition metals, 15-lipoxygenase, myeloperoxidase-generated hypochlorous acid (HOCl), and reactive nitrogen species such as peroxynitrite (ONOO\(^-\)).\(^7\)

Antioxidants are potential antiatherogenic agents because they can inhibit LDL oxidation. Most attention has focused on vitamin E (\( \alpha \)-tocopherol, TOH), as it is the major lipid-soluble antioxidant in LDL, yet the outcome of interventions with TOH supplements has been overall disappointing.\(^8\) Also, in vitro mechanistic studies have revealed a complex role for TOH during lipoprotein lipid oxidation. Thus, in the absence of compounds capable of reducing the \( \alpha \)-tocopheroxy radical (TO·) and exporting the radical from LDL, the vitamin can promote lipid oxidation by the process of tocopherol-mediated peroxidation (TMP).\(^9,\(^10,\(^11\)

Despite some evidence,\(^12\) there is surprisingly little direct evidence establishing that TOH attenuates lipid oxidation in the artery wall. One approach to resolve this issue is to examine the extent and type of TOH oxidation and how this relates to the extent of lipid oxidation in lesions. Because TOH is one of the most reactive components of LDL, such measurements should also provide a sensitive gauge for the extent of LDL oxidation in the artery wall. Furthermore, by measuring the distribution of specific TOH oxidation products, important information can be obtained on the nature of the oxidant causing LDL oxidation in the artery wall. At present, information on TOH oxidation in human atherosclerotic lesions is limited to the measurement of \( \alpha \)-tocopherylquinone (TQ) in carotid plaque.\(^1,\(^4\)

We therefore measured TOH and its oxidation products—TQ, 5,6-epoxy-\( \alpha \)-tocopherylquinone (TQE1), and 2,3-epoxy-\( \alpha \)-tocopherylquinone (TQE2) (Figure 1) in human atherosclerotic lesions representing different stages of the disease. We also compared the profile of TOH oxidation products in these lesions with that obtained for LDL exposed to various oxidants in vitro. We show that TQ is the major oxidation product in both lesions and LDL oxidized by the two-electron oxidation process.
Materials and Methods
Isolation of LDL
Human plasma was obtained from heparinized blood of healthy volunteers with approval of the local ethics committee and the LDL isolated by ultraacentrifugation as described.\textsuperscript{13,14}

Human Lesion Material
All human lesion material was obtained with approval of the local ethics committee. Plaque specimens (approximately 1 gram wet weight) were obtained from patients undergoing carotid endarterectomy. All samples were typical of advanced lesions\textsuperscript{4} and were homogenized in sodium carbonate buffer (pH 11) according to established methods.\textsuperscript{4}

Human aortic specimens (from men or women 20 to 60 years old; 10 to 50 grams) were obtained postmortem (<48 hours) and washed immediately with ice-cold, argon-flushed phosphate-buffered saline (PBS) containing 300 μmol/L ethylenediaminetetraacetic acid, 100 μmol/L diethylenetriaminepentaacetic acid, 10 mmol/L 3-amino-1,2,4-triazole, 20 μmol/L butylated hydroxytoluene, and 2 mmol/L benzamide hydrochloride. Specimens were stored at \(-80°C\) for no longer than 6 months before processing. Aortic homogenates were prepared in sodium carbonate buffer as described above for plaque samples using \(\sim1\) gram of intima resected from an appropriate area of aorta depending on the lesion type. Specimens were classified macroscopically into four distinct groups as defined by Stary et al\textsuperscript{15}:

- no/initial lesion (I\textsuperscript{-}III), fibrofatty lesions (IV\textsuperscript{-}V), and complex/ulcerated lesions (V\textsuperscript{VI}).
- A small piece of aorta depending on the lesion type. Specimens were classified into four distinct groups as defined by Stary et al\textsuperscript{15}:
- \(\mathrm{I}\)–\(\mathrm{III}\), \(\mathrm{IV}\)–\(\mathrm{V}\), and \(\mathrm{VI}\). A small piece of aorta depending on the lesion type. Specimens were classified into four distinct groups as defined by Stary et al\textsuperscript{15}:
- \(\mathrm{I}\)–\(\mathrm{III}\), \(\mathrm{IV}\)–\(\mathrm{V}\), and \(\mathrm{VI}\).

