Oxidation of Low Density Lipoprotein Leads to Particle Aggregation and Altered Macrophage Recognition

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Oxidized (ox-) low density lipoproteins (LDL) is characterized by the formation of lipid peroxides and their decomposition to reactive aldehydes which covalently link to apoB in LDL. These chemical changes are believed to be responsible for the enhanced recognition of ox-LDL by receptors on macrophages in culture. When oxidation is extensive, particle aggregation also occurs. The aim of this study was to characterize aggregation formation and how this influences the interaction of ox-LDL with macrophages in culture. When LDL was oxidized by incubating at 500 μg of protein/ml with 10 μM Cu²⁺ at 20 °C for up to 25 h, time-dependent increases in thiobarbituric acid reactive substances, conjugated diene content, electrophoretic mobility, and fluorescence at 360 excitation/430 emission were found. Particle aggregation increased in parallel with several parameters of oxidation and increased with increasing incubation temperatures and LDL concentrations used. When evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, apoB fragments of reproducible sizes and higher molecular weight species appeared after mild oxidation of LDL. The percent of total apoB remaining aggregated in sodium dodecyl sulfate was 50–80% at high degrees of oxidation, whereas it was far less in LDL that had been aggregated without chemical modification. This suggested that intermolecular cross-linking of apoB had occurred during oxidation of LDL at high concentrations. Degradation of ox-LDL in mouse peritoneal macrophages (MPM) increased in parallel with the degree of oxidation and with particle aggregation that reached a plateau after 12 h. Results from cross-competition studies in MPM with soluble and insoluble portions of extensively ox-LDL and with acetyl-LDL were consistent with uptake of soluble ox-LDL via both the scavenger receptor and another receptor on MPM, and uptake of the insoluble ox-LDL by an alternative mechanism.

Oxidation of LDL¹ has gained in interest in recent years (1–3), based in large part on evidence suggesting that it may exist in vivo, either in atherosclerotic lesions (4–8) and/or in the circulation under specific situations (9). Lipid peroxidation in LDL has been shown to be mediated in vitro by free radicals (10, 11) and/or by lipoxygenases (12, 13) produced by different vascular cells. The resultant hydroperoxy fatty acids can be detected by measuring the content of conjugated dienes in oxidized (ox-) LDL (14) or the amount of malondialdehyde liberated by hydrolysis in the thiobarbituric acid (TBARS) assay (10, 11). These hydroperoxides rapidly undergo decomposition yielding a number of reactive aldehydes (15–17), some of which can interact with lysine residues on apoB-100 in LDL (15, 16). This results in a net increase in the surface charge of the LDL particle as measured by agarose electrophoresis (15), and in an increase in the fluorescence of apoB-100 at 360 ex/430 em (16, 17), characteristic of Schiff base adducts formed in both Cu²⁺-oxidized LDL (11, 16, 17) and LDL modified directly with 4-hydroxynonenal (HNE) (18, 19). ApoB in ox-LDL was also shown to undergo extensive fragmentation (20, 21), the result of nonenzymatic scission of apoB induced by the lipid alkoy radicals formed by the decomposition of lipid hydroperoxides (22). Particle aggregation has also been briefly reported in ox-LDL (23), but little information was provided on the factors responsible for this event. Ox-LDL was shown to be recognized by both the scavenger receptor (24) as well as other sites on macrophages (25, 26).

Although several reports have identified some of the chemical modification of LDL during oxidation responsible for enhanced cellular recognition and uptake (2, 15, 16, 24), no systematic study has been reported identifying the relative changes in the chemical and structural characteristics of ox-LDL, especially particle aggregation, and how they might affect cellular recognition. In this study we have demonstrated that particle aggregation occurs at higher degrees of Cu²⁺-induced oxidation, that it increases with increasing incubation temperature and LDL concentration, and that it is associated with apoB aggregation, presumably the result of intermolecular cross-linking by reactive aldehydes formed during oxidation. Furthermore, we have obtained data from cross-competition studies of ¹²⁵I-ox-LDL with mouse peritoneal macrophages that are consistent with a concept that soluble ox-LDL is recognized by the scavenger receptor and an additional receptor, whereas aggregated ox-LDL is recognized by an alternative uptake mechanism.

