Role of α-Tocopheroxyl Radical in the Initiation of Lipid Peroxidation in Human Low-Density Lipoprotein Exposed to Horse Radish Peroxidase†

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ABSTRACT: Heme-containing (per)oxidases including horse radish peroxidase (HRP)/H_2O_2 have been shown to oxidatively modify isolated low-density lipoprotein (LDL) *in vitro* and oxidized LDL is implicated in the early events leading to atherosclerosis. The role of α-tocopherol (α-TOH) in the oxidation of LDL by HRP/H_2O_2 is unclear, although α-tocopheroxyl radical (α-TO•), which is formed during this process, can act as a chain transfer agent of lipid peroxidation in LDL. By combining HPLC and EPR spectroscopy, we hereby show that during HRP/H_2O_2-induced oxidation of human LDL: (i) the accumulation of cholesteryl linoleate hydroperoxides and hydroxides (CE-O(O)H) occurs concomitantly with the formation of α-TO• and consumption of α-TOH in the absence of other detectable organic ($g \approx 2$) radicals; (ii) the rates of α-TO• formation and subsequent decay reflect the rates of both α-TOH consumption and CE-O(O)H accumulation; (iii) CE-O(O)H accumulation is directly dependent on the level of endogenous α-TOH, and vitamin E supplementation results in increased lipid oxidizability; (iv) the inhibition of HRP activity by catalase plus urate results in a persistent α-TO• signal, the decay ($t_{1/2} \approx 20$ min) of which is accompanied by continued accumulation of CE-O(O)H, with complete cessation of lipid peroxidation upon loss of the chromanoxyl signal. These results demonstrate a direct correlation between α-TOH/α-TO• and the extent of HRP/H_2O_2-induced LDL lipid peroxidation, and that this type of oxidative modification can occur in the absence of $g \approx 2$ radicals other than α-TO•. Together, the results support a role for tocopherol-mediated peroxidation but not the involvement of a protein radical in the initiation of LDL lipid peroxidation induced by HRP/H_2O_2.

The oxidative modification of low-density lipoprotein (LDL)[1] is implicated as an early event in atherogenesis (Steinberg et al., 1989; Steinbrecher et al., 1990; Witztum & Steinberg, 1991; Ross, 1993) though the precise mechanism(s) by which LDL becomes oxidized remains unclear. Oxidatively modified LDL may be taken up by macrophages via the scavenger receptor to form lipid-laden or "foam" cells, indicative of early atherosclerotic lesions (Goldstein & Brown, 1977). As a result of the likely physiological relevance of oxidized LDL recent interest has focused on mechanisms of LDL lipid oxidation initiated by a variety of oxidants.

Studies employing strongly oxidizing conditions to initiate LDL lipid peroxidation have indicated that α-tocopherol (α-TOH) is an efficient *antioxidant* for LDL [for a review see Esterbauer et al. (1992)]. Indeed, when Cu^{2+} is used at high Cu^{2+} to LDL ratios (10−16:1) relatively small amounts of lipid hydroperoxides are accumulated until α-TOH has been depleted (Esterbauer et al., 1987).[2] Upon consumption of α-TOH, lipid peroxidation enters a rapid, "uninhibited" phase (Jürgens et al., 1987; Esterbauer et al., 1989). Under these conditions, enrichment of LDL with vitamin E increases the resistance of the lipoprotein lipid toward oxidation (Dieber-Rotheneder et al., 1991; Reaven & Witztum, 1993).

By contrast, when lower fluxes of oxidants are used to oxidize LDL (for example either aqueous or lipophilic peroxyl radicals (ROO•), Cu^{2+}:LDL ratios ≤ 3, Ham's F-10 medium in the absence and presence of human macrophages, hydroxyl radicals, or soybean 15-lipoxygenase) α-TOH acts as a *pro-oxidant* in the initial stages of lipid peroxidation of isolated, ascorbate- and ubiquinol-10-free LDL (Bowry et al., 1992a; Bowry & Stocker, 1993; Witting et al., 1995; Kontush et al., 1996; Neuzil et al., 1997; Thomas et al., 1996). That is, LDL lipid peroxidation proceeds via a radical-chain reaction in the presence of α-TOH, is enhanced by α-TOH enrichment and markedly suppressed in LDL depleted of α-TOH, and is faster in the presence of α-TOH than immediately after its depletion.

These seemingly contradicting observations can be explained readily by the oxidant-induced conversion of α-TOH to α-tocopheroxyl radical (α-TO•), which in turn acts as a chain-transfer agent by initiating and propagating LDL lipid

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[1] Abbreviations: AAPH, 2,2′-azo*bis*(2-amidinopropane); AU, absorbance units; apo B, apolipoprotein B 100; apo B•, apo B-derived radical; HTAC, cetyltrimethylammonium chloride; CE-O(O)H, cholesteryl linoleate hydroperoxides and hydroxides; EPR, electron paramagnetic resonance spectroscopy; HRP, horse radish peroxidase; H_2O_2, hydrogen peroxide; LDL, low-density lipoprotein; LOO•, lipid peroxyl radicals; LH, bisallylic hydrogen-containing polyunsaturated lipid; LOOH; lipid hydroperoxides; protein•, protein-derived radical; R_p, rate of propagation; R_{pmax}, maximal rate of propagation; TPI, 2,2,5,5-tetramethyl-4-phenylimidazolin-3-oxide-1-oxyl; α-TOH, α-tocopherol; TMP, tocopherol-mediated peroxidation; α-TO•, α-tocopheroxyl radical; α-TQ; α-tocopherol quinone.

[2] Recent studies (Neuzil et al., 1997) demonstrate, however, that in the absence of preformed lipid hydroperoxide, α-TOH is required for Cu^{2+}-induced LDL lipid peroxidation, thereby questioning the ability of the vitamin to act as an antioxidant.

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peroxidation via tocopherol-mediated peroxidation (TMP) (Bowry et al., 1992a; Bowry & Stocker, 1993; Waldeck & Stocker, 1996). Under conditions of high radical flux this chain-transfer activity of α-TO$^{\bullet}$ is inhibited due to preferential radical−radical termination reactions, resulting in the consumption of and overall antioxidant effect by the vitamin.