In Vitro LDL Oxidation
Oxidation of LDL was performed in PBS under air at \(37°C\) for the indicated period of time and using the oxidant specified.\textsuperscript{16}

Peripheral blood monocytes were isolated by elutriation, and monocyte-mediated LDL oxidation was performed in Hanks’ balanced salt solution containing 100 μmol/L diethylenetriaminepentaacetic acid, as described previously by others.\textsuperscript{17}

HPLC Sample Workup and Analysis
Aliquots of LDL (100 μL) or tissue homogenate (100 to 400 μL) were extracted and analyzed for cholesterol (FC), cholesteryl esters (CE), TOH, and CE-O(O)H by reversed-phase HPLC with UV, electrochemical, and postcolumn chemiluminescence detection, respectively, as described.\textsuperscript{14}

Gas Chromatography–Mass Spectrometry (GC-MS) Sample Workup and Analysis
Analysis of TOH and its oxidation products—TQ, TQE1, and TQE2—was achieved using a stable-isotope dilution GC-MS method,\textsuperscript{18} with an Agilent 5973N GC-MS instrument. Run parameters were as described previously\textsuperscript{15} except that injections were carried out in a split-splitless inlet operating at 250°C in the pulsed-splitless mode with a pulse pressure of 23 psi for 0.8 minutes and a splitless period of 1 minute.

Control Experiments
Lesion samples were spiked with deuterated TOH, TQ, and TQE to assess the extent of artificial TOH oxidation and the recovery and stability of oxidation products during tissue homogenization and sample extraction. These experiments showed that 85% of all species were recovered and were stable in the homogenate (see the online data supplement for a detailed description).

Statistical Analyses of Data
Statistical analyses were performed by one-way ANOVA with a Newman-Keuls posttest. A probability value of <0.05 was considered significant.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results
In Vitro LDL Oxidation
We first established the profiles of TOH oxidation products in LDL exposed in vitro to different chemically defined oxidized (Figure 2). Because lipoproteins isolated from human lesions still contain TOH,\textsuperscript{3,4} we used “mild” oxidation conditions and determined both the extent of lipid oxidation and the profile of TOH oxidation products while vitamin E remained present.

We first used a typical radical oxidant. Incubation of LDL under conditions where aqueous peroxy radicals were generated at a constant rate led to the steady increase of all three TOH oxidation products (Figure 2A). Overall, the oxidation products measured accounted for \(90\%\) of the TOH consumed. TQE2 was the major product formed followed by TQE1 and TQ, accounting for \(55\%, 20\%, \text{and} 15\%\) of the TOH consumed, respectively. After 6 hours of oxidation, when all eight molecules of TOH present initially in LDL were consumed, 60 molecules of CE-O(O)H were formed per LDL particle (Figure 2A). CE-O(O)H are the single major lipid oxidation products detected in in vitro–oxidized LDL\textsuperscript{19} and in atherosclerotic lesions.\textsuperscript{3} Thus, lipid oxidation proceeded in a chain reaction despite the presence of TOH, an observation that can be explained readily by TMP.\textsuperscript{9}

We used copper ions (\(\mathrm{Cu}^{2+}\)) as an additional radical oxidant. \(\mathrm{Cu}^{2+}\) efficiently catalyzes LDL lipid oxidation and...
causes the generation of lipid alkoxyl and peroxyl radicals.20
Similar to the situation with aqueous peroxyl radicals, large
quantities of CE-O(O)H accumulated under these conditions,
and TQE2 was again the major TOH oxidation product
(Figure 2B). However, all TOH oxidation products together
could account for only $\frac{1}{10}$ of the vitamin consumed,
although the oxidation profile suggests that this relatively low
yield was not the result of degradation of the known TOH
oxidation products. These results show that although Cu$^{2+}$
generates presently unidentified TOH oxidation products,
the exposure of LDL to radical oxidants is generally character-
ized by the preferential accumulation of TQE2, and the total
extent of lipid oxidation exceeds that of TOH oxidation
by 10- to 40-fold.