MATERIALS AND METHODS

Cells and Reagents—C57BL/6 mice (9–12 weeks of age) were purchased from the Trudeau Institute (Saranac Lake, NY). Rosewell Park Memorial Institute (RPMI) medium and fetal calf serum were obtained from Gibco Laboratories (Grand Island, NY). Bovine serum albumin (fatty acid-free), polyinosinic acid, trichloroacetic acid, vitamin E, and EDTA were purchased from Sigma. Na-Carbobenzoyl-L-lysine and malonaldehyde bis(dimethyl acetal) (MDA), were ob-
tained from Aldrich Chemicals, and thiobarbituric acid was purchased from Fisher Chemicals (Fair Lawn, NJ). Bicinchoninic acid assay reagents were purchased from Pierce Chemical Co. Tissue culture plates were from Costar (Cambridge, MA). Carrier-free Na" was obtained from Aldrich Chemicals, and thiobarbituric acid was purchased from Smith et al. (32) except that a 1-h 60 °C heating step was used. Bovine serum albumin was used as a standard. This technique was used because of its greater sensitivity than the procedure of Lowry et al. (33), and by the observation that it is less prone to interference by the presence of commonly encountered biochemical reagents in protein samples. This was determined by the procedure of Roeschlaub et al. (34). Transmission electron microscopy, using phosphoglutamic acid as a negative stain (35), was used to determine lipoprotein morphology. The protein components of reduced, denatured, and delipidated ox-LDL were evaluated by SDS-PAGE using a 3-8% discontinuous gradient (36) and diacryltetra- piperazine (Integrated Separation Systems, Hyde Park, MA) as a cross-linker. Following overnight fixation of the polyacrylamide gels in 25% methanol, 10% acetic acid, proteins were stained with the Daichi II Silver Stain (Tokyo, Japan) according to the manufacturer's instructions. The films were exposed to Kodak RADI- film cassette with K film (Kodak Rochester, NY). The distribution of label in each band was determined by slicing each band from parallel gels not subjected to autoradiography and measuring the amount of radiolabel in individual slices.

**Results**

**Chemical Characterization**—When LDL (500 µg of protein/ ml) was oxidized with Cu" as described under "Materials and Methods," time-dependent increases were found in TBARS and conjugated dienes (Fig. 1a), as well as in electrophoretic mobility relative to LDL (REM), and in fluorescence at 360 ex/430 em (Fig. 1b). The increases were greatest between 6 and 12 h for all parameters. However, between 12 and 25 h they diverged, TBARS actually decreased and conjugated dienes increased only slightly (Fig. 1a), as contrasted to the continued increase in REM and fluorescence (Fig. 1b). No further increase in absorbance of conjugated dienes was seen after 1 week when these samples were stored in 0.3 mM EDTA, suggesting that no further oxidation was occurring under these conditions. When particle aggregation was also assessed in the above experiment by measuring the precipitation of 125I-ox-LDL at 10,000 x g, little aggregation was found up to 8 h, but a sharp increase was seen between 10 and 12 h, paralleling the other parameters of oxidation (Fig. 1c). This was followed by a more gradual increase between 12 and 15 h. We noted in...
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To assess whether any smaller aggregates were present after centrifugation at 10,000 × g for 10 min in a supernatant fraction of LDL that had been extensively oxidized (24 h), we subjected such a sample to transmission electron microscopy after negatively staining, comparing its morphology to a sample of ox-LDL in which no pellet was formed by centrifuging at 10,000 × g, e.g. ox-LDL (8 h), and to a sample of unmodified LDL. Only the supernatant fraction of ox-LDL (24 h) still represented LDL particles. This has been previously observed in ox-LDL (23) and in LDL modified with 4-hydroxynonenal (19).

