Published in final edited form as:

Free Radic Biol Med. 2014 November ; 0: 61–68. doi:10.1016/j.freeradbiomed.2014.07.031.

Absence of an effect of vitamin E on protein and lipid radical formation during lipoperoxidation of LDL by lipoxygenase

Douglas Ganini^{a,*} and Ronald P. Mason^a

^aFree Radical Metabolism Section, Laboratory of Toxicology and Pharmacology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709, USA

Abstract

LDL oxidation is the primary event in atherosclerosis, where LDL lipoperoxidation leads to modifications in the apolipoprotein B-100 (apo B-100) and lipids. Intermediate species of lipoperoxidation are known to be able to generate amino acid-centered radicals. Thus, we hypothesized that lipoperoxidation intermediates induce protein-derived free radical formation during LDL oxidation. Using DMPO and immuno spin-trapping, we detected the formation of protein free radicals on LDL incubated with Cu²⁺ or the soybean lipoxidase (LPOx)/phospholipase A₂ (PLA₂). With low concentrations of DMPO (1 mM), Cu²⁺ dose-dependently induced oxidation of LDL and easily detected apo B-100 radicals. Protein radical formation in LDL incubated with Cu²⁺ showed maximum yields after 30 minutes. In contrast, the yields of apo B-100-radicals formed by LPOx/PLA₂ followed a typical enzyme-catalyzed kinetics that was unaffected by DMPO concentrations of up to 50 mM. Furthermore, when we analyzed the effect of antioxidants on protein radical formation during LDL oxidation, we found that ascorbate, urate and Trolox dose-dependently reduced apo B-100-free radical formation in LDL exposed to Cu²⁺. In contrast, Trolox was the only antioxidant that even partially protected LDL from LPOx/PLA₂. We also examined the kinetics of lipid radical formation and protein radical formation induced by Cu²⁺ or LPOx/PLA₂ for LDL supplemented with α-tocopherol. In contrast to the potent antioxidant effect of α-tocopherol on the delay of LDL oxidation induced by Cu²⁺, when we used the oxidizing system LPOx/PLA₂, no significant protection was detected. The lack of protection of α-tocopherol on the apo B-100 and lipid free radical formation by LPOx may explain the failure of vitamin E as a cardiovascular protective agent for humans.

Keywords

Free radicals; LDL oxidation; DMPO; immuno-spin trapping; copper; lipoxidase; lipoxygenase; lipoperoxidation; protein-derived free radicals; atherosclerosis; vitamin E

*Corresponding author: Douglas Ganini, Free Radical Metabolism Section, Laboratory of Toxicology and Pharmacology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709, USA. Telephone number: (919) 541-7573. ganinidasilvad@niehs.nih.gov.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

INTRODUCTION

The low density lipoprotein (LDL) is the main carrier of cholesterol in the blood of mammals [1]. This particle is synthesized in the liver, and its main physiological function is providing cholesterol to extra-hepatic tissues [1]. The lipoprotein comprises a molecule of apolipoprotein B-100 (apoB-100) that wraps around a monolayer of phospholipids with a nonpolar core of esterified cholesterol [2].

This macromolecule is widely studied because its oxidation plays a central role in the pathogenesis of atherosclerosis [3–5]. Cells take up LDL through a receptor-mediated process using LDL receptors for native LDL [6] or scavenger receptors for the oxidized LDL [7]. The pathogenesis of atherosclerosis starts with the native or minimally oxidized LDL entering the artery wall and penetrating the subendothelial region where the lipoprotein is further oxidized [4]. This theory is supported by the detection of higher levels of oxidized lipids and proteins of LDL in human arterial atherosclerotic plaques [8–10].

Macrophages are very effective at capturing oxidized LDL due to the high abundance of scavenger receptors, mainly CD36 [11, 12] and LOX-1 [7, 13]. The active uptake of the oxidized LDL by macrophages leads to their transition to foam cells, initiating plaque formation [4]. This process is largely mediated and supported by the metabolism of endothelial and smooth muscle cells in response to oxidized LDL, concomitant with the release of pro-inflammatory cytokines from emerging foam cells [4, 13].

The most physiologically relevant source of oxidants for the oxidation of LDL is still under debate [3–5]. *In vitro*, the classic protocol for oxidizing LDL is the use of Cu^{2+} salts [14–16]. However, the enzyme-catalyzed oxidizing system with lipoxidase V (LPOx) and phospholipase A₂ (PLA₂) has been proposed as a physiological model of LDL oxidation [17, 18], while transition metals may have importance during later stages in the development of atheromas [4, 19]. Incubation of LDL with Cu^{2+} induces the oxidation of the lipoprotein, increasing the content of lipid peroxidation products [16, 20], negative charge [15], density [14], and the ability to be taken up by macrophages [20, 21]. The ability to be taken up by macrophages can be mimicked by acetylation of LDL, which suggests that protein modifications contribute to recognition by the macrophage scavenger receptors [21–23]. However, the consensus is that oxidized LDL is recognized by the immune system through different epitopes that are formed as a result of the oxidation of LDL [24, 25].

Apolipoprotein B-100 is a large protein consisting of 4,536 amino acid residues and a molecular mass of 550 kDa for its glycosylated form [2]. The protein is composed of 5 distinct flexible domains [26], is very insoluble in aqueous solution [21] and is easily oxidized and fragmented [27]. The three-dimensional molecular structure of the native protein is still under extensive study since its chemical properties make challenging crystallographic studies of the entire protein in its native state [2].

Since lipoperoxidation and protein modifications are responsible *in vivo* for the atherogenic capacity of oxidized LDL, we studied the formation of protein-derived free radicals on apolipoprotein B-100 induced in LDL exposed to Cu^{2+} or LPOx and PLA₂. Using immuno-spin trapping, we show that during LDL oxidation, apo B-100 radicals are formed by the

intermediate free radicals of lipoperoxidation. Moreover, in contrast to the LDL oxidation induced by Cu^{2+} , LPOx/PLA₂ mediates free radical formation on apo B-100 that is not prevented by ascorbate, urate or α -tocopherol.