We next exposed LDL to two-electron oxidants. For this
we first used sydnonimine-1, a chemical that generates a
steady flux of ONOO$^-$ (Figure 2C). LDL exposed to reagent HOCl also generated TQ as the principal
TOH oxidation product and with only traces of TQEs formed
(Figure 2D). Eighty percent of TOH consumed was ac-
counted for by TQ alone. For both two-electron oxidants, the
extent of lipid oxidation exceeded that of TOH oxidation by
only 2-fold (Figures 2C and 2D).

Together, these results show that the TOH oxidation profiles
induced by one- and two-electron oxidants markedly differ
in that the former predominantly yield TQEs whereas
the latter almost exclusively yield TQ. In addition, the two
types of oxidation pathways differ in that radical oxidants
give rise to substantially higher ratios of lipid to TOH
oxidation products compared with two-electron oxidants.

**Cell-Mediated LDL Oxidation**
Activated leukocytes generate superoxide anion that dis-
mutes to H$_2$O$_2$ and is utilized by myeloperoxidase to
produce HOCl. In the presence of nitrite (NO$_2^-$), several
additional reactive nitrogen species are formed.21-23 The
various species have been implicated in LDL oxidation in
the artery wall, as relevant markers such as 3-chlorotyrosine,
$p$-hydroxyphenylacetaldehyde, and 3-nitrotyrosine are elevated in atherosclerotic lesions.5,6,24
We therefore measured TOH and lipid oxidation in LDL
exposed to phorbol myristate acetate (PMA)-stimulated human mono-
cytes. LDL (~0.5 $\mu$mol/L) were suspended in Hanks’ balanced
salt solution (pH 7.4) containing sodium nitrite (500 $\mu$mol/L) and
$\approx1 \times 10^6$ cells/mL at 37°C under a 5% CO$_2$ atmosphere. A, Time
course of TOH oxidation, TQ, TQEs, and CE-O(O)H production
(symbols provided in Figure 2 legend). B, Changes to TQ
(hatched bars) and TQE1+TQE2 (open bars) and C, CE-O(O)H
(filled bars) production after 4 hours of oxidation with additions
and deletions as indicated. Data are expressed as mol% of ini-
tial TOH (B) or number of molecules per LDL (C), and initial TOH
levels were ~8.7 molecules per LDL. Final concentrations of
catalase and butylated hydroxytoluene were 300 nmol/L and
1 mmol/L respectively. Mean±SEM (n=3). *P<0.05 vs complete
system.

**Figure 2.** TOH oxidation and lipid peroxidation in LDL exposed
to various oxidants. LDL (~1 $\mu$mol/L) suspended in PBS (pH 7.4) were exposed to 4 different oxidants and incubated at
37°C. A, 2 mmol/L 2,2-$'$-azobis(2-amidinopropane) dihydrochloride. B, 2 $\mu$mol/L CuSO$_4$. C, 40 $\mu$mol/L SIN-1, 25°C. D, 400
$\mu$mol/L NaOCl. TOH (▫), TQ (µ), TQE1 (•), TQE2 (•), and
CE-O(O)H (○). TOH and TQ(E) are expressed as mol% of initial
TOH content of LDL (~8 TOH per LDL). Mean±SEM (n=3).

**Figure 3.** TOH oxidation and lipid peroxidation in LDL exposed
to phorbol myristate acetate (PMA)-stimulated human mono-
cytes. LDL (~0.5 $\mu$mol/L) were suspended in Hanks’ balan-
ced salt solution (pH 7.4) containing sodium nitrite (500 $\mu$mol/L) and
$\approx1 \times 10^6$ cells/mL at 37°C under a 5% CO$_2$ atmosphere. A, Time
course of TOH oxidation, TQ, TQEs, and CE-O(O)H production
(symbols provided in Figure 2 legend). B, Changes to TQ
(hatched bars) and TQE1+TQE2 (open bars) and C, CE-O(O)H
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and deletions as indicated. Data are expressed as mol% of ini-
tial TOH (B) or number of molecules per LDL (C), and initial TOH
levels were ~8.7 molecules per LDL. Final concentrations of
catalase and butylated hydroxytoluene were 300 nmol/L and
1 mmol/L respectively. Mean±SEM (n=3). *P<0.05 vs complete
system.