Peroxidase-mediated LDL lipid oxidation has been suggested as an *in vitro* model for transition-metal independent oxidation of LDL (Wieland et al., 1993), and a number of studies have employed peroxidases to initiate the oxidation of LDL lipid (Braun & von Bruchhausen, 1994; Panasenko et al., 1994; Savenkova et al., 1994; Santaman & Partharsarthy, 1995). Kalyanaraman and co-workers (1995) reported that upon treatment with horse radish peroxidase (HRP) and hydrogen peroxide (H_2O_2) conjugated dienes were formed rapidly with concomitant consumption of LDL's α-TOH and detection of α-TO$^{\bullet}$. Following complete decay of α-TO$^{\bullet}$, another free radical was detected and tentatively assigned to an apo B radical (apo B$^{\bullet}$). Although lipid peroxidation and α-TO$^{\bullet}$ were observed before the putative apo B$^{\bullet}$ was detected, the authors concluded that the latter initiated lipid peroxidation. A role for α-TOH was discounted because enrichment of LDL with vitamin E afforded LDL with increased resistance to HRP/H_2O_2-induced lipid oxidation (Kalyanaraman et al., 1995). Inconsistent with this observation, others (Braun & von Bruchhausen, 1994; Santaman & Partharsarthy, 1995) reported that supplementation of LDL with low levels of α-TOH afforded a pro-oxidant effect on LDL lipid oxidation induced by HRP/H_2O_2. Furthermore, we recently observed that LDL devoid of α-TOH was completely resistant to LDL lipid oxidation induced by HRP/H_2O_2 (Neuzil et al., 1997). In light of these contrasting results we reinvestigated the role of α-TOH in HRP/H_2O_2-induced LDL lipid oxidation.

EXPERIMENTAL PROCEDURES

Materials

Phosphate buffer (pH 7.4, 50 mM in phosphate) was prepared from nanopure water and stored over Chelex-100 (Bio-Rad, Richmond, CA) at 4 °C for at least 24 h, effectively removing contaminating transition metals, as verified by the ascorbate autoxidation method (Buettner, 1990). Stock solutions of HRP (Grad 1, 2500 units/mL, Boehringer, Mannheim, Germany) and catalase (10^6 units/mL, CALBIOCHEM, La Jolla, CA) were prepared from their respective lyophilized salts using phosphate buffer and stored at 4 °C prior to use. H_2O_2 (30% w/v) was obtained from British Drug Houses (Melbourne, Australia) and diluted to 88.2 mM with phosphate buffer. α-TOH (purity 96%) was obtained as a gift (Henkel Corporation, Sydney, Australia) and was prepared as either an 18 mM stock solution in DMSO or a 200 mM stock solution in ethanol. The nitroxide spin label 2,2,5,5-tetramethyl-4-phenylimidazolin-3-oxide-1-oxyl (TPI) was a gift from Dr. Vitaly Roginsky, (Russian Academy of Sciences, Moscow) and was used without further purification. Cholesteryl linoleate, ascorbic acid, uric acid, and cetyltrimethylammonium chloride (HTAC) were obtained from Sigma (St. Louis, MO). Stock solutions of HTAC micelles (100 mM) were prepared in phosphate buffer, and dispersions of α-TOH in such micelles were obtained by diluting the 200 mM ethanolic solution of α-TOH (Bisby & Parker, 1991) to a final concentration of either 500 or 15 μM. α-TOH-containing micelles were sonicated (47 ± 2 kHz, 30 W) for 15 s at which time the solutions were completely homogeneous. 2,2′-Azo*bis*(2-amidinopropane) (AAPH) was obtained from Polysciences (Warrington, PA). Authentic samples of cholesteryl linoleate hydroperoxide (Ch18:2-OOH) were prepared as described (Yamamoto et al., 1987) and stored as a stock solution in ethanol at −20 °C.

Methods

Preparation of Native and α-TOH-Enriched LDL. Blood was obtained from four different non-fasted healthy male and female donors (28−40 years of age) and drawn into heparin-containing vacutainers (Becton Dickinson, Rutherford, NJ). Plasma was prepared by centrifuging freshly obtained blood at 900*g* at 4 °C for 20 min and was used undiluted. LDL (density ≈1.06 g/mL) was isolated from plasma by 2 h ultracentrifugation using a TL-100 benchtop centrifuge with a TL-100.4 rotor (Beckman, Palo Alto, CA) (Sattler et al., 1994). Incubation of freshly isolated plasma with DMSO (≤2% v/v) containing various concentrations of α-TOH (0, 180, 360, and 720 μM) for 3 h at 37 °C (Esterbauer et al., 1991) prior to lipoprotein isolation, afforded LDL with increasing levels of α-TOH. LDL was then stored at 4 °C for 19−26 h, resulting in the oxidation of most of the endogenous ubiquinol-10. Immediately prior to use, excess KBr and remaining low molecular weight water-soluble antioxidants were removed from the LDL preparations by passage through a gel filtration column (PD-10, Pharmacia, Uppsala, Sweden). Where α-TOH-enriched and hydroperoxide-containing LDL was required, a sample of LDL (∼0.5 mg of protein/mL) was treated with 2 mM AAPH for 20 min at 37 °C, the reaction mixture chilled to 4 °C, and the AAPH removed by passage through two consecutive PD-10 columns. The resulting LDL was divided into four aliquots, each treated with an equal volume of lipoprotein deficient plasma (Neuzil et al., 1997), the reconstituted plasma incubated for 3 h at 37 °C with various concentrations of α-TOH, and the LDL finally reisolated as described above. The resulting LDL (∼0.5 mg of protein/mL) contained ∼15 μM Ch18:2-OOH (corresponding to ∼15 molecules Ch18:2-OOH/LDL particle) and varying levels of α-TOH, and was used immediately. Where indicated pooled LDL, obtained from at least two different donors, was concentrated to 3−4 mg of protein/mL using Centriprep-30 concentrator tubes (molecular weight cut-off 30 kDa, Amicon Inc., Beverly, MA). LDL concentrations were determined from the cholesterol content (Esterbauer et al., 1992), assuming that each lipoprotein particle contains 550 molecules of free cholesterol (Bowry et al., 1992b).

