Inverse deuterium kinetic isotope effect for peroxidation in human low-density lipoprotein (LDL): a simple test for tocopherol-mediated peroxidation of LDL lipids

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Abstract **α-Tocopherol (α-TOH) can act as a pro- or anti-oxidant for isolated ubiquinol-10-free human low density lipoprotein (LDL). We demonstrate that α-TOH is a more potent pro-oxidant than other forms of vitamin E for LDL peroxidation initiated by mild fluxes of aqueous peroxyl radicals and low concentrations of Cu^{2+}. A simple deuterium exchange test shows that α-TOH switches from pro- to anti-oxidant at Cu^{2+}:LDL ratios >2.5. The results suggest that this test may be useful to distinguish 'inhibited' peroxidation of emulsion lipids propagated via the lipid peroxyl radical from that mediated via the antioxidant radical.**

Key words: Antioxidant; Atherosclerosis; Oxidation; Vitamin E

1. Introduction

The formation of oxidized low-density lipoprotein (LDL) is strongly implicated as an initiating event in the development of atherosclerosis, the major cause of death in Western society. This has led to a veritable explosion of research over the last decade into oxidation of LDL and its prevention by antioxidants, particularly α-tocopherol (α-TOH) [1], i.e. the most active and abundant phenolic antioxidant in the lipids of human plasma [2] and extracts of LDL [3]. However, despite scores of publications on the role of α-TOH in modulating the peroxidation of LDL (for reviews see [4,5]) there is no consensus as to whether enrichment of the lipoprotein with α-TOH will retard the 'oxidative modification' of LDL under physiologically feasible conditions (for the affirmative case see [6–11]; for the contrary see [12–15]).

Our investigations into the kinetics of LDL peroxidation in vitro suggest that this lack of agreement is probably due to a consequence of the lipid particle size [15,16] which results in tocopherol-mediated peroxidation (TMP), a mechanism of peroxidation that will be manifested in various ways according to experimental conditions (vide infra and [17]). Here we introduce a simple deuterium exchange test for the 'mode of antioxidant' activity, apply it to peroxyl radical-induced and Cu^{2+}-initiated LDL oxidation, and also examine the relative potency of α-TOH vs other forms of vitamin E for accelerating peroxyl radical-initiated LDL peroxidation.

2. Materials and methods

2.1. Materials

Phosphate buffer (50 mM, pH 7.4) was prepared from either nanopure water or deuterium oxide, and stored over Chelex-100 (Bio-Rad, Hercules, CA) at 4°C for at least 24 h. This treatment effectively removed contaminating transition metals, as verified by the ascorbate autoxidation method [18]. 2,2′-Azo*bis*(2-amidinopropane) (AAPH) and 2,2′-azo*bis*(2,4-dimethylvaleronitrile) (AMVN) were purchased from Polysciences (Warrington, PA) and diluted in freshly prepared phosphate buffer and ethanol, respectively. α-TOH was obtained as a gift from Henkel Corporation (La Grange, IL) and was analytically pure by HPLC analysis. The γ- and δ-tocopherols (γ-TOH and δ-TOH, respectively) were obtained from Eastman Kodak (Rochester, NY) and 98% and 86% pure, as determined by the HPLC method described below. γ-TOH contained <1% α-TOH, and δ-TOH contained 0.2% α-TOH and 8.6% γ-TOH. Vitamin E solutions were prepared as 18 mM stock solutions in DMSO. Cholesteryl linoleate, deuterium oxide and glutathione were purchased from Sigma Chemicals (St. Louis, MO). Authentic cholesteryl linoleate hydroperoxide (Ch18:2-OOH) was prepared from the purified lipid by oxidation with AMVN [19], and stored as a stock solution in ethanol.

2.2. Preparation of native and tocopherol-enriched LDL

Fresh blood was obtained from a non-fasted healthy male donor (27 years of age) and drawn into heparinized vaccutainers (Becton Dickinson, Rutherford, NJ). Plasma was prepared by centrifugation and used immediately. For enrichment of LDL with the various vitamin E isomers [20], separate aliquots of plasma (5 ml) were supplemented with 400 μl of DMSO (control) or stock solution of either α-, γ- and δ-TOH, incubated for 6 h at 37°C, before LDL was isolated by the 2 h ultracentrifugation method using the TL100.4 rotor [21]. Excess KBr and remaining water-soluble antioxidants were then removed from the LDL by gel filtration using a single PD-10 column (Pharmacia, Uppsala, Sweden). The protein content of the resulting LDL (H_2O-LDL) was obtained as described in [22].

