α-Tocopherol does not inhibit hypochlorite-induced oxidation of apolipoprotein B-100 of low-density lipoprotein

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Abstract The amount of α-tocopherol (α-TOH) can dramatically alter the extent of radical-induced oxidation of low density lipoprotein (LDL) lipids, a process generally thought to be important in atherogenesis. However, LDL with atherogenic features can also be formed in vitro by exposure to the strong non-radical oxidant hypochlorite (HOCl), which preferentially oxidises LDL apolipoprotein B-100. Here we show that varying LDL content of α-TOH by vitamin supplementation or depletion has no effect on the extent of HOCl-induced oxidation of apolipoprotein B-100 as measured by the loss of lysine and tryptophan residues, and the alteration in relative electrophoretic mobility of the lipoprotein particle.

Key words: Vitamin E; Low-density lipoprotein; Hypochlorous acid; Protein oxidation; Atherosclerosis

1. Introduction

Oxidation of low-density lipoprotein (LDL) is thought to be an important early event in atherogenesis [1] and may be modulated by the content of antioxidants within and surrounding LDL. Indeed, lipophilic antioxidants that associate with LDL are of interest as potential therapeutic agents [2]. However, despite some recent promising results [3], prospective randomised trials on the effect of supplementation with α-tocopherol (α-TOH, quantitatively the major lipid-soluble antioxidant in LDL extracts) on cardiovascular outcome in humans have overall been disappointing [4].

There is convincing evidence that LDL or LDL-like particles isolated from human atherosclerotic lesions are oxidatively modified, both in lipid and in protein moieties. While the precise nature of the oxidant(s) active in vivo remains uncertain, recent evidence suggests that hypochlorite (HOCl) could be involved. Thus, both active myeloperoxidase [5] and HOCl-oxidised proteins [6] have been identified in human atherosclerotic lesions, and HOCl can convert LDL into an atherogenic form in vitro [7,8]. HOCl-induced oxidation of LDL differs from at least some radical-mediated oxidation (e.g. copper-induced oxidation) in that the strong nucleophilic oxidant HOCl preferentially oxidises the protein moiety of LDL rather than the lipid moieties or lipophilic antioxidants [7–10]. What is not known to date is whether α-TOH can affect HOCl-induced LDL and protein modification. Clarification of this could have implications for the design of anti-atherogenic drugs based on antioxidant activity. We therefore tested the effect of supplementation with, and depletion of, α-TOH on the extent of HOCl-mediated oxidation of LDL.

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Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) hydrochloride; apo B-100, apolipoprotein B-100; CE-O(O)H, the sum of cholesteryl ester hydroperoxides and cholesteryl ester hydroxides; DMSO, dimethyl sulphoxide; EDTA, ethylenediamine tetraacetic acid; HOCl, an equilibrium mixture of hypochlorous acid and hypochlorite at pH 7.4; LDL, low-density lipoprotein; REM, relative electrophoretic mobility; α-TOH, α-tocopherol

2. Materials and methods

Nanopure water was used for all buffers and aqueous solutions, which were subsequently treated with Chelex-100 (Bio-Rad, Hercules, CA, USA) to remove contaminating transition metals. Heparinised vacutainers were obtained from Becton-Dickinson (Lincoln Park, NJ, USA); bicinchoninic acid assay reagents, reduced glutathione and fluorescamine from Sigma Diagnostics (Castle Hill, NSW, Australia); α-TOH from Henkel (Sydney, Australia) and reagent HOCl (5% available chlorine minimum) from Aldrich (Castle Hill, NSW, Australia). The concentration of HOCl was determined spectrophotometrically at pH 13.0 using $\varepsilon_{292\ nm} = 350\ M^{-1}\ cm^{-1}$ [11]. 2,2′-Azobis(2-amidinopropane) hydrochloride (AAPH) was purchased from Polysciences (Warrington, PA, USA). Ebselen (2-phenyl-1,2-benzoisoselenazol) was a generous gift from Daiichi Pharmaceuticals (Tokyo, Japan). Phosphate-buffered saline (PBS) contained 150 mM NaCl and 50 mM $(Na_2H$ and $NaH_2)PO_4$ at pH 7.4 and was used for gel filtration and resuspension of LDL after its isolation. For LDL isolation, 0.1% (w:v) ethylenediamine tetraacetic acid (EDTA) was added to the PBS. Organic solvents were of HPLC quality (EM Science, Gibbstown, NJ, USA) or the highest grade available. Hexane was washed with nanopure water prior to its use. Collection of blood for this work was approved by the local human ethics committee.

