TECHNICAL NOTE

EFFECT OF EXPOSURE TIME ON THE OXIDATION OF HUMAN LDL WITH COPPER ION

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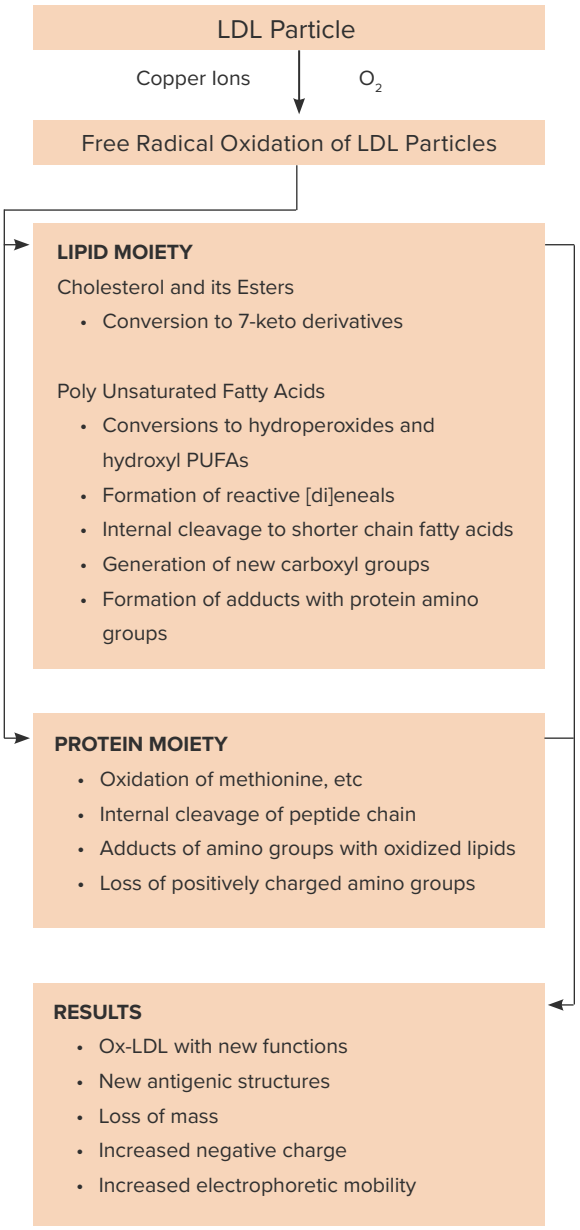
Oxidation of LDL causes changes in both the lipid and the protein moieties of the lipoprotein particle (Figure 1). Polyunsaturated fatty acids are cleaved by oxidation to generate shorter chain and lower molecular weight components. Some of the oxidized lipids and phospholipids may form adducts with protein (1, 2); the protein moiety can also be broken down by oxidation (3). Previously (4) we reported on the physicochemical characteristics of human LDL following 18 hours of oxidation with concentrations of copper sulfate ranging from 0.01 to 40 µM. We estimated oxidation changes in the lipid moiety by the appearance of TBARS (5,6) 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. These results showed notably different levels of TBARS, blocked amino groups, and electrophoretic migration for the isolated Ox-LDL. Of note, the lipoprotein associated TBARS values were small at 0.1 uM Cu, reached a maximum at 1 uM Cu and then decreased at higher Cu concentrations.

Herein we report on the physicochemical changes which occur during oxidation of human LDL at various time intervals with copper ion concentrations of 1 and 20 µM.

Figure 1. Changes during Oxidation of LDL



METHODS

LDL with a density of 1.019 – 1.063 g/mL was prepared from human plasma by serial isopycnic ultracentrifugation in solutions of potassium bromide (7). Prior to oxidation, the concentration of native LDL was adjusted to 1 mg/mL in Dulbecco's Phosphate Buffered Saline (DPBS). A 2-mL aliquot of the material was placed into each of three vials. One aliquot was used as the un-oxidized control while lipoproteins in the other two were oxidized with copper (II) sulfate at a concentration of either 1 or 20 µM. Reaction mixtures were incubated at 37°C in the presence of oxygen for 48 hours. At various time intervals, samples from each reaction mixture were removed and stored at -20°C. Samples were then thawed and analyzed for (a) TBARS which represents the oxidized lipid components, (b) TNBSA which measures remaining free amino groups and (c) electrophoretic mobility.