EXPERIMENTAL PROCEDURES

Materials

OptiPrep® (iodixanol 60% (w/v)), CuSO_4 , lipoxidase V from *Glycine max* (EC 1.13.11.12), phospholipase A₂ from porcine pancreas (EC 3.1.1.4), CaCl_2 , ascorbic acid, α -tocopherol (96%), and barbital buffer were from Sigma Aldrich (St. Louis, MO, USA). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was from Dojindo (Kumamoto, Japan). Sephacryl S200 was from GE Healthcare Lifescience (Pittsburg, PA, USA). Agarose and BODIPY^{581/591} C11 were from Invitrogen (Grand Island, NY, USA). The BCA protein assay was from Pierce (Rockford, IL, USA). Chelex C-100 and Commassie Blue staining solution were from BioRad (Hercules, CA, USA). Phosphate and Tris buffers were prepared and Chelex-treated for removal of contaminant trace metal ions.

LDL isolation

LDL was isolated from human plasma (American Red Cross, Charlotte, NC, USA) using a self-generating iodixanol gradient essentially as described by Billington and co-workers [28]. Briefly, ultracentrifuge tubes were loaded sequentially with 2 mL of 20% (v/v) iodixanol in PBS, 20 mL of human plasma with 12% (v/v) iodixanol, and approximately 2.0 mL of PBS. After ultracentrifugation using a 60Ti rotor for 3h, 350,000 g at 16°C, the tubes were fractionated (1.0 mL per fraction) from the bottom. The two before the last iodixanol fractions were LDL-enriched and free of contaminant VLDL, HDL or other plasma proteins (data not shown). The LDL in the pooled fractions was freed of iodixanol by passing the sample through a Sephacryl S200 column pre-equilibrated with 10 mM phosphate buffer, pH 7.4. The pure LDL eluted in the void volume of the column was assayed for protein content.

Detection and quantification of protein radical formation

LDL samples (0.5 mg/mL) with CuSO_4 or LPOx/PLA₂ were prepared in the presence of the spin trap DMPO. At specific time points, samples were diluted 1:10 (v/v) with PBS, and 2.5 μg of protein was bound to nitrocellulose membranes using a slot blot apparatus coupled to a vacuum pump (Amersham Biosciences, Pittsburg, PA, USA). After the samples were immobilized on the membrane, two washes with 500 μL of PBS per slot were made. The membranes were immunostained using the standard protein western blot protocol [29, 30], a monoclonal anti-DMPO primary antibody from mice (5 $\mu\text{g}/\text{mL}$) for 2h and a secondary anti-mouse antibody (1:10,000, v/v) conjugated to IRDye 800CW from goats (LiCor Bioscience, Lincoln, NE, USA) for 1h. The fluorescence in the membranes was detected using an Odyssey scanner (LiCor Bioscience, Lincoln, NE, USA), and the densitometries of the bands were quantified using the software ImageJ, version 1.45s. After optimizations, the dot blot-based assay was chosen instead of an ELISA format because artifactual formation of DMPO-protein adducts is not possible after the fast immobilization of the samples on the membranes, which separates the LDL from the DMPO. Even more, in contrast to an ELISA-

based assay, the use of detergents in the subsequent washing solutions did not remove or interfere with the binding of the proteins to the support, but indeed assured that our dot blot-based assay was specifically detecting radicals formed on apo B-100, as shown in Fig. 1C.

Preparation of iodinated LDL

Iodinated LDL was prepared according to method C described by Sobal *et al.* (2004) [31] with the following modifications: (i) NaI was used at 100 mM; (ii) LDL was incubated in the reaction vessel for 15 min; (iii) after iodination, LDL was desalted using a PD-10 column, followed by a Zebaspin column, both previously equilibrated with 10 mM phosphate buffer, pH 7.4. TBARs determination on control, sham- and NaI-treated samples showed no significant increase in lipid oxidation products, namely malondialdehyde. Experiments regarding the protein free radical formation on LDL were prepared with the sham- and the NaI-treated LDL.

BODIPY^{581/591} C11 oxidation

An increase in the fluorescence of oxidized BODIPY^{581/591} C11 (excitation = 500 nm, emission = 520 nm) was used for the specific assessment of the lipid radical formation [32] in LDL exposed to Cu²⁺ or LPOx/PLA₂. The reduced probe ($\lambda_{\text{max}} = 595$ nm) has a nonpolar BODIPY fluorochrome center conjugated to a phenyl group by a diene interconnection. Upon oxidation, the conjugation is lost, which results in increased fluorescence at shorter wavelengths, with a maximum at 520 nm [33]. As expected, incubation of BODIPY^{581/591} C11 (2 μ M) with LDL (0.5 mg/mL) for 5 min resulted in total incorporation of the probe into the lipoprotein (Supplemental Fig. 1). The fluorescence of the reduced probe is highly quenched when the probe is diluted in neat buffer; however, samples of LDL incubated with BODIPY^{581/591} C11 had a bright red fluorescence, consistent with the unoxidized probe. Samples of LDL and BODIPY^{581/591} C11 subjected to ultrafiltration showed complete retention of the fluorochrome in the particles retained (LDL), but not in the flowthrough (phosphate buffer).

The stock solution of BODIPY^{581/591} C11 (2 mM) was prepared using DMSO, and this solvent at 0.1% (v/v) did not induce any change in the protein-derived free radical formation in LDL exposed to Cu²⁺ or LPOx/PLA₂ (data not shown).

LDL supplementation with vitamin E

LDL enriched with vitamin E was prepared as described by Esterbauer and co-workers [34]. Human plasma was spiked with 10 μ L/mL of a 1 mM α -tocopherol solution prepared in DMSO and incubated for 3h at 37°C. LDL was then isolated as described. The authors describe an enrichment of approximately 20 mol of vitamin E per mol of LDL [34].

RESULTS

With the immuno-spin-trapping technique [29, 30] it is possible to detect and semi-quantify the formation of protein-derived free radicals using the spin-trapping agent DMPO. In this methodology the protein free radicals are trapped by DMPO, generating covalent adducts of the protein and the spin trap that can be detected and quantified using standard

immunological techniques with antibodies raised against the nitron of DMPO [29, 30]. For the detection of protein radicals in LDL, we used the immuno-spin-trapping technique in a newly developed dot blot-based assay (Fig. 1). It is clear that protein radicals are formed during the incubation of LDL with CuSO_4 (Fig. 1A). Protein radicals were also detected in LDL exposed to LPOx/PLA₂ (Fig. 1B). Samples of LDL (0.5 mg/mL) oxidized by CuSO_4 (100 μM) or LPOx/PLA₂ (2.5 kU/mL and 5 U/mL) were delipidated and probed for DMPO-staining (Fig. 1C) [21]. As expected, it was found that apolipoprotein B-100, the protein fraction of LDL, was the only species contributing to the DMPO-staining in our dot blot-based assay, since lipid-derived DMPO adducts would be lost in the washing steps.