2.3. Preparation of LDL containing deuterium-labelled tocopherol (D_2O-LDL)

Preliminary ^1H NMR spectroscopic experiments, performed using a Brucker AC200, 200 MHz spectrometer at 20°C, showed that the labile phenolic proton of α-TOH (3 mM in $CDCl_3$) was exchanged completely with deuterium after 2 min equlibration with D_2O (400 μl). This observation is consistent with the reported rapid deuterium exchange of α-TOH [23]. Similarly, ^1H NMR analysis of a $CDCl_3$ solution of the lipid fraction of α-TOH-enriched LDL extract, obtained by the method of Bligh and Dyer [24], showed a broad proton resonance in the region 3.5–4.0 ppm, assigned to the phenolic proton of α-TOH. This proton readily and completely exchanged with deuterium upon mixing of the $CDCl_3$ solution (300 μl) with D_2O (400 μl). Given the efficacy of this exchange process, LDL containing deuterium labelled tocopherol was obtained by percolating the freshly isolated lipoprotein (2 ml of ≈0.2 mg protein/ml) through a PD-10 column equilibrated immediately prior to use with 10 ml of 50 mM phosphate buffer prepared with D_2O, and eluting the LDL with 3 ml of the same buffer. The LDL thus obtained

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(D_2O-LDL) did not contain detectable amounts of Ch18:2-OOH and was used immediately for subsequent oxidation experiments.

2.4. Peroxyl radical mediated oxidation of LDL particles

Purified control or tocopherol-enriched D_2O- and H_2O-LDL (0.35–0.7 mg protein/ml, equivalent to 0.7–1.4 μM in lipoprotein particle) were oxidised by exposure to AAPH (4 mM final concentration) at 37°C under air. AAPH thermally decomposes and generates aqueous peroxyl radicals (ROO•) at a known and constant rate. Aliquots of the reaction mixture (100 μl) were withdrawn at the time points indicated, extracted with a mixture of methanol and 0.1% acetic acid (2 ml) and hexane (10 ml), the hexane layer separated, evaporated to dryness under reduced pressure, and the residue redisolved in isopropyl alcohol (200 μl) for HPLC analyses of antioxidants, cholesterol and Ch18:2-OOH (see below).

2.5. Peroxyl radical mediated oxidation of LDL lipid extracts in homogeneous solution

The lipid soluble components of freshly prepared H_2O-LDL (18 ml of ≈0.2 mg protein/ml) were extracted with chloroform and ethanol by the method of Bligh and Dyer [24]. The lipid extract was redissolved in benzene to a final volume of 2 ml and aliquots (1 ml), underlayered with 0.2 ml D_2O or H_2O, respectively, and oxidized with AMVN (0.5 mM) at 37°C under air. Aliquots of the reaction mixture (100 μl) were withdrawn at the time points indicated and extracted as described above for subsequent HPLC analyses.

2.6. Cu^{2+}-mediated oxidation of LDL particles

The concentration of H_2O- or D_2O-LDL, prepared as described above, was adjusted carefully to 0.7 or 1.4 μM (in particle) by standardising the free cholesterol content for each sample assuming 550 molecules of cholesterol per lipoprotein particle [25]. H_2O- or D_2O-LDL dispersions were then immediately treated with appropriate volumes of a 1 mM stock solution of $CuSO_4$ to give the final Cu^{2+}/LDL particle ratios indicated in the legends to Figs. 3 and 4. Time-dependent lipid peroxidation was monitored by removing aliquots of the reaction mixture at the time points indicated, and HPLC analyses of lipids and antioxidants (see below).