The protein concentration was determined with the BCA Protein Assay Kit (Cat. 23227, Pierce, Rockford, IL) with bovine serum albumin as the reference. 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 of apo B100). The blockage of amino groups was calculated by comparing the free amino groups remaining in the oxidized product to those of the un-oxidized control.

The electrophoretic migration 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 migration (REM) of oxidized LDL relative to native LDL was calculated as the distance that oxidized LDL migrated divided by that of a native LDL reference.

RESULTS

The results presented herein were obtained using a single preparation of KALEN Human LDL that was oxidized with copper sulfate at 1 or 20 µM for up to 48 hours. Results of analyses using TBARS, TBNSA, and electrophoretic mobility are presented below.

Effect of time and copper ion concentration on TBARS. The results of TBARS analyses, which measure all of the oxidized lipids present in the reaction mixture, are shown in Figures 2a and 2b. The oxidized lipids present in the reaction mixtures are those associated with the lipoprotein as well as those that have been cleaved from the lipoprotein, *i.e.*, free in solution.

Figure 2a. Total TBARS at 1 µM Cu

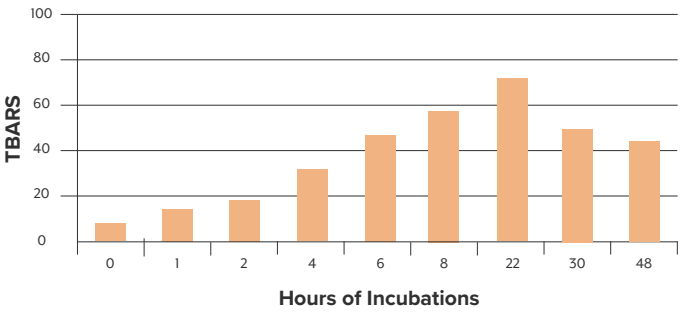
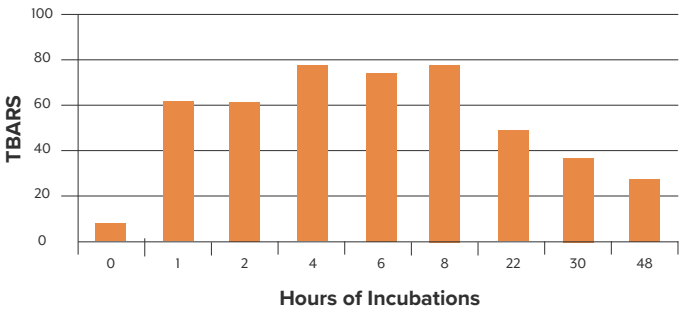


Figure 2b. Total TBARS at 20 µM Cu



With 1 µM copper ion, TBARS increased in a curvilinear manner during the first hours of exposure and reached a peak by 22 hours. Subsequently, the TBARS level declined. After 48 hours of exposure, the level was about 60% of the peak level. At a concentration of 20 µM copper ion, TBARS levels also increased to a peak and then declined. However, the increase in TBARS level at 20 µM was more rapid than at 1 µM and the value declined to a lower level, about 40% of the peak value.

Effect of time and copper ion concentration on blocked amino groups. The results of the determination of the percent of blocked amino groups in the protein moiety of Ox-LDL are presented in Figures 3a and 3b.

Figure 3a. Blocked Amino Groups at 1 µM Cu

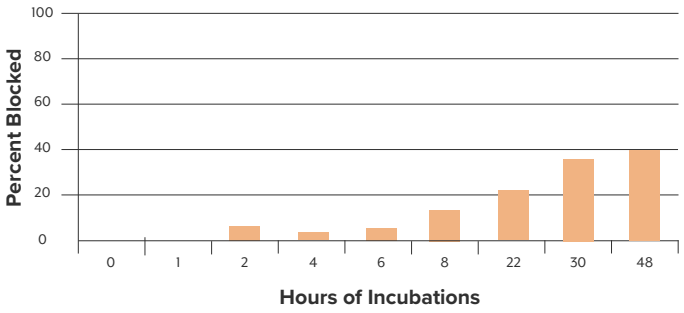
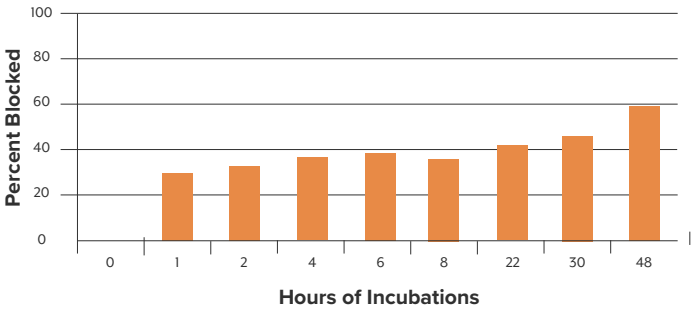