It is noteworthy that the optimal concentrations of DMPO used to detect the protein radicals were distinctly different for the two oxidizing agents (Fig. 1B). The highest yield of protein-free radicals was detected in LDL (0.5 mg/mL) and CuSO_4 (100 μM) with DMPO at a concentration of 5 mM, and higher concentrations of the spin trap were inhibitory. However, using LPOx/PLA₂ (2.5 kU/mL and 5 U/mL, respectively), the detection of protein-free radicals was higher with DMPO concentrations up to 50 mM (Fig. 1B). These results indicate an antioxidant effect of DMPO during the oxidation of LDL by Cu^{2+} , but not by LPOx/PLA₂. Analyses with the antibody E06 (Fig. 2), an antibody that binds to oxidized LDL, revealed that LDL exposed to CuSO_4 or LPOx/PLA₂ was significantly oxidized, but high concentrations of DMPO inhibited the formation of the oxidized LDL in both systems. In classical experiments, the inhibitory effect of DMPO on the LDL oxidation was also observed for changes in the net charge of LDL by subjecting the samples to agarose native electrophoresis (Supplementary Fig. 2).

DMPO is known to form stable adducts with tyrosyl radicals in proteins, so we wanted to verify the extension of tyrosine-centered apo B-100-DMPO formed in LDL oxidized by Cu^{2+} . Iodination of tyrosine prevents the formation of DMPO-trappable tyrosyl radicals; thus, the difference in DMPO-adducts of the neat protein compared to the iodinated protein is equivalent to the contribution of DMPO-adducts centered on tyrosines. Iodinated LDL and its sham control were prepared using a gentle procedure with Iodogen® (1,3,4,6-tetrachloro-3 α ,6 α -diphenyl glycoluril, see Materials and Methods), which in fact resulted in no accumulation of malondialdehyde, a lipid oxidation product (data not shown), or loss in the protein or antigenicity for the apo B-100 antibody used here (Fig. 3A); however, iodinated LDL showed 50% less formation of DMPO-adducts (Fig. 3B), which indicates that tyrosine is one of the major sites of protein free radical formation during LDL oxidation.

We next examined the time course of apo B-100-free radicals in LDL exposed to Cu^{2+} or LPOx/PLA₂ (Fig. 4). Treatment of LDL with Cu^{2+} induced the formation of apo B-100-DMPO in the first 30 minutes of incubation at 37°C. In contrast, LDL incubated with LPOx/PLA₂ showed a typical enzyme-catalyzed kinetics, with linearly increasing levels of apo B-100-DMPO for up to 2h at 37°C. The lipid oxidation of LDL exposed to Cu^{2+} or LPOx/PLA₂ was studied using the classical formation of conjugated dienes, but also using the oxidation-sensitive probe for lipid radical formation BODIPY^{581/591} C11, which shifts in the fluorescence from red to green upon its oxidation (see Materials and Methods) [32, 33]. In samples with CuSO_4 , both methodologies had the same kinetic formation of

chromophores (Supplemental Fig. 3A). However, LDL incubated with LPOx/PLA₂ showed a very rapid and high formation of conjugated dienes (Supplemental Fig. 3C) but much slower BODIPY^{581/591} C11 oxidation. Indeed, the lipoxidase activity would be expected to generate mostly lipid hydroperoxides [35] that absorb UV light at 234 nm. Furthermore, experiments using different concentrations of DMPO to follow the formation of conjugated dienes could not be prepared since the spin trap strongly absorbs UV light ($\epsilon_{228\text{nm}} = 7800 \text{ M}^{-1}.\text{cm}^{-1}$ [36]). In contrast, the BODIPY^{581/591} C11 oxidation assay for lipid radical formation in LDL samples exposed to Cu²⁺ or LPOx/PLA₂ could be used in the presence of DMPO. Experiments with DMPO indicated that even at low concentrations of the spin trap (1 mM), it inhibited the Cu²⁺-mediated LDL lipid radical formation, with a maximum inhibition at 5–10 mM (Supplemental Fig. 3B). DMPO had only a modest effect on the enzymatic lipid radical formation induced by LPOx/PLA₂ (Supplemental Fig. 3D).

The antioxidants ascorbic acid, uric acid and Trolox were evaluated for their effect on the protein-derived free radical yield (Fig. 5). Ascorbic acid and uric acid were highly effective in inhibiting the formation of apo B-100-free radicals in the Cu²⁺-mediated LDL oxidation, but did not have any effect on the LPOx/PLA₂ treatment (Fig. 5A). However, the water-soluble vitamin E-analogue Trolox was an efficient antioxidant in Cu²⁺-mediated protein radical formation and partially effective in the LPOx/PLA₂ system (Fig. 5B). We prepared LDL supplemented with α -tocopherol and performed kinetic experiments for lipid radical formation and protein radical generation (Fig. 6). As expected, the LDL supplemented with vitamin E was indeed more resistant to lipid radical formation and protein-derived free radical formation mediated by Cu²⁺ (Fig. 6A, B). The LDL supplemented with vitamin E exposed to Cu²⁺ had nearly three times the lag-phase time for the lipid radical formation compared to controls (Fig. 6B). The effect of vitamin E supplementation was even stronger for the protein radical formation induced by Cu²⁺, which showed a four times longer lag phase (Fig. 6B). It is noteworthy that for the control LDL, the protein radicals were formed just before the end of the lipid radical formation lag phase, but in the vitamin E-supplemented LDL, protein radicals occur simultaneously with the lipid radical propagation phase. However, when LPOx/PLA₂ was used, the antioxidant effect of vitamin E in LDL was very modest with only 18% lower initial lipid radical formation rate. The lag phase for the protein radical formation was nearly the same between the control and the vitamin E-supplemented LDL. The lipid radical formation induced by LPOx/PLA₂ is modest but did not show a distinguished lag phase and, as expected, the time course for protein radical formation was nearly the same for control and vitamin E-supplemented LDL.

