KALEN Human OxLDL™

Technical Note 2

Effect of Copper Ion Concentration on the Oxidation of Human LDL

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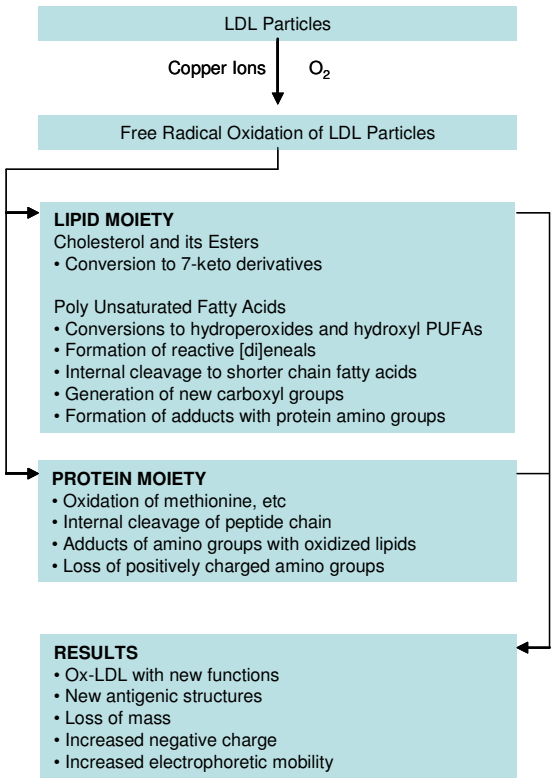
Introduction

Oxidation of Low Density Lipoprotein (LDL) causes changes in both the lipid and the protein moieties of the lipoprotein particle (Figure 1). Some of the polyunsaturated fatty acids are cleaved by oxidation to generate shorter chain and lower molecular weight components. A portion of these oxidized lipids and phospholipids can form adducts with protein (1, 2); the protein moiety can also be broken down by oxidation (3).

Figure 1. Changes during Oxidation of LDL



Lipid peroxidation has commonly been estimated by the measurement of thiobarbituric acid reactive substances (TBARS) (4, 5). These reactive substances are produced by the oxidation and subsequent breakdown of poly-unsaturated fatty acids in the lipid moiety of the LDL particle.

Spectral changes, especially an increase in absorption at 234 nm, have been reported as early events in lipoprotein oxidation. The rate of spectral changes is dose dependent at low concentrations of the copper (II) ion. The rate has been reported to approach a maximum at approximately 5 µM copper ion (6, 7).

In the current investigations, the oxidation reaction mixture was subjected to gel filtration prior to conducting the analyses. Consequently, any small molecules generated by the oxidations were removed during the isolation procedure. These small molecules could be detected by analysis of the oxidation mixture and may be responsible for some spectral changes (unpublished results).

Here we present some of our observations on the oxidation of human LDL with different concentrations of Cu (II) ions. Because all the analyses were conducted on oxidized LDL separated from the reaction mixtures, all of the results reflect the state of the oxidized lipoprotein itself.

We estimated oxidation changes in the lipid moiety by the appearance of TBARS and changes in the protein by loss of free amino groups. The changes in both the lipid and protein components of the oxidized LDL particle were evaluated by gel electrophoresis.

Methods

LDL with a density of 1.019 – 1.063 g/mL was prepared from human plasma by serial isopycnic ultracentrifugation in potassium bromide (8). Prior to oxidation, the concentration of native LDL was adjusted to 1.1 mg/ml in Dulbecco's Phosphate Buffered Saline (DPBS). A 2-mL aliquot of the material was placed into each of seven vials. Material in six of the vials was oxidized for 18 hours, each with a concentration of copper (II) sulfate ranging from 0.1 to 40 µM. Material in the seventh vial was used as the unoxidized control. Reaction mixtures were incubated at 37°C in the presence of oxygen for 18 hours. Each mixture was then subjected to gel filtration chromatography using a Sephadex G25 column and DPBS. Pooled fractions containing the lipoprotein were used for analysis of TBARS, TNBSA, protein concentration, and electrophoretic mobility.

TBARS were measured using the TBARS Assay Kit (Cat No. 10009055, Cayman Chemical Company, Ann Arbor MI). TNBSA (2,4,6-TriNitroBenzene Sulfonic Acid, Cat. 28997, Pierce Rockford IL) was used to measure free amino groups (primarily the epsilon-amino group of lysine) compared to the unoxidized control. TNBSA reacts with primary amines to form a chromogenic product. The protein concentration was determined with the BCA Protein Assay Kit (Cat. 23227, Pierce, Rockford, IL) with bovine serum albumin as the reference.

The electrophoretic mobility of oxidized LDL was determined by agarose gel electrophoresis in sodium barbital buffer (Titan Gel Lipoprotein Kit 3045, Helena Laboratories, Beaumont, TX). Lipoprotein was visualized by staining with Fat Red 7B. The electrophoretic mobility of oxidized LDL relative to native LDL (REM) was

calculated as follows: [the distance that oxidized LDL migrated] divided by [the distance that a native LDL reference migrated].

Results

-The level of TBARS in the control material was undetectable, and the level of TBARS in the sample treated with copper sulfate at 0. 1 µM was only slightly higher than that measured for the control (Figure 2). Increasing the copper concentration to 1 µM resulted in a large increase in the level of TBARS in the sample. Interestingly, oxidation of LDL with copper concentrations in excess of 1.0 µM resulted in reduced levels of TBARS associated with the lipoprotein after 18 hours of oxidation.