μmol/L nitrite yielded the same distribution of oxidation products, although the overall extent of oxidation was less (data not shown). We next examined the amounts of TOH oxidation (Figure 3B) and lipid oxidation products (Figure 3C) formed after 4 hours under varying oxidizing conditions. The complete system was comprised of the same conditions as for Figure 3A. TOH oxidation was significantly less in the absence of NO2 and was negligible for nonactivated cells (minus phorbol myristate acetate [PMA]). Where oxidation was observed, TQ always remained the major TOH oxidation product, except in the presence of catalase that suppressed TQ formation completely. The absence of nitrite decreased the production of TQ but not that of TQEs (Figure 3B). Lipid peroxidation was only partially nitrite-dependent, whereas catalase and butylated hydroxytoluene significantly suppressed such oxidation (Figure 3C). Together, these results indicate that in the presence of nitrite, activated human monocytes cause LDL TOH and lipid oxidation similar to that seen with chemically defined two-electron oxidants.

**Atherosclerotic Lesions**

Next we measured the amounts of FC, CE, CE-O(O)H, TOH, and its oxidation products in homogenates of human aortic tissue exhibiting varying degrees of disease. FC and the sum of the two major CE in human LDL, cholesteryl linoleate and cholesteryl arachidonate, were higher in advanced than in early lesions when expressed per milligram of total tissue protein (Table 1), indicating lipid loading in the diseased vessel wall. TOH increased commensurately (per mg protein), consistent with the fact that it is delivered to the vessel wall along with other lipids in LDL. Therefore, when normalized to CE or FC, the concentration of TOH remained unaltered independent of lesion severity, except for a 62% decrease in the TOH/CE value in fatty streaks (Table 1). Compared with native LDL isolated from plasma, the TOH-to-FC ratio in lesions was lower (26.3±8.3 versus 7 to 12 mmol TOH/mol FC for LDL and lesions, respectively). However, the TOH-to-CE ratio was comparable in lesions and plasma LDL (16.2±4.5 versus 11 to 30 mmol TOH/mol CE for LDL and lesions, respectively). Considering that CE are more readily oxidized than FC and are the major class of lipid in LDL, these data indicate that on average, lesion lipids are not depleted of TOH.

Absolute amounts of protein-standardized oxidized lipid and TOH increased with increasing disease severity (Figure 4). At all stages, TQ was the major TOH oxidation product, present at 15- to 20-fold higher concentration than TQE1 or TQE2. In early lesions (type I–II), there was ∼10 times more TQ than CE-O(O)H (Table 2). The content of oxidized lipids increased markedly in more advanced

![Figure 4](http://circres.ahajournals.org/doi/fig/10.1161/01.RES.89.2.334)  
Figure 4. TOH and CE oxidation in human atherosclerotic lesions. Postmortem human aorta specimens were classified into groups as defined by Stary et al,15 no/initial lesions (I–II), fatty streak lesions (II–III), fibrofatty lesions (IV–V), and complex/ulcerated lesions (V–VI). A, Concentrations (nmol per mg protein) of CE-O(O)H (□) and TQ (□□). B, TQE1 (▲) and TQE2 (●). C, Each oxidation product is expressed as mol% of total (nonoxidized parent plus related products). Mean±SEM (n=5 for each lesion type). *P<0.05 vs type I–II or II–III; **P<0.05 vs all other types.
lesions, where it was \( \approx 7 \)-fold higher than the total amount of oxidized TOH (Table 2).

The proportion of CE oxidized to CE-O(O)H also increased significantly in advanced lesions, with up to \( \approx 2\% \) of total CE oxidized (Figure 4C). In contrast, the proportion of TOH oxidized to TQ was highest in early lesions where it accounted for \( \approx 17\% \), but did not increase thereafter (Figure 4C). TQEs were not prevalent (<1\%) across lesion groups, with only a slight increase in the advanced (type V–VI) lesions (Figure 4C).