DISCUSSION

The exact mechanism of LDL oxidation by copper is still under debate [4, 16, 19, 37], but it is known that the redox-cycling of copper takes place during LDL oxidation [15, 16, 19, 20]. Various theories have been formulated to explain the initial species that triggers LDL oxidation. Vitamin E [15, 38, 39] and apo B-100 [3, 40] have been shown to reduce Cu²⁺, while lipid hydroperoxides have been shown to oxidize Cu²⁺ [41]. Interestingly, LDL has multiple reduction sites with different affinities for Cu²⁺ [16], which shows that the high association and integration of the different components of LDL make the analysis of the contribution of each individual component difficult. However, *in vivo* evidence suggests that

the initial LDL oxidation occurs primarily by endogenous lipoxygenases, namely lipoxygenase 15 [8–10, 42, 43]; thus, we also used the combination of lipoxidase and phospholipase A₂ as an *in vitro* model of the physiological LDL oxidation system [17]. Lipoxidases are known to catalyze the conversion of polyunsaturated free fatty acids to their hydroperoxides [35]. The catalysis involves intermediate lipid-derived carbon- and oxygen-centered radicals that have been detected with ESR [44–47]. In this context, phospholipase A₂ is used to increase the free fatty acid content of LDL, essential for the lipoxidase activity [35]. Using immuno-spin trapping in a dot blot-based assay, it is clear that LDL peroxidation induced protein radical formation on apolipoprotein B-100 by both oxidant systems (Fig. 1). We also show that 50% of total protein radicals were produced on tyrosines of apo B-100 in LDL oxidized by CuSO₄. Interestingly, the level of 3-nitrotyrosine on LDL *in vivo* correlates with atherosclerotic development, which shows higher levels of 3-nitrotyrosine-rich LDL in the intima wall of atherosclerotic arteries [48]. Nitration of tyrosine depends on the formation of this amino acid-centered free radical before reacting with NO₂, the nitrating agent produced by ONOO[−] or peroxidases and NO₂[−] [49]. Therefore, our results indicate that the oxidation of LDL could facilitate the nitration of apo B-100 and might explain the higher 3-nitrotyrosine content on LDL of patients under risk factors usually associated with oxidative stress such as atherosclerosis [48], smoking [50] and diabetes [51].

The extension of LDL oxidation was followed by the binding of the E06 antibody (Fig. 3) and also by the classical analyses of changes in the net charge of LDL (Supplemental Fig. 2). LDL incubated with Cu²⁺ was highly oxidized, and the LDL modifications were completely inhibited by high concentrations of DMPO. High concentrations of DMPO are expected to inhibit the LDL oxidation mediated by Cu²⁺ not only by trapping intermediate free radicals but also by binding copper directly [52, 53].

Furthermore, in a series of works from Kalyanaraman and co-workers [54–57], LDL lipid oxidation was shown to be preferentially inhibited by hydrophobic nitrones that intercept lipid and apolipoprotein B-100-derived free radicals. Following this argument, DMPO was expected to have a relatively minor negligible effect on the lipid oxidation of LDL due to its low hydrophobicity (log*P* = −1.0 [58]). In agreement with those arguments, the maximum protein radical formation in LDL exposed to Cu²⁺ was detected with low concentrations of DMPO, which resulted in oxidized LDL that showed difference neither for the binding of the E06 antibody nor for the net charge modification.

The apolipoprotein B-100-derived free radical formation in LDL and LPOx/PLA₂ follows typical enzyme-catalyzed kinetics (Fig. 4). The protein radical formation (Fig. 4), lipid radical formation (Fig. 5C) and E06 antibody binding (Fig. 5) corroborate the notion that under the conditions used, LPOx/PLA₂ is a moderate oxidizer of LDL in comparison to Cu²⁺. It is obvious that the apo B-100 radical formation by LPOx/PLA₂ was unaffected by DMPO (Fig. 1), in contrast to Cu²⁺. The spin trapping had only a minor effect on the lipid radical formation kinetics of LDL exposed to LPOx/PLA₂ (Supplemental Fig. 3D), suggesting that the bound lipoxidase generates an enzyme-bound free radical that DMPO is not able to trap. The site-specificity in the generation of the protein-derived radical by LPOx is supported by the reduced yield of E06 binding in samples with high DMPO

concentrations (Fig. 2) that still have the highest levels of LDL-DMPO nitron adduct formation.

Our results suggest that LDL oxidation by Cu^{2+} follows an unspecific lipid radical formation, which ultimately leads to protein radical generation. The oxidation of LDL by LPOx may occur mainly by the accidental formation of lipid radicals by the enzyme, which ultimately induces protein radical formation.

In the literature, some reports propose that protein radicals on apo B-100 are the initiating species of LDL oxidation [40, 59]. However, using our specific immuno-spin-trapping assay, the kinetics of protein-radical formation show that apo B-100-free radicals are formed following the kinetics of the lipid radical formation on LDL induced by Cu^{2+} or LPOx/PLA₂ (Fig. 4 and Fig. 6). Another fact that supports the notion that LDL oxidation and apo B-100 modifications are mediated by lipid radicals is the known inhibition of LDL oxidation by NO. Nitric oxide acts as an antioxidant, inhibiting the lipoperoxidation in LDL by reacting with lipid-derived radicals [60, 61].

Different antioxidants were evaluated for their ability to prevent the formation of apolipoprotein B-100-derived free radicals (Fig. 5). The polar antioxidants ascorbic acid and uric acid were effective at preventing protein radical formation in samples of LDL incubated with Cu^{2+} but not with LPOx/PLA₂. However, the moderately hydrophobic synthetic antioxidant Trolox ($\log P = 3.23$ [62]) was effective in preventing protein-derived free radical formation induced by Cu^{2+} or LPOx/PLA₂.

It is well accepted that hydrophobic molecules are the main antioxidants in LDL [63], and α -tocopherol plays a central role as an antioxidant but can also exert a prooxidant effect [64–66]. This effect is seen in LDL [64] and is ultimately dependent on low fluxes of lipid radicals [65, 66]. In this study the high concentrations of copper (approximately 100-fold excess of copper to LDL) and lipoxygenase (2.5 kU/mL) used reduce the importance of the α -tocopherol-mediated lipoperoxidation. The role of α -tocopherol in our assays would be mainly as an antioxidant, prolonging the inhibitive phase of productive lipoperoxidation or *lag phase* [66]. Indeed, LDL enriched with α -tocopherol was much less susceptible to lipid radical formation and protein radical generation induced by Cu^{2+} (Fig. 6A,B) but of little effect in the LPOx/PLA₂ system (Fig. 6C,D).