2.1. LDL preparation

Fresh blood was obtained from non-fasted, healthy male or female subjects (20–40 years old) and drawn into heparin vacutainers. The blood was centrifuged at 4°C ($1000 \times g$ for 15 min) to separate cells from plasma. LDL was isolated from plasma by ultracentrifugation by a rapid analytical method ($417000 \times g$, 2 h, 15°C) using 5.1 ml tubes and a TL100.4 rotor [12]. All subsequent handling of the samples was carried out at 4°C. Low molecular weight compounds were removed by gel filtration through a PD-10 column (Pharmacia, Uppsala, Sweden), and the LDL resuspended in PBS. For supplementation and replenishment procedures (see below), lipoprotein-depleted plasma was obtained by removing 1.7 ml of the bottom fraction of the plasma following its density ultracentrifugation described above.

2.2. Supplementation, depletion or replenishment of α-TOH in LDL

Freshly isolated LDL was depleted of α-TOH by rapid oxidation with AAPH at 37°C [10]. The reaction was stopped by cooling to 4°C upon approx. 90% TOH depletion (as assessed by HPLC analysis; see below) and the AAPH removed by gel filtration. This method consumes α-TOH with little lipid peroxidation [10] and no significant loss of amino acid residues (see Section 3). Any small amounts of lipid hydroperoxides that formed were reduced to the nonreactive hydroxides by treatment of the LDL with ebselen and reduced glutathione, followed by gel filtration [13]. LDL referred to as 'native' or 'supplemented' was treated similarly with the exception that AAPH was replaced with PBS for the 37°C incubation.

Subsequently, native LDL and depleted LDL were supplemented and replenished with α-TOH, respectively, by incubation in lipopro-

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tein-deficient plasma (2:1, v:v) with 20 mM α-TOH in DMSO [10] at 37°C for 4 h [14]. In addition, aliquots of native LDL and depleted LDL were incubated with lipoprotein-deficient plasma treated with DMSO alone under the same conditions as those used for supplementation. The final concentration of DMSO in all samples was 3% (v:v). Following supplementation or replenishment, LDL was reisolated by ultracentrifugation as described for native LDL and then gel-filtered. Replenished LDL, with α-TOH at a comparable concentration to that found in native LDL, has been shown to behave similarly to native LDL upon oxidation with a range of different oxidants under mild oxidising conditions [10], indicating that the above in vitro manipulations do not affect LDL oxidisability. LDL was oxidised with HOCl within 2 days of collection of fresh blood. The concentration of LDL in the resulting solutions was standardised using the bicinchoninic acid assay [15]. The protocol described in the manufacturer's instructions was modified by the addition of SDS [16]; bovine serum albumin (Sigma Diagnostics) was used as a standard.

2.3. HOCl-mediated oxidation of LDL

Oxidation of LDL with reagent HOCl was carried out on ice by the addition of 1 vol of HOCl, freshly diluted in PBS, to 4 vol of LDL solution (0.08–0.4 μM, final apo B-100 concentration). After addition of HOCl, the reaction mixture was mixed briefly (< 1 s) and left on ice for 15 min.

2.4. Quantification of amino acid residues

Unmodified lysine residues in LDL were quantified by fluorescamine fluorescence (excitation 390 nm, emission 475 nm) using the manual procedure of Böhlen [17] described in [7]. Tryptophan fluorescence of LDL was measured (in the absence of SDS) using excitation 280 nm and emission 335 nm [18]. For both lysine and tryptophan residues, the fluorescence of HOCl-oxidised samples was expressed as a percentage of the fluorescence of the corresponding LDL sample not treated with HOCl.

2.5. Agarose gel electrophoresis

LDL (≈1 μg protein) was loaded on pre-cast agarose gels (Ciba Corning) and the gels run in barbitone buffer (pH 8.6) at 90 V for 45 min. Following electrophoresis, the gels were fixed in methanol for 30 s, dried at 50°C for 15 min, stained with Fat Red 7B stain, destained in 70% (v/v) methanol and dried overnight at room temperature.