2.7. Analytical methods

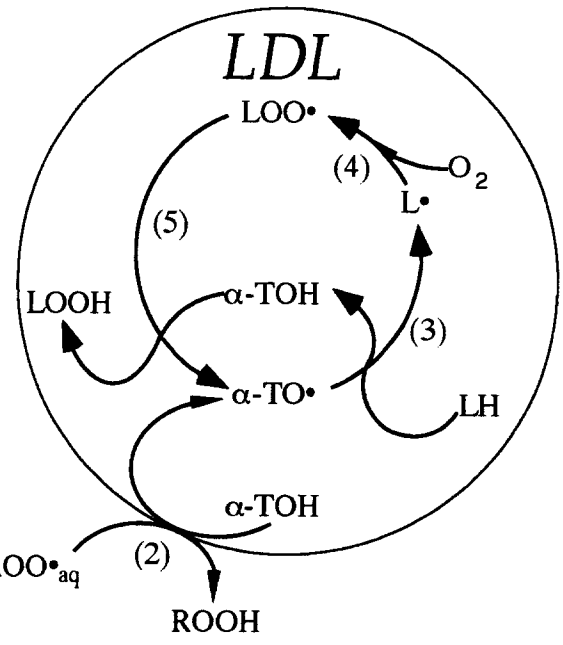
O_2 consumption was determined using a Yellow Springs Instruments Model 5300 Biological Oxygen Monitor (Yellow Springs, OH) with a Clark electrode as a sensor at 37.0 ± 0.2°C. The lipid-soluble components (free cholesterol, α-TOH and Ch18:2-OOH) present in the redisolved hexane extract were analysed as described in detail in [21] using reversed-phase HPLC with UV (210 nm), electrochemical and post-column chemiluminescence detection, respectively. The detection limit of the chemiluminescence assay for Ch18:2-OOH was 1–5 pmol. Free cholesterol was employed as the internal standard for all lipid-soluble components analysed. Where needed, LDL extracts were also analysed for δ- and γ-TOH by HPLC with electrochemical detection [26].

3. Results and discussion

α-TOH is a powerful antioxidant in homogeneous solution, where even very small amounts strongly inhibit the oxidation of lipids containing bisallylic hydrogens (LH) by rapidly trapping the chain-propagating lipid peroxyl radical (LOO•) ($k_H^{30°C} \approx 3 \times 10^6\ M^{-1} \cdot s^{-1}$ in non-polar solvent):

$$LOO^{\bullet} + \alpha\text{-TOH} \xrightarrow{k_H} LOOH + \alpha\text{-TO}^{\bullet}$$

Detailed kinetic investigations [15] into the radical-mediated aerobic peroxidation of ascorbate- and ubiquinol-10-free LDL show, however, that under conditions of very low radical peroxidation initiation (rate R_i <~5 nM·s^{-1}) endogenous α-TOH acts as a pro-oxidant in the lipoprotein — the rate of lipid hydroperoxide (LOOH) formation (R_p = d[LOOH]/dt) is *faster* in the presence of α-TOH than in the uninhibited period following its consumption ($R_p^{inh} > R_p^{uninh}$). Our explanation for this remarkable 'about face' is that LDL particles are physically too small to support more than one radical at a time, and an α-tocopheroxyl radical (α-TO•) formed (via reaction 2, Scheme 1) is too water-insoluble to escape from that particle into the surrounding aqueous environment. Under these conditions,



Scheme 1

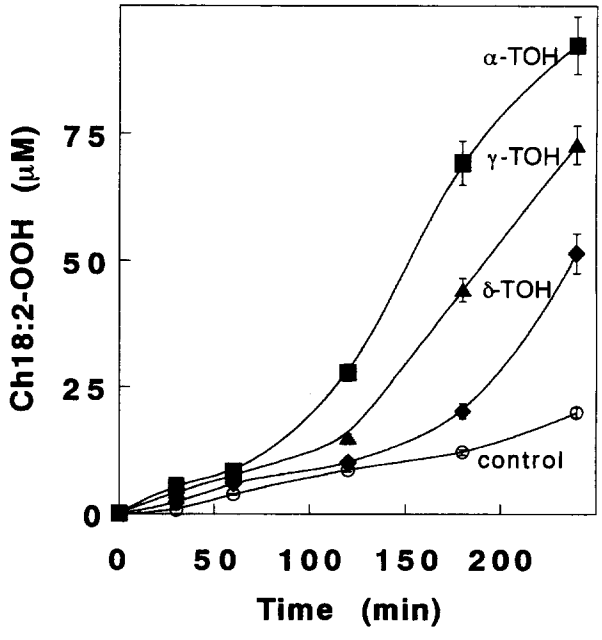


Fig. 1. α-TOH is the best pro-oxidant form of vitamin E for LDL. Purified LDL (0.35–0.7 mg protein/ml) isolated from native human plasma or plasma enriched in vitro [20] with either α-, γ- or δ-TOH, was oxidized with AAPH and analyzed for Ch18:2-OOH formation [21]. The results show mean values ± S.E.M. of three separate experiments. Initial concentrations of α-, γ- and δ-TOH for enriched and control LDL samples were 52, 51, 47 and 3.2 μM, respectively, while residual [α-TOH] for γ- and δ-TOH-enriched LDLs were 3.4 and 3.3 μM, respectively.