In vivo, plaques of human patients are found to be rich in protein and lipid oxidation products of LDL characteristically formed by lipoxygenases but not metals [8, 9]. Indeed, there is much evidence in the literature that lipoxygenase 15 is probably the main LDL-oxidizing agent *in vivo* [8–10, 42, 43], despite the fact that transition metals and myeloperoxidase may have a role, particularly in later stages of atheroma formation [4, 19]. In this work we used the soybean LPOx V (EC 1.13.11.12), which shows a different specificity of product generation when compared to mammalian lipoxygenase 15; however, both enzymes, to some extension and especially in LDL, show a lipoperoxidation activity which occurs through a free radical mechanism not within the enzyme [67]. Enrichment with α -tocopherol had only a minor effect on the lipid radical formation and protein radical generation in LDL incubated with LPOx/PLA₂ (Fig. 6). Based on our results, the absence of

any beneficial effect of oral supplementation with vitamin E in humans for the prevention of cardiovascular disease [68, 69] is not surprising because, *in vivo*, the oxidation of LDL is mainly mediated by lipoxygenases and, as shown here with the soybean enzyme, α -tocopherol is unable to prevent the free radical formation of lipids or protein in LDL by LPOx/PLA₂.

In summary, we show that the formation of apo B-100 free radicals accompanies the LDL lipid radical formation induced by Cu²⁺ or the physiologically relevant system LPOx/PLA₂. In the Cu²⁺-mediated LDL oxidation, lipid radicals are responsible for the apo B-100 free radical formation. In contrast, LDL oxidation by LPOx/PLA₂ induces an antioxidant-resistant apo B-100 free radical. Supplementation of LDL with α -tocopherol was very inhibitory when the oxidation was induced by Cu²⁺ but produced little effect when the oxidation was initiated by the physiologically relevant system LPOx/PLA₂. Our study may explain the lack of beneficial effects of vitamin E supplementation for cardiovascular complications in humans [68, 69].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors gratefully acknowledge Dr. Thomas Eling, Dr. Ann Motten, Mary Mason and Jean Corbett for their valuable help in the preparation and revision of the manuscript. We also acknowledge Jean Corbett for her valuable technical assistance. This research was supported by the Intramural Research Program of the NIEHS, National Institute of Environmental Health Sciences/NIH.

References

1. Brown MS, Kovanen PT, Goldstein JL. Regulation of plasma cholesterol by lipoprotein receptors. *Science*. 1981; 212:628–635. [PubMed: 6261329]
2. Prassl R, Laggner P. Molecular structure of low density lipoprotein: current status and future challenges. *European Biophysics Journal with Biophysics Letters*. 2009; 38:145–158. [PubMed: 18797861]
3. Esterbauer H, Gebicki J, Puhl H, Jurgens G. The role of lipid-peroxidation and antioxidants in oxidative modification of LDL. *Free Radical Biology and Medicine*. 1992; 13:341–390. [PubMed: 1398217]
4. Yoshida H, Kisugi R. Mechanisms of LDL oxidation. *Clinica Chimica Acta*. 2010; 411:1875–1882.
5. Steinberg D, Witztum JL. History of discovery oxidized low-density lipoprotein and atherosclerosis. *Arteriosclerosis Thrombosis and Vascular Biology*. 2010; 30:2311–2316.
6. Osono Y, Woollett LA, Herz J, Dietschy JM. Role of the low density lipoprotein receptor in the flux of cholesterol through the plasma and across the tissues of the mouse. *J Clin Invest*. 1995; 95:1124–1132. [PubMed: 7883961]
7. Greaves DR, Gordon S. Recent insights into the biology of macrophage scavenger receptors. *Journal of Lipid Research*. 2005; 46:11–20. [PubMed: 15548472]
8. Folcik VA, Nivavaristy RA, Krajewski LP, Cathcart MK. Lipoxygenase contributes to the oxidation of lipids in human atherosclerotic plaques. *Journal of Clinical Investigation*. 1995; 96:504–510. [PubMed: 7615823]
9. Leeuwenburgh C, Rasmussen JE, Hsu FF, Mueller DM, Pennathur S, Heinecke JW. Mass spectrometric quantification of markers for protein oxidation by tyrosyl radical, copper, and

- hydroxyl radical in low density lipoprotein isolated from human atherosclerotic plaques. *Journal of Biological Chemistry*. 1997; 272:3520–3526. [PubMed: 9013599]
10. Fu S, Davies MJ, Stocker R, Dean RT. Evidence for roles of radicals in protein oxidation in advanced human atherosclerotic plaque. *Biochemical Journal*. 1998; 333:519–525. [PubMed: 9677308]
 11. Hrboticky N, Draude G, Hapfelmeier G, Lorenz R, Weber PC. Lovastatin decreases the receptor-mediated degradation of acetylated and oxidized LDLs in human blood monocytes during the early stage of differentiation into macrophages. *Arterioscler Thromb Vasc Biol*. 1999; 19:1267–1275. [PubMed: 10323779]
 12. Nicholson AC, Han J, Febbraio M, Silverstein RL, Hajjar DP. Role of CD36, the macrophage class B scavenger receptor, in atherosclerosis. *Ann N Y Acad Sci*. 2001; 947:224–228. [PubMed: 11795270]
 13. Mitra S, Deshmukh A, Sachdeva R, Lu J, Mehta JL. Oxidized low-density lipoprotein and atherosclerosis implications in antioxidant therapy. *Am J Med Sci*. 2011; 342:135–142. [PubMed: 21747278]
 14. Steinbrecher UP, Parthasarathy S, Leake DS, Witztum JL, Steinberg D. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc Natl Acad Sci U S A*. 1984; 81:3883–3887. [PubMed: 6587396]
 15. Lynch SM, Frei B. Reduction of copper, but not iron, by human low density lipoprotein (LDL) - Implications for metal ion-dependent oxidative modification of LDL. *Journal of Biological Chemistry*. 1995; 270:5158–5163. [PubMed: 7890625]
 16. Ziouzenkova O, Sevanian A, Abuja PM, Ramos P, Esterbauer H. Copper can promote oxidation of LDL by markedly different mechanisms. *Free Radical Biology and Medicine*. 1998; 24:607–623. [PubMed: 9559873]
 17. Sparrow CP, Parthasarathy S, Steinberg D. Enzymatic modification of low-density lipoprotein by purified lipoxygenase plus phospholipase-A₂ mimics cell-mediated oxidative modification. *Journal of Lipid Research*. 1988; 29:745–753. [PubMed: 3139813]
 18. Parthasarathy S, Wieland E, Steinberg D. A role for endothelial-cell lipoxygenase in the oxidative modification of low-density lipoprotein. *Proceedings of the National Academy of Sciences of the United States of America*. 1989; 86:1046–1050. [PubMed: 2536929]
 19. Burkitt MJ. A critical overview of the chemistry of copper-dependent low density lipoprotein oxidation: roles of lipid hydroperoxides, alpha-tocopherol, thiols, and ceruloplasmin. *Arch Biochem Biophys*. 2001; 394:117–135. [PubMed: 11566034]
 20. Morgan J, Leake DS. Oxidation of low density lipoprotein by iron or copper at acidic pH. *Journal of Lipid Research*. 1995; 36:2504–2512. [PubMed: 8847477]
 21. Parthasarathy S, Fong LG, Otero D, Steinberg D. Recognition of solubilized apoproteins from delipidated, oxidized low-density-lipoprotein (LDL) by the acetyl-LDL receptor. *Proceedings of the National Academy of Sciences of the United States of America*. 1987; 84:537–540. [PubMed: 3467373]
 22. Steinbrecher UP, Loughheed M, Kwan WC, Dirks M. Recognition of oxidized low-density lipoprotein by the scavenger receptor of macrophages results from derivatization of apolipoprotein-B by products of fatty-acid peroxidation. *Journal of Biological Chemistry*. 1989; 264:15216–15223. [PubMed: 2768257]
 23. Zhang HF, Yang YH, Steinbrecher UP. Structural requirements for the binding of modified proteins to the scavenger receptor of macrophages. *Journal of Biological Chemistry*. 1993; 268:5535–5542. [PubMed: 8383674]
 24. Horkko S, Bird DA, Miller E, Itabe H, Leitinger N, Subbanagounder G, Berliner JA, Friedman P, Dennis EA, Curtiss LK, Palinski W, Witztum JL. Monoclonal autoantibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low-density lipoproteins. *Journal of Clinical Investigation*. 1999; 103:117–128. [PubMed: 9884341]
 25. Chang MK, Binder CJ, Torzewski M, Witztum JL. C-reactive protein binds to both oxidized LDL and apoptotic cells through recognition of a common ligand: Phosphorylcholine of oxidized