2.6. Measurement of antioxidants and lipid content of LDL

To determine the content of LDL antioxidants, unoxidised lipids and hydroperoxides and hydroxides of cholesteryl esters (the latter two collectively referred to as CEO(O)H), a 100 μl aliquot of the reaction mixture was extracted with 1 ml of cold methanol containing 0.02% (v:v) acetic acid and 5 ml hexane. The vacuum-dried hexane phase was redissolved in propan-2-ol for analysis. Cholesterol, cholesteryl esters and CE-O(O)H were separated by HPLC and quantified by ultraviolet detection as described [12]. α-TOH was determined by HPLC with electrochemical detection [12]. Lipids and α-TOH were internally standardised to cholesterol, the content of which changed only marginally in the different LDL samples as a result of treatment with HOCl (mean percent loss ± S.D. at 600 molecules of HOCl per LDL was 5.6 ± 4.5).

3. Results

The α-TOH contents of the differently treated LDL samples varied up to 100-fold (Table 1). CE-O(O)H were barely detectable in native and supplemented LDL, and < 10 molecules were present per particle in depleted and replenished LDL (Table 1). The concentration of cholesteryl linoleate (Table 1) and cholesteryl arachidonate (not shown), the two major cholesteryl esters in LDL, was not significantly different in the various samples. Similarly, neither LDL's lysine and tryptophan residues nor its relative electrophoretic mobility (REM) were significantly different between native and treated samples (Table 1).

Upon exposure of these various LDL samples to reagent HOCl, lysine and tryptophan residues were lost in a concentration dependent manner, to an extent independent of the amount of α-TOH present in the LDL (Fig. 1A,B). Oxidation of lysine and tryptophan residues together accounts for ≈75% of the HOCl added to LDL [7], which makes these reactions quantitatively important. The concentration of α-TOH in the lipoprotein particle did not influence the HOCl-induced increase in the REM of LDL (Fig. 2). A substantial increase in REM, together with modification of the lysine amino group, are indicative of conversion of LDL into a particle recognised by the scavenger receptor [19] and hence denote the potential of the modified lipoprotein particle to be atherogenic by causing lipid accumulation in macrophages.

As expected [7], treatment with HOCl resulted in a small and concentration-dependent decrease in the mean values of α-TOH in native LDL (Table 2). Likewise, α-TOH-supplemented, -replenished and -depleted LDL showed a small extent of oxidation of the vitamin (Table 2). However, even with the highest initial α-TOH concentration the extent of vitamin E consumption corresponded to less than 3% of the HOCl added, assuming a 1:1 stoichiometry. Supplemented and replenished LDL had a larger absolute loss of α-TOH than native LDL (Table 2). However, when measured as a proportion of the initial amount of α-TOH present, the extent of vitamin oxidation in these samples was less than that in native LDL (not shown). Conversely, depleted LDL (which gave rise to a smaller absolute loss of α-TOH than native LDL, Table 2) showed an increased proportional loss of the vitamin compared with native LDL (not shown). These results indicate that LDL's α-TOH is not a preferred substrate for reagent HOCl.

Exposure of LDL to HOCl resulted in minimal formation

Table 1
Comparison of native, α-TOH-supplemented, -depleted and -replenished LDL prior to HOCl oxidation

	Treatment of LDL			
	Native	Supplemented	Depleted	Replenished
α-TOH[a]	7.68 ± 3.04	59.5 ± 34.1	1.41 ± 0.85	43.9 ± 30.1
	(5.68-11.2)	(39.6-98.9)	(0.81-2.38)	(22.9-78.3)
CE-O(O)H[ab]	0.09 ± 0.08	0.36 ± 0.21	9.20 ± 1.26	9.70 ± 1.34
Ch18:2[b]	1.00 ± 0.00	1.04 ± 0.06	0.98 ± 0.03	1.03 ± 0.03
Lysine[b]	1.00 ± 0.00	1.03 ± 0.09	0.93 ± 0.07	0.93 ± 0.14
Tryptophan[b]	1.00 ± 0.00	1.16 ± 0.25	0.85 ± 0.17	0.98 ± 0.10
REM[b]	1.00 ± 0.00	1.06 ± 0.01	1.03 ± 0.11	1.10 ± 0.10

The values show mean ± S.D. of three independent experiments using LDL isolated from blood of three different donors. CE-O(O)H, cholesteryl ester hydro(pero)xides; Ch18:2, cholesteryl linoleate; REM, relative electrophoretic mobility.
[a]Units used are number per LDL particle or equivalently mole per mole LDL. The range is given in parentheses.
[b]Fraction of that detected in native LDL.