α-TO• propagates the chain reaction (reactions 3, 4 and 1) depicted in the inner circle of Scheme 1.

Our previous studies addressed the kinetics of propagation of such tocopherol-mediated peroxidation (TMP) of LDL lipids [15] and its inhibition by co-antioxidants [17,27] without examining the important first step of TMP - the transfer of radical centers from the aqueous into the lipid phase of the LDL dispersion (reaction 2 in Scheme 1). We therefore compared the peroxidation of LDL enriched with either α- or the less reactive γ- or δ-TOH [1,2,23]. Fig. 1 shows enrichement with any of these forms of vitamin E accelerates the rate of AAPH-initiated peroxidation. An increase in the loading of α-TOH from its normal 8–10 to 45 molecules of α-TOH/per LDL particle accelerated R_p by a factor of 4.6 ± 2.1 (mean ± S.D., n = 3), whereas smaller rate increases were produced by similar enrichment of the LDL with γ-TOH or δ-TOH, i.e. factors of 3.4 ± 1.7 and 2.0 ± 0.7, respectively (Fig. 1). Thus, we find that under these conditions, α-TOH, the most reactive peroxyl radical scavenger of the vitamin E family, was a better pro-oxidant for isolated LDL than the less reactive peroxyl radical scavengers γ-TOH or δ-TOH.

A potential problem with the interpretation of above experiments is that the different LDL samples varied in their composition and that the lipoproteins enriched with γ-TOH or δ-TOH also contained relative large concentrations of α-TOH. We therefore sought an additional way to test the importance of the phase transfer activity of α-TOH for LDL lipid peroxidation without altering the composition of the lipoprotein. It is well known that phenolic protons rapidly exchange with deuterium in the presence of D_2O [28]. In the case of α-TOH, the resulting α-TOD is ≈4-fold less reactive than α-TOH, when assessed by kinetic peroxidation tests in homogeneous solution [23] (k_H = 4.0 ± 0.5k_d in chlorobenzene at 30°C). Our own experiments with LDL lipid extract (Fig. 2A) were consistent with these earlier studies since R_p^{inh} in an azo-initiated benzene solution of LDL extract was faster for D_2O-underlayered than for the H_2O-underlayered LDL extract.

In contrast to results in lipid extracts, replacement of the usual H_2O-buffer of an LDL *dispersion* by one based on D_2O led to a marked deceleration in the rate of peroxyl radical-initiated LDL peroxidation (Fig. 2B). Thus, R_p was 2.0 ± 0.7 times slower in D_2O- than H_2O-LDL, as estimated from the average ratio of Ch18:2-OOH measured in D_2O- vs. H_2O-LDL (Fig. 2, inset). The rate of α-TOH consumption was also slower for D_2O- than H_2O-LDL, the difference being a factor of 1.6–1.7 for the initial 30 min of oxidation. The lower R_p^{inh} was not due to some (unprecedented) effect of D_2O on the rate of generation of radicals from AAPH since the rate of O_2 uptake in the absence of LDL was the same (± <5%) in D_2O- and H_2O-buffer. Moreover, the lower R_p^{inh} was not due to a slower rate of radical initiation since the same R_p^{inh} was found when the initiator concentration was doubled to compensate for lower initiation efficiency (not shown).

