Immunological and functional properties of in vitro oxidized low density lipoprotein

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Abstract  We studied the effect of in vitro moderateoxidization on low density lipoprotein (LDL) conformation and metabolism. LDL was modified with either copper ions or phospholipase A2 plus lipooxygenase and, in both cases, mild oxidative conditions were used. The resulting conformational changes were investigated by studying immunological and biological properties of oxidized LDL. The immunoreactivity of apolipoprotein (apo) B-100 was examined using seven monoclonal antibodies. The biological implications of conformational changes were provided by cell-lipoprotein interaction studies using human fibroblasts and mouse peritoneal macrophages. Enzymatically treated LDL presented a relatively less oxidative degree of modification because it generated lower levels of TBARS, and displayed a lower electonegativity and more comparable cellular interactions with those of native LDL. Nevertheless, dramatic immunological changes were measured on both forms of LDL, i.e., a significant increase in the immunoreactivity of an epitope located in the B/E receptor binding domain, but also at epitopes far from this site and located in the N-terminal part of the apoB-100 molecule. The immunoreactivity of the C-terminal region was, in contrast, decreased. Yet, as compared with enzymatically oxidized LDL, much more dramatic structural changes with chemically modified LDL were observed, resulting in such a particular conformation of lipoprotein that its interaction with the macrophagical scavenger receptor was favored, but its recognition by the B/E receptor of fibroblasts was abolished. In contrast, despite a lower interaction between enzymatically modified LDL and the B/E receptor, the metabolism of this lipoprotein was quite comparable with that of native LDL and its degradation with cultured macrophages was poor. The use of in vitro models is common for study of the relationship between oxidized LDL and atherosclerosis in humans. The choice of the more appropriate way to modify lipoproteins is of interest and is discussed.—Harduin, P., A. Tailleux, S. Lestavel, V. Clavey, J-C. Fruchart, and C. Fievet. Immunological and functional properties of in vitro oxidized low density lipoproteins. J. Lipid Res. 1995. 36: 919-950.

Supplementary key words copper ions • phospholipase A2 • lipooxygenase • apoB-100 epitopes • apoB/E receptor • macrophagical scavenger receptor

Several studies suggest that the oxidative modification of low density lipoprotein (LDL) may play an important role in the initiation and progression of atherosclerotic lesions (1, 2). This relationship may be explained through physico-chemical changes that accompany the oxidation of LDL, as well as a number of biological properties of oxidized lipoproteins and alterations in gene expression of arterial cells (2, 3).

The LDL particle consists of a hydrophobic core of cholesterol esters and triglycerides, with a surface composed primarily of amphiphilic phospholipids, cholesterol, and apolipoprotein (apo) B-100, which is the ligand for cell surface receptor-mediated uptake. As a result of the changes occurring on LDL during the oxidative process, oxidized LDL differ greatly from native counterparts, and when oxidized to a sufficient extent, they preferentially recognize receptors which, unlike the B/E receptor, are not subject to down-regulation (4). This functional alteration of LDL can be related not only to specific changes induced by the oxidative process, such as the presence of additional reactive peroxidative products and the derivatization of apoB-100 (5, 6), but also to structural rearrangements of the lipoprotein due to changes in lipid composition and size that may also occur (1). The conformation of the apoB-100 molecule on the surface of LDL is a determinant factor for an effective interaction with its receptor, and alterations of the accessibility of apoB-100 might, therefore, induce perturbed cellular recognition. Because epitopes of apoB-100 are expressed as a function of LDL conformation, immunological approaches are a good means of studying structural changes on LDL (7).
Although Steinbrecher et al. (8) reported that immunoreactivity of LDL apoB-100 did not change upon oxidation when assessed with polyclonal antiserum, the expression of some well-defined apoB-100 epitopes has been reported to be altered on the surface of oxidized LDL (9-12). Nevertheless, the most convincing result that the oxidative process can indeed induce immunochemical changes is the characterization of specific markers generated as a result of LDL oxidation (13-15).

There is some evidence for the presence of oxidized LDL in vivo. Its isolation is still difficult and often the lipoproteins present only moderate physical, chemical, or functional changes indicative of oxidation (16-19).

Much of the information accumulated on the oxidative process is, therefore, based on the use of models of in vitro oxidized LDL. These models can be generated by the incubation of LDL with oxygen-containing buffer, copper or iron ions, endothelial cells, smooth muscle cells, macrophages, enzymes, or by aging (2). Copper-catalyzed oxidation is a commonly used experimental procedure though it does not occur physiologically. However, the oxidative modification induced by two specific enzymes (lipoxygenase plus phospholipase A2) that endothelial cells do contain, better imitates the more biological cell-mediated oxidative process (20). The purpose of this study is to examine the structural changes occurring on copper-oxidized and enzymatically modified LDL, both prepared in mild modificate conditions, and the induced perturbations of their metabolism. Structural changes were studied through the immunological properties of oxidized LDL preparations and the use of anti-apoB-100 monoclonal antibodies (MAbs) whose epitopes have been previously characterized. To assess the physiological relevance of these oxidized LDLs, we have analyzed their interaction with the B/E receptor of fibroblasts and their metabolism through the macrophagic scavenger receptor pathway.