Since some published studies on Cu²⁺-induced oxidation of LDL had been performed at 37 °C (16, 26) as contrasted to our study described in Fig. 1, which was performed at 20 °C, we made a direct comparison of oxidation-induced aggregation of ¹²⁵I-LDL (500 μg/ml) at 20 and 37 °C (separate LDL sample

**Fig. 1.** Time-dependent increases in Cu²⁺-induced LDL oxidation over a 25-h time interval. ¹²⁵I-LDL at 500 μg of protein/ml was subjected to oxidation by dialyzing against 10 μM Cu²⁺ as described under "Materials and Methods" for periods as indicated. Following further dialysis in PBS for 2 h to remove Cu²⁺ ions, samples were assayed for the following parameters as described under "Materials and Methods," conjugated dienes, and thiobarbituric acid reactive substances (TBARS) (a), REM on 1% agarose, and fluorescence at 360 ex/430 em (b), and degree of particle aggregation estimated by the percent of ¹²⁵I-LDL precipitated by centrifugation at 10,000 × g (O), or the percent of label remaining at the origin following electrophoresis (●) (c).

**Fig. 2.** Small aggregates of unlabeled particles present in soluble fractions of oxidized LDL. Samples of unmodified LDL (a), LDL subjected to oxidation for 8 h (b), and a sample of the supernatant fraction after centrifugation at 10,000 × g of LDL subjected to oxidation for 24 h (c), were treated with 2% phosphotungstate as described under "Materials and Methods" and viewed by transmission electron microscopy.
from that used in Fig. 1). Aggregation increased much more abruptly after 3 h of incubation with Cu2+ at 37 °C than at 20 °C (Fig. 3). Moreover, the maximum level of aggregation achievable over a 24-h period was greater at 37 °C than at 20 °C. The earlier appearance of aggregates in this sample of LDL at 20 °C, than in the sample studied in Fig. 1, may reflect differences in the levels of natural occurring antioxidants in the individual LDL samples (22).

Since in preliminary studies we had found major differences in the percent of LDL particles that were aggregated by oxidation when different concentrations of LDL were used, we sought to determine the concentration dependence of LDL aggregation during oxidation. 125I-LDL was oxidized by incubating with 10 μM Cu2+ for up to 24 h at LDL protein concentrations of 50 μg/ml, 200 μg/ml, and 2 mg/ml at 20 °C, and the degree of aggregation assessed as previously described. No aggregation was found for concentrations of LDL of 200 μg/ml and below, whereas at 2 mg/ml extensive aggregation occurred between 10 and 24 h (Fig. 4). Thus, it appears that aggregation during oxidation is highly dependent on the concentration of LDL.

When each of the samples of 125I-LDL oxidized as described in Fig. 1 were subjected to SDS-PAGE plus autoradiography, we observed a time-dependent increase in the fragmentation of apoB-100, eventually resulting in its disappearance (Fig. 5, a and b). The size of the fragmentation products was reproducible over several separate samples of LDL subjected to oxidation (not shown), as was previously shown by Fong et al. (21). Most noteworthy was the observation that higher molecular weight bands (greater than apoB-100) were seen at the earlier times of oxidation (Fig. 5a) suggesting covalent

![Fig. 3. Dependence of aggregation on the temperature during Cu2+ oxidation of LDL.](image)

![Fig. 4. Dependence of aggregation on the concentration of LDL used for Cu2+-induced oxidation.](image)

![Fig. 5. SDS-PAGE of 125I-ox-LDL demonstrating the distribution of apoB in polyacrylamide gel (a) and quantification of the relative distribution of label from 125I-ox-LDL (b).](image)
cross-linking of a small number of apoB molecules. Similar bands were seen for LDL modified with malondialdehyde (38) or with HNE (19). Furthermore, significantly less of the total applied label entered into the 3–8% polyacrylamide gel during SDS-PAGE for 125I-ox-LDL oxidized for 12 and 25 h than for 6 h for control 125I-ox-LDL (Fig. 5b), suggesting even a further increase in size of the apoB aggregates. It is unlikely that any loss of label from fragmented apoB occurred during SDS-PAGE, since electrophoresis was terminated before the dye front reached the bottom of the gel. Furthermore, treatment of the gel with methanol/acetic acid led to a loss of only 1% of the label entering the gel.