Previous studies from this laboratory [12,15] (J. Neuzil, S.R. Thomas and R.S., unpublished) have demonstrated that the extent to which the pro-oxidant activity of α-TOH in ascorbate- and ubiquinol-10-free LDL is seen depends largely on the *rate* of peroxidation initiation (R_i) rather than the nature of the

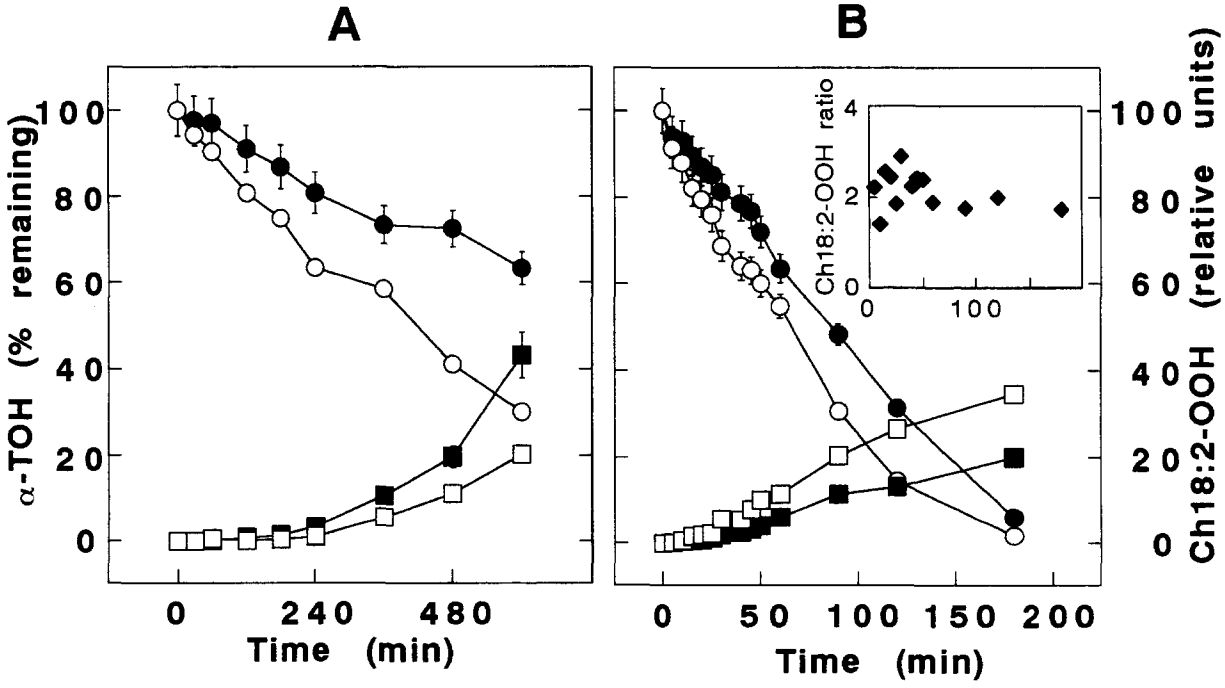


Fig. 2. Peroxyl radical-initiated peroxidation of LDL lipid extract (A) and LDL lipid dispersion (B). (A) LDL (18 ml, 0.2 mg protein/ml) was isolated, extracted [24], dried and redissolved in benzene. Aliquots of the lipid extract were underlayered with D_2O (filled symbols) or H_2O (open symbols), respectively and oxidized with AMVN. An experiment using a higher radical flux (1.0 mM AMVN) gave similar results to those shown. (B) LDL (0.3–0.4 mg protein/ml) isolated from and suspended in deuterated or protonated buffer was oxidized with AAPH in D_2O (filled sybols) or H_2O (open symbols). Results show mean values ± S.E.M. of two (A) and three (B) separate experiments carried out with different LDL preparations. Where error bars are not seen they are smaller than the symbol. Initial concentrations of α-TOH (circles) varied from 52–53 μM (A) and 4.3–5.5 μM (B). α-TOH and Ch18:2-OOH (squares) were determined as described [21]. The units for Ch18:2-OOH in (A) and (B) are 0.025 and 1 μM, respectively. The inset shows the ratio of Ch18:2-OOH formed in protonated over deuterated systems.

