Interaction of peroxynitrite with carotenoids and tocopherols within low density lipoprotein

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Abstract Peroxynitrite is a powerful oxidising and nitrating agent generated in vivo by the combination of nitric oxide and superoxide. Previous studies have shown that on exposure to peroxynitrite, low density lipoprotein (LDL) is modified resulting in both a time- and concentration-dependent change to lipid and protein components. The present investigation highlights the reaction between carotenoids and tocopherols, present within the lipophilic phase of LDL, and peroxynitrite at varying concentrations. It was observed that the carotenoids were consumed by a significantly greater proportion than that of the tocopherols with lycopene (87.2 ± 11%) being more reactive than β-carotene (68.2 ± 5.8%) when exposed to peroxynitrite (50 μM) for 1 min. Among the tocopherols, α-tocopherol (54.9 ± 20.2%) was more extensively depleted than γ-tocopherol (14.7 ± 1.09%) at peroxynitrite concentration of 500 μM. It was also observed that peroxynitrite, unlike copper ions, does not lead to significant peroxidation of LDL as determined by the formation of conjugated dienes and thiobarbituric acid-reactive substances.

Key words: Carotenoid; Low density lipoprotein oxidation; Peroxynitrite; Tocopherol; Lipid peroxidation

1. Introduction

Peroxynitrite is an important biological oxidant generated in vivo by the combination of nitric oxide and superoxide [1,2]. The rate constant for this reaction has been measured as 6.7×10^9 M^{-1} s^{-1} [3]. Stimulated macrophages, neutrophils and endothelial cells have all been shown to generate peroxynitrite [4–6]. Peroxynitrite at physiological pH (p*K*a −6.8) protonates to form peroxynitrous acid which decays rapidly to form a mixture of reactive products. Peroxynitrite and products derived from it have been reported to induce lipid peroxidation, oxidise thiol groups and modify amino acyl groups on proteins [7]. This suggests that peroxynitrite may play a significant role in atherogenesis [8,9] since oxidative modification of low density lipoproteins (LDL) is thought to play a key role in the development of atherosclerotic lesions [10].

Vitamin E, the major lipophilic antioxidant, consists of two major forms, α-tocopherol (α-TOH) and γ-tocopherol (γ-TOH). The only structural difference between these forms of tocopherol is the presence of an additional methyl group at the 5-position of α-tocopherol (Fig. 1). α-TOH has a higher antioxidant activity in vitro [11]. Levels of α-TOH in vivo (in humans) are several fold higher than those of γ-TOH [12]. This is believed to be due to preferential re-incorporation of α-TOH into VLDL [13] and its decreased cellular turnover compared to γ-TOH. The majority of the evidence generated to date suggests that α-TOH acts as an antioxidant by electron donation and can be converted to the corresponding α-tocopheroquinone [14,15]. In addition, γ-TOH, unlike α-TOH, can also undergo substitution reactions. For example, the corresponding nitrated derivative of γ-TOH is formed when reacted with peroxynitrite [15].

The carotenoids are mainly present in human plasma associated with LDL [16,17]. Considerable evidence has been gathered which suggests that carotenoids such as β-carotene and lycopene (Fig. 1) are effective scavengers of reactive oxygen species and modulate free radical processes in vitro. The mode of action of carotenoids as antioxidants has been linked to their ability to quench singlet oxygen and prevent lipid peroxidation induced by this reactive oxygen species [18]. Recent studies employing pulse radiolysis and rapid time-resolved spectrophotometry have shown that carotenoids react with oxidising species by either electron transfer to generate the radical cation or by radical addition to generate a radical adduct (reviewed in [17]). Another study employing exposure of β-carotene to reactive nitrogen species has demonstrated its total destruction from spectrophotometric investigations [19].

The present study investigates the extent of carotenoid and tocopherol utilisation within LDL following exposure to various concentrations of peroxynitrite. Comparative studies using copper ions to oxidise LDL were also conducted. Sensitive and specific methods were applied to distinguish between carotenoid and tocopherol consumption following exposure to oxidants and also to establish differences between the two major classes of antioxidants within LDL.