This increased aggregation of apoB from extensively oxidized LDL resembled the aggregation of apoB found in LDL modified directly with HNE (19), a major long-chain aldehyde formed during scission of hydroperoxides (16–18). We had suggested that the particle aggregation found in HNE-modified LDL was the result of intermolecular cross-linking of apoB induced by the bifunctional HNE (19). It is possible that a similar process was occurring in extensively oxidized LDL at high LDL concentrations. We asked whether major differences in aggregation of apoB would be found between apoB in 125I-ox-LDL after extensive oxidation, and unmodified 125I-ox-LDL, which had been induced to aggregate by vortexing as previously described (37) in the presence of butylated hydroxytoluene to inhibit any oxidation. We found that after oxidation of 125I-ox-LDL for 24 h and separation of the insoluble fraction by centrifugation of 10,000 × g, only 21% of the label was recovered in the 3–8% polyacrylamide gel following SDS-PAGE. By contrast, when the sample of 125I-ox-LDL that had been vortexed for 30 s to induce 100% aggregation was subjected to the same procedure, 68% was recovered in the gel. Under the same conditions unmodified 125I-ox-LDL gave values ranging from 80 to 100% recovered in the gel (not shown). These results suggested that oxidation of LDL at high concentrations was inducing intermolecular cross-linking of apoB, resulting in its irreversible aggregation as reflected by its inability to enter into a 3–8% polyacrylamide gel. The similarity of the results of extensively oxidized LDL and of HNE-modified LDL further suggest that such bifunctional aldehydes may be responsible for cross-linking apoB molecules from individual LDL particles during oxidation, provided the concentration of LDL is sufficiently high.

Interaction with Macrophages—To assess the degree and mode of interaction with macrophages of LDL samples oxidized as described in the legend to Fig. 1, we incubated each sample with MPM in culture and determined its uptake and degradation rate. Degradation of 125I-ox-LDL remained constant until 6 h, increasing abruptly between 6 and 12 h, and then remaining the same between 12 and 25 h (Fig. 6, inset). To investigate whether each sample of ox-LDL was recognized by the scavenger receptor, we also determined its degradation rate in MPM in the absence or presence of unlabelled acetyl-LDL. When we determined the ability of 5-fold excess amounts of acetyl-LDL to inhibit the degradation of 125I-ox-LDL oxidized for 8, 12, or 25 h, designated 125I-ox-LDL(8 h), - (12 h), and - (25 h) respectively, we found that 125I-ox-LDL(8 h) was inhibited by 50%, 125I-ox-LDL(12 h) by 22%, but 125I-ox-LDL(25 h) by 0% (Fig. 6). Since polyinosinic acid (poly-I) at 25 μg/ml was previously shown to completely inhibit MPM degradation of a sample of 125I-ox-LDL that had been oxidized by incubating with endothelial cells, whereas excess acetyl-LDL was able to only partially inhibit such a sample (25), we also assessed the ability of poly-I and acetyl-LDL to inhibit the degradation of 125I-ox-LDL oxidized to different degrees with Cu2+. However, inhibition of MPM degradation by poly-I of 125I-ox-LDL samples from Fig. 1 differed little from that induced by excess acetyl-LDL. We also determined whether the excess amounts of supernatant or soluble fraction and pellet or insoluble fraction of a separate sample of LDL oxidized with Cu2+ for 24 h, e.g. ox-LDL (24 h), could inhibit the degradation of 125I-ox-LDL(8 h), 125I-ox-LDL(12 h), 125I-ox-LDL(25 h). A 5-fold excess concentration of unlabelled soluble ox-LDL(24 h) was an effective inhibitor of all three labelled ox-LDL fractions (Fig. 6). Excess insoluble ox-LDL(24 h) was less effective. The above-described inhibition of degradation is unlikely the result of cytotoxicity, since no loss of cell protein was found after the 4.5-h incubation, nor were any increases relative to control LDL found in lactate dehydrogenase release by the macrophages, or decreases found in incorporation of [3H]leucine into trichloroacetic acid-precipitable label (not shown).