Finally, we measured TOH and lipid oxidation products in homogenates of human carotid plaque specimens. Overall, the results obtained were similar to those of type V–VI aortic lesions, with comparable absolute concentrations of FC, CE, and TOH (Table 1). TQ was the major (11\% of total TOH) and TQE1 and TQE2 the minor oxidation products (Figure 5), and the extent of lipid oxidation exceeded that of TOH oxidation by \( \approx 7 \)-fold (Table 2).

**Discussion**

The present study shows that TOH remains essentially intact in the human vessel wall as atherosclerotic lesions develop. Only a fraction of the vitamin is oxidized, and TQ is the single major oxidation product. Maximal, albeit limited, TOH oxidation is observed at the earliest stage of atherogenesis, at a time when the extent of lipid oxidation is minimal and overall less than that of the vitamin. The preferential formation of TQ and the low ratio of lipid to TOH oxidation closely resemble the situation when LDL is oxidized in vitro by reagent two-electron oxidants or those produced by activated human monocytes. Therefore, our findings indicate that oxidative events taking place in the artery wall during early atherogenesis are initiated primarily by two-electron oxidants.

Our observation that TOH oxidation exceeds lipid oxidation during early atherogenesis seems to suggest that TOH effectively protects arterial lipids from oxidation. Indeed, TOH is a highly effective scavenger of one-electron (ie, radical) oxidants, although for LDL lipids this is not necessarily associated with effective protection against lipid oxidation.9,11 Also, the vitamin fails to protect LDL from two-electron oxidants that give rise to TQ in preference over TQEs, as our in vitro studies with isolated LDL and chemically defined oxidants (Figure 2) clearly establish, and as is seen in human lesions. In contrast, radicals generally cause more substantial oxidation of LDL lipid than TOH and preferentially give rise to TQEs. The observed low extent of lipid oxidation and high TQ to TQE ratio in early lesions are therefore best explained with TOH engaging in two-electron oxidation reactions rather than acting as radical-scavenging antioxidant protecting LDL lipids from oxidation.

The fact that two-electron oxidants give rise primarily to TQ rather than TQEs is explained readily on the basis of the underlying chemistry. Thus, two-electron oxidants convert TOH to the intermediate tocopheroxylium cation (Figure 1)27 that hydrolyzes rapidly to TQ, rather than giving rise to the radical intermediate TO. Although TO- is a precursor to both TQ and TQEs, formation of the latter also requires the presence of peroxyl radicals (Figure 1). The most likely source of peroxyl radicals is LDL lipids undergoing lipid peroxidation, which itself is a radical chain reaction that is effectively initiated by one- but not two-electron oxidants.28 Hence, it is the lack of coexisting TO- and peroxyl radicals that explains the low concentration of TQEs when LDL is exposed to two-electron oxidants.

There are two obvious candidates for the two-electron oxidants implicated in the TOH and lipid oxidation seen in early atherosclerosis, ONOO\(^-\) and HOCl. Although our results do not allow us to distinguish between different types of two-electron oxidants, the concomitant formation of nitric oxide and superoxide, eg, by vascular cells,29 is a potential source of ONOO\(^-\), and there is chemical6 and immunohistochemical10 evidence for its involvement in LDL oxidation in the artery wall. Alternatively, or in addition, HOCl or related oxidant(s) produced by activated phagocytes may be involved. Indeed, there is growing evidence for this.31 We showed earlier that HOCl-modified proteins, including apolipoprotein B–containing lipoproteins accumulate in human lesions.32 Consistent with this, Heinecke and coworkers showed preferential accumulation of lipid and protein products derived from oxidation with HOCl or myeloperoxidase/H\(_2\)O\(_2\)/Cl\(^-\) in human lesion LDL.5,24 Our in vitro studies indicate that activated human monocytes oxidize LDL predominantly via processes mediated by two-electron oxidants.

