Direct Evidence for apo B-100-Mediated Copper Reduction: Studies with Purified apo B-100 and Detection of Tryptophanyl Radicals

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Copper binding to apolipoprotein B-100 (apo B-100) and its reduction by endogenous components of low-density lipoprotein (LDL) represent critical steps in copper-mediated LDL oxidation, where cuprous ion (Cu(I)) generated from cupric ion (Cu(II)) reduction is the real trigger for lipid peroxidation. Although the copper-reducing capacity of the lipid components of LDL has been studied extensively, we developed a model to specifically analyze the potential copper reducing activity of its protein moiety (apo B-100). Apo B-100 was isolated after solubilization and extraction from size exclusion-HPLC purified LDL. We obtained, for the first time, direct evidence for apo B-100-mediated copper reduction in a process that involves protein-derived radical formation. Kinetics of copper reduction by isolated apo B-100 was different from that of LDL, mainly because apo B-100 showed a single phase-exponential kinetic, instead of the already described biphasic kinetics for LDL (namely α-tocopherol-dependent and independent phases). While at early time points, the LDL copper reducing activity was higher due to the presence of α-tocopherol, at longer time points kinetics of copper reduction was similar in both LDL and apo B-100 samples. Electron paramagnetic resonance studies of either LDL or apo B-100 incubated with Cu(II), in the presence of the spin trap 2-methyl-2-nitroso propane (MNP), indicated the formation of protein-tryptophanyl radicals. Our results supports that apo B-100 plays a critical role in copper-dependent LDL oxidation, due to its lipid-independent-copper reductive ability.

Key Words: LDL oxidation; apo B-100; tryptophan-centered radical; protein oxidation; lipid oxidation; copper; free radicals; nitric oxide.

A number of clinical and experimental evidences have been obtained indicating a dose association between low-density lipoprotein (LDL)2 oxidation and the onset and progression of atherosclerosis (1–5). Although the relevant mechanisms for LDL oxidation in vivo are not fully identified, it is well known that transition metals (i.e., copper and iron) are critical for cell-mediated oxidative modification of LDL (6–8). In fact, cell-mediated LDL oxidation can be mimicked by incubation of LDL with cupric ions Cu(II) (1, 9), leading to the formation of an oxidized form of LDL that shares many structural and functional properties of either the LDL oxidized by cells (10) or the LDL extracted from atherosclerotic plaques (11). Moreover, Cu(II) mediated oxidation is frequently used to assess the susceptibility of LDL to oxidation as a possible risk factor for atherosclerosis (12).

The mechanisms by which copper initiates LDL oxidation have been only partially defined. Copper binding primarily to apolipoprotein B-100 (apo B-100) (13–15) and its reduction by endogenous components of LDL (i.e., lipid hydroperoxides, α-tocopherol and apo

2 Abbreviations used: Cu(II), cupric ion; Cu(I), cuprous ion; LDL, low-density lipoprotein; apo B-100, apolipoprotein B-100; MNP, 2-methyl-2-nitroso propane; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BC, bathocuproinedisulfonic acid; EPR, electron paramagnetic resonance; NO, nitric oxide.
apo B-100 residues (16–22) have been implicated in this process. Although the binding of Cu(II) to apo B-100 is not completely understood, it has been shown that copper binds to histidine residues on apo B-100, but is reduced at other protein sites (23). In this sense, aromatic amino acids have been implicated in protein binding of cationic ligands. In fact, the combination of both hydrophobic and polar properties in phenylalanine, tyrosine and tryptophan facilitates the creation of cation recognition sites in proteins (24).

Herein, studies in LDL and isolated apo B-100 have been carried out to investigate the role of apo B-100 on copper reduction. We report direct evidence demonstrating that apo B-100 represents a copper reductant within the LDL particle in a lipid-independent process that involves the formation of tryptophanyl radicals.