2. Materials and methods

2.1. Chemicals

Sodium dihydrogen orthophosphate dihydrate, disodium hydrogen orthophosphate dihydrate, potassium hydroxide and hydrochloric acid were obtained from BDH (Poole, Dorset, UK). Lycopene, β-carotene, α-tocopherol, γ-tocopherol, δ-tocopherol, hydrogen peroxide, sodium nitrite, trichloroacetic acid and thiobarbituric acid were obtained from Sigma-Aldrich Chemical Company (Poole, Dorset, UK). Ethyl β-apo-8′-carotenoate (*trans*) was purchased from Fluka Chemika Biochemika (Dorset, UK). High performance liquid chromatography (HPLC) grade acetonitrile was purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland). All other reagents used were of analytical grade. All the reagents were prepared using deionised water (Waters Milli-Q system).

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Abbreviations: LDL, low density lipoprotein; α-TOH, α-tocopherol; α-TQ, α-tocopheroquinone; γ-TOH, γ-tocopherol; BHT, butylated hydroxy toluene; TBARS, thiobarbituric acid reactive substances; CV%, percentage coefficient of variation; M%D, mean percentage difference; MDA, malondialdehyde

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2.2. Peroxynitrite synthesis

Peroxynitrite synthesis was carried out as described previously [20]. Briefly, acidified hydrogen peroxide (1 M) and sodium nitrite (0.2 M) were drawn into two separate syringes and injected simultaneously into an ice-cold potassium hydroxide (1.5 M) solution. Concentration was estimated by measuring the absorbance at 302 nm ($\varepsilon = 1670$ $M^{-1} \cdot cm^{-1}$).

2.3. Isolation and exposure of LDL to peroxynitrite

Low density lipoprotein was isolated from fresh human plasma obtained from a healthy volunteer. Density gradient ultracentrifugation, as described by Chung et al. [21], was utilised to isolate LDL using a Beckman L-70 centrifuge with a fixed angle rotor (Ti-70) spun at $150\,000 \times g$. After isolation, LDL was filtered through a 0.22-μm pore size filter (Millipore) followed by dialysis in phosphate buffered saline (10 mM, pH 7.4) containing 10 μM EDTA at 4°C. Protein concentration was estimated using modified Lowry method with bovine serum albumin used as the standard [22]. LDL was used at a final concentration of 1 mg LDL protein/ml for all experiments conducted.

The extent of modification of the carotenoids and tocopherols in LDL following exposure to varying concentrations of peroxynitrite was determined by reversed phase HPLC. A 20-μl aliquot of peroxynitrite (0–500 μM) was added to LDL (0.5 mg of protein) in 0.2 M phosphate buffer giving a total volume of 0.5 ml. Samples containing decomposed peroxynitrite were also tested to estimate the effect on antioxidants. A 20-μl aliquot of peroxynitrite was added to 0.2 M phosphate buffer and allowed to stand for 10 min at ambient temperature followed by the addition of LDL to give a total volume of 0.5 ml. The reaction was allowed to take place for 1 min followed by the addition of methanol (1 ml) containing the internal standards ethyl β-apo-8′-carotenoate (*trans*) (5 nmol/ml) and δ-tocopherol (25 nmol/ml). The addition of methanol also leads to the precipitation of apolipoprotein B100. Samples were subsequently cyclomixed and allowed to stand for 5 min at room temperature. The carotenoids and tocopherols, including the internal standards, were then extracted by the addition of 3 ml of hexane followed by cyclomixing for 10 min and finally centrifuged at $1000 \times g$ for 20 min. The hexane layer was then transferred into a separate extraction tube and evaporated to dryness under a stream of nitrogen. The samples were finally reconstituted in 200 μl of acetone and analysed by HPLC using a Waters-Novopak C18 column (15 cm × 3 mm i.d., 4 μm particle size). A Hewlett Packard Model 1090M-II HPLC system with an autoinjector, autosampler, diode array detector and a Hewlett Packard 1046A-programmable fluorescence detector linked to a Hewlett Packard 900-300 data station was used to analyse the samples. An isocratic system consisting of 85% methanol and 15% acetonitrile was utilised as the mobile phase. Control samples without the addition of peroxynitrite were analysed to estimate the basal levels of the tocopherols and carotenoids in LDL.