**TABLE 2. Relative Amounts of CE-O(O)H and TQ(E) in Human Atherosclerotic Lesions**

<table>
<thead>
<tr>
<th>Lesion Type*</th>
<th>CE-O(O)H†</th>
<th>TQ(E)‡</th>
<th>CE-O(O)H/TQ(E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I–II aorta</td>
<td>0.07</td>
<td>0.88</td>
<td>0.08</td>
</tr>
<tr>
<td>II–III aorta</td>
<td>0.99</td>
<td>0.62</td>
<td>1.6</td>
</tr>
<tr>
<td>IV–V aorta</td>
<td>5.2</td>
<td>1.0</td>
<td>5.2</td>
</tr>
<tr>
<td>V–VI aorta</td>
<td>6.9</td>
<td>0.93</td>
<td>7.4</td>
</tr>
<tr>
<td>Carotid plaque</td>
<td>4.7</td>
<td>0.66</td>
<td>7.1</td>
</tr>
</tbody>
</table>

*Stary classification.15
†Average number per LDL, assuming 550 molecules of FC per LDL.
‡TQE = TQE1 + TQE2 + TQ.

**Figure 5.** TOH, TOH oxidation products, and CE-O(O)H in human carotid atherosclerotic plaque. Data are expressed as nmol per mg of protein and mol% of total (nonoxidized parent plus related products) in parentheses above each bar. Mean ± SEM (n = 11).
Formation of TQ was largely nitrite-dependent (Figure 3B), suggesting a significant involvement of reactive nitrogen species such as nitryl chloride and ONOO⁻. This conclusion seems at odds with an earlier report by Hazen et al⁵ that suggested the primary involvement of the one-electron oxidant nitrogen dioxide, although a participation of nitryl chloride and ONOO⁻ was not excluded. The present findings therefore indicate that further studies are warranted to more clearly establish the major oxidants produced by activated human monocytes in the presence of NO₃⁻.

The ratio of oxidized lipid to oxidized TOH increased steadily from 0.1 to 7 as the severity of the lesions increased (Table 2). Thus, lipid oxidation becomes more prevalent as lesions develop. However, even at the most advanced stage, the ratio of oxidized lipid to TOH still more closely resembled that seen with two-electron oxidants, suggesting that, overall, radical oxidants play a less important role in LDL oxidation in the vessel wall than has been generally assumed.

As indicated, the concentrations of CE-O(O)H detected in fibrofatty and advanced lesions were higher than the total amount of TOH oxidation products. This seems surprising considering that TOH is 10⁻⁵-fold more reactive toward peroxyl radicals than CE, and lesions contain only 50- to 100-fold more CE than TOH. Thus, on purely kinetic grounds, one would expect at least 10⁴-fold more oxidation of TOH than lipid. The observed ratio of CE-O(O)H to TOH oxidation products of around seven in advanced lesions is however fully consistent with TMP.⁹,¹¹ This model predicts that TOH can react more frequently with LDL lipids (to generate CE-O(O)H) than it reacts with other radicals, such as lipid peroxyl radicals, to inhibit lipid peroxidation. If TOH were to predominantly react with lipid peroxyl radicals, we would expect TQEs to be formed in high yields (Figure 1), whereas we detected only trace levels of these TOH oxidation products at all stages of the disease.

The above explanations imply that TOH does not effectively inhibit lipid oxidation in the vessel wall, and that TMP operates in vivo. Indeed, there is additional support for the idea that TMP contributes to lipid oxidation during atherogenesis. Thus, the distribution of configurational isomers of CE-O(O)H suggests that in human lesions most of the oxidized lipids are formed in the presence of TOH.¹¹ Also, in animal models of atherosclerosis, compounds that inhibit TMP (ie, co-antioxidants)³⁵–³⁷ significantly decrease the accumulation of oxidized lipids in the vessel wall. Therefore, our present observations suggest that it is the lack of availability of co-antioxidants rather than depletion of vitamin E that explains why lipids become increasingly oxidized as lesions develop. The fact that advanced human atherosclerotic lesions contain ascorbate,¹ a powerful co-antioxidant,¹⁰ suggests that vitamin C may be localized within cells, whereas lipoprotein lipid oxidation may occur outside cells.