- phospholipids. Proceedings of the National Academy of Sciences of the United States of America. 2002; 99:13043–13048. [PubMed: 12244213]
26. Kumar V, Butcher SJ, Oorni K, Engelhardt P, Heikkonen J, Kaski K, Ala-Korpela M, Kovanen PT. Three-dimensional cryoEM reconstruction of native LDL particles to 16Å resolution at physiological body temperature. PLoS One. 2011; 6:e18841. [PubMed: 21573056]
 27. Fong LG, Parthasarathy S, Witztum JL, Steinberg D. Nonenzymatic oxidative cleavage of peptide-bonds in apoprotein-B-100. Journal of Lipid Research. 1987; 28:1466–1477. [PubMed: 3323390]
 28. Billington D, Maxwell E, Graham JM, Newland P. Large-scale preparation of human low- and high-density lipoproteins by density gradient centrifugation using iodixanol. Anal Biochem. 2007; 367:137–139. [PubMed: 17559788]
 29. Gomez-Mejiba SE, Zhai Z, Akram H, Deterding LJ, Hensley K, Smith N, Towner RA, Tomer KB, Mason RP, Ramirez DC. Immuno-spin trapping of protein and DNA radicals: “tagging” free radicals to locate and understand the redox process. Free Radic Biol Med. 2009; 46:853–865. [PubMed: 19159679]
 30. Mason RP. Using anti-5,5-dimethyl-1-pyrroline N-oxide (anti-DMPO) to detect protein radicals in time and space with immuno-spin trapping. Free Radical Biology and Medicine. 2004; 36:1214–1223. [PubMed: 15110386]
 31. Sobal G, Resch U, Sinzinger H. Modification of low-density lipoprotein by different radioiodination methods. Nucl Med Biol. 2004; 31:381–388. [PubMed: 15028251]
 32. Drummen GP, van Liebergen LC, Op den Kamp JA, Post JA. C11-BODIPY(581/591), an oxidation-sensitive fluorescent lipid peroxidation probe: mspectroscopic characterization and validation of methodology. Free Radic Biol Med. 2002; 33:473–490. [PubMed: 12160930]
 33. Nicolescu AC, Li Q, Brown L, Thatcher GRJ. Nitroxidation, nitration, and oxidation of a BODIPY fluorophore by RNOS and ROS. Nitric Oxide-Biology and Chemistry. 2006; 15:163–176.
 34. Esterbauer H, Dieber-Rotheneder M, Striegl G, Waeg G. Role of vitamin E in preventing the oxidation of low-density lipoprotein. Am J Clin Nutr. 1991; 53:314S–321S. [PubMed: 1985404]
 35. Andreou A, Feussner I. Lipoxygenases - Structure and reaction mechanism. Phytochemistry. 2009; 70:1504–1510. [PubMed: 19767040]
 36. Buettner GR. On the reaction of superoxide with DMPO/OOH. Free Radic Res Commun. 1990; 10:11–15. [PubMed: 2165980]
 37. Thomas SR, Stocker R. Molecular action of vitamin E in lipoprotein oxidation: Implications for atherosclerosis. Free Radical Biology and Medicine. 2000; 28:1795–1805. [PubMed: 10946221]
 38. Yoshida Y, Tsuchiya J, Niki E. Interaction of α-tocopherol with copper and its effect on lipid peroxidation. Biochimica Et Biophysica Acta. 1994; 1200:85–92. [PubMed: 8031846]
 39. Proudfoot JM, Croft KD, Puddey IB, Beilin LJ. The role of copper reduction by α-tocopherol in low-density lipoprotein oxidation. Free Radic Biol Med. 1997; 23:720–728. [PubMed: 9296448]
 40. Bathiany C, Santos CXC, Botti H, Cervenansky C, Radi R, Augusto O, Rubbo H. Direct evidence for apo B-100-mediated copper reduction: Studies with purified apo B-100 and detection of tryptophanyl radicals. Archives of Biochemistry and Biophysics. 2000; 384:335–340. [PubMed: 11368321]
 41. Jones CM, Burkitt MJ. EPR spin-trapping evidence for the direct, one-electron reduction of tert-butylhydroperoxide to the tert-butoxyl radical by copper(II): paradigm for a previously overlooked reaction in the initiation of lipid peroxidation. J Am Chem Soc. 2003; 125:6946–6954. [PubMed: 12783547]
 42. Cyrus T, Witztum JL, Rader DJ, Tangirala R, Fazio S, Linton MF, Funk CD. Disruption of the 12/15-lipoxygenase gene diminishes atherosclerosis in apo E-deficient mice. J Clin Invest. 1999; 103:1597–1604. [PubMed: 10359569]
 43. Harats D, Shaish A, George J, Mulkins M, Kurihara H, Levkovitz H, Sigal E. Overexpression of 15-lipoxygenase in vascular endothelium accelerates early atherosclerosis in LDL receptor-deficient mice. Arterioscler Thromb Vasc Biol. 2000; 20:2100–2105. [PubMed: 10978255]
 44. Chamulitrat W, Mason RP. Lipid peroxy radical intermediates in the peroxidation of polyunsaturated fatty acids by lipoxygenase. Direct electron spin resonance investigations. J Biol Chem. 1989; 264:20968–20973. [PubMed: 2556389]