**MATERIALS AND METHODS**

**LDL isolation**

Whole blood obtained by venipuncture from five healthy volunteers after 12 h of fasting was collected into Vacutainer tubes containing preservatives as follows: D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) (1 μM), ethylenediamine tetraacetic acid (EDTA) (3.2 mM), sodium azide (NaN3) (2% wt/vol), gentamycin (0.08%), aprotinin (10,000 U/l), and sodium chloride (NaCl) (0.15 M). Blood cells were sedimented by low speed centrifugation for 20 min at 4°C. Phenylmethanesulfonyl fluoride (PMSF) (1 mM) and benzamidine (1 μM) were then added to the plasma that was stored at 4°C and processed for LDL separation within 1 day. LDL (1.025<d<1.050 g/ml) were isolated by sequential ultracentrifugation in KBr solutions containing EDTA (2.7 mM). This density range was chosen because there is no contamination by very low density lipoprotein (VLDL) and lipoprotein [a]. Isolated LDL were extensively dialyzed at 4°C in the dark against oxygen-free and EDTA-free 10 mM phosphate-buffered saline (PBS), pH 7.2. From a pool of fresh normolipemic plasma, LDL was likewise ultracentrifugally purified and then dialyzed at 4°C in the dark against oxygen-free PBS that contained 300 μM EDTA. All LDL preparations were sterilized by filtration through a 0.22-μm filter and stored at 4°C in sterile aliquots under nitrogen gas in the dark.

**Lipoprotein iodination**

Some aliquots from the LDL standard and from native and modified LDL were radioiodinated with Na125I according to a modification of McFarlane’s procedure (21). 125I-labeled LDL preparations used in immunoassays and cellular studies were kept in tightly capped vials in the dark under nitrogen at 4°C for no longer than 2 weeks. We needed to use five different preparations of LDL standard to carry out the study. The mean specific activity of 125I-labeled LDL was 500 cpm/ng protein and, in each preparation, more than 90% of the radioactivity was precipitated by 10% trichloroacetic acid.

**Lipoprotein modification**

LDL (100 μg/ml) was oxidized by incubation with 1.66 μM CuCl2 in EDTA-free PBS at 25°C for 8 h as previously described (22). Enzyme-mediated lipoprotein modification was performed according to Sparrow, Parthasarathy, and Steinberg (20), except that the incubation was reduced from 20 h to 100 min. Briefly, LDL (100 μg/ml) was incubated in 50 mM borate, pH 9.0, containing NaCl (0.077 M), CaCl2 (1 mM), EDTA (0.1 mM) in the presence of phospholipase A2 (20 ng/ml) (EC 3.1.1.4, Ref P 9279, Sigma Chemical Co., St. Louis, MO) and lipoxygenase (4 μg/ml) (EC 1.13.11.12, type V, Ref L-6632, Sigma Chemical Co., St. Louis, MO) for 100 min at 37°C.

Oxidative processes were stopped by the addition of EDTA (1 mM) and the modified LDL were purified by gel permeation chromatography on a Sephacryl S-300 HR column. The column (2.5 cm x 70 cm) was equilibrated at 4°C with 10 mM PBS (pH 7.2) containing EDTA (300 μM) and run at a constant flow rate (12 ml/h). After concentration by ultrafiltration in a 43-mm diameter Amicon cell PM 10 (Diafloor membrane, Amicon Division, Beverly, MA), the lipoproteins were filtered and kept as previously described for freshly isolated LDL. LDL modified by copper ions or enzymes are hereafter referred to respectively as Cu-LDL or E-LDL. In both cases, negative controls were performed, omitting oxidative agents in the incubation medium; these are noted as Cu-LDL control and E-LDL control. The term N-LDL refers...
to native LDL that was not incubated. For some experiments, LDL was acetylated (Ac-LDL) by sequential additions of acetic anhydride (23).

Physical and chemical lipoprotein analysis

Native and modified LDL lipoprotein sizes were determined by electrophoresis under nonreducing conditions on a 2-10% polyacrylamide gradient gel using an SE 250 Hoefer Mighty II electrophoresis apparatus (150 V, 45 min, 15°C). Protein bands were stained with Coomassie Blue R-250. Increases in electrophoretic mobility of modified lipoproteins were measured on Cellogel strips from Sebia (Issy les Moulineaux, France) following the manufacturer's directions for electrophoretic conditions, gel staining, and destaining. Results were expressed as $R_f$ relative to native LDL.

LDL protein content was determined according to Lowry et al. (24) with bovine serum albumin (BSA) used as a standard. Total cholesterol (TC), triglycerides (TG), and phospholipids (PL) were automatically measured by enzymatic test kits from Boehringer-Mannheim (Mannheim, Germany). Free cholesterol (FC) was determined manually with a modified Boehringer-Mannheim kit in which cholesteryl ester hydrolase was omitted. Cholesteryl esters (CE) were calculated as 1.68 × (TC – FC).

Lipids were extracted with chloroform–methanol 2:1 (vol/vol), and phospholipids were further isolated by high performance thin-layer chromatography (Silica Gel H, Merck, Germany) in a migrating solvent as previously described (25). Phospholipid subfractions were visualized by iodine vapors. Lecithin, lyssolecithin, and sphingomyelin bands were scraped and assayed for phosphorus content (26).

Thiobarbituric acid-reactive substances (TBARS) were quantified as previously described (27) and expressed as the malondialdehyde equivalent content in nanomoles per milligram of protein by using freshly diluted tetramethoxypropane standard. For all chemical analyses, a statistical comparison between respective control or modified and native LDLS was performed using the Mann-Whitney test (U-test).

To study the breakdown of the apoB-100 moiety, LDL were submitted to sodium dodecylsulfate 4–15% polyacrylamide gel electrophoresis (SDS-PAGE) and the separated proteins were then transferred to nitrocellulose paper. The replicas were used for immunochemical detection of apoB with rabbit polyclonal antibodies. The protocol for electrophoresis, transfer, and immunological revelation has been detailed previously (18).