There are several limitations to our study. Given the ethical implications involved, it was not possible to obtain and analyze the postmortem material within a substantially shorter period of time. This leaves open the possibility for postmortem artifacts, the most likely of which would be artifactual oxidation of TOH and lipids. Two lines of evidence argue against this. First, the overall extent of oxidation of TOH, the most readily oxidizable substance, is low, and normal levels of nonoxidized vitamin E remain present. Second, if significant artifactual oxidation had occurred, we would have expected more oxidized TOH and lipid to be present in the most advanced aortic lesions than in the comparably diseased carotid material that we obtained freshly (see however Figure 5). Another limitation to our study is the fact that we examined snapshot views of the disease process only, so that we cannot rule out the possibility that some of the TOH and/or lipid oxidation products measured had undergone metabolism. To address this issue, we spiked lesion homogenates with labeled TQ or TQEs before incubation at 37°C for up to 18 hours. Only marginal degradation of the labeled compounds was observed (see experimental procedures and supporting data in the online supplement available at http://www.circresaha.org). Also, topical application of TQEs to mice followed by analysis of the epidermis showed that TQEs are stable in vivo.³⁸ and to date, there are no metabolic processes known to degrade TQEs. This argues against a preferential metabolism of TQEs over TQ that, if occurring, would limit the validity of our conclusion that oxidative events in the artery wall are primarily initiated by two-electron oxidants. TQ can be reduced to TOH in vitro,³⁹ although this requires harsh chemical conditions unlikely to exist in vivo. Finally, our results would be confounded if TOH oxidation products other than those measured were formed in vivo. Indeed, our in vitro LDL oxidation experiments with Cu²⁺ show that this is feasible. However, there is no good evidence to suggest that Cu²⁺ participates in LDL oxidation in the vessel wall, and if it were involved, we would have expected more TQE than TQ to accumulate (Figure 2B). Because this was not the case and the TOH oxidation products detected accounted for most of the TOH lost in the other in vitro LDL oxidation conditions, substantial formation of unknown oxidation products of vitamin E seems unlikely. This is particularly so, as there is no evidence that a substantial amount of TOH becomes oxidized in the vessel wall as lesions develop.

In conclusion, we show that in human atherosclerotic lesions, most of the endogenous vitamin E remains intact and that the small extent of oxidation of the vitamin takes place early in atherogenesis and is primarily caused by two-electron oxidants. Lipid oxidation becomes more prevalent at later developmental stages, although this process does not appear to be inhibited by vitamin E. This together with the coexistence of TOH and oxidized lipid suggests that vitamin E supplements are not likely to be beneficial in preventing LDL lipid oxidation in the vessel wall. This view is supported by the overall disappointing outcome of human intervention studies aimed at lowering coronary heart disease with vitamin E supplements. Our findings suggest that co-antioxidants and/or antioxidants that scavenge two-electron oxidants should instead be considered as alternative antiatherogenic supplements.

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References


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Vitamin E Oxidation in Human Atherosclerotic Lesions

Terentis et al.

Preparation of LDL Enriched with Deuterium-Labeled TOH

5-[\textsuperscript{2H}\textsubscript{3}-Methyl]-\textalpha-tocopherol (d\textsubscript{3}-TOH) was prepared from the corresponding acetate ester by lithium aluminium hydride reduction.\textsuperscript{1} d\textsubscript{3}-TOH in dimethylsulfoxide was added to freshly prepared plasma to a final concentration of 500 \(\mu\text{mol/L}\) d\textsubscript{3}-TOH and 0.3\% v/v dimethylsulfoxide. The plasma was then incubated at 37\(^\circ\)C for 5 h before LDL (enriched with d\textsubscript{3}-TOH) was isolated by centrifugation.