**RESULTS**

**Chromatographic Purification of LDL and apo B-100**

Previous studies demonstrated that Cu(II) must be reduced to Cu(I) in order to trigger the oxidation of unsaturated fatty acids in LDL (14, 16–22). To analyze the potential copper reducing activity of apo B-100 it is critical to have a pure sample of the protein avoiding the presence of the other endogenous LDL copper reductants. For this purpose, we developed a protocol that includes further purification of LDL by size exclusion-HPLC (Fig. 1A) followed by extraction, salubrification and isolation of SDS-solubilized apo B-100 (Fig. 1B). Apo B-100 purity and integrity was confirmed by gradient SDS–PAGE, showing a single band with the expected molecular weight (Fig. 1C, line 3). As expected, the protein moiety of HPLC-purified LDL also appears as a single band (line 1), which is completely degraded upon incubation with Cu(II) (line 2).

Since α-tocopherol was already proposed as the major copper reductant in the LDL particle (18, 19, 22), we determined its content in LDL (3.75 ± 0.08 mol/mol of apo B), HPLC purified LDL (3.58 ± 0.11 mol/mol of apo B), and in isolated apo B-100 (nondetectable). Similarly, the absence of contaminant lipid hydroperoxides in the isolated apo B-100 fraction was also confirmed by RP-HPLC.

**Copper Reduction by LDL and apo B-100**

We studied the Cu(II) reduction activities of both LDL and isolated apo B-100. Figure 2 shows Cu(I) formation after incubation of 10 μM Cu(II) with increasing concentrations of LDL or isolated apo B-100 (0–0.2 μM) in the presence of 360 μM bathocuproine (BC). This figure shows that both LDL and isolated apo B-100 reduce Cu(II) in a dose-dependent manner. However, at low protein concentrations (0.01–0.04 μM) the LDL copper reducing activity was three- to sixfold higher than that of isolated apo B-100. Importantly, SDS (1.5 mM), present in the isolated apo B-100 preparation, did not change LDL copper reducing activity (data not shown). In addition, the time course of copper reduction by isolated apo B-100 was different from that of LDL (Fig. 3). In LDL, high Cu(II)/LDL ratios determined the presence of a biphasic kinetic during Cu(II) reduction (Fig. 3A), including an early α-tocopherol-dependent phase followed by a plateau and a second
reduction phase, as previously described by Perugini et al. (22). In contrast, at the same Cu(II)/protein ratio, copper-reduction by apo B-100 showed a single phase-exponential-kinetic, where, as expected, no \( \alpha \)-tocopherol-dependent early phase of copper reduction was detected (Fig. 3B). These results clearly indicate that apo B-100 is a copper reductant per se, in addition to other copper reductants present in LDL.

Electron Paramagnetic Resonance Detection of apo B-100 Tryptophan-Centered Radical Generated by Copper