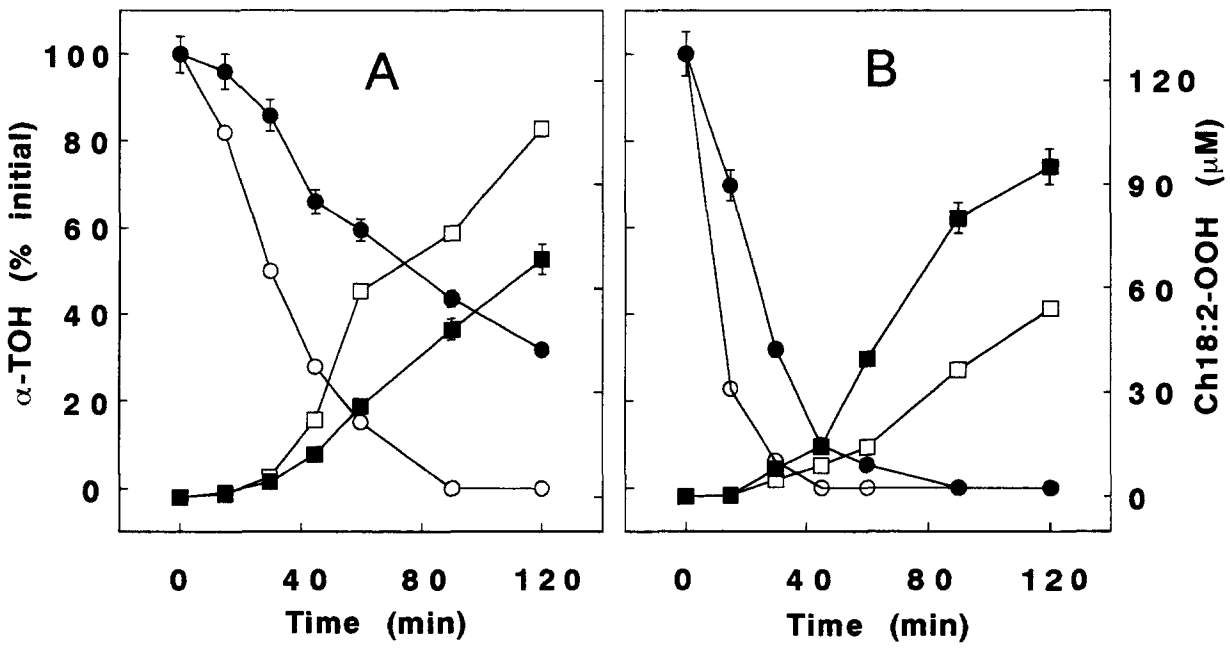


Fig. 3. Cu^{2+}-mediated peroxidation of LDL dispersion. LDL (0.7 or 1.4 μM) isolated from and suspended in D_2O- (filled symbols) or H_2O-buffer (open symbols) buffer was oxidized with Cu^{2+} to give a final ratio of (A) 2 or (B) 5 Cu^{2+}: LDL. Results show mean values ± S.E.M. of two separate experiments carried out with different LDL preparations. Where error bars are not seen they are smaller than the symbol. Initial concentrations of α-TOH (circles) varied from 5.2–6.3 μM for all LDL samples used for oxidation experiments. α-TOH and Ch18:2-OOH (squares) were determined as described [21].

radical oxidant. That is, under conditions of low R_i (when α-TOH is consumed slowly) the pro-oxidant activity is most pronounced, whereas α-TOH acts as an anti-oxidant ($R_p^{inh} < R_p^{uninh}$) under conditions of high R_i (when α-TOH is consumed rapidly). The results in Fig. 3 support this for Cu^{2+}-mediated oxidation of H_2O-LDL, where R_p^{inh} was significantly higher at a Cu^{2+}/LDL ratio of 2 than 5. At the low Cu^{2+}/LDL ratio, substantial amounts of LOOH were formed in the presence of α-TOH (Fig. 3A), indicative of LDL oxidation occurring by a TMP mechanism [15].

These findings raised the possibility of using the deuterium exchange as a simple test to assess the importance of TMP for LDL lipid peroxidation: an inverse deuterium kinetic isotope effect (DKIE) is expected only if R_p^{inh} is mediated largely by α-TO•, whereas R_p^{inh} is expected to be greater in D_2O- than H_2O-LDL if LOO• is the main chain carrying radical. To test this, we compared LDL oxidation rates in H_2O- vs D_2O-buffer using increasing Cu^{2+}/lipoprotein ratios, and expressing LOOH formation rates as the ratio of Ch18:2-OOH produced in the H_2O- over D_2O-buffer system. Replacement of the usual H_2O-buffer by one based on D_2O led to a 2-fold slower rate of α-TOH consumption irrespective of the Cu^{2+}/LDL ratio employed (Fig. 3A and B). In contrast, R_p^{inh} was affected by the Cu^{2+}/LDL ratio used (Figs. 3 and 4). Thus, oxidation of LDL with ≤2.5 Cu^{2+} per LDL particle proceeded slower in D_2O- than H_2O-LDL resulting in Ch18:2-OOH ratios of >1 (Fig. 4), consistent with the results observed for AAPH-initiated oxidation of H_2O- vs D_2O-LDL. At ≥3 Cu^{2+} per LDL particle the Ch18:2-OOH ratio was <1, indicating that the activity of α-TOH switched from pro- to anti-oxidant. The Ch18:2-OOH ratio remained at ≈0.5 for up to 5 Cu^{2+} per LDL; at higher Cu^{2+}/LDL particle ratios Ch18:2-OOH could not be determined with confidence for the inhibited peroxidation periods due to their short duration under these strongly oxidizing conditions.