Since Sparrow et al. (25) had demonstrated complete competition of 125I-ox-LDL by poly-I, whereas we obtained maximally 50% inhibition, we sought to determine whether higher concentrations of poly-I might be more effective. Furthermore, we studied the ability of poly-I to inhibit the degradation in MPM of both the soluble and insoluble fractions of 125I-ox-LDL(24 h). However, at concentrations of up to 80 μg/ml of poly-I, inhibition of soluble 125I-ox-LDL(24 h) degradation still did not exceed 50%, whereas no inhibition of insoluble 125I-ox-LDL degradation was found (Fig. 7). Consistent with the data of Sparrow et al. (25), poly-I at 25 μg/ml effectively inhibited the degradation of 125I-acetyl-LDL.

We also compared the ability of excess concentrations of unlabelled acetyl-LDL to inhibit the degradation in MPM of the same samples of soluble and insoluble 125I-ox-LDL(24 h) as studied in Fig. 7, with their ability to inhibit degradation of 125I-acetyl-LDL. We found that even at 30-fold excess concentrations, acetyl-LDL had no effect on the degradation of insoluble 125I-ox-LDL(24 h), inhibited degradation of soluble 125I-ox-LDL(24 h) by no more than 50%, while inhibiting the degradation of 125I-acetyl-LDL by about 80% (Fig. 8). A 20-fold excess of LDL in a separate study had no effect on
we had shown previously (19). However, the soluble forms of degradation by excess acetyl-LDL, consistent with recognition of degradation compared to unmodified LDL and inhibition of HNE-modified 125I-LDL showed both an increased MPM soluble (supernatant) fraction and 125I-acetyl-LDL. 125I-LDL was oxidized for 24 h and the degradation in MPM of either soluble or insoluble 125I-LDL was determined in incubation media in the absence or presence of increasing concentrations of poly-I as indicated. Lipoprotein degradation was determined as described under "Materials and Methods." Data points represent the mean of triplicate determinations. MPM degradation (micrograms of protein/mg of all protein) values in the absence of competitors were: soluble 125I-ox-LDL(24 h) = 2.62; insoluble 125I-ox-LDL(24 h) = 2.75; and 125I-acetyl-LDL = 4.73.

Our results clearly show a direct association between the aggregation of LDL following extensive oxidation and changes in macrophage recognition, as evidenced by changes in the ability of ox-LDL degradation to be inhibited by competitors of the scavenger receptor. We sought to better understand the underlying mechanism by using HNE-modified LDL as a model system. We speculated that aggregation of LDL during oxidation may be the result of cross-linking of LDL particles induced by reactive aldehydes formed during lipid peroxidation such as HNE (17, 18). Since our current results demonstrated more aggregation of LDL during oxidation at higher LDL concentrations than at lower ones (Fig. 4), we asked whether less aggregation would occur when LDL was modified with HNE at low rather than at high LDL concentrations, but at similar HNE to LDL ratios. We, therefore, compared the level of aggregation (precipitation at 10,000 g) of 125I-LDL modified at different concentrations of HNE at both 50 and 500 µg of LDL protein/ml. We also assessed the ability of a 30-fold excess of unmodified acetyl-LDL to inhibit the degradation in MPM of the different forms of HNE-modified LDL. As seen in Fig. 9, modification of LDL with HNE at a 10-fold lower LDL concentration resulted in no aggregation as contrasted to >90% aggregation of LDL at 500 µg/ml modified with HNE at a similar LDL to HNE ratio. The soluble HNE-modified LDL appeared to be chemically modified, since it possessed an increased REM (not shown).