Oxidation of LDL and α-TOH Using HRP/H_2O_2. Briefly, LDL was chilled to 4 °C before addition of H_2O_2 (1 mM) and HRP (5−50 units/mg of LDL protein), the mixtures were incubated at 37 °C, and the time-dependent consumption of antioxidants and formation of lipid oxidation products were determined (see below). Where appropriate, two aliquots of the reaction mixtures were taken; the first was used immediately for EPR spectroscopic analyses (see below) and the second for corresponding HPLC analyses. The time-dependent consumption of α-TOH (15 μM) dispersed in HTAC micelles in the presence of HRP/H_2O_2 was monitored at 37 °C by using reversed-phase HPLC as described (Pascoe et al., 1987).

Inhibition of the HRP/H_2O_2-Oxidizing System. To observe the formation of compounds I and II of HRP by UV spectroscopy (Chance, 1949), a solution of the enzyme was prepared in phosphate buffer (125 units/mL), chilled to 4 °C, and treated consecutively with H_2O_2 (1 mM), catalase (1000 units/mL), and urate (100 μM) with changes to the Soret band recorded (500−320 nm) before and after each treatment. Addition of appropriate volumes of phosphate buffer served as controls for these studies.

This protocol was employed to study HRP/H_2O_2-induced oxidation and inhibition thereof, in LDL and α-TOH-containing micelles. For this, HRP/H_2O_2-oxidizing reaction mixtures were treated with catalase (1000 units/mL) before addition of urate (100 μM), ascorbate (50 μM), or phosphate buffer (control). Aliquots of the reaction mixture were taken in duplicate and used for HPLC and EPR spectroscopy (see below).

Enzyme Activity Tests. HRP activity was determined from the oxidation of scopoletin, at 37 °C by fluorescence spectroscopy (Root et al., 1975). Briefly, a stock solution of scopoletin (25 mM, Fluka, Switzerland) was prepared in 33% (v/v) acetic acid and used within 1 day of preparation. HRP (2.5 units/mL) was prepared in phosphate buffer before addition of H_2O_2 (1 mM) followed by scopoletin (80 μM). Where "preincubated enzyme" was required, HRP and H_2O_2 were incubated for 2 h at 37 °C prior to addition of scopoletin, and the decay of the probe was followed as before.

EPR Spectroscopic Assays. X-band EPR spectra were obtained at 9.41 GHz using a Bruker ESP-300 spectrometer at 20 °C, except for the results shown in Figure 3 where a Bruker EMX-Benchtop spectrometer was used. At the times indicated aliquots (200 μL) of HRP/H_2O_2-oxidizing LDL or α-TOH (500 μM)-containing micelles were placed into an aqueous flat cell (500 μL, Wilmad, Buena, NJ) and the formation of α-TO$^\bullet$ monitored. The time lag between obtaining the reaction aliquot, tuning of the sample in the cavity and carrying out the EPR analysis was consistently 2−3 min. Unless specified otherwise, the time-dependent increase in α-TO$^\bullet$ intensity was measured using the following parameters (method 1): range, 60 G; power, 20 mW; modulation amplitude, 1.0 G; modulation frequency, 12.5 kHz; gain, 1×10^5; time constant, 163 ms; and sweep time, 20.5 s. Employing these EPR parameters and averaging the output of five cumulative scans gave α-TO$^\bullet$ with acceptable signal to noise ratio that allowed the determination of time-dependent changes in concentration of the chromanoxyl signal. For optimal signal to noise, required to maximize the possibility for the detection of other organic radicals, scans were accumulated over ∼16 min and EPR settings were optimized (method 2): power, 100 mW; modulation frequency, 100 kHz; modulation amplitude, 3.2 G; time constant, 2.5 s; and sweep time, 5.5 min. Concentrations of α-TO$^\bullet$ were estimated by comparison of the total peak area for the α-TO$^\bullet$ signal with that obtained from a 5 μM solution of the nitroxide TPI, measured under identical spectrometer conditions in the absence of LDL.

Analysis of Lipid Hydroperoxides, α-TOH, and α-Tocopheryl Quinone. The methods employed for analyzing oxidized and non-oxidized lipids by HPLC have been described previously (Sattler et al., 1994) except that UV_{234nm} rather than post-column chemiluminescence detection was used to measure both cholesteryl linoleate hydoperoxides and corresponding hydroxides (CE-O(O)H). Where experiments were carried out using concentrated LDL, 20 μL aliquots of the reaction mixtures were extracted into 5 mL of hexane and 1 mL of methanol/acetic acid (0.2% v/v) for lipid analyses. For lower concentrations of LDL (0.2−0.5 mg of protein/mL) 80−100 μL aliquots were taken for extraction. Unesterified cholesterol, which remained unoxidized throughout the experiments, was employed as the internal standard for all lipid-soluble components analyzed. Determination of α-tocopheryl quinone in the reductive mode (α-TQ) was carried out using HPLC with electrochemical detection (Pascoe et al., 1987). Monitoring of LDL oxidations using UV_{234nm} was performed discontinuously at 37 °C.

