Time-course studies by neutron solution scattering and biochemical assays of the aggregation of human low-density lipoprotein during Cu²⁺-induced oxidation

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The oxidative modification of low-density lipoproteins (LDL) is recognized to be a key event in the development of atherosclerotic plaques on artery walls. The characteristics of LDL oxidized by cells of the artery wall can be imitated by the addition of Cu²⁺ ions to initiate lipid peroxidation in LDL. Neutron scattering of LDL in ²H₂O buffers enables the time course of changes in the gross structure of LDL during oxidation to be continuously monitored under conditions close to physiological. Oxidation of LDL [2 mg of apolipoprotein B (apoB) protein/ml] was studied in the presence of 6.4, 25.6 and 51.2 µmol of Cu²⁺/g of apoB by incubation at 37 °C for up to 70 h. Neutron Guinier analyses showed that the radius of gyration *R_g* (indicative of size) and the forward-scattered intensity at zero angle *I*(0) (indicative of *M_r*) continuously increased during oxidation, indicating that LDL had aggregated. Both the rate of aggregation and the change in *R_g* and *I*(0) values after 10 and 50 h increased with Cu²⁺ concentration. Distance-distribution functions *P*(*r*) showed that, within 4 h, the maximum dimension of LDL increased from 23 to 55 nm. The *P*(*r*) curves of oxidatively modified LDL exhibited two peaks at 10–12 nm and 26 nm. The 10–12 nm peak corresponds to native LDL, and the 26 nm peak is assigned to the initial formation of LDL dimers and trimers and their

progression to form higher oligomers. The growth of the 26 nm peak depended on Cu²⁺ concentration. Particle-size-distribution functions *D_v*(*r*) suggested that the polydisperse spherical structure of LDL ceased to exist after 30 h, at which point the LDL samples underwent a phase separation. Related, but not identical, changes in the *I*(*Q*) and *P*(*r*) curves were observed when native LDL was self-aggregated by brief vortexing. Parallel assessment of LDL protein modification by SDS/PAGE showed increased aggregation and degradation of apoB with increased Cu²⁺ concentrations, and that the main apoB protein band had diminished after 2–8 h, depending on the amount of Cu²⁺ added. The uptake and degradation of oxidized ¹²⁵I-labelled LDL by mouse peritoneal macrophages occurred maximally within the first 10 h, and increased in proportion to the Cu²⁺ concentration. ApoB protein broke down within the first 10 h of oxidation, and this is the period when scavenger receptors on macrophages can recognize and internalize oxidized LDL. Within 10 h, the protein–lipid interactions responsible for the spherical LDL structure became destabilized by protein fragmentation. After 30 h, LDL formed large vesicular aggregates with no regular structure. In summary, neutron-scattering data show that LDL aggregation is continuous during oxidation.

INTRODUCTION

Low-density lipoproteins (LDL) are a polydisperse assembly of a single molecule of apolipoprotein B (apoB) of *M_r* 512 000 with varying proportions of lipid, adding to an overall particle *M_r* of 2 × 10⁶–3 × 10⁶. LDL are central to the development of atherosclerosis, initiated by the formation of cholesterol-rich deposits in the intimal space of arteries. There is strong evidence that LDL accumulate within the intimal space of arteries and becomes oxidized, possibly by contact with macrophages which also invade the area [1–4]. The accumulation of intracellular cholesteryl esters from LDL within macrophages in the intima is a characteristic feature of human atherosclerosis [5]. However, native LDL when isolated from the blood of healthy subjects fail to induce elevated lipid levels in cultured macrophages. Macrophages down-regulate the high-affinity receptors for LDL lipid accumulation in macrophages is only observed in the presence of chemically and oxidatively modified LDL through different classes of receptors that recognize these modified forms [6–8]. A broad range of these modifications cause the same response at the cellular level, i.e. the accumulation of lipids within macrophages, which are then converted into foam cells.