Competition by excess unlabeled acetyl-LDL of aggregated 125I-HNE-LDL showed no inhibition of MPM degradation as we had shown previously (19). However, the soluble forms of HNE-modified 125I-LDL showed both an increased MPM degradation compared to unmodified LDL and inhibition of degradation by excess acetyl-LDL, consistent with recognition by the scavenger receptor. Thus, at high LDL concentrations intermolecular cross-linking of LDL by reactive aldehydes formed during lipid peroxidation is likely responsible for LDL aggregation and the apparent lack of scavenger receptor recognition.

**DISCUSSION**

Although published studies have documented the increases in TBARS (10, 11, 14), conjugated dienes (14), REM (15), fluorescence (16, 17), aggregation (23), and recognition by the scavenger receptor on macrophages (24) of LDL following oxidation, none have systematically documented all of these parameters concurrently with increasing degrees of oxidation. We focused our attention on the underlying mechanism leading to oxidation-induced aggregation of LDL and how such aggregation modified macrophage recognition. We found that aggregation increases with time of Cu2+-induced oxidation, with the temperature at which oxidation takes place, and with the concentration of LDL used.
that oxidation induces structural changes in apoB as evidenced by the presence of apoB with higher molecular weight than B-100 at short times of oxidation, and larger aggregates at longer times of oxidation. This aggregation of apoB was sufficient to prevent migration into a 3–5% polyacrylamide gel during SDS-PAGE. These results were the same as those obtained for LDL modified directly with HNE, a major decomposition product of linoleate hydroperoxide (16, 17, 24), at high concentrations of both LDL and HNE (19). We proposed that HNE by forming Schiff-base reactions with lysyl residues in apoB (16), as well as possibly other covalent bonds, was causing LDL particle aggregation by inducing intermolecular cross-bridges formed when the bifunctional aldehyde HNE interacts covalently with apoB on separate particles. The similarity between LDL oxidation and aldehyde-modified LDL with respect to these structural changes, strongly suggests that direct modification of LDL with HNE models the critical events occurring during extensive oxidation. This assumption is further strengthened by the observation that LDL particle aggregation occurs both in oxidation and HNE modification, provided LDL concentrations are high.

Based on several lines of circumstantial evidence, there is reason to believe that aggregation of LDL occurs in the artery wall, especially in atherosclerotic lesions (7, 39–41), and that such aggregation may be induced by the mechanisms described in the report during oxidation of LDL in such lesions. Recently, oxidation of LDL in lesions was identified by positive reactivity using monoclonal antibodies (4, 7), by increases during the cell incubation may modify the labeled ligands, the data of Sparrow who used extensively Cu2+-oxidized LDL. Another explanation of the degree of aggregation of the original ox-LDL sample, the data of Sparrow et al. (25) who showed complete inhibition of '251-ox-LDL degradation in MPM by 25 pg/ml of poly-I, a 50% inhibition of degradation in MPM in soluble 1Z5I-ox-LDL(24 h). One possible explanation for this apparent discrepancy could be the fact that Sparrow et al. (25) used endothelial cell–oxidized LDL while we used the soluble portion of extensively Cu2+-oxidized LDL. Another explanation is that small LDL aggregates were probably still present in our samples (as indicated from the data in Fig. 2) that could have been taken up by phagocytosis rather than receptor-mediated endocytosis. We were able to rule out cytotoxicity to MPM by ox-LDL as a possible explanation of our competition data, since such incubation did not affect the incorporation of [H]leucine into cell protein or the release of lactate dehydrogenase.

In summary, the data from this study indicate that LDL particle aggregation is an important structural modification of LDL during extensive oxidation, especially if oxidation is performed at high LDL concentrations. Furthermore, our data indicate that macrophage recognition and uptake mode changes with increasing degrees of oxidation, presumably the result of this aggregation, from one involving the scavenger receptor and possibly an additional receptor, to an alternative one, presumably involving phagocytosis.

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