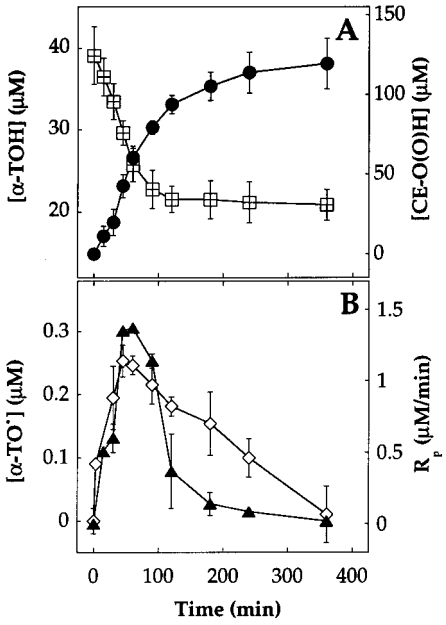

FIGURE 1: Treatment of LDL (3.2−3.6 mg of protein/mL) with H_2O_2 (1 mM) and HRP (5 units/mg of protein) at 37 °C resulted in consumption of α-TOH (crossed boxes) and accumulation of CE-O(O)H (●) (A). Concurrent EPR analyses of the reaction mixture showed the formation and subsequent decay of α-TO$^\bullet$ (◇) which correlated with the time-dependent changes in R_p (▲) (B). EPR spectra were obtained using the parameters described for method 1, accumulating five successive sweeps. Data shown are the mean ± SD of three independent experiments performed in duplicate using different preparations of pooled LDL.

RESULTS

Oxidation of LDL by HRP/H_2O_2. Incubation of LDL with HRP/H_2O_2 at 37 °C resulted in the time-dependent and initially rapid consumption of α-TOH and accumulation of CE-O(O)H (Figure 1A). After ∼2 h, the consumption of α-TOH and accumulation of CE-O(O)H slowed markedly, with no significant further changes after 4 h. The apparent kinetic chain length (ν, $\nu = R_{pmax}/R_i$) for the reaction was 1.5−2, where R_{pmax} = maximum rate of lipid peroxidation, and R_i = the rate of initiation, as estimated by -2Δ[α-TOH]/ΔT (ie, by the method of Hammond as applied to LDL; Boozer et al., 1955; Bowry & Stocker, 1993). The rate of CE-O(O)H accumulation showed two distinct phases but no clear "lag-period" (Figure 1B); this is consistent with previous reports (Braun & von Bruchhausen, 1994; Kalyanaraman et al., 1995; Neuzil et al., 1997). The concentration of α-TO$^\bullet$ increased during the first 30 min of incubation and remained at ∼0.25 μM for approximately the following 1 h before decaying slowly (Figure 1B). No other organic free

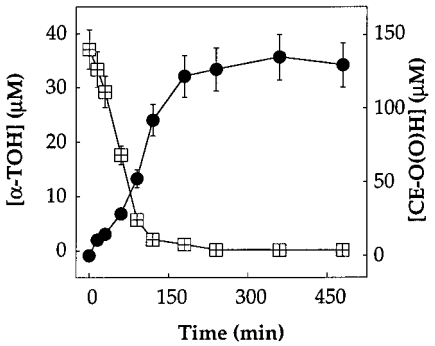

FIGURE 2: Treatment of LDL (3.0−3.5 mg of protein/mL) with H_2O_2 (1 mM) and HRP (10 units/mg of protein) at 37 °C resulted in relatively high rates of α-TOH consumption (crossed boxes) and accumulation of CE-O(O)H (●). Data shown are the mean ± SD of two independent experiments each performed in duplicate using different preparations of pooled LDL.

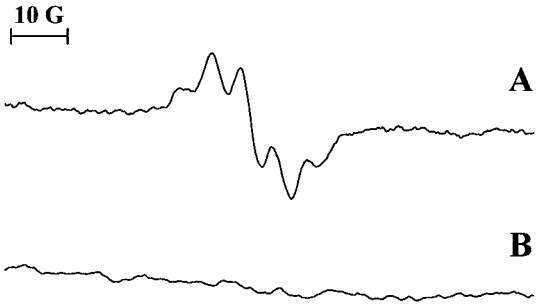

FIGURE 3: Treatment of LDL (3.5 mg of protein/mL) with H_2O_2 (1 mM) and HRP (10 units/mg of protein) at 37 °C resulted in the immediate formation of α-TO• (A) which decayed to below the detection limit after 4 h (B). No other $g \approx 2$ radical was detected for up to 6 h after complete decay of α-TO•. Spectra were obtained using the optimized EPR parameters (method 2), with the receiver gain initially set at 1×10^5 and increased to 2×10^6 after loss of α-TO•. Spectra shown are representative of three independent experiments performed in duplicate with different preparations of pooled LDL.

radical ($g \approx 2$ radical) was detected during the 6 h period for which the oxidation was monitored. The time over which α-TO• remained at a near steady state level was similar to the period where R_{pmax} was sustained (Figure 1B). There was a reasonable correlation between the time to onset and subsequent rate of decrease in both the rate of peroxidation (R_p) and the α-TO• concentration. The relatively large error in the measurement of R_p between 100 and 180 min is inherent to the estimation in the tangential rates at a point of inflexion. The depletion of α-TO•, however, coincided with cessation of detectable lipid peroxidation.

We next employed a higher HRP:LDL ratio to duplicate the oxidant:LDL ratio for which the putative apo B• was reported (Kalyanaraman et al., 1995) (Figure 2). Under these comparatively harsh oxidizing conditions CE-O(O)H accumulation again exhibited biphasic kinetics, while the rate and extent of α-TOH consumption was enhanced (*cf.* Figure 2 vs Figure 1), resulting in a lower kinetic chain length ($\nu \approx 1$). The maximum concentration of α-TO• detected was marginally lower, but the steady state level was also maintained for ∼1 h (not shown). The decay of α-TO• to below the detection limit however, was more rapid and coincided with depletion of α-TOH to <3% of its starting level (not shown). However, $g \approx 2$ signals other than α-TO• were not detected, either during or for up to 6 h post α-TOH depletion (not shown), even when the signal to noise ratio was optimized (see Methods), the entire experiment was performed inside the EPR cavity, three different preparations of LDL pooled from several different subjects were employed, or a different EPR spectrometer was used (Figure 3).