45. Iwahashi H, Parker CE, Mason RP, Tomer KB. Radical Adducts of nitrosobenzene and 2-methyl-2-nitrosopropane with 12,13-epoxylinoleic acid radical, 12,13- epoxylinolenic acid radical and 14,15-epoxyarachidonic acid radical. *Biochemical Journal*. 1991; 276:447–453. [PubMed: 1646600]
46. Iwahashi H, Deterding LJ, Parker CE, Mason RP, Tomer KB. Identification of radical adducts formed in the reactions of unsaturated fatty acids with soybean lipoxygenase using continuous flow fast atom bombardment with tandem mass spectrometry. *Free Radic Res*. 1996; 25:255–274. [PubMed: 8889492]
47. Dikalov SI, Mason RP. Spin trapping of polyunsaturated fatty acid-derived peroxyl radicals: reassignment to alkoxyl radical adducts. *Free Radic Biol Med*. 2001; 30:187–197. [PubMed: 11163536]
48. Leeuwenburgh C, Hardy MM, Hazen SL, Wagner P, Oh-ishi S, Steinbrecher UP, Heinecke JW. Reactive nitrogen intermediates promote low density lipoprotein oxidation in human atherosclerotic intima. *J Biol Chem*. 1997; 272:1433–1436. [PubMed: 8999808]
49. Nakai K, Mason RP. Immunochemical detection of nitric oxide and nitrogen dioxide trapping of the tyrosyl radical and the resulting nitrotyrosine in sperm whale myoglobin. *Free Radic Biol Med*. 2005; 39:1050–1058. [PubMed: 16198232]
50. Yamaguchi Y, Haginaka J, Morimoto S, Fujioka Y, Kunitomo M. Facilitated nitration and oxidation of LDL in cigarette smokers. *Eur J Clin Invest*. 2005; 35:186–193. [PubMed: 15733073]
51. Rabbani N, Chittari MV, Bodmer CW, Zehnder D, Ceriello A, Thornalley PJ. Increased glycation and oxidative damage to apolipoprotein B100 of LDL cholesterol in patients with type 2 diabetes and effect of metformin. *Diabetes*. 2010; 59:1038–1045. [PubMed: 20068133]
52. Hanna PM, Chamulitrat W, Mason RP. When are metal ion-dependent hydroxyl and alkoxyl radical adducts of 5,5-dimethyl-1-pyrroline *N*-oxide artifacts. *Archives of Biochemistry and Biophysics*. 1992; 296:640–644. [PubMed: 1321591]
53. Burkitt MJ, Tsang SY, Tam SC, Bremner I. Generation of 5,5-dimethyl-1-pyrroline *N*-oxide hydroxyl and scavenger radical adducts from copper/H₂O₂ mixtures: effects of metal ion chelation and the search for high-valent metal-oxygen intermediates. *Arch Biochem Biophys*. 1995; 323:63–70. [PubMed: 7487075]
54. Kalyanaraman B, Joseph J, Parthasarathy S. Site-specific trapping of reactive species in low-density-lipoprotein oxidation - Biological implications. *Biochimica Et Biophysica Acta*. 1993; 1168:220–227. [PubMed: 8389205]
55. Thomas CE, Ku G, Kalyanaraman B. Nitron spin trap lipophilicity as a determinant for inhibition of low-density-lipoprotein oxidation and activation of interleukin-1- β release from human monocytes. *Journal of Lipid Research*. 1994; 35:610–619. [PubMed: 8006515]
56. Thomas CE, Ohlweiler DF, Kalyanaraman B. Multiple mechanisms for inhibition of low-density-lipoprotein oxidation by novel cyclic nitron spin traps. *Journal of Biological Chemistry*. 1994; 269:28055–28061. [PubMed: 7961741]
57. Kalyanaraman B, Darleyusmar V, Struck A, Hogg N, Parthasarathy S. Role of apolipoprotein B-derived radical and α -tocopheroxyl radical in peroxidase-dependent oxidation of low-density-lipoprotein. *Journal of Lipid Research*. 1995; 36:1037–1045. [PubMed: 7658151]
58. Janzen EG, West MS, Kotake Y, DuBose CM. Biological spin trapping methodology. III. Octanol-water partition coefficients of spin-trapping compounds. *J Biochem Biophys Methods*. 1996; 32:183–190. [PubMed: 8844325]
59. Giebauf A, van Wickern B, Simat T, Steinhart H, Esterbauer H. Formation of *N*-formylkynurenine suggests the involvement of apolipoprotein B-100 centered tryptophan radicals in the initiation of LDL lipid peroxidation. *FEBS Letters*. 1996; 389:136–140. [PubMed: 8766816]
60. Hogg N, Kalyanaraman B, Joseph J, Struck A, Parthasarathy S. Inhibition of low-density lipoprotein oxidation by nitric oxide. Potential role in atherogenesis. *FEBS Lett*. 1993; 334:170–174. [PubMed: 8224243]
61. O'Donnell VB, Chumley PH, Hogg N, Bloodsworth A, DarleyUsmar VM, Freeman BA. Nitric oxide inhibition of lipid peroxidation: Kinetics of reaction with lipid peroxyl radicals and comparison with α -tocopherol. *Biochemistry*. 1997; 36:15216–15223. [PubMed: 9398249]