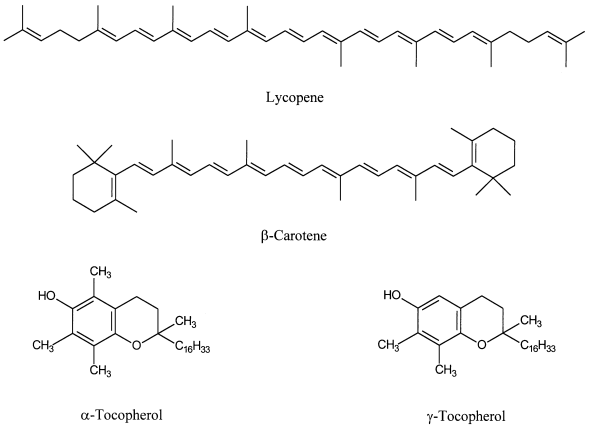


Fig. 1. Chemical structures of carotenoids and tocopherols.

The amounts of tocopherols and carotenoids present within controls and samples following exposure to peroxynitrite were determined from calibration plots constructed using authentic samples. Lycopene, β-carotene and ethyl β-apo-8′-carotenoate (*trans*) were monitored at 450 nm. α-, γ- and δ- tocopherols were monitored by fluorescence using an excitation wavelength of 285 nm and an emission wavelength of 315 nm. Calibration plots of α-tocopherol (range 0–50 nmol/ml), γ-tocopherol (range 0–10 nmol/ml), β-carotene (range 0–5 nmol/ml) and lycopene (range 0–5 nmol/ml) were constructed. Known concentrations of the tocopherol and carotenoid solutions prepared in ethyl acetate were spiked into LDL (0.5 mg of protein/0.5 ml) in pH 7 phosphate buffer. The samples were then treated in a manner identical to that described for the preparation of test samples. Linear behaviour with correlation coefficient values ≥0.995 were obtained. The assays developed for the quantification of both carotenoids and tocopherols were found to be both precise and accurate with CV% and M%D of ≤5%.

2.4. Measurement of lipid peroxidation

2.4.1. Conjugated diene study. Formation of conjugated dienes was observed by exposing LDL to the oxidants copper [23] and to peroxynitrite and monitoring the optical density at 234 nm for 4 h. Aliquots of 100 μl of peroxynitrite (100 μM final concentration) and copper (3 μM final concentration) were transferred into two separate cuvettes. LDL (0.125 mg of protein/ml) in a total volume of 0.9 ml was then added to the cuvettes. Absorbance was measured immediately at 234 nm and then every 5 min for 4 h using a Hewlett Packard 8453 diode array spectrophotometer with a multiple cell unit. The