Oxidation of α-TOH-Supplemented LDL with HRP/H_2O_2. To further test a potential involvement of α-TO• in LDL lipid peroxidation by HRP/H_2O_2, we reinvestigated the effect of α-TOH supplementation. Independent of the LDL concentration and HRP:LDL ratio employed, increasing the content of LDL's α-TOH increased R_{pmax} and the extent of CE-O(O)H accumulation (Figure 4). R_i values for the individual LDL samples were similar; e.g., after 90 min, 4.5, 4.4, 4.7, and 4.5 μM α-TOH were consumed for the highest to lowest α-TOH-supplemented LDL's, respectively (Figure 4B). Using UV_{234nm} spectroscopy instead of HPLC analyses also showed increased peroxidation rates with increasing α-TOH content (data not shown). Furthermore, oxidation of α-TOH-supplemented LDL containing 15 μM CE-O(O)H (see Methods) also showed enhanced LDL lipid oxidizability (data not shown), demonstrating that the observed prooxidant activity of α-TOH was independent of both the method used for analysis and the presence of preformed hydroperoxides and directly supporting the involvement of α-TOH in the initiation of oxidation of LDL lipid by HRP/H_2O_2.

Above results indicated that the rate and extent of α-TOH consumption was dependent on the ratio of HRP:LDL. To further test this, LDL was divided into four aliquots treated with 1 mM H_2O_2 and either 0, 5, 10, or 50 units of HRP/mg of protein. In the absence of HRP, a slow consumption of α-TOH was observed without CE-O(O)H accumulating. Increasing the ratio of HRP:LDL resulted in the stepwise increase in the rate and extent of α-TOH consumption. By contrast, R_{pmax} and the extent of CE-O(O)H accumulation remained largely unaffected (Figure 5), resulting in an inverse relationship between ν and R_i, with no "lag-period". These features cannot be explained by the conventional model of lipid peroxidation but are fully consistent with peroxidation proceeding via TMP (Bowry & Stocker, 1993). Increasing the HRP:LDL ratio also resulted in a stepwise decrease in the initial level of α-TOH, as measured at $t = 0$ min, with little or no lipid oxidation. Only marginal increases in α-TQ content, a maximum of 0.8 μM at the highest HRP:LDL ratio employed, were observed, indicating that this type of two-electron oxidation of vitamin E was not a primary reaction pathway, consistent with a recent report (Nakamura & Hayashi, 1992).

Inactivation of HRP. To test whether the cessation in α-TOH consumption noted above was due to inactivation of HRP or depletion of H_2O_2, the oxidation of scopoletin was examined with and without preincubation of HRP/H_2O_2 at 37 °C for 2 h. Preincubation decreased HRP activity, whereas addition of a second aliquot of H_2O_2 to fresh or preincubated HRP/H_2O_2 did not effect HRP activity (data not shown). By contrast, addition of HRP (5 units/mg of protein) after 90 min of LDL lipid oxidation resulted in a marked increase in both the rate of α-TOH consumption and accumulation of CE-O(O)H (Figure 6). These results indicate that HRP becomes inactivated upon reaction with H_2O_2 in the absence or presence of LDL.

Elimination of Redox Active Forms of HRP/H_2O_2 by Urate. Treatment of HRP (125 units/mL) with H_2O_2 (1 mM) at 4

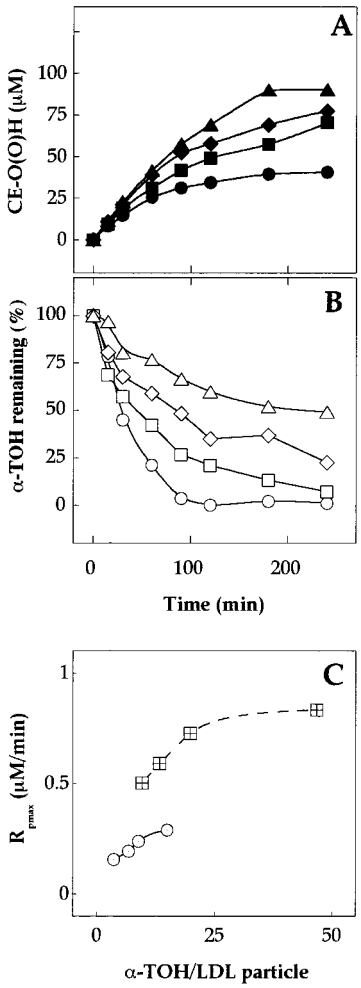

FIGURE 4: α-TOH-supplemented LDL showed enhanced susceptibility to HRP/H_2O_2-induced lipid oxidation. LDL (0.25−0.29 mg of protein/mL) with 4.6 (circles), 6.7 (squares), 8.8 (diamonds), or 14.9 (triangles) α-TOH/LDL particle was incubated at 37 °C with 14 units of HRP/mg of protein and H_2O_2 (1 mM), the accumulation of CE-O(O)H (A) and consumption of α-TOH (B) were monitored, and the dependence of R_{pmax} on increasing α-TOH (C) was determined. The broken line in C refers to the dependence of R_{pmax} on α-TOH content in a separate set of experiments using LDL (0.5−0.6 mg of protein/mL) containing increasing amounts of the vitamin and oxidized with 5 units of HRP/mg of protein at 37 °C. Data shown are representative of three independent experiments carried out in duplicate using different preparations of α-TOH-supplemented LDL.

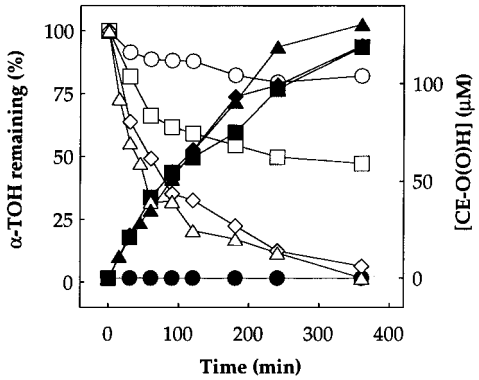

FIGURE 5: Rate of α-TOH consumption, but neither the rate nor the extent of CE-O(O)H accumulation is dependent on the HRP:LDL ratio. LDL (3.5−3.6 mg of protein/mL) was divided into four aliquots to which 1 mM H_2O_2 was added, followed by 0 (circles), 5 (squares), 10 (diamonds), or 50 (triangles) units of HRP/mg of protein, and both α-TOH consumption and CE-O(O)H accumulation were monitored at 37 °C. Initial α-TOH levels were 46, 43, 41, and 38 μM for the lowest to highest HRP:LDL ratios, respectively. Data are representative of three independent experiments carried out in duplicate and using different preparations of pooled LDL.