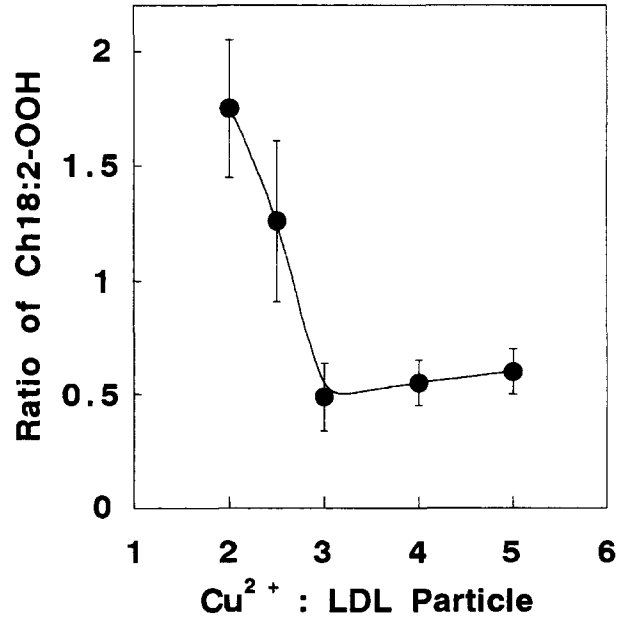


Fig. 4. The Cu^{2+}:LDL ratio determines the DKIE observed during Cu^{2+}-mediated LDL lipid peroxidation. Results show mean values ± S.E.M. of two separate experiments carried out with different LDL dispersions (0.7 or 1.4 μM) suspended in D_2O- or H_2O-buffer in the presence of increasing Cu^{2+}:LDL ratios as indicated in the figure. The ratios of LOOH were determined for individual experiments by dividing the rate of Ch18:2-OOH formation in H_2O over D_2O-buffer throughout the two hours each oxidation experiment was monitored.

This study clearly shows that we can have a situation in which the usual paradigms of inhibited autoxidation are inverted: the most effective phenolic antioxidant for lipids — α-TOH — becomes the best pro-oxidant for LDL and the apparent DKIE measured as $(R_p)_{H_2O}/(R_p)_{D_2O}$ is ≈2.0. These unusual findings are readily explained by the TMP mechanism [15]: α-TOH can be a pro-oxidant for LDL because it actively scavenges $ROO^{\bullet}_{aq}$ at the particle surface (reaction 2, Scheme 1), thereby facilitating the 'import' of radicals from the aqueous phase into the lipid phase of the LDL dispersion and initiating TMP radical chains (reaction 3, Scheme 1). The increased phase transfer activity in tocopherol-enriched LDL therefore leads to a greater steady-state population of α-TO$^{\bullet}$ and thus to faster LOOH-formation. α-TOH is a stronger pro-oxidant than γ-TOH or δ-TOH *because* α-TOH is a more reactive peroxyl radical scavenger than γ- or δ-TOH [23]. Peroxidation of D_2O-LDL is slower *because* α-TOD is less reactive (toward $ROO^{\bullet}_{aq}$) than α-TOH.

Our findings with Cu^{2+}-mediated LDL lipid peroxidation strongly support that the TMP mechanism can also be important for this oxidant, and suggest that the DKIE experiment reported can be used as a simple test whether 'inhibited' peroxidation of a lipid dispersion is being propagated via the peroxyl or mainly via the antioxidant radical. In the former case the deuterium exchange (that rapidly occurs in the presence of D_2O) accelerates peroxidation whereas with phenol-mediated peroxidation the exchange will retard the rate of peroxidation.

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