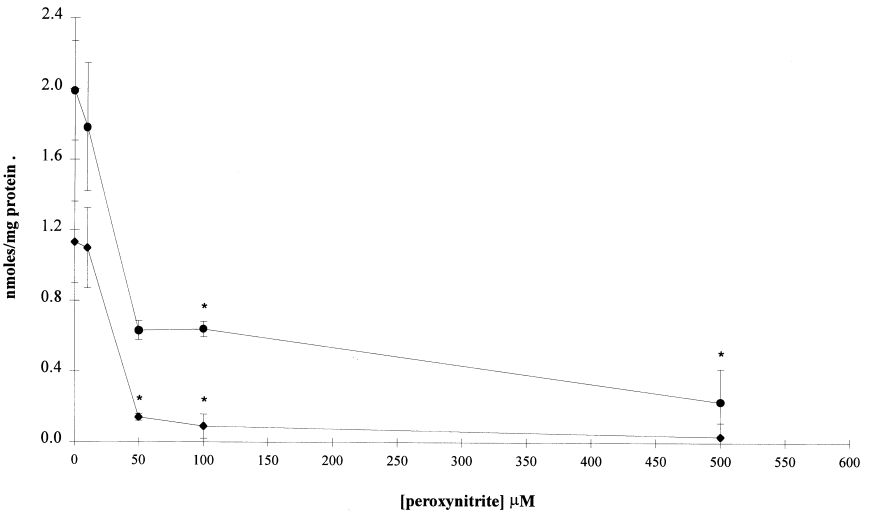


Fig. 2. Concentration dependent decrease in β-carotene (●) and lycopene (♦) levels in LDL exposed to increasing concentrations of peroxynitrite for one minute as described in Section 2. Lycopene was found to be consumed more rapidly than β-carotene ($^*P \leq 0.01$, unpaired *t*-test, $n = 5$).

absorbance was corrected to zero at the first time point (blank) with the respective sample.

2.4.2. Measurement of thiobarbituric acid reactive substances (TBARS). Time- and concentration-dependent lipid peroxidation of LDL was estimated by measuring the thiobarbituric acid reactive substances (TBARS). LDL (0.125 mg of protein/ml) was either exposed to increasing concentrations (0–1000 μM) of peroxynitrite for 1 min (concentration-dependent study, ambient temperature) or exposed to fixed concentrations of peroxynitrite (100 μM and 1000 μM) and copper (3 μM) for up to 3 h (time-dependent study, 37°C). The reaction was stopped by the addition of 5 μl of BHT in ethanol. Protein was precipitated by the addition of 0.5 ml of 10% trichloroacetic acid followed by the addition of 0.5 ml of 0.75% thiobarbituric acid in 0.1 N HCl. The samples were heated at 80°C for 45 min followed by centrifugation for 10 min. The supernatant was removed and the absorbance measured at 532 nm. The extent of TBARS formation was calculated based on its molar extinction coefficient 1.56×10^5 M^{-1} cm^{-1}. Results obtained are expressed as nmoles of MDA/ml.

3. Results

Short duration (1-min) exposure of LDL (1 mg/ml) to varying concentrations of peroxynitrite at pH 7 (ambient temperature) led to extensive consumption of β-carotene and lycopene (Fig. 2). At 50 μM peroxynitrite concentration, the concentration of β-carotene within LDL decreased from 1.99 nmol/mg protein to 0.63 nmoles/mg protein (by $68.2\pm5.8\%$) and lycopene from 1.13 nmol/mg protein to 0.14 nmoles/mg protein (by $87\pm11\%$) ($P\leq0.01$). Increasing the concentration of peroxynitrite to 100 μM did not significantly enhance consumption of either β-carotene or lycopene. Even at the highest concentration of peroxynitrite (500 μM), there was only a small further increase in carotenoid consumption.