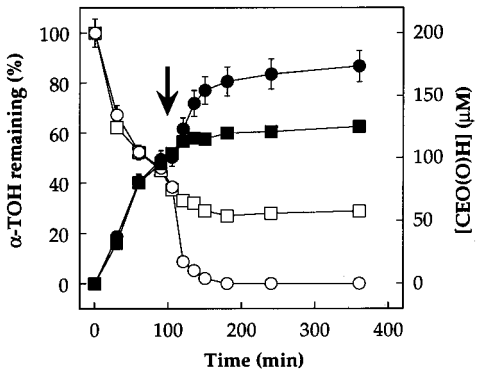

FIGURE 6: HRP is inactivated on prolonged incubation with H_2O_2 and LDL at 37 °C. LDL (3.0 mg of protein/mL) was treated with H_2O_2 (1 mM) and 5 units of HRP/mg of protein. Where indicated (arrow), a second aliquot of HRP (∼5 units/mg of protein) was added resulting in increased rates of α-TOH consumption (open circles) and lipid peroxidation (closed circles) compared with a control (open and closed squares, respectively). The initial α-TOH level was 41.2 ± 1.2 μM. Data shown are mean ± SD of a triplicate study representative of two independent studies using different preparations of pooled LDL. Where SD are not shown the symbols are larger than the error.

°C resulted in a shift in the Soret band from 407 to 399 nm with a decrease in intensity by 0.1 absorbance units (AU). Addition of catalase (1000 units/mL) neither affected λ_{max} nor peak intensity. After an additional 2 min, addition of urate (100 μM) temporarily shifted both the Soret band (from 399 to 418 nm) and intensity (from 0.2 to 0.3 AU). This complex was relatively unstable and decayed to resting HRP, but at lower concentration (0.2 compared with originally 0.3 AU), in support of enzyme inactivation. These spectral changes are consistent with cycling of HRP between the rest state via compounds I and II upon sequential addition of H_2O_2 and urate (Chance, 1949; George, 1952; Job & Dunford, 1976), and suggests that enzyme inactivation occurs during this cycling. To test whether this protocol is useful to completely halt HRP activity, a dispersion of α-TOH (15 μM) in HTAC micelles was first treated with HRP (2.5 units/mL) and H_2O_2 (1 mM). This resulted in the time-dependent consumption of α-TOH and formation of α-TO$^\bullet$ (data not shown). Addition of catalase followed (after 2 min) by either ascorbate (50 μM) or urate (100 μM) resulted in complete prevention of further α-TOH consumption, confirming that this protocol effectively eliminated the redox active forms of HRP. Importantly, parallel EPR studies directly demonstrated that α-TO$^\bullet$ persisted in the presence of urate but not ascorbate, where the ascorbyl radical [doublet signal with $A^H \approx 1.75$ G (Kalyanaraman et al., 1992; Sharma & Buettner, 1993)] was formed immediately upon addition (data not shown).

Differential Effect of Urate and Ascorbate on HRP/H_2O_2-Oxidizing LDL. Treatment of HRP/H_2O_2-oxidizing LDL with catalase followed by either ascorbate or urate as described above, resulted in immediate and slow decay of α-TO$^\bullet$, respectively, compared with the control sample (addition of buffer instead of catalase followed by the antioxidant) (Figures 7A−G and 8A). In the case of ascorbate, the immediate decay of α-TO$^\bullet$ coincided with detection of ascorbyl radicals (data not shown). In the case

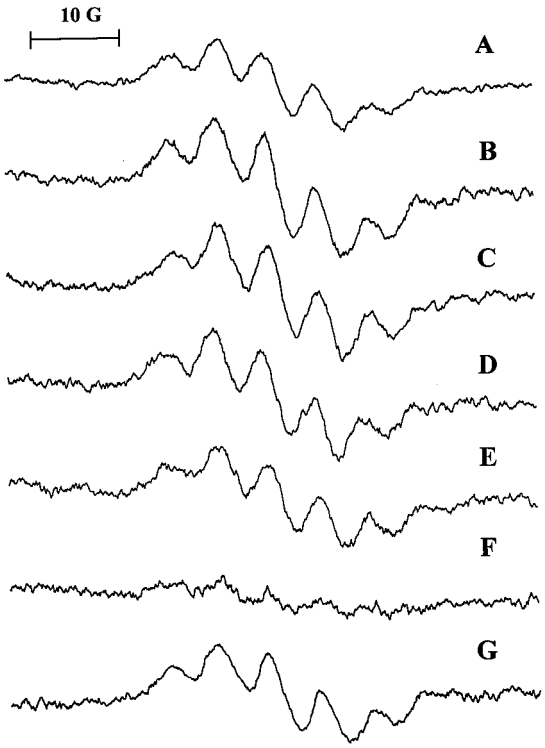

FIGURE 7: Time-dependent changes in α-TO• concentration in LDL treated sequentially with HRP/H_2O_2 then either phosphate buffer (control) or catalase followed by urate. LDL (3.6 mg of protein/mL) was treated with 1 mM H_2O_2 and 5 units of HRP/mg of protein at 37 °C. After 20 min, catalase (1000 units/mL) was added and after a further 2 min urate (100 μM) was added to the mixture, while monitoring continued. At the times indicated, (A) 5, (B) 15, (C) 20, (D) 25, (E), 30, and (F) 45 min, aliquots were removed for EPR spectroscopic analyses. A sample taken from a control oxidation at 90 min is shown in G. EPR parameters are as for Figure 1. Data shown are representative of three independent experiments carried out in duplicate and using different preparations of pooled LDL.