Electron paramagnetic resonance (EPR) spin trapping studies of the oxidation of LDL by Cu(II) in the presence of MNP have been reported before (13, 37, 38). High LDL concentrations (5 mg ml\(^{-1}\)) and long incubation times (\( \geq 10 \) h) were used and lipid derived radicals were the only species detected (38). We confirmed these results (data not shown) and changed the experimental conditions in order to look for protein-derived radicals. Incubations of LDL (0.95 mg ml\(^{-1}\)), Cu(II) (100 \( \mu \)M), and the spin trap MNP (50 mM) for 30 min led to the detection of an immobilized EPR signal (Fig. 4A) that was not seen in the controls (not shown). The features of the signal (2\( \alpha \)Nzz = 63 G) are consistent with a macromolecule adduct, although the resolved central triplet indicates more mobile radicals than those previously reported in longer incubation times (38). After scanning of the EPR spectrum (Fig. 4A), the incubation mixture was immediately extracted with chloroform and both the organic and aqueous phases examined by EPR (see Materials and Methods). The EPR signal present in the organic phase (Fig. 4B), which contained 10% of the initial protein present...
in LDL, was consistent with the previously described lipid-derived radical adduct \((a_N = 14.8 \, \text{G}; a_H = 1.85 \, \text{G})\) produced in incubations of LDL with Cu(II) and lipoxygenase (13, 38). The aqueous phase also presented a relatively immobile EPR signal (Fig. 4C) whose dominant triplet \((a_N = 15.7 \, \text{G})\) is consistent with a protein-derived radical. In agreement, the aqueous phase contained 78% of the initial LDL protein. Moreover, incubations of purified apo B-100 \((0.5 \, \text{mg} \cdot \text{ml}^{-1})\) with Cu(II) in the presence of MNP \((50 \, \text{mM})\) for 30 min led to the detection of a similar triplet signal \((a_N = 15.7 \, \text{G})\) (Fig. 4D), whose nearly isotropic characteristics are likely to be due to the detergent present in apo B-100 solutions. These results confirm that upon Cu(II) addition to LDL and apo B-100 the metal ion is reduced with concomitant production of a protein-derived free radical. This radical should be a protein-tryptophanyl radical due to the hyperfine splitting constant of the obtained MNP adduct \((a_N = 15.7 \, \text{G})\), which is in agreement with previously reported values for the MNP-tryptophanyl adduct (39, 40).

**DISCUSSION**

Apo B-100 has been postulated to be essential for copper-catalyzed LDL oxidation by providing the ligand binding sites for the formation of LDL-Cu complexes (14). Herein, we demonstrate that apo B-100 plays an additional triggering role in copper-mediated LDL oxidation. We report direct evidences for apo B-100 mediated copper reduction in a lipid-independent process that involves protein-tryptophanyl radical formation.

**FIG. 3.** Time course of Cu(II) reduction by LDL and apo B-100. Different concentrations of (A) HPLC-purified LDL \((0.01, 0.02, 0.04, \text{ and } 0.08 \, \mu\text{M})\) or (B) isolated apo B-100 \((0.01, 0.02, 0.04, 0.08, \text{ and } 0.2 \, \mu\text{M})\) were incubated in sodium phosphate buffer, 50 mM, pH 7.4, with Cu(II) \((10 \, \mu\text{M})\) at 37°C for 90 min in the presence of 360 \, \mu\text{M} \text{BC}. Cu(I) formation was monitored at 480 nm. Cu(II)/LDL ratios were 125, 250, 500, and 1000; Cu(II)/apo B-100 ratios were 50, 125, 250, 500, and 1000, respectively.

**FIG. 4.** EPR spectra of MNP adducts obtained from oxidation of LDL or apo B-100 with Cu(II). EPR spectra of MNP adducts obtained during 30 min incubations of LDL \((0.95 \, \mu\text{g} \cdot \text{ml}^{-1})\) or isolated apo B-100 \((0.5 \, \text{mg} \cdot \text{ml}^{-1})\) with Cu(II) and 50 mM MNP at room temperature. (A) LDL; (B) organic phase obtained from the extraction of LDL whose protein contents were 0.11 \, \mu\text{g} \cdot \text{ml}^{-1}; (C) aqueous phase obtained from the extraction of LDL whose protein contents were 0.74 \, \mu\text{g} \cdot \text{ml}^{-1} \text{ protein}; (D) Apo B. The incubation mixtures were performed in 150 mM potassium phosphate buffer, pH 7.2. Incubations and extraction were performed as described under Materials and Methods. Instrumental conditions: microwave power, 20 mW; modulation amplitude, 2.7 G except for (B) for which 1 G was used; scan time, 82 ms for (A) and (B) and 328 ms for (C) and (D); gain, \(1 \times 10^6\). Spectra (C) and (D) were the accumulation of four scans.
Different studies demonstrated that isolated LDL is definitively able to reduce copper (16, 19, 21, 22, 41). Low-density lipoprotein-copper complexes have been described (42–44), where: (a) more than 80% of copper primarily binds to apo B-100 (14), (b) the protein-bound copper is able to initiate LDL oxidation (14), and (c) there is a finite number of saturable copper binding sites on the LDL particle (45). In this work, it is shown for the first time that a purified preparation of the protein moiety of LDL is able to reduce copper directly (Fig. 2). Moreover, copper reduction kinetics and extents of Cu(I) formation by apo B-100 are different from that of LDL (Fig. 3). It was previously suggested that the first copper reduction phase in LDL was α-tocopherol-dependent while the second phase was lipid-peroxide dependent (22). While at early time points, the LDL copper reducing activity was higher due to the presence of α-tocopherol, at longer time points kinetics of copper reduction was similar in both LDL and apo B-100 samples (Fig. 3). Our results support the idea that in addition to lipid peroxides, apo B-100 would be an important copper reductant during the second α-tocopherol-independent phase. The lower copper reducing activity of the apo B-100 compared with LDL is in agreement with the presence of other endogenous copper reductants in the intact LDL particle (16–22).