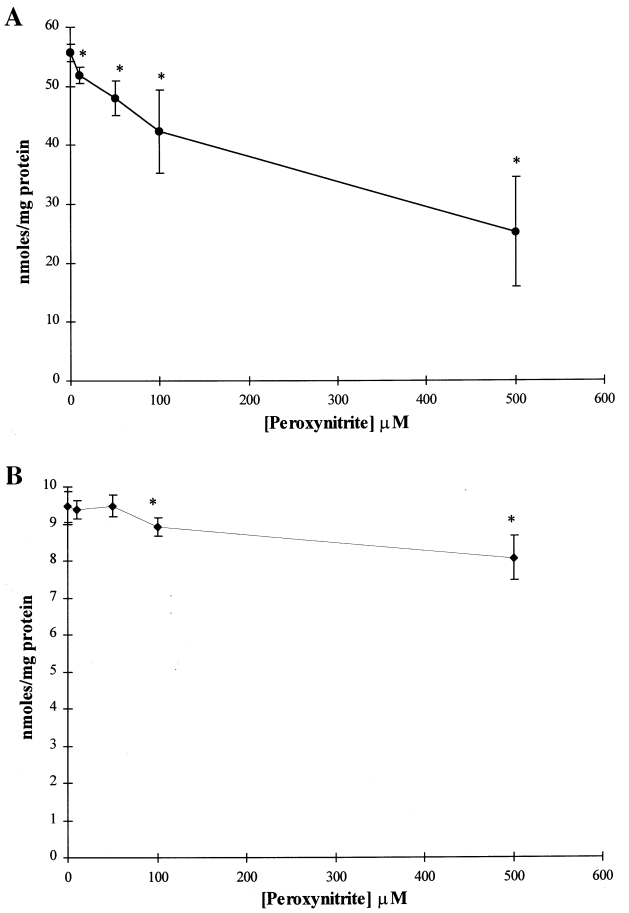


Fig. 3. Extent of tocopherol decrease in LDL following exposure to increasing concentrations of peroxynitrite. There was a significant loss in the levels of α-tocopherol (A) with greater than 50% loss at 500 μM peroxynitrite. In contrast, however, γ-tocopherol (B) was not depleted to the same extent with only a 15% decrease at 500 μM peroxynitrite concentration (*$P\leq0.05$, unpaired *t*-test). Data shown are mean values ± S.D. of five complete sets of experiments.

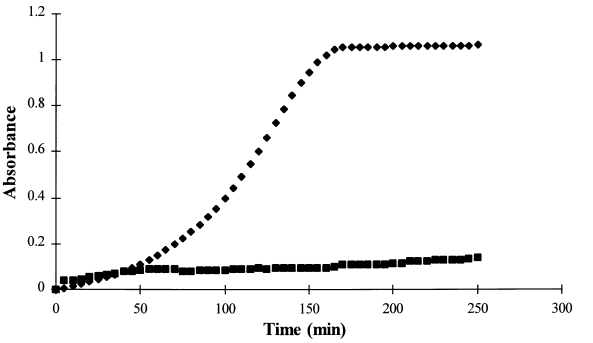


Fig. 4. Formation of conjugated dienes on exposing LDL to copper (3 μM, ♦) and peroxynitrite (100 μM, ■). Results indicate the characteristic increase in the formation of dienes induced by copper compared to rapid and limited formation of the same when induced by peroxynitrite.

The profile for the consumption of tocopherols by peroxynitrite was, however, in total contrast to that of the carotenoids (Fig. 3). Percentage consumption of tocopherols was lower than the percentage consumption of carotenoids, although in absolute amount the extent of α-tocopherol consumption was far greater. For example, at 500 μM peroxynitrite concentration (the highest concentration of peroxynitrite used), the levels of α-tocopherol decreased by 55% from 55.6 nmoles/mg protein to 25.09 nmol/mg protein while the levels of γ-tocopherol decreased from 9.46 nmol/mg protein to 8.07 nmol/mg protein (14.7% decrease). This decrease was, by and large, in proportion to the concentration of peroxynitrite added to LDL. The decrease in the levels of α-tocopherol was statistically significant ($P\leq0.05$) at all concentrations of peroxynitrite used. Even at the lowest concentration of peroxynitrite (10 μM) the decrease was by ~6.5% (Fig. 3A). In contrast, at low concentrations of peroxynitrite (10 and 50 μM), there was only a negligible decrease in γ-tocopherol levels ($<0.5\%$) (Fig. 3B). Statistically significant ($P\leq0.05$) reductions were only observed at peroxynitrite concentrations of 100 μM (5.5%) and 500 μM (14.7%). Exposure of LDL to decomposed peroxynitrite did not lead to the depletion of antioxidants.