Immunological analysis

The immunoreactivity of apoB epitopes was determined in each LDL preparation by a radioimmunoassay using seven specific anti-apoB MAbS produced in rats or mice by immunization with intact LDL (28, 29). The localization of the specific epitopes along the apoB molecule has been mapped between residues 405 and 559 (MAb B1), 1854 and 1879 (MAb B4), at residues 2331 (MAb BL7), 3506 (MAb BAL1), and 4355 (MAb BL3) (30). The epitope recognized by MAb DA7 is not clearly assigned as two different fusion proteins can react with the antibody. Nevertheless, it seems to be located close to the N-terminal part of the B/E receptor binding domain (R. W. Milne and Y. L. Marcel, unpublished results). The antibody MAb BL5 failed to react with any fragments, suggesting that it only recognizes conformationally expressed epitopes.

The radioimmunoassay was based on a competition between a fixed amount of $^{125}$I-labeled LDL and serial triplicate increasing concentrations of the competitors (LDL standard, control, and modified LDL) to be tested, for each MAb immobilized on polystyrene wells, as described earlier (18).

B/Bo ratios (where B and Bo are specific cpm bound in the presence and absence of competitors, respectively) versus lipoprotein concentrations were plotted. The displacement curves were linearized by logit-log transformation of the data and the slopes were calculated. These correspond to the apparent affinity of the LDL preparations for each antibody. These slopes were compared using a test for heterogeneity based on the general linear model procedure (31). The apparent apoB content (number of epitopes) was calculated from competitive displacement curves. The results were expressed as a percentage of the standard to which an arbitrary expression of 100% was assigned to each epitope. Therefore, a percentage value below 100% indicates that less competitor is needed to achieve the same degree of displacement of the labeled LDL standard and reflects a better accessibility of the measured epitope. In contrast, a percentage value above 100% indicates a lower immunological accessibility of an epitope for its MAb. A statistical comparison of apoB contents among the different LDL preparations was performed using the Mann-Whitney test (U-test).

Cell interaction studies

Binding of LDL on fibroblasts. Human skin fibroblasts from a normocholesterolemic donor were routinely grown in Dulbecco's modified Eagle's medium (DMEM) containing penicillin (100 units/ml), streptomycin (100 µg/ml), and 10% (vol/vol) fetal calf serum (FCS). At day 0, cells were seeded into dishes (30 mm diameter) containing 2 ml of the culture medium. At day 5, the cells were preincubated for 48 h at 37°C in a culture medium containing 10% (vol/vol) of lipoprotein-deficient calf serum (LPDS; prepared from d > 1.25 g/ml infranatant of serum) (DMEM/LPDS) in order to enhance the B/E receptor expression. The medium was then removed and 700 µl fresh DMEM/LPDS containing increasing concentrations (0, 2.5, 5, 10, 25 µg/ml) of $^{125}$I-labeled lipoproteins (N-LDL,

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Cu-LDL, E-LDL) was added to each well. After incubation for 2 h at 4°C, the wells were washed twice with Tris (20 mM), NaCl (0.15 M), BSA (0.2%, wt/vol), and twice with Tris (20 mM), NaCl (0.15 M) pH 7.4. The cells were extracted with 1 ml NaOH (0.1 N) and transferred to tubes. The radioactivity associated with the cells reflected the amount of lipoprotein bound to the cell surface. Aliquots of 100 µl from the cell extraction were used to measure cellular protein (24). Nonspecific binding was determined by adding to the binding assay system a 20-fold excess of unlabeled native LDL at each concentration of 125I-labeled lipoproteins, and subtracting from the total binding to obtain the specific binding by the B/E receptor pathway. Each stage was carried out in triplicate.

Uptake and degradation of LDL by fibroblasts. Cells were grown and treated as for the binding study. After 48 h incubation in a culture medium containing LPDS, the medium was removed and 700 µl of fresh DMEM/LPDS containing increasing concentrations (0, 2.5, 5, 10, 25 µg/ml) of 125I-labeled LDL (native or modified) was added to each well. After incubation for 4 h at 37°C, 0.5-ml aliquots of each incubation medium were treated with Tris (20 mM), NaCl (0.15 M), pH 7.4. The cells were washed as for the binding study. Lipoproteins bound to the cell surface were detached by treatment with 0.5 ml of trypsin at 0.05% (wt/vol) in PBS for 5 min at 37°C. Trypsinized cells were transferred to tubes and centrifuged for 3 min at 4°C. Radioactivity associated with the cells was measured by treating 0.5 ml of each incubation medium with 175 µl trichloracetic acid (50%, wt/vol) to remove precipitable lipoproteins. Free iodine was eliminated by treatment of the acid-soluble material with potassium iodide (40%, wt/vol) and H₂O₂ (30%, vol/vol) followed by extraction with chloroform. The aqueous phase was counted and corresponded to the lipoproteins degraded during the 4-h incubation and released by the cells to the medium. Nonspecific metabolism was determined by adding to the binding assay system a 20-fold excess of unlabeled native LDL at each concentration of 125I-labeled lipoproteins, and subtracting from the total degradation to obtain the specific metabolism by the scavenger receptor pathway. Each stage was carried out in triplicate.