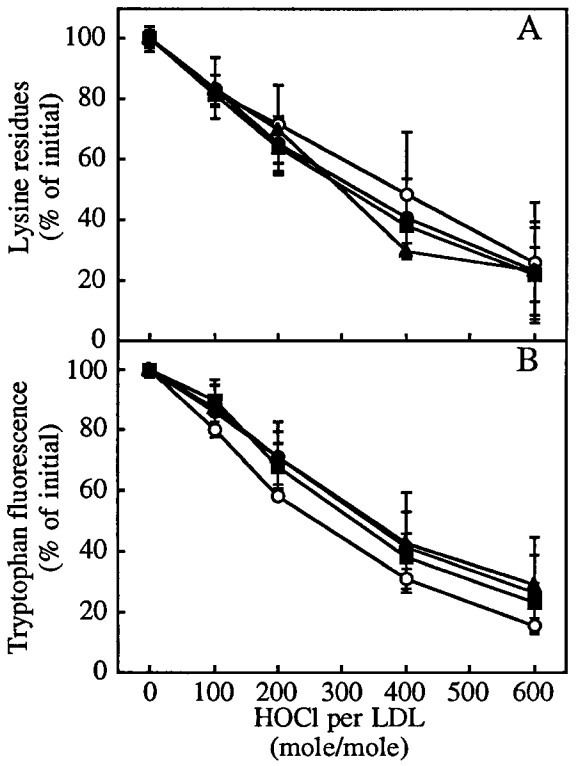

Fig. 1. HOCl-induced loss of lysine residues (A) and tryptophan fluorescence (B) of apo B-100 is independent of the amount of α-TOH in LDL. Native LDL (open circles), LDL supplemented with α-TOH (filled circles), LDL depleted of α-TOH (filled squares) and depleted LDL replenished with α-TOH (filled triangles) were oxidised with reagent HOCl at 4°C for 15 min. The remaining lysine and tryptophan residues were assayed by the fluorescamine assay and fluorescence (excitation 280 nm, emission 335 nm), respectively. The results shown represent the mean ± S.D. from three independent experiments with LDL isolated from three separate donors.

of CE-O(O)H, and the extent of this was indistinguishable for the various LDL samples. Thus, 0.21 ± 0.21, 0.41 ± 0.34, 0.08 ± 0.25, and 0.93 ± 0.59 molecules of CE-O(O)H were formed per native, α-TOH-supplemented, -depleted, and -replenished LDL particle, respectively as a result of exposure to 600 molecules HOCl per lipoprotein particle. These results are comparable with our previous findings [7]. Also, independent of the content of α-TOH, exposure of LDL to HOCl did not result in detectable loss of cholesteryl esters (not shown).

4. Discussion

The methods used in this study to prepare LDL with various amounts of α-TOH had minimal effect on the protein and lipid components of the lipoprotein particle (Table 1), and this allowed the examination of the role of α-TOH in HOCl-induced oxidation of LDL. We found that neither the extent of oxidation of lysine and tryptophan residues nor the changes in REM of LDL correlated with the α-TOH concentration of the lipoprotein. In addition, even at low concentrations of HOCl, there was no dramatic inhibition of protein oxidation which could correspond to a timeline 'lag' phase. The lack of effect of α-TOH on HOCl-induced oxidative modification of LDL apo B-100 is in contrast to the conspicuous effect of the vitamin when lipid peroxidation in LDL is induced by radical oxidants. Using initially peroxide-free LDL and either AAPH, Cu^{2+} or soybean lipoxygenase as the oxidant under low radical flux conditions, depletion of α-TOH to an extent similar to that used in this study inhibited lipid peroxidation by > 80% [10]. Under conditions of high radical flux and highly reactive radical oxidants such as hydroxyl radicals, depletion of the vitamin from LDL increased the overall extent of lipid peroxidation [10]. In addition, under such strongly oxidising conditions, supplementation of LDL with α-TOH decreased the extent and delayed the onset of rapid lipid peroxidation [10,20,21]. Thus, independent of the oxidant flux, α-TOH controls radical-induced LDL lipid peroxidation.

In contrast to this, α-TOH had little effect on HOCl-induced LDL lipid peroxidation, as indicated by the lack of a large effect on both the formation of CE-O(O)H and the loss of cholesteryl esters. This is not surprising given that HOCl is expected to catalyse two-electron oxidation reactions rather than the one-electron reactions required for lipid peroxidation. We have shown previously that the concentrations of HOCl used here (< 3 mmole/g protein) do not directly oxidise substantial amounts of lipid in LDL; rather apo B-100 is the preferred target [7,8]. For this reason, the effect of α-TOH on radical-induced oxidation of apo B-100 would be more meaningful for comparison. In this context, direct protein oxidation needs to be distinguished from that induced by secondary reactions derived from breakdown of lipid peroxides.