Figure 2. LDL-associated TBARS

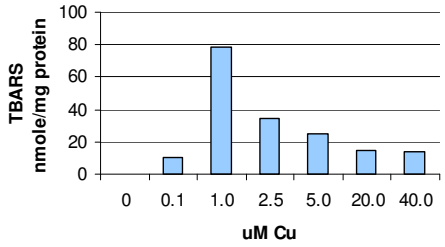


Figure 3. Blocked Amino Groups

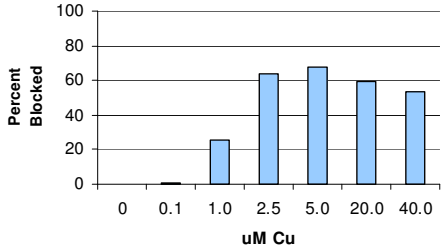
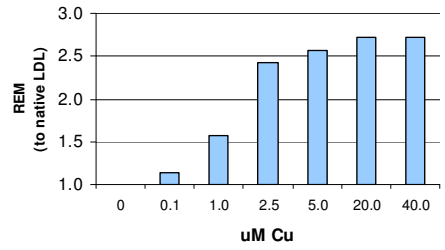


Figure 4. REM



The percentage of blocked amino groups in the protein moiety of oxidized LDL, primarily in apo B100, is presented in Figure 3. At a concentration of 0.1 µM copper sulfate, the percentage of blocked amino groups was less than 1%. Increasing the copper concentration in the reaction mixture from 0.1 to 5.0 µM resulted in an increase in the percentage of blocked amino groups to more than 60%. With higher concentrations of copper sulfate, the percentage of blocked amino groups declined to about 50%.

The effect of increasing copper concentration on the electrophoretic migration of oxidized LDL is depicted in Figure 4. As expected, increasing concentrations of copper sulfate yielded increases in the migration of oxidized LDL. Note that increasing the copper concentration from 20 to 40 µM did not change the migration.

Discussion

Oxidation of lipoproteins with copper sulfate is largely concentration dependent as measured by the electrophoretic mobility and percentage of free amino groups in the resulting lipoprotein after 18 hours. The electrophoretic mobility of oxidized LDL particles increases with increases in copper ion concentration approaching a maximum at a copper ion concentration of 20 uM. Mobility does not increase further at 40 µM. The increase in electrophoretic mobility of the oxidized LDL particle is likely due to a combination of factors. These include a decrease in positive charges as a result in the loss of free amino groups on the protein moiety and an increase in negative charges from oxidation of the poly unsaturated lipids. A reduction in the molecular weight of the particle due to protein and lipid breakdown resulting from the oxidation may also contribute to the increase in electrophoretic mobility.

As the concentration of copper ion increases, the percentage of blocked amino groups also increases up to a concentration of 5 µM. The reduction in the percentage of free amino groups with increasing copper ion concentration was expected and is likely to be largely due to the formation of adducts between protein amino groups and lipid peroxide decomposition products (2). The small decrease in amino group loss at higher copper ion concentrations may not be significant as it may be due to the imprecision of the measurements.

Unexpectedly, the level of TBARS does not increase proportionately with increases in the concentration of copper ion after 18 hours of oxidation. A copper ion concentration of 0.1 µM results in minimal levels of lipoprotein-associated TBARS. However, lipoprotein-associated TBARS values reach a maximum at a 1.0 µM copper ion concentration but decline at higher oxidative conditions. These observations suggest that the reactive substances in the oxidized lipoprotein particle may be transitory. If the production of TBARS at higher copper concentrations occurs more quickly than at lower concentrations (7) and if some of the TBARS are transitory, then the TBARS level at higher concentrations might be expected to be less than at lower concentrations. For this reason, measurement of lipoprotein-associated TBARS may not provide an accurate picture of lipoprotein changes with oxidation. In fact, of the three analyses, the electrophoretic migration pattern

of the oxidized LDL particle may be the best method to evaluate the extent of lipoprotein oxidation. The time course of the appearance and loss of lipoprotein-associated TBARS will be addressed in a subsequent technical note.

Based on the results presented herein and elsewhere, the characteristics of oxidized lipoproteins can vary greatly depending on the conditions used to oxidize the lipoprotein (6,9,10). The observed differences are likely due to a cascade of events in which reactive substances are formed. These in turn mediate the formation of adducts and the breakdown of both the constituent lipids and protein. Given the complexity of these events and the variation in the physicochemical properties of oxidized lipoproteins produced by various methods, the description of oxidized lipoprotein based on metal ion (or other oxidant) concentration, may be inadequate. The adjectives used by various authors to describe the levels of resulting oxidation appear wide ranging and inconsistent. For clarity, we will describe our oxidized lipoproteins as based on their physicochemical properties as follows:

- Minimally Oxidized: No obvious changes in physicochemical measurements are observed although changes in spectral characteristics and lipid content may be evident.
- Mildly Oxidized: Small but definite changes in physicochemical measurements are observed in LDL oxidized under mild conditions. These include a small reduction in the percentage of free amino groups and a small increase in electrophoretic mobility.
- Oxidized: Major changes in physicochemical measurements are observed. In these particles about 50% of the free amino groups are blocked. Major increases in electrophoretic mobility are also observed and the band is compact.
- Extensively Oxidized: Extensively oxidized lipoproteins exhibit extensive changes in physicochemical measurements. Approximately 50% of the free amino groups are blocked in these particles. Additionally, extensive oxidation is accompanied by a multifold increase in electrophoretic mobility, and the band is quite diffuse.

These differences in the physicochemical characteristics of oxidized lipoproteins may correlate with functional changes. For example, minimally oxidized LDL continues to bind to LDL receptors but does not bind to scavenger receptors (9). It also stimulates the spreading of macrophages (11). The conditions for oxidation of lipoproteins and their physicochemical characterization are key elements in the production of consistent, high quality lipoproteins needed for cardiovascular research.

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Ordering Information

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