62. Tafazoli S, Wright JS, O'Brien PJ. Prooxidant and antioxidant activity of vitamin E analogues and troglitazone. *Chem Res Toxicol*. 2005; 18:1567–1574. [PubMed: 16533021]
63. Laranjinha J, Cadenas E. Redox cycles of caffeic acid, α -tocopherol, and ascorbate: implications for protection of low-density lipoproteins against oxidation. *IUBMB Life*. 1999; 48:57–65. [PubMed: 10791916]
64. Alessi M, Paul T, Scaiano JC, Ingold KU. The contrasting kinetics of peroxidation of vitamin E-containing phospholipid unilamellar vesicles and human low-density lipoprotein. *Journal of the American Chemical Society*. 2002; 124:6957–6965. [PubMed: 12059219]
65. Waldeck AR, Stocker R. Radical-initiated lipid peroxidation in low density lipoproteins: Insights obtained from kinetic modeling. *Chemical Research in Toxicology*. 1996; 9:954–964. [PubMed: 8870982]
66. Bowry VW, Ingold KU. The unexpected role of vitamin E (α -tocopherol) in the peroxidation of human low-density lipoprotein. *Accounts of Chemical Research*. 1999; 32:27–34.
67. Noguchi N, Yamashita H, Hamahara J, Nakamura A, Kuhn H, Niki E. The specificity of lipoxygenase-catalyzed lipid peroxidation and the effects of radical-scavenging antioxidants. *Biol Chem*. 2002; 383:619–626. [PubMed: 12033451]
68. Yusuf S, Dagenais G, Pogue J, Bosch J, Sleight P. Vitamin E supplementation and cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. *N Engl J Med*. 2000; 342:154–160. [PubMed: 10639540]
69. Lonn E, Bosch J, Yusuf S, Sheridan P, Pogue J, Arnold JM, Ross C, Arnold A, Sleight P, Probstfield J, Dagenais GR. Effects of long-term vitamin E supplementation on cardiovascular events and cancer: a randomized controlled trial. *JAMA*. 2005; 293:1338–1347. [PubMed: 15769967]

- Protein radical formation accompanies the LDL oxidation induced by copper or soybean lipoxidase (LPOx)/phospholipase A₂ (PLA₂).
- Vitamin E does not protect LDL from the protein radical formation induced by LPOx.
- This may explain the failure of α -tocopherol as a cardiovascular protective agent for humans.

Fig. 1. Anti-DMPO slot blots to apo B-100 of LDL exposed to Cu²⁺ and lipoxidase/phospholipase A₂

Anti-DMPO slot blots were developed using 2.5 µg of protein per slot. LDL samples (0.5 mg/mL) were incubated with CuSO₄ in phosphate buffer, pH 7.4, for 1h at 37°C, or LPOx and 5 U/mL PLA₂ in Tris buffer, pH 8.0, and 5 mM CaCl₂ for 2h at 37°C. In (A) a representative slot blot of samples prepared with LDL, CuSO₄ and 1 mM DMPO is shown for anti-DMPO, and after stripping the membranes, for anti-apo B. In (B) different concentrations of DMPO were used. In (C) samples were prepared incubating LDL (0.5 mg/mL) for 1h at 37°C in the presence of 100 µM CuSO₄ with 1 mM DMPO, or for 2h at 37°C in the presence of 2.5 kU/mL LPOx, PLA₂ with 50 mM DMPO. Immediately after

incubation, the samples were delipidized [21] and the protein (resuspended as 0.5 mg/mL using 60 mg/mL octyl glucoside in water [21]) was subjected to the slot blot assay for DMPO staining using the nonextracted sample for comparison.

Fig. 2. E06 antibody binding to LDL oxidized by Cu^{2+} or lipoxidase/phospholipase A_2
 The binding of the murine autoantibody E06 to LDL was used as an index of the oxidation status of the particles [24]. The ELISA experiments were prepared according to the procedure described by Hörkkö *et al.* [24]. LDL samples (0.5 mg/mL) were incubated with 100 μM CuSO_4 in phosphate buffer, pH 7.4, for 1 h at 37°C, or with 2.5 kU/mL LPOx and 5 U/mL PLA₂ in Tris buffer, pH 8.0, and 5 mM CaCl_2 for 2 h at 37°C.

Fig. 3. Contribution of tyrosine-centered free radicals on apo B-100 of LDL oxidized by Cu²⁺ Anti-DMPO slot blots were developed using 2.5 μg of protein per slot. Sham or iodinated LDL samples (0.5 mg/mL) were incubated with CuSO₄ in phosphate buffer, pH 7.4, for 1h at 37°C. In (A) a representative slot blot of samples prepared with LDL, CuSO₄ and 1 mM DMPO is shown for anti-DMPO and, after stripping the membranes, for anti-apo B. In (B) quantifications for the densitometry of the anti-DMPO relative to the apo-B staining are shown.

Fig. 4. Kinetics of protein radical formation of LDL oxidized by Cu^{2+} or lipoxidase/phospholipase A_2

Anti-DMPO slot blots were developed using 2.5 μg of protein per slot. LDL samples (0.5 mg/mL) were incubated with 100 μM CuSO_4 in phosphate buffer, pH 7.4, at 37°C , or with 2.5 kU/mL LPOx and 5 U/mL PLA_2 in Tris buffer, pH 8.0, and 5 mM CaCl_2 at 37°C . At the indicated time points, aliquots were diluted in PBS and immediately immobilized on nitrocellulose membranes.

Fig. 5. Effect of ascorbic acid, uric acid and Trolox on the protein radical formation of LDL exposed to Cu^{2+} or lipoxidase/phospholipase A_2

Anti-DMPO slot blots were developed using 2.5 μg of protein per slot. LDL samples (0.5 mg/mL) in the presence of the antioxidants (A) ascorbic acid and uric acid (1–100 μM), or (B) Trolox (1–100 μM) were incubated with 100 μM CuSO_4 in phosphate buffer, pH 7.4, for 1h at 37°C, or with 2.5 kU/mL LPOx and 5 U/mL PLA_2 in Tris buffer, pH 8.0, and 5 mM CaCl_2 for 2h at 37°C.

Fig. 6. Effect of α -tocopherol supplementation on the lipid radical formation and protein radical generation of LDL oxidized by Cu^{2+} or lipoxidase/phospholipase A₂

In (A) and (C) anti-DMPO slot blots were developed using 2.5 μg of protein per slot.

Control and α -tocopherol-supplemented LDL samples (0.5 mg/mL) were incubated with (A–B) 100 μM CuSO_4 in phosphate buffer, pH 7.4, or (C–D) 2.5 kU/mL LPOx and 5 U/mL PLA₂ in Tris buffer, pH 8.0, and 5 mM CaCl_2 . LDL samples in the presence of the oxidizing agents (A, C) had aliquots collected at the time points for the time-course of protein radical generation. For the lipid radical formation (B, D), the fluorescence of the oxidized BODIPY^{581/591} C11 (Ex 510 nm; Em 520 nm) was followed. The time to half-maximum ($t_{1/2}$) was obtained after regression of the data using the Boltzmann equation.