Oxidation of LDL has been demonstrated to occur *in vitro* by incubation with cultures of macrophages or endothelial cells provided that the medium contains the transition metal ions Fe²⁺ or Cu²⁺. Metal ions alone are frequently used to initiate LDL oxidation, as the resulting LDL have very similar biological activities and chemical compositions to that of LDL modified by macrophages, endothelial and smooth-muscle cells [3,4,9]. The effects of Cu²⁺ on LDL oxidation have been well characterized, and are dependent on the presence of small amounts of lipid peroxides. In summary, the antioxidant content of LDL (α-tocopherol and β-carotene) is first consumed, followed by lipid peroxidation of the unsaturated fatty acyl chains to generate aldehydes, including malondialdehyde. The amounts of the four main lipid classes in LDL (cholesterol, cholesteryl esters, triacylglycerols and phospholipids) change during oxidation. These aldehydes are known to form Schiff bases with the lysine residues of the apoB protein of LDL. This results in the recognition of apoB by the macrophage scavenger receptor and the increase in electrophoretic mobility of apoB on agarose gels because of the rise in electronegativity [4]. Fragmentation and aggregation of apoB can be observed by SDS/PAGE during the time course of oxidation [10–12]. Despite the extensive biochemical charac-

terization of the changes seen in LDL during oxidation, relatively few physical studies of the structural changes have been reported. These include the use of neutron and X-ray solution scattering, and Fourier-transform IR, UV, ESR and NMR spectroscopy [11,13–18]. None of these have involved time-course studies of the development of structural changes in LDL during oxidation in which these changes may be related to the altered composition of the lipoproteins.

Neutron scattering of native LDL in 100% $^2\text{H}_2\text{O}$ buffers is a diffraction process that characterizes the LDL structure as a polydisperse assembly of spheres of mean ($\pm$ S.D.) radius $10 (\pm 2)$ nm [19]. The high negative solute/solvent contrast masks out most of the internal structure of lipids and protein, and results in a view of LDL as an object of almost uniform internal scattering density. Neutron scattering thus becomes highly advantageous as a monitor of the averaged overall structure that is measured under conditions that are close to physiological. As full neutron-scattering curves can be measured in 10–20 min during an oxidation time course using the new LOQ instrument at the neutron spallation source ISIS [20], this offers the possibility of continuous monitoring of the structure of LDL during oxidation over 50–70 h. Previous neutron-scattering work on the D17 instrument at the Institut Laue-Langevin, Grenoble, France was based on LDL samples where oxidation had to be stopped in order that the three subcurves required for the full neutron curve could be measured [11]. In this study, we describe the structural changes that occur in LDL during oxidation at three Cu^{2+} concentrations using a range of LDL samples prepared from different donors. As these experiments indicate that LDL aggregation has occurred, a comparison is made with neutron-scattering data from native LDL spontaneously aggregated by vortexing [21,22]. In order to correlate the observed structural changes with literature data on the time course of biochemical oxidation, complementary biochemical assays of oxidized LDL under conditions identical with those of neutron-scattering work are described. These are based on the use of SDS/PAGE to monitor the structural integrity of the apoB protein at the surface of LDL (which stabilizes the spherical LDL structure) and macrophage uptake assays which monitor the extent of oxidation in LDL. These experiments indicate for the first time how the overall physical structure of LDL changes with respect to shape and size during Cu^{2+} -initiated oxidation of LDL.

Preliminary accounts of this work were presented at the 645th meeting of the Biochemical Society, London, U.K. and the IXth International Conference on Small Angle Scattering, Saclay, France [22a,22b].

MATERIALS AND METHODS

Preparation of human LDL for oxidation experiments

LDL (density range 1.025–1.055 g/ml) were isolated by ultracentrifugation [23] from normal human blood collected in citrate dextrose (113.8 mM glucose, 29.9 mM trisodium citrate, 72.6 mM NaCl and 2.8 mM citric acid) [19]. LDL oxidation was inhibited during sample preparation by the addition of $2 \mu\text{M}$ diethylenetriaminepenta-acetic acid and $300 \mu\text{M}$ EDTA, and LDL were stored in the dark for no more than 2 days at 4°C . LDL were dialysed against 12.5 mM Tris/HCl/140 mM NaCl, pH 7.4, in $^2\text{H}_2\text{O}$ solvents for neutron-scattering experiments or H_2O solvents for SDS/PAGE and macrophage-degradation analysis. LDL protein concentrations were determined by the Lowry method with BSA as a standard [24].