Degradation of LDL by the macrophages. Mouse peritoneal macrophages were prepared as described by Goldstein et al. (32). The degradation of native and modified LDL by cells was measured at 37°C by incubating the cells with 25 µg/ml of the corresponding 125I-labeled lipoproteins. After a 5-h incubation, 0.5 ml of the culture medium was removed and treated as described for degradation of lipoproteins by fibroblasts. The nonspecific metabolism was determined by adding a 20-fold excess of unlabeled acetylated-LDL to the 125I-labeled lipoproteins and subtracting from the total degradation to obtain the specific metabolism by the scavenger receptor pathway. Each stage was carried out in triplicate.

RESULTS

Compared to native LDL, both forms of oxidized LDL demonstrated a decreased size with polyacrylamide gradient gel electrophoresis (Fig. 1). The electrophoretic pattern also revealed the presence of high molecular weight aggregates of LDL in copper ion-treated lipoproteins.

As shown in Fig. 2, the SDS-PAGE followed by the immunoblotting technique confirmed that both oxidative treatments induced a breakdown of the apoB-100 moiety. Nevertheless, fewer lower molecular weight fragments were produced by enzymes than by copper ions and some native apoB-100 material remained in E-LDL as compared with totally degraded protein from Cu-LDL. This suggests a more extensive degradation of apoB-100 in Cu-LDL in comparison with E-LDL. It is interesting that we did not observe apoB aggregates from Cu-LDL under denaturing conditions.

Fig. 1. Coomassie blue staining of PAGE (2-10%) under nondenaturing conditions; lane 1, native LDL; lane 2, control for copper ion treated-LDL; lane 3, copper ion treated-LDL; lane 4, control for enzymatically treated-LDL; and lane 5, enzymatically treated-LDL. Each lane was loaded with 20 µg protein. Oxidative treatment of LDL with copper ions or enzymes (phospholipase A₂ plus lipoxygenase) was performed as described in Materials and Methods.
The modification of LDL by copper ions or enzymes caused an increased mobility on cellulose acetate gel electrophoresis as compared to native LDL (Fig. 3). Cu-LDL showed a higher increase in its net negative charge than E-LDL (1.45 ± 0.15 vs. 1.25 ± 0.09, respectively, \( P < 0.05 \)). On Figs. 1, 2, and 3, no gross differences were detected between native and control LDL, ensuring that the modification procedures we performed did not produce artifactual modifications of LDL.

The chemical composition of the LDL preparations is reported in Table 1. Native and control LDL had virtually the same composition. Compared to native LDL, incubation with copper ions increased the lipoprotein content of protein by 9.7%, and of phospholipids by 14.1%, whereas it decreased the content of cholesteryl esters by 20.8%. Incubation with enzymes increased the LDL content of free cholesterol by 22.2% and of triglycerides by 47.8%, but decreased the content of protein by 12.1% and of phospholipids by 10.6%.

As expected, oxidation of LDL in the presence of enzymes and, to a lesser extent, of copper ions resulted in substantial changes in phospholipid composition (Table 2). Lecithin content decreased by 30.5% and 74.5% whereas lysolecithin increased by 129.4% and 641.2% and sphingomyelin by 40.6% and 19.6% in LDL incubated with copper ions and enzymes relative to native LDL, respectively.

Both forms of modified LDL showed a significant increase in TBARS content (\( P < 0.001 \)) as compared to native or control LDL content (Table 3), consistent with lipid peroxidation occurring in oxidative procedures. It

### Table 1. Chemical composition of copper ion- or phospholipase A2 plus lipoxygenase-oxidized LDL

<table>
<thead>
<tr>
<th></th>
<th>N-LDL</th>
<th>Cu-LDL Control</th>
<th>Cu-LDL</th>
<th>E-LDL Control</th>
<th>E-LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins</td>
<td>24.8 ± 1.2</td>
<td>25.0 ± 0.7</td>
<td>27.2 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.2 ± 0.5</td>
<td>21.8 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Free cholesterol</td>
<td>9.9 ± 0.5</td>
<td>9.2 ± 0.7</td>
<td>10.3 ± 0.5</td>
<td>10.1 ± 0.3</td>
<td>12.1 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cholesteryl esters</td>
<td>40.8 ± 1.1</td>
<td>40.4 ± 0.5</td>
<td>32.3 ± 3.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41.1 ± 0.8</td>
<td>41.3 ± 1.4</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>4.6 ± 0.5</td>
<td>4.5 ± 0.5</td>
<td>7.5 ± 2.9</td>
<td>4.2 ± 0.9</td>
<td>6.8 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>19.9 ± 0.6</td>
<td>20.9 ± 0.8</td>
<td>22.7 ± 1.7</td>
<td>20.4 ± 0.4</td>
<td>17.8 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Results are expressed as a mass percentage of LDL. Values are mean ± standard deviation. N-LDL, native LDL; Cu-LDL, copper ion-oxidized LDL; E-LDL, phospholipase A2 plus lipoxygenase-oxidized LDL.  
<sup>a</sup>\( P < 0.05 \), <sup>b</sup>\( P < 0.01 \), as compared to N-LDL (U-test).
must be pointed out that this chemical change appeared to be more important with Cu-LDL than with E-LDL.