Recovery and Stability of TOH Oxidation Products in Lesion Samples

The efficiency of recovery of TOH and its oxidation products through the GCMS sample extraction process was shown to be \(>90\%\) for all species.\textsuperscript{1} However, we also tested the recoveries of TOH and its oxidation products added to atherosclerotic lesion specimens prior to homogenization. Thus, d\textsubscript{3}-TOH-enriched LDL was incubated at 37\(^\circ\)C with 2,2\'-azobis(2-amidinopropane) dihydrochloride (10 mmol per \(\mu\text{mol LDL, 5 h}\)) until the LDL particles were depleted of d\textsubscript{3}-TOH and contained d\textsubscript{3}-TQE2, d\textsubscript{3}-TQE1 and d\textsubscript{3}-TQ. The oxidized, labeled LDL was passed through a PD-10 gel filtration column and concentrated before addition to fresh carotid plaque specimens suspended in sodium carbonate buffer to a final concentration of 0.5 \(\mu\text{mol/L}\) spiked LDL. Specimens were then homogenized and the resulting homogenates assessed by GCMS using the standard extraction procedures. This showed recovery of 86 \(\pm\) 11\% d\textsubscript{3}-TQE1, 86 \(\pm\) 13\% d\textsubscript{3}-TQE2 and 92 \(\pm\) 2\% d\textsubscript{3}-TQ (mean \(\pm\) SD, \(n = 3\)).

In addition, spiked homogenates were incubated at 37\(^\circ\)C for 18 h in sodium carbonate buffer (pH = 11) or phosphate-buffered saline (pH = 7) to assess the stability of TOH oxidation...
products in the samples. This showed a decrease of 10 ± 1% ($d_3$-TQ and $d_3$-TQE2) or 20 ± 5% ($d_3$-TQE1) in the concentration of these species with no dependence on pH. Overall, these control experiments demonstrate that TOH oxidation products are relatively stable and efficiently recovered from the lesion samples.

**TOH Oxidation During Sample Work-up**

Previous work established that artefactual oxidation of TOH during tissue storage and homogenization is suppressed by the presence of >100-fold excess of butylated hydroxytoluene over TOH in the extraction mixtures and the use of chelex-treated buffers containing butylated hydroxytoluene, aminotriazole (an inhibitor of myeloperoxidase), ethylenediaminetetraacetic acid and diethylenetriaminepentaacetic acid.\textsuperscript{1-3} We carried out additional control experiments in which $d_3$-TOH-enriched LDL was added to the lesion material before mincing and homogenizing, and the extent of oxidation of the labeled tocopherol measured. The relative amount (mol% of total) of each species measured after workup was 95.9 ± 1.6% $d_3$-TOH, 2.7 ± 1.9% $d_3$-TQ, 0.3 ± 0.1% $d_3$-TQE1 and 1.1 ± 0.9% $d_3$-TQE2 (mean ± SD, n = 3). Thus, <5% of TOH is oxidized during sample preparation.

As a positive control, we also carried out ‘autoxidation experiments’ in which spiked samples were incubated under air at 37°C for 18 hours. This resulted in measurable oxidation of TOH with the content of TQ and TQE2 increasing by 33 and 193% respectively (Online Table 1). This demonstrates that our method is suitable to detect tocopherol oxidation when it occurs, and also shows that autoxidation leads to preferential accumulation of TQE2 over TQ, in contrast to the distribution of endogenous products seen in non-incubated plaque samples.
Online Table 1. Human carotid plaque specimens were spiked with $d_3$-TOH-enriched LDL and homogenized using the same procedure as non-spiked samples. The homogenates were then incubated at 37°C for 18 h before $d_3$-TOH and $d_3$-TQE1, $d_3$-TQE2 and $d_3$-TQ were measured by GCMS. Data represent the concentration ($\mu$mol/L) of each deuterated species in the samples at the time points indicated, mean ± SD for 3 separately spiked specimens.

<table>
<thead>
<tr>
<th>species</th>
<th>t = 0 (post-homogenization)</th>
<th>+ 18hr</th>
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</thead>
<tbody>
<tr>
<td>$d_3$-TOH</td>
<td>5.9 ± 1.5</td>
<td>4.5 ± 1.4</td>
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<tr>
<td>$d_3$-TQ</td>
<td>0.6 ± 0.5</td>
<td>0.8 ± 0.4</td>
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<tr>
<td>$d_3$-TQE1</td>
<td>0.07 ± 0.01</td>
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<tr>
<td>$d_3$-TQE2</td>
<td>0.14 ± 0.05</td>
<td>0.41 ± 0.15</td>
</tr>
</tbody>
</table>

REFERENCES