As far as we are aware, there is no strong evidence that α-TOH can substantially prevent *direct* oxidation of isolated proteins induced by one-electron oxidants. Dean and coworkers showed that the water-soluble analogue of α-TOH, Trolox, efficiently inhibited AAPH-induced loss of tryptophan residues in isolated BSA [22]. However, methanolic solutions of α-TOH were without effect, and when α-TOH was added in soybean phosphatidylcholine liposomes, the inhibitory effect on tryptophan loss observed was not clearly distinguished from that of the vitamin-free liposomes [22]. α-TOH can, however, repair tyrosine phenoxy radicals in partially unfolded lysozyme [23].

The situation becomes more complicated with biological protein-lipid mixtures. With regard to LDL, Giessauf et al. [24] reported that supplementing the lipoprotein with vitamin E had no effect on the initial rate of Cu^{2+}-induced tryptophan

Table 2
Oxidation of α-TOH upon treatment of the various LDL samples with HOCl

LDL	HOCl/LDL particle				
	0	100	200	400	600
Native	7.7 ± 3.0	7.2 ± 3.5	6.8 ± 3.7	5.7 ± 1.4	4.2 ± 1.9
Supplemented	60 ± 34	50 ± 22	51 ± 25	48 ± 26	4 ± 26
Depleted	1.4 ± 0.8	1.2 ± 0.8	1.1 ± 0.8	0.8 ± 0.6	0.3 ± 0.2
Replenished	44 ± 30	41 ± 27	41 ± 25	38 ± 24	36 ± 24

The values shown are from three independent experiments and are expressed as the mean ± S.D. of α-TOH per LDL particle (mole/mole).

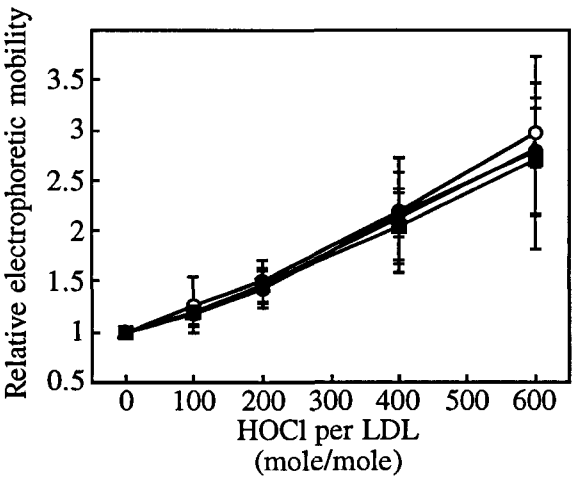

Fig. 2. α-TOH does not influence the HOCl-induced increase in REM of LDL. Symbols and oxidation conditions are the same as for Fig. 1. The REM was assessed by agarose gel electrophoresis. The results shown represent the mean ± S.D. from three independent experiments with LDL isolated from three separate donors.

loss, even though α-TOH consumption occurred more rapidly than that of tryptophan. Also, blocking the thiol groups of apo B-100 increases the rate of α-TOH loss induced by peroxyl radicals [25] and Cu^{2+} [26], indicating that the thiol residues compete with the vitamin for the initiating oxidants and/or those derived from them. However, one cannot conclude from these results that the vitamin prevents direct oxidation of apo B's amino acids.

α-TOH can clearly affect protein oxidation dependent on secondary lipid peroxidation products [22,27], by affecting the formation and/or degradation of lipid hydroperoxides. Generally speaking, this type of protein oxidation is attenuated by α-TOH under conditions where the vitamin decreases lipid peroxidation (see e.g. [9]). By contrast, under conditions where vitamin E promotes lipid peroxidation (see above), protein oxidation may not necessarily be enhanced in parallel due to α-TOH inhibiting the breakdown of lipid hydroperoxides [28]. However, such inhibition of such secondary protein oxidation by α-TOH does not appear to be relevant for oxidation of LDL by reagent HOCl, as under the conditions studied in this work neither lipid peroxidation nor the breakdown of lipid hydroperoxides are major reactions.