The abilities of the different preparations to compete with ^125I-labeled standard LDL for binding to the anti-apoB MAbs were compared (Table 4). We observed that the slopes of the displacement curves of the antibodies presented low standard deviation values and were identical within each competitor, thus demonstrating a good immunochemical homogeneity and a comparable apparent affinity in all these preparations. We could, therefore, determine the apparent apoB content of each epitope in LDL preparations, reflecting their accessibility (Table 4). Control LDL did not differ from native LDL in its immunoreactivity for any specific epitopes recognized by the MAbs, and the number of each epitope expressed was about 100%. However, some altered reactivities were obtained for modified LDL. Copper ion treatment induced a marked decrease, to the point of virtual destruction, in the accessibility of epitopes recognized by MAbs BL3, BL5, and BL7, and, to a lesser extent, of epitopes for MAbs B4 and DA7. Under the same experimental conditions, the accessibility of epitopes for MAbs B1 and BAII was enhanced. Less drastic immunological changes were obtained with phospholipase A2 plus lipoxygenase-treated LDL. Nevertheless, a significant decrease in the accessibility of epitopes recognized by MAbs BL3, BL5, and DA7 was measured, and a similarly enhanced expression like that for Cu-LDL was obtained for epitopes for MAbs B1 and BAII. No changes were observed for the accessibility of epitopes for MAbs B4 and BL7.

The binding of native and oxidized LDL on the B/E receptor of fibroblasts at 4°C was studied by incubating the cells with increasing amounts of each ^125I-labeled lipoprotein. Specific binding is shown in Fig. 4. Results are expressed in nanograms of labeled LDL bound per milligram of cellular protein and represent the mean ± standard deviation of assays performed in triplicate. The nonspecific binding of N-LDL, E-LDL, and Cu-LDL represented, respectively, 12, 24, and 27% of the total binding. E-LDL bound less efficiently than N-LDL, and Cu-LDL hardly bound to fibroblasts. Indeed, the specific binding of E-LDL and Cu-LDL represented, respectively, about 68 and 30% of values obtained with N-LDL.

The binding, internalization, and degradation of oxidized LDL through the B/E receptor pathway were studied by incubating each labeled lipoprotein with human skin fibroblasts at 37°C for 4 h (Fig. 5). Results are expressed in nanograms of lipoproteins bound, internalized, or degraded, respectively, per nanogram of cellular proteins, and correspond to the mean ± standard deviation of triplicate assays. The nonspecific uptake and degradation were 16, 32, and 29% for N-LDL, Cu-LDL, and E-LDL, respectively. Enzymatically modified LDL was bound and internalized by the B/E receptor pathway at the same rate as native LDL, but its degradation appeared lower than that obtained with N-LDL. On the other hand, Cu-LDL barely bound to the fibroblasts and its metabolism through the B/E receptor pathway was reduced by 50% compared with N-LDL.

The degradation of modified LDL through the macrophagic scavenger pathway was studied by incubating the different ^125I-labeled lipoproteins at 37°C for 5 h with the macrophages, in the presence or absence of a 20-fold excess of unlabeled acetylated LDL. Results are shown in Fig. 6 and represent the specific degradation of lipoproteins. The nonspecific degradation values were 81,

**TABLE 2.** Phospholipid composition of copper ion- or phospholipase A2 plus lipoxygenase-oxidized LDL.

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>N-LDL</th>
<th>Cu-LDL Control</th>
<th>Cu-LDL</th>
<th>E-LDL Control</th>
<th>E-LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyssolecithin</td>
<td>6.8 ± 4.5</td>
<td>6.9 ± 3.2</td>
<td>13.6 ± 2.9</td>
<td>6.2 ± 4.6</td>
<td>50.4 ± 3.5</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>27.6 ± 2.9</td>
<td>26.7 ± 2.4</td>
<td>38.8 ± 2.2</td>
<td>26.6 ± 3.5</td>
<td>33.0 ± 3.3</td>
</tr>
<tr>
<td>Lecithin</td>
<td>65.6 ± 7.1</td>
<td>66.4 ± 4.3</td>
<td>45.6 ± 4.8</td>
<td>67.2 ± 3.9</td>
<td>16.6 ± 3.1</td>
</tr>
</tbody>
</table>

Results are expressed as percentage of total phospholipids. Values are mean ± standard deviation. N-LDL, native LDL; Cu-LDL, copper ion-oxidized LDL; E-LDL, phospholipase A2 plus lipoxygenase-oxidized LDL.

**TABLE 3.** Generation of TBARS by copper ion- or phospholipase A2 plus lipoxygenase-mediated oxidation of LDL.

<table>
<thead>
<tr>
<th>TBARS (nmol MDA/mg of LDL)</th>
<th>N-LDL</th>
<th>Cu-LDL Control</th>
<th>Cu-LDL</th>
<th>E-LDL Control</th>
<th>E-LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8 ± 0.8</td>
<td>2.4 ± 1.3</td>
<td>22.1 ± 2.0&quot;</td>
<td>1.3 ± 1.1</td>
<td>6.0 ± 0.7&quot;</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation. N-LDL, native LDL; Cu-LDL, copper ion-oxidized LDL; E-LDL, phospholipase A2 plus lipoxygenase-oxidized LDL.

*P < 0.001 as compared to N-LDL (U-test)
56, 9, and 15% for N-LDL, E-LDL, Cu-LDL, and Ac-LDL, respectively. These low percentages for Cu-LDL and Ac-LDL were due to the exclusive interaction of these lipoproteins with the scavenger receptor, while high percentages for N-LDL and E-LDL indicated their preferential interaction with the few B/E receptors expressed at the cell surface despite a down-regulation. N-LDL, as a negative control, was not degraded by the macrophagic scavenger receptor. On the other hand, acetylated-LDL, as a positive control, was highly degraded (12.7 ± 0.66 μg of protein degraded/mg of cellular protein), while an excess of unlabeled acetylated-LDL inhibited this degradation and native LDL did not (data not shown). Cu-LDL was highly catabolized by the macrophages (4.21 ± 0.40 μg of protein degraded/mg of cellular protein), as compared with E-LDL (0.34 ± 0.07 μg of protein degraded/mg of cellular protein) which in turn were only degraded a fraction more than N-LDL (0.02 ± 0.04 μg of protein degraded/mg of cellular protein).

