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Original Contribution

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

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ABSTRACT

Low-density lipoprotein (LDL) oxidation is the primary event in atherosclerosis, and LDL lipoperoxidation leads to modifications in 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 Cu^{2+} or the soybean lipoxidase (LPOx)/phospholipase A_2 (PLA_2). With low concentrations of DMPO (1 mM), Cu^{2+} dose-dependently induced oxidation of LDL and easily detected apo B-100 radicals. Protein radical formation in LDL incubated with Cu^{2+} showed maximum yields after 30 min. In contrast, the yields of apo B-100 radicals formed by LPOx/PLA_2 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 Cu^{2+}. In contrast, Trolox was the only antioxidant that even partially protected LDL from LPOx/PLA_2. We also examined the kinetics of lipid radical formation and protein radical formation induced by Cu^{2+} or LPOx/PLA_2 for LDL supplemented with α-tocopherol. In contrast to the potent antioxidant effect of α-tocopherol on the delay of LDL oxidation induced by Cu^{2+}, when we used the oxidizing system LPOx/PLA_2, 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.

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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 extrahepatic tissues [1]. The lipoprotein comprises a molecule of apolipoprotein B-100 (apo B-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 protein of LDL in human arterial atherosclerotic plaques [8–10].

Macrophages are very effective at capturing oxidized LDL because of their 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 proinflammatory 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 A_2 (PLA_2) has been proposed as a physiological model of LDL oxidation [17,18], whereas 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 the

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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 4536 amino acid residues and a molecular mass of 550 kDa for its glycosylated form [2]. The protein is composed of five 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 because its chemical properties make challenging crystallographic studies of the entire protein in its native state [2].

Because 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 PLA_2. 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/PLA_2 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 A_2 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 Coomassie blue staining solution were from Bio-Rad (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 phosphate-buffered saline (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 3 h, 350,000 g at 16 °C, the tubes were fractioned (1.0 ml per fraction) from the bottom. The two iodixanol fractions before the last 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 preequilibrated 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/PLA_2 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 μg/ml) for 2 h, and a secondary anti-mouse antibody (1:10,000, v/v) conjugated to IRDye 800CW from goats (LiCor Bioscience, Lincoln, NE, USA) for 1 h. The fluorescence in the membranes was detected using an Odyssey scanner (LiCor Bioscience), and the densitometries of the bands were quantified using the software ImageJ, version 1.45 s. 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. Furthermore, 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 ensured 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. [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. Thiobarbituric acid-reactive substances 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 lipid radical formation [32] in LDL exposed to Cu^{2+} or LPOx/PLA_2. The reduced probe (λ_{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 μM) with LDL (0.5 mg/ml) for 5 min resulted in total incorporation of the probe into the lipoprotein (Supplementary 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 dimethyl sulfoxide (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 Cu^{2+} or LPOx/PLA_2 (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 μl/ml of a 1 mM α-tocopherol solution prepared in DMSO and incubated for 3 h at 37 °C. LDL was then isolated as described. The authors describe an enrichment of approximately 20 moles of vitamin E per mole of LDL [34].