HOCl-exposed LDL shows atherogenic characteristics [7,8], the production of which is not dependent on lipid peroxidation. The results presented in this work show that α-TOH is unable to prevent at least some of the oxidative protein modifications that may lead to these characteristics. Our findings could help explain the lack of clearly positive results in intervention studies using α-TOH to inhibit coronary heart disease in humans [3,4,29]. Our results also suggest that studies on antioxidants as potential antiatherogenic agents may need to consider inhibitors of HOCl-mediated protein damage rather than exclusively focusing on effective radical scavengers and/or inhibitors of lipid peroxidation.

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References

[1] Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C. and Witztum, J.L. (1989) New Engl. J. Med. 320, 915–924.
[2] Steinberg, D. (1995) Lancet 346, 36–38.
[3] Stephens, N.G., Parsons, A., Schofield, P.M., Kelly, F., Cheeseman, K., Mitchinson, M.J. and Brown, M.J. (1996) Lancet 347, 781–786.
[4] Jha, P., Flather, M., Lonn, E., Farkouh, M. and Yusuf, S. (1995) Ann. Intern. Med. 123, 860–872.
[5] Daugherty, A., Dunn, J.L., Rateri, D.L. and Heinecke, J.W. (1994) J. Clin. Invest. 94, 437–444.
[6] Hazell, L.J., Arnold, L., Flowers, D., Waeg, G., Malle, E. and Stocker, R. (1996) J. Clin. Invest. 97, 1535–1544.
[7] Hazell, L.J. and Stocker, R. (1993) Biochem. J. 290, 165–172.
[8] Hazell, L.J., van den Berg, J.J.M. and Stocker, R. (1994) Biochem. J. 302, 297–304.
[9] Esterbauer, H., Gebicki, J., Puhl, H. and Jürgens, G. (1992) Free Radical Biol. Med. 13, 341–390.
[10] Neuzil, J., Thomas, S.R. and Stocker, R. (1997) Free Radical Biol. Med. 22, 57–71.
[11] Morris, J.C. (1966) J. Phys. Chem. 70, 3798–3805.
[12] Sattler, W., Mohr, D. and Stocker, R. (1994) Methods Enzymol. 233, 469–489.
[13] Frei, B. and Gaziano, J.M. (1993) J. Lipid Res. 34, 2135–2145.
[14] Esterbauer, H., Dieber-Rotheneder, M., Striegl, G. and Waeg, G. (1991) Am. J. Clin. Nutr. 53, 314S–321S.
[15] Smith, P.K., Krohn, R.J., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
[16] Morton, R.E. and Evans, T.A. (1992) Anal. Biochem. 204, 332–334.
[17] Böhlen, P., Stein, S., Dairman, W. and Udenfriend, S. (1973) Arch. Biochem. Biophys. 155, 213–220.
[18] Teale, F.W.J. (1960) Biochem. J. 76, 381–388.
[19] Haberland, M.E., Olch, C.L. and Fogelman, A.M. (1984) J. Biol. Chem. 259, 11305–11311.
[20] Dieber-Rotheneder, M., Puhl, H., Waeg, G., Striegl, G. and Esterbauer, H. (1991) J. Lipid Res. 32, 1325–1332.
[21] Kontush, A., Finckh, B., Karten, B., Kohlschütter, A. and Beisiegel, U. (1996) J. Lipid Res. 37, 1436–1448.
[22] Dean, R.T., Hunt, J.V., Grant, A.J., Yamamoto, Y. and Niki, E. (1991) Free Radical Biol. Med. 11, 161–168.
[23] Hoey, B.M. and Butler, J. (1984) Biochim. Biophys. Acta 791, 212–218.
[24] Giessauf, A., Steiner, E. and Esterbauer, H. (1995) Biochim. Biophys. Acta 1256, 221–232.
[25] Bowry, V.W. and Stocker, R. (1993) J. Am. Chem. Soc. 115, 6029–6044.
[26] Ferguson, E., Singh, R.J., Hogg, N. and Kalyanaraman, B. (1997) Arch. Biochem. Biophys. 341, 287–294.
[27] Zhang, H., Yang, Y. and Steinbrecher, U.P. (1993) J. Biol. Chem. 268, 5535–5542.
[28] Lauraux, C., Therond, P., Bonnefont-Rousselot, D., Troupel, S.E., Legrand, A. and Delattre, J. (1997) Free Radical Biol. Med. 22, 185–194.
[29] The Alpha-Tocopherol Beta Carotene Cancer Prevention Study Group (1994) New Engl. J. Med. 330, 1029–1035.