### DISCUSSION

In this study, we have observed the conformational changes occurring in LDL after in vitro copper- or phospholipase A₂ plus lipoxygenase-mediated oxidation. The structural alterations have been studied through the immunological accessibility of apoB-100 epitopes and the interaction of lipoproteins with cellular receptors.

The immunoreactivity of oxidized LDL has previously been studied, but a conclusive result is difficult to find from the available data. Until now, immunoreactivity of oxidized LDL was often studied after drastic oxidation

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**TABLE 4. Immunological accessibility of copper ion- or phospholipase A₂ plus lipoxygenase-oxidized LDL**

<table>
<thead>
<tr>
<th>Monoclonal Antibodies</th>
<th>N-LDL</th>
<th>Cu-LDL</th>
<th>E-LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>Apparent apoB content (%)</td>
<td>101 ± 3</td>
<td>45 ± 5*</td>
</tr>
<tr>
<td>Slope</td>
<td>1.90 ± 0.07</td>
<td>1.86 ± 0.05</td>
<td>1.68 ± 0.05</td>
</tr>
<tr>
<td>B4</td>
<td>Apparent apoB content (%)</td>
<td>97 ± 6</td>
<td>148 ± 11*</td>
</tr>
<tr>
<td>Slope</td>
<td>4.35 ± 0.13</td>
<td>4.42 ± 0.17</td>
<td>4.17 ± 0.19</td>
</tr>
<tr>
<td>BL7</td>
<td>Apparent apoB content (%)</td>
<td>99 ± 1</td>
<td>570 ± 199*</td>
</tr>
<tr>
<td>Slope</td>
<td>1.85 ± 0.10</td>
<td>1.60 ± 0.10</td>
<td>1.85 ± 0.05</td>
</tr>
<tr>
<td>BA11</td>
<td>Apparent apoB content (%)</td>
<td>100 ± 0</td>
<td>78 ± 7*</td>
</tr>
<tr>
<td>Slope</td>
<td>1.87 ± 0.06</td>
<td>1.89 ± 0.04</td>
<td>1.89 ± 0.07</td>
</tr>
<tr>
<td>BL3</td>
<td>Apparent apoB content (%)</td>
<td>101 ± 2</td>
<td>479 ± 123*</td>
</tr>
<tr>
<td>Slope</td>
<td>2.30 ± 0.16</td>
<td>1.73 ± 0.07</td>
<td>2.30 ± 0.08</td>
</tr>
<tr>
<td>DA7</td>
<td>Apparent apoB content (%)</td>
<td>103 ± 1</td>
<td>121 ± 6*</td>
</tr>
<tr>
<td>Slope</td>
<td>1.93 ± 0.04</td>
<td>1.96 ± 0.13</td>
<td>1.92 ± 0.07</td>
</tr>
<tr>
<td>BL5</td>
<td>Apparent apoB content (%)</td>
<td>99 ± 2</td>
<td>311 ± 71*</td>
</tr>
<tr>
<td>Slope</td>
<td>1.90 ± 0.09</td>
<td>1.64 ± 0.08</td>
<td>1.92 ± 0.11</td>
</tr>
</tbody>
</table>

Data are the apparent apoB contents (expressed as percentage of the LDL standard to which an arbitrary expression of each epitope was assigned as 100%) and slopes calculated from competitive displacement curves (as described in Materials and Methods). They correspond to five LDL preparations and are the compiled results of triplicate experiments. The respective slopes (mean ± SD) obtained for LDL standard were 1.88 ± 0.08 (B1), 4.32 ± 0.17 (B4), 1.87 ± 0.08 (BL7), 1.82 ± 0.07 (BA11), 2.31 ± 0.10 (BL3), 1.90 ± 0.02 (DA7), and 1.93 ± 0.03 (BL5). N-LDL, native LDL; Cu-LDL, copper ion-oxidized LDL; E-LDL, phospholipase A₂ plus lipoxygenase-oxidized LDL.