Copper-induced oxidation of LDL [23], when analysed by the conjugated diene formation, showed the three characteristic phases (Fig. 4). In contrast, peroxynitrite-induced formation of conjugated dienes exhibited a very slow rise in absorbance up to 0.14 absorbance units after 240 min (Fig. 4). Exposure to higher concentrations of peroxynitrite did not significantly alter the result. In parallel with this it was observed that peroxynitrite (100 μM) leads to a small but instantaneous increase in the formation of the aldehydic decomposition products of lipid hydroperoxides, as analysed by the TBARS assay (Fig. 5). The reaction was observed to be complete within the first few seconds since no further change took place with increasing duration of time. Increasing the concen-

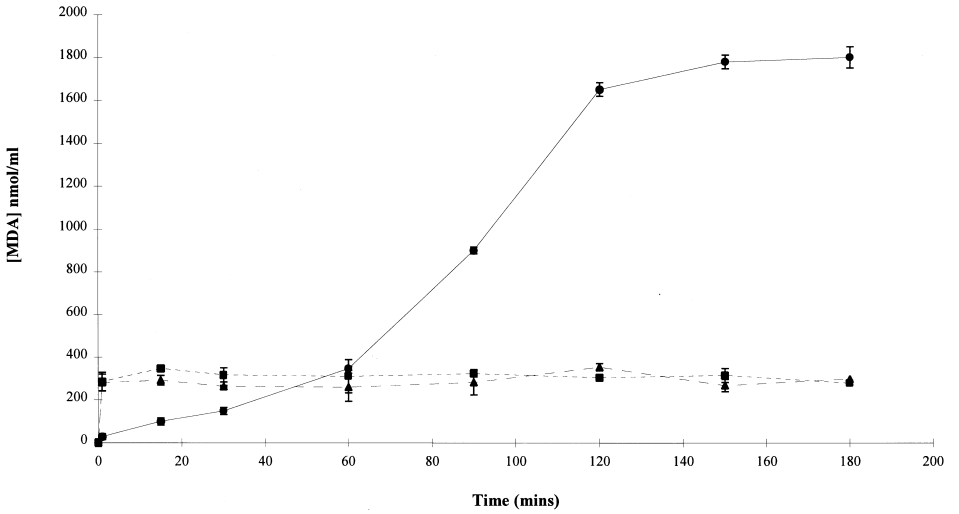


Fig. 5. Peroxynitrite (100 μM (▲) and 1000 μM (■)) and copper (3 μM (●)) induced lipid peroxidation of LDL as measured by the formation of TBARS and expressed in terms of MDA in nmol/ml. Results indicate a rapid but reduced extent of peroxidation when exposed to peroxynitrite compared to copper ($n = 3$).

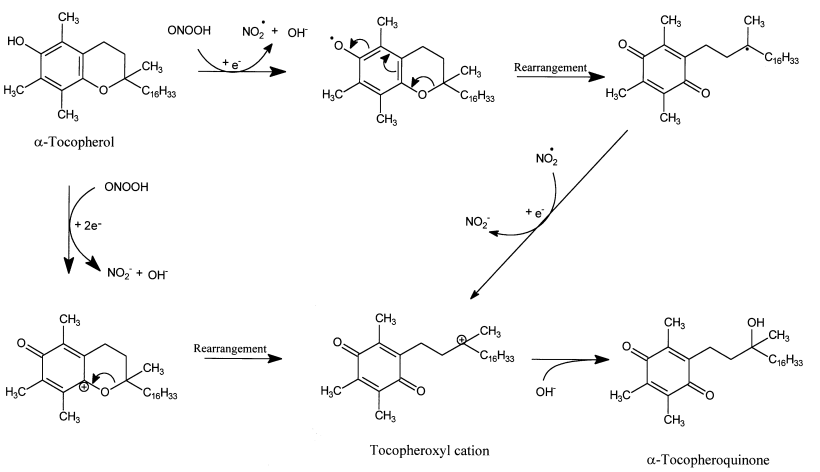
tration of peroxynitrite to 1 mM did not increase the extent of lipid peroxidation. There was only a minimal increase (about 5-fold) in the formation of TBARS (1 min exposure) with increasing peroxynitrite concentration from 10 μM to 1000 μM (data not shown). In contrast, copper-induced oxidation of LDL over 3 h (Fig. 5) exhibited the lag phase of oxidation followed by the propagation of lipid peroxidation consistent with the conjugated diene study [23].