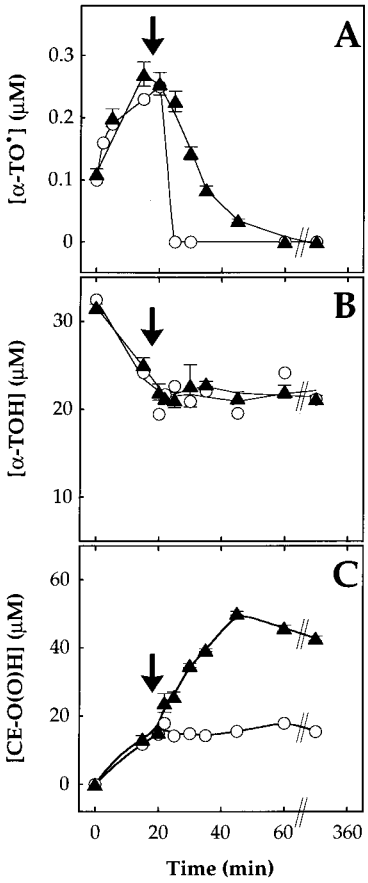

FIGURE 8: Time-dependent changes in the concentration of α-TO•, α-TOH, and CE-O(O)H in HRP/H_2O_2-oxidizing LDL treated sequentially with catalase followed by either urate or ascorbate. Experimental conditions are as described in the legend to Figure 7. The concentration of α-TO• (A), α-TOH (B) and the accumulation of CE-O(O)H (C) were measured in the presence of either 50 μM ascorbate (○) or 100 μM urate (▲). The data shown represent mean values of three independent studies carried out in duplicate and using different preparations of pooled LDL. SD are shown in the case of urate only, for reason of clarity. SD in the case of ascorbate were ≤8.6% of the values shown.

of urate, α-TO• decayed with an estimated half-life of ∼20 min (Figure 8A). Corresponding HPLC analyses indicated that addition of catalase followed by addition of either urate or ascorbate resulted in the immediate cessation of α-TOH consumption (Figure 8B). Addition of ascorbate, which can reduce α-TO• in oxidizing LDL (Kagan et al., 1992; Witting et al., 1996), also resulted in the complete prevention of further accumulation of CE-O(O)H (Figure 8C). By contrast, addition of urate, which cannot reduce chromanoxyl radicals in aqueous solutions (Davies et al., 1988), failed to both immediately eliminate α-TO• in oxidizing LDL (Figures 7 and 8A) and to prevent further accumulation of CE-O(O)H (Figure 8C). The sustained accumulation of CE-O(O)H, in the absence of an active enzyme, persisted only while α-TO• was detected (*cf.* Figure 8A and 8C). As expected, oxidizing LDL treated with catalase/urate peroxidized at slower rates and for shorter periods of time than oxidizing LDL in the absence of both catalase and urate (not shown).

DISCUSSION

On the basis of the lack of supporting literature, direct oxidation of LDL lipids by HRP/H_2O_2 is unlikely, suggesting that LDL oxidation by this enzymic system occurs indirectly, either via apolipoprotein B or non-proteinaceous compounds associated with LDL other than its lipids. Kalyanaraman and co-workers recently suggested (1995) that HRP/H_2O_2 oxidizes LDL lipids containing bisallylic hydrogens (LH) via the initial generation of apoB•, and scavenging of the lipid peroxyl radical (LOO•) by α-TOH to give α-TO• and lipid hydroperoxides (LOOH) (reactions 1−4).

$$\text{apo B} + \text{HRP/H}_2\text{O}_2 \rightarrow \text{apo B}^\bullet + \text{rest state enzyme} \quad (1)$$

$$\text{apo B}^\bullet + \text{LH} \rightarrow \text{apo B} + \text{L}^\bullet \quad (2)$$

$$\text{L}^\bullet + \text{O}_2 \rightarrow \text{LOO}^\bullet \quad (3)$$

$$\text{LOO}^\bullet + \alpha\text{-TOH} \rightarrow \text{LOOH} + \alpha\text{-TO}^\bullet \quad (4)$$

There is evidence that protein radicals (protein•) are formed during the action of HRP/H_2O_2 or enzymes with peroxidase activity and that these radicals can be translocated intra- (Wilks & Ortiz de Montellano, 1992) and intermolecularly (Ortiz de Montellano & Catalano, 1985; Ortiz de Montellano & Grab, 1987; Heinecke et al., 1993; Moreau et al., 1995). The latter may result in the co-oxidation of low molecular weight substrates or protein cross-links. In direct support of a protein• being involved in the oxidation of LDL's LH by HRP/H_2O_2, Kalyanaraman and co-workers (1995) re-

ported a $g \approx 2$ radical different to that of α-TO•; this was detected only upon prolonged incubation of LDL with HRP/H_2O_2 and the species that gave rise to this signal was found to be present in the protein pellet after a Folch extraction of the reaction mixture. The assignment of this radical to apo B (Kalyanaraman et al., 1995) rather than to HRP itself, and the nature of this species remained inconclusive (hence the distinction of protein• *vs* apo B• made here). Also, the proposed role of apo B• in the *initiation* of LDL lipid peroxidation (reaction 2) appears to contradict the authors' own observation [Figure 8 in Kalyanaraman et al. (1995)] that for the first 100 min of the reaction α-TO• was the only radical detected, and its detection clearly preceeded that of the other $g \approx 2$ radical. These latter observations could be explained if α-TOH (rather than LH) were to rapidly scavenge the apo B• initially formed, although this would also be inconsistent with the mechanism proposed (Kalyanaraman et al., 1995) while supporting TMP. Alternatively, apo B• and α-TO• could be formed in different LDL particles (with only the latter radical species being detectable) and each could contribute to the initiation of lipid peroxidation. However, inconsistent with this proposed role of apo B• is our recent observation that LDL devoid of tocopherol (and lipid hydroperoxides) is completely resistant to HRP-induced lipid peroxidation (Neuzil et al., 1997).