*P < 0.05, as compared to N-LDL (U-test).
Marcel (10) reported that the immunoreactivity of one epitope mapped to the C-terminal part of copper-oxidized LDL apoB-100 increased markedly during the first time of oxidation, the effect being partially reversed upon prolonged incubation with copper, whereas there was a decrease in immunoreactivity of some other epitopes located in the middle or N-terminal part of the protein. Kleinman et al. (9) demonstrated that during lipolysis of LDL with phospholipase A₂, one epitope localized near the carboxyl end of the apoB-100 molecule became less immunoreactive, while the accessibility of other parts of apoB-100 remained unchanged. Gandjini et al. (11) demonstrated that the immunoreactivity of one epitope located in the N-terminal region of apoB-100 was little affected by oxidative treatments of LDL (copper ions or lipoxygenase plus phospholipase A₂), whereas the immunoreactivity of the C-terminal or the middle part decreased significantly. Finally, Negri et al. (12) recently showed that the immunoaccessibility of apoB-100 was spared upon oxidation of LDL, except for one epitope located between amino acids 2152 and 2377 for which the reactivity was lost. When all these results are considered together, some controversy appears, particularly when LDL is oxidized by exposure to copper. This is really not surprising because very different experimental oxidative conditions have been used, depending on the copper concentrations, incubation length, incubation temperature, and/or LDL concentrations, leading to LDL with varying degrees of modification. It is, therefore, very important to interpret each set of data according to experimental conditions that have to be detailed. Some works evidently state this caution, and the physical, chemical, or biological procedures whereas in the present study, we assessed the immunological conformation of moderately modified LDL as we have conducted both the oxidative procedures in relatively mild conditions. Zawadski, Milne, and Marcel (10) reported that the immunoreactivity of one epitope mapped to the C-terminal part of copper-oxidized LDL apoB-100 increased markedly during the first time of oxidation, the effect being partially reversed upon prolonged incubation with copper, whereas there was a decrease in immunoreactivity of some other epitopes located in the middle or N-terminal part of the protein. Kleinman et al. (9) demonstrated that during lipolysis of LDL with phospholipase A₂, one epitope localized near the carboxyl end of the apoB-100 molecule became less immunoreactive, while the accessibility of other parts of apoB-100 remained unchanged. Gandjini et al. (11) demonstrated that the immunoreactivity of one epitope located in the N-terminal region of apoB-100 was little affected by oxidative treatments of LDL (copper ions or lipoxygenase plus phospholipase A₂), whereas the immunoreactivity of the C-terminal or the middle part decreased significantly. Finally, Negri et al. (12) recently showed that the immunoaccessibility of apoB-100 was spared upon oxidation of LDL, except for one epitope located between amino acids 2152 and 2377 for which the reactivity was lost. When all these results are considered together, some controversy appears, particularly when LDL is oxidized by exposure to copper. This is really not surprising because very different experimental oxidative conditions have been used, depending on the copper concentrations, incubation length, incubation temperature, and/or LDL concentrations, leading to LDL with varying degrees of modification. It is, therefore, very important to interpret each set of data according to experimental conditions that have to be detailed. Some works evidently state this caution, and the physical, chemical, or biological
cal changes that could take place in LDL on exposure to oxidative conditions depend on the mode or degree of lipoprotein oxidation (8, 10). It is also possible that LDL preparations differ in factors that control their susceptibility to oxidation such as, for example, their content in antioxidants or their fatty acid composition (2).

In the present study, copper ion treatment (1.66 μM CuCl₂, 25°C, 8 h) of LDL (100 μg/ml) from normolipidemic subjects and, to a lesser extent, enzymatic treatment increased the immunoreactivity of the N-terminal part of apoB-100. Copper ion treatment of LDL induced a markedly decreased accessibility, if not a total destruction, of the middle and C-terminal parts of apoB-100, except for the B/E receptor recognition domain whose accessibility was enhanced. Immunoreactivity of epitopes located in the median fragment of apoB-100 was little affected by LDL oxidation with phospholipase A₂ plus lipoxygenase, while immunoreactivity of the epitopes in the C-terminal part was markedly decreased. Once again, the accessibility of the epitope located in the B/E receptor recognition domain of apoB-100 at residue 3506 (specific for MAb BA11) was enhanced in E-LDL. It has previously been shown that LDL aggregation occurs at high degrees of oxidation (33) and a possible explanation for the immunological changes we observed could reside in this physical alteration, particularly upon copper treatment, as we observed high molecular weight material by native electrophoresis of treated LDL. This appears not to be the case because the slopes of the competitive displacement curves of the antibodies for the various competitors, including native LDL and LDL standard, were similar (Table 4). This result is in agreement with Gandjini et al. (11) who found that aggregated and nonaggregated LDL had the same affinity towards anti-apoB MAB among which two were identical to ours.

We observed an enhanced activity of the epitope located in the B/E receptor recognition domain of apoB-100 upon LDL oxidation and this could appear to contradict our biological investigations as we demonstrated a lower metabolism of E-LDL, and practically no interactions of Cu-LDL, with the B/E receptor of fibroblasts as seen with N-LDL. This apparently conflicting result might be explained by the fact that one epitope involves a smaller region than the B/E receptor recognition domain, and that conformational changes could modify a punctual region, without necessarily altering neighboring epitopes. Thus, Keidar et al. (34) have shown that a high carbohydrate fat-free diet modulated epitope expression of the LDL-apoB-100 region spanning amino acids 3214-3506 but not the region that encompasses residues 3506-3635. Also, more recently, Negri et al. (12) have demonstrated that among four MABs that recognize close epitopes in the middle part of apoB-100, only one presented an altered immunoreactivity upon LDL oxidation.

Previous studies have indicated that in apoB-100, long-range conformational interactions may be required for appropriate expression of the apolipoprotein area responsible for binding activity, and that both regions, close to and distant from the receptor binding site, can influence the interactions of the protein with its receptor (35-38). The bases for these observations are quite complex and several interpretations are possible. As the C-terminal part of apoB-100 has been demonstrated to have a negative effect on the B/E receptor interaction (35, 36), a possible steric hindrance might occur in this conformational mechanism. Furthermore, the derivatization or the substitution of some critical lysine residues might be another important factor for determining the receptor binding (39, 40). Finally, any structural changes might increase the molecular flexibility of the protein, therefore bringing regions distant from each other on the peptidic sequence closer to the B/E receptor binding domain. Here, we report an overall gradual decrease in the immunoreactivity from the N- to C-terminal part of apoB-100 upon LDL oxidation. This loss of resistance to oxidation could be due to change in the lipid environment of LDL as Marcel et al. (41) have demonstrated that the lipid required for conformation increased along the apoB-100 molecule from the N- to the C-terminal part. However, variations in lipid composition cannot completely explain the immunological changes. Indeed, Harduin et al. (42) have demonstrated, for example, that the higher the content of free cholesterol, cholesteryl esters, and triglycerides in LDL, the more the epitope recognized by the MAb BA11 was accessible. In the present study, the increased accessibility of the epitope recognized by MAb BA11 was concomitant with a decreased cholesteryl ester content and no changes in free cholesterol and triglyceride levels in Cu-LDL.