4. Discussion

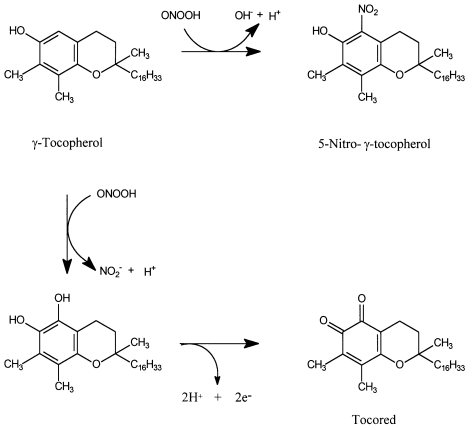
Comparison of the sequence of degradation of carotenoids as they exert their antioxidant actions in a variety of biological systems in vitro reveals remarkably consistent results. In LDL exposed to ex vivo oxidation [23] promoted by copper, the order of antioxidant consumption after 1 h oxidation is shown to be: α-tocopherol > γ-tocopherol > lycopene > β-cryptoxanthin > lutein/zeaxanthin > α,β-carotene. Studies involving model lipid systems, peroxyl radical initiators and model radical systems under a variety of conditions broadly mirror the above findings (reviewed in [17]). The direct interaction of carotenoids with singlet oxygen or with the radical cation $ABTS^{\bullet+}$ indicate a greater reactivity with lycopene than β-carotene [24,25].

The present study, interaction of LDL with peroxynitrite provides further supporting evidence for the differential reactivity of the carotenoids. Although both β-carotene and lycopene were extensively consumed following exposure to peroxynitrite, lycopene appeared to be marginally more reactive – as indicated by the more extensive decrease in its level, within LDL, compared to β-carotene. Reactivity of the carotenoids depends on their location and orientation within the phospholipid bilayers [26]. Both lycopene and β-carotene are located within the hydrophobic core, but their rate of reaction with peroxynitrite entirely depends on their structural orientation. It is possible that due to its open structure (Fig. 1) lycopene has the capability to form more readily a delocalised carbon-centred radical cation than β-carotene [17], which may account for its more rapid consumption, assuming peroxynitrite induced attack is radical mediated.

LDL α-tocopherol was consumed to a much greater extent than γ-tocopherol under the reaction conditions with peroxynitrite described here. It has been reported that the major product of α-tocopherol modification following exposure to



Scheme 1. Two electron oxidation of α-tocopherol by peroxynitrite to form α-tocopheroquinone. (Adapted from Hogg et al. [27].)



Scheme 2. Apparent nitration and quinone formation of γ-tocopherol following exposure to peroxynitrite. (Adapted from Christen et al. [15].)

peroxynitrite is the formation of α-tocopheroquinone (α-TQ) (Scheme 1) [14,15,27]. This can occur either by a one-step two electron-donation pathway or a two-step single electron-donation pathway. The formation of α-tocopheryl cation, via the formation of the tocopheroxyl radical through a single electron donation, has been reported to be less likely to occur, based on the low yield ($< 2\%$) of the radical species detected by ESR [27].

Recent studies conducted by Christen et al. have shown that the predominant reaction products of γ-tocopherol and peroxynitrite are 5-nitro-γ-tocopherol and 5-hydroxy-γ-tocopherol which can undergo further oxidation to form its corresponding quinone-tocored (Scheme 2) [15]. In principle, γ-TOH like α-TOH can also take part in the two electron-donation pathway to form the corresponding paraquinone. However, this product was not detected [15].

In summary, following exposure of LDL to peroxynitrite, a higher proportion of the carotenoids was consumed than the tocopherols, although the absolute loss of α-tocopherol was greater. A point of particular significance is the fact that peroxynitrite under these conditions, unlike copper ions, does not lead to extensive lipid peroxidation of LDL.

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