Figure 3b. Blocked Amino Groups at 20 µM Cu



During the first eight hours of exposure to 1 µM copper ion, the percent of blocked amino groups increased by about 10% and continued to increase during the ensuing 40 hours of exposure reaching a maximum of about 40%. With exposure to 20 uM, the increase in blocked amino groups was more rapid than with the lower concentration of copper ion. Within the first hour, the level increased to about 30% and eventually reached about 60% at 48 hours.

Effect of time and copper ion concentration on electrophoretic mobility. The increases in electrophoretic migrationof the Ox-LDL with copper ion at either 1 or 20 µM levels are presented in Figures 4a and 4b.

Figure 4a. REM at 1 µM Cu

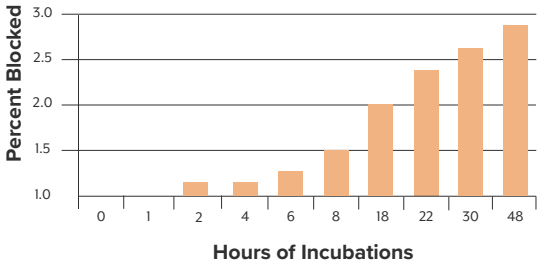
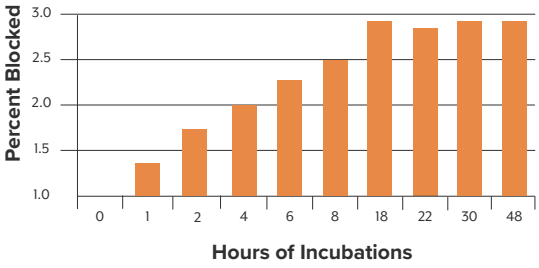


Figure 4b. REM at 20 µM Cu



As expected from preliminary experiments and production runs, the REMs of the two samples were quite different at 18 hours, the time point previously used for comparisons (7). With 1 µM copper ion, the REM increased slowly over 48 hours reaching 2.9. For the 20 µM sample, the REM increased rapidly over the first 8 hours. The REM reached a maximum of 2.9 at 18 hrs; no changes were observed during the remaining 30 hours.

DISCUSSION

The time course of the results presented herein are summarized in the Table 1. Both concentrations of copper ion produced nearly the same peak levels of TBARS. As expected, the rate of increase in TBARS values was much slower at 1 µM than at 20 µM. The most interesting observation is that the TBARS values reach a peak and then decline significantly with further exposure time (Figure 2). Much of the TBARS materials in the reaction mixtures can be fractionated from the OxLDL by gel filtration.

Table I. Reaction Time (hours) to Reach ½ Maximum and Maximum values

Copper Ion Concentration	TBARS Value at:		Blocked Amino Groups at:		REM at:	
	½ Maximum	Maximum	½ Max	Maximum	½ Max	Maximum
1 µM Cu	5	20	22	30-48	18	48
20 µM Cu	< 1	About 8	< 1	30-48	4	18

Unlike the transitory values observed using TBARS as a measure of lipoprotein oxidation, measurements based on the percent of blocked amino groups and the increase in electrophoretic mobility reached their maximums during exposure to copper ion and did not decline significantly.

As we reported previously (4), blockage of free amino groups increases the negative charge on the lipoprotein particle and increases its electrophoretic migration. This is due, in large part, to the formation of adducts between protein amino groups and lipid peroxide decomposition reactive molecules (2).

The changes observed in electrophoretic migration (REM) at both copper ion concentrations were slower than, and did not parallel, the results of TBARS or the percent of amino group. The increase in migration is likely due to a combination of factors. These include the decrease of positive charges (from the loss of free amino groups) on the protein moiety and the addition of negative charges from oxidation of the poly unsaturated lipids. In addition, the loss of mass from the breakdown of some proteins and lipids caused by the oxidation would reduce the molecular weight of oxidized LDL. All of these factors taken together should account for the observed increase in the electrophoretic migration of oxidized LDL.

As we shall discuss in the next Kalen Technical Note, this increase in electrophoretic migration can continue over days or weeks after isolation of the oxidized LDL and removal of the Cu catalyst. This observation may help to account for the instability of oxidized LDL preparations available from some suppliers.

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