Not only chemical changes but also physical parameters such as the size of lipoproteins can modulate apoB-100 epitope expression (43). We have previously shown that MABs B4 and BA11 bind all lipoproteins with the same affinity, while B1 recognizes VLDL to a lesser extent than LDL (28). Others have reported a high affinity of BL3 for VL DL and LDL, and a poor recognition of VLDL by BL5 (29). Therefore, in the present study, the changes in the immunoreactivity of apoB-100 epitopes in oxidized LDL could not be related entirely to alterations in the size of LDL.

The oxidation of LDL by copper ions or phospholipase A₂ plus lipoxygenase resulted in modifications in cellular interaction, i.e., an increase in the rate of macrophage degradation and a decrease in recognition by fibroblasts. A similar tendency has already been noted (8, 12, 20), but unfortunately no study has compared the effect of both kinds of modification within the same experiment. We observed that upon copper oxidative treatment, LDL underwent much more pronounced changes than upon en-
zymatic treatment, and this despite relatively mild conditions for copper oxidation (1.66 μM Cu²⁺, 8 h, 25°C vs. 5, 10, or 20 μM Cu²⁺, 24 h, 37°C) generally described in literature (8, 12).

Owing to its chemical composition, relative electrophoretic mobility, and TBARS content, the designed Cu-LDL herein resembles LDL oxidized by incubation with 5 μM CuSO₄ during approximately 3-6 h at 37°C, as described by Steinbrecher et al. (8). In agreement with these authors, and despite the fact that the lipoprotein has probably undergone relatively modest degrees of oxidation, we observed no binding of Cu-LDL to the B/E receptor of fibroblasts. In contrast, in our study, its uptake in macrophages was greatly enhanced, despite its electrophoretic mobility being increased only 1.5-fold that of native LDL. We cannot explain this discrepancy, and perhaps, some difference has even been generated in the nature of modified LDL, as for example in the free amino-groups availability on LDL. Indeed, Haberland, Olch, and Fogelman (44) have shown that the expression of a scavenger receptor recognition site on human macrophages might depend upon the charge modification of critical lysine residues of the LDL protein rather than the net negative charge of the lipoprotein.

The functional properties we found for E-LDL may be compared with those described by Sparrow et al. (20). In both studies, the results agreed one with the other, and despite a lower incubation time with enzymes, in our experiment, we report a tendency for phospholipase A₂ plus lipoxygenase-oxidized LDL to be slightly more and slightly less recognized by macrophages and fibroblasts, respectively, as compared with native LDL. Kleinman et al. (9) have shown that lipolysis of LDL with phospholipase A₂ alone altered the cellular interaction of the lipoproteins, inducing a nonspecific binding on fibroblasts. According to the authors, this aberrant interaction could be due to the loss of surface phospholipids and the uncovering of core lipids that react nonspecifically with cell surface components. Under our experimental conditions, we obtained only moderate nonspecific binding values. This difference may be explained by a lower hydrolysis of phospholipids due to a shorter incubation with enzymes, and/or by the presence of lipoxygenase which would modify free fatty acids because their release would occur in such a way that they would remain linked to the lipoproteins, thus maintaining a structure compatible with a specific cellular interaction.

Under physiological conditions, copper does not occur in vivo in free form while it has been shown that LDL expressed an intrinsic phospholipase A₂ activity (43) and that the oxidation of lipoproteins by some cells depended on cellular lipoxygenase activity (46). It was also previously shown that the addition of phospholipase A₂ to the lipoxygenase was required to further modify the LDL so that scavenger receptors could recognize it (20). We would therefore be tempted to set up an enzymatic modification of LDL as a more physiological model than copper treatment to study the oxidative mechanism, and to define enzymatically modified LDL as a potential biological ligand for the scavenger receptors, as Fogelman et al. (47) have proposed for malondialdehyde-modified LDL. Cultured endothelial cells and smooth muscle cells can also modify LDL in a manner that could lead to a biologically modified form of LDL. However, although it is relatively easy to perform reproducible modifications for a given preparation of LDL by following an enzymatic protocol, it is not so easy for cell-induced oxidation, despite well-described cell types and conditions for culture.

As for an in vitro situation, upon in vivo oxidation, LDL generates a heterogeneous mixture of particles containing molecules that have been modified to different degrees (33). This heterogeneity may account for much of the confusion surrounding the cellular responses to lipoprotein extracted from atherosclerotic plaque (16, 48), therefore hindering the choice of the best experimental model for the understanding of the oxidative process in relation to atherogenesis in humans.

In this study, we have examined immunological and biological properties of LDL oxidized by copper ions and by phospholipase A₂ plus lipoxygenase under mild conditions. We therefore confirm some previously published findings, i.e., changes in the immunoreactivity of apoB-100, functional alterations of oxidized LDL, and the existence of a long-range conformational interaction for determining the best conformation for biological activity of B/E receptor domain. The new data presented in this paper, and not considered in previous reports, are that even when relative mild conditions for oxidative modification were used on LDL, conformational and biological changes occurred. As copper-oxidized LDL is a common in vitro model for studying atherogenesis in humans, it was of interest to compare the effects of this nonphysiological chemical model to an enzymatic model that could appear more biological. This comparison clearly suggests some caution regarding pathophysiological implications of oxidized LDL from in vitro models for the formation of foam cells in vivo.

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