Apo B-100 is one of the largest proteins known, consisting of 4,536 amino acid residues and is the sole protein component of LDL (46–49). It contains 37 tryptophan residues where 8–9 apo B-100 tryptophan residues are accessible to Cu(II) and may be available for one-electron reduction of Cu(II) (4, 34, 50). The changes occurring in apo B-100 during LDL oxidation have been considered to result from the interaction of lipid oxidation products with amino acid residues (51). However, by using an apo B-100 preparation without the lipid components of LDL, we showed a direct oxidation of the protein by copper. In fact, we demonstrate the formation of tryptophan-centered radicals not only during the incubation of LDL with Cu(II), but also by using pure apo B-100 (Figs. 1 and 4).

It should be noted that the MNP-tyrosyl radical adduct (52) has EPR parameters similar to those of MNP-tryptophanyl radical adduct (39, 40) and it would be difficult to discriminate between both adducts only on the basis of EPR spin trapping experiments. However, the EPR data reported here (Fig. 4) taken together with the observation of an early destruction of tryptophan residues of apo B-100 and that kynurenine is produced during copper-mediated LDL oxidation (34, 50, 53), clearly establishes that protein-tryptophanyl radicals are produced during the interaction of Cu(II) with LDL and apo B-100. Although these results point to a direct one-electron reaction between Cu(II) and apo B-100 tryptophan residues, it should be noted that electron transfer within proteins has been described and so, the initial reactive site could not necessarily involve tryptophan. However, EPR analysis showed that tryptophan-radical formation in apo B-100 incubated with Cu(II) has a similar time-course than copper-reduction (data not shown).

Previous detection of tryptophan oxidation products during copper-mediated LDL oxidation suggested that one electron-oxidized tryptophan residues of apo B could ultimately initiate lipid oxidation (53). However, tryptophan-centered radical formation during copper-mediated LDL oxidation was not directly evidenced. The present demonstration of apo B-100 tryptophan-centered radical formation by a direct reaction between the protein and Cu(II) in a lipid oxidation independent process, support the idea that the initiating lipid oxidation species might be Cu(II) as well as tryptophan-centered radicals (37, 40, 53). In addition to provide an alternative mechanism for initiating lipid oxidation, tryptophanyl radicals may also be targets for nitric oxide (NO) during LDL oxidation. Nitric oxide has been reported to have a protective role during LDL oxidation through its diffusion-limited reaction with lipid radicals (i.e., alcoxyl and peroxy radical) leading to the inhibition of lipid oxidation processes (54–57). In addition, NO rapidly reacts with aminoacyl radicals, including tyrosyl and tryptophanyl radicals in amino acids, peptides and proteins with rate constants of (1–2) × 10³ M⁻¹ · s⁻¹ (58, 59). The demonstration of the apo B-100 tryptophan-centered radical formation in both LDL and apo B-100 open the possibility for a novel antioxidant role of NO during LDL oxidation.

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**REFERENCES**


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