We repeatedly failed to detect any $g \approx 2$ radical other than α-TO•, independent of the HRP:LDL ratio, the EPR spectrometer, the blood donors for LDL, and whether the LDL used contained preformed LOOH or not. The experimental conditions used here are similar though not identical to those of Kalyanaraman et al. (1995). While we cannot rule out significant differences in the LDL,[3] it appears unlikely to us that the difference in the enzyme supplier was responsible for the different results obtained. It is also unlikely that our inability to detect a protein• was due to a lack of sensitivity, as the signal to noise ratio for α-TO• measured using the optimal EPR settings (see Methods) was comparable to that obtained elsewhere [*cf.* Figure 3A in this work with Figure 8A Kalyanaraman et al. (1995)]. In any case, we observed LDL lipid peroxidation initiation in the absence of a detectable protein• (Figures 1 and 2), consistent with the data shown by, but not the interpretation of, Kalyanaraman and co-workers (1995). From our findings we conclude that HRP/H_2O_2-induced initiation of LDL lipid peroxidation does not require a protein• and that formation of a detectable protein• is not a general feature of this type of LDL oxidation, at least as long as the lipoprotein contains α-tocopherol.

Evidence for α-TO• in HRP/H_2O_2-Induced LDL Lipid Oxidation. The protein-independent oxidation of phenols such as α-TOH, by HRP/H_2O_2 is well established (Nakamura, 1991; Nakamura & Hayashi, 1992), and α-TOH is by far the most abundant, reactive phenol present in human LDL (Esterbauer et al., 1992). Our EPR spectroscopic results from micellular dispersions and LDL treated with HRP/H_2O_2 are consistent with these reports, and show that direct oxidation of α-TOH by HRP/H_2O_2 readily occurs even when the vitamin is presented in complex emulsions. The latter is directly supported by the observation that anaerobic incubation of LDL with HRP/H_2O_2 caused the oxidation of α-TOH with concomitant formation of α-TO• (Kalyanaraman et al., 1995) (reaction 5). Once formed, α-TO• may react with LH to initiate lipid oxidation via TMP (reactions 6, 3, and 4) (Bowry & Stocker, 1993):

$$\alpha\text{-TOH} + \text{HRP/H}_2\text{O}_2 \rightarrow \alpha\text{-TO}^\bullet + \text{rest state enzyme} \quad (5)$$

$$\alpha\text{-TO}^\bullet + \text{LH} \rightarrow \alpha\text{-TOH} + \text{L}^\bullet \quad (6)$$

Several lines of evidence support this mechanism, while they cannot be explained by that described in reactions 1−4 or the conventional mechanism of lipid peroxidation and antioxidation by α-TOH (Bowry & Stocker, 1993; Waldeck & Stocker, 1996). Thus, there was a reasonable correlation between α-TO• and R_p, particularly during the early phase of the oxidation (Figure 1B), and R_{pmax} was independent of R_i (Figure 5). Also, supplementation with α-TOH enhanced the oxidizability of LDL lipid (Figure 4) independent of the presence of CE-O(O)H, whereas the lipids in α-TOH-depleted, LOOH-free lipoprotein were completely resistant to HRP/H_2O_2-induced oxidation (Neuzil et al., 1997). In addition, CE-O(O)H continued to accumulate as long as α-TO• could be detected, even in the absence of active HRP (Figures 1, 2, and 8). In particular, the ability of urate to eliminate active forms of HRP combined with its inability to both eliminate α-TO• and to prevent CE-O(O)H accumulation (Figures 7 and 8) is fully consistent with the "urate paradox" described in (Bowry & Stocker, 1993) and provides the most direct evidence to date for α-TO• acting as the lipid peroxidation chain-carrying radical in oxidizing LDL. Clearly, the relative long half-life in oxidizing LDL does not reflect an intrinsic property of α-TO•, as upon addition of ascorbate to the catalase-treated LDL/HRP system, the radical signal decayed immediately.

We have no explanation for the apparent opposite results obtained from supplementation of LDL with α-TOH between the present study and that reported by Kalyanaraman et al. (1995). Whether α-TOH acts as an anti- or pro-oxidant for LDL is dependent, in part, on the flux of radicals to which the lipoprotein is exposed (Bowry & Stocker, 1993; Neuzil et al., 1997). However, even when high HRP:LDL ratios or similar experimental conditions described in (Kalyanaraman et al., 1995) were employed, no reduction in R_{pmax} was observed upon α-TOH supplementation, nor was this affected by the presence of preformed CE-O(O)H or the method used for peroxidation measurement.

While our results strongly support a role for TMP in HRP/H_2O_2-induced LDL lipid oxidation, they do not unambiguously prove that this mechanism is operative. Indeed, the observed rapid initial depletion of α-TOH without CE-O(O)H accumulation at high HRP:LDL ratios could be seen as evidence against TMP. However, a rapid loss of α-TOH is expected to occur under these strongly oxidizing conditions which favor the presence of more than one radical per LDL particle (Bowry & Stocker, 1993; Neuzil et al., 1997). Under these conditions radical−radical termination reactions occur rapidly, thereby effectively preventing reaction 6, resulting in the inhibition of CE-O(O)H accumulation. Such termination reactions are likely to involve α-TO•, causing consumption of the vitamin without formation of LOOH. Therefore, a lack of correlation between α-TOH consumption and

[3] Kalyanaraman et al. (1995) do not describe the length of time for which LDL was stored prior to the various experiments, and this could affect the results reported, as the content of LDL's antioxidants and LOOH changes during storage at 4 °C.

LOOH accumulation does not argue *per se* against TMP. The isolation and characterization of such oxidation products of α-TOH, however, could provide useful information of the nature of the radicals involved in these reactions.

In summary, the results presented largely support a role for TMP in HRP/H_2O_2-induced LDL lipid oxidation. If this mode of lipid oxidation is applicable to other heme-containing (per)oxidases, our previous results (Bowry et al., 1995) suggest that the prevention of TMP may reduce this type of oxidative modification of LDL *in vivo*.

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