LDL oxidation was initiated by the addition of typically 12.8, 51.2 or $102.4 \mu\text{M}$ Cu^{2+} as CuSO_4 to 0.4 ml of LDL from the same donor at 2 mg of apoB/ml in the quartz Hellma cells used for

neutron-scattering-data acquisition. The Cu^{2+} /LDL samples and a blank were then incubated at 37°C in a water bath for up to 3 days. Oxidation was ineffective in PBS (12.5 mM phosphate/140 mM NaCl, pH 7.4) or modified Tyrode's buffer [11], and this was attributed to Cu^{2+} precipitation by phosphate. To ensure uniformity during oxidation, samples were inverted gently whenever they were measured. To prepare vortexed LDL, 0.4 ml of native LDL at 2 mg of apoB/ml was pipetted into Eppendorf tubes and vortexed using a Fisons Whirlmixer for times between 5 and 300 s. The vortexed sample was immediately transferred to a quartz Hellma cell for neutron-scattering-data acquisition.

Neutron-scattering-data acquisition and reduction

Neutron-scattering data on oxidized LDL at 15 or 37°C were obtained in parallel for up to four different samples measured in cyclic fashion at 8–14 time points during oxidation for up to 45–70 h on instrument LOQ at ISIS (Rutherford-Appleton Laboratories) [20]. The oxidation time course was defined from data obtained in three separate beamtime sessions with eight LDL preparations at 2 mg of apoB/ml. This apoB concentration or less was recommended to avoid concentration effects [19]. Details of data acquisition are as in the preceding paper [19]. Acquisition times were close to 15 min for this apoB concentration. Further controls included the use of the same LDL sample for all Cu^{2+} concentrations as well as a blank without Cu^{2+} to circumvent structural polydispersity and differential oxidation effects between different samples. The stages of raw data reduction to obtain the final scattering curves $I(Q)$ [25], together with the use of Guinier analyses to obtain the radius of gyration R_g and the intensity of forward scattering $I(0)$ at low scattering angles Q ($Q = 4\pi\sin\theta/\lambda$; where 2θ is the scattering angle), and the use of Glatter's indirect transformation procedure (ITP) to determine the distance-distribution function $P(r)$ and size-distribution function $D_v(r)$ are also given elsewhere [19,26].

Time course of apoB breakdown in oxidized LDL analysed by SDS/PAGE

To assess both sample purity using overloaded gels before oxidation and the breakdown of apoB during oxidation, SDS/PAGE analyses [27] were carried out on 2.5–16% linear gradient polyacrylamide gels in 0.3% Tris/1.44% glycine/0.1% SDS buffer (Isolab) at pH 8.3 using a Pharmacia Mini Gel apparatus. At time points during Cu^{2+} oxidation over 20 h, LDL equivalent to $100 \mu\text{g}$ of protein in a volume of $50 \mu\text{l}$ was pipetted into a microcentrifuge tube, and $25 \mu\text{l}$ of sample buffer was added (30 mM Tris, 3 mM EDTA, 3% SDS, 15% glycerol, 15% 2-mercaptoethanol). The sample was denatured by incubation at 80°C for 15 min before application to the gel. Gels were stained using Coomassie Blue, and a high- M_r calibration kit (Pharmacia) was used as markers. Non-denatured native and oxidized LDL, having been prestained for lipid with 0.085% Tetrazolium Blue/9 mM NADH/1.14 mM phenazine methosulphate [28], were also applied to 3–8% gradient gels of polyacrylamide at pH 8.3 (0.3% Tris/1.44% glycine running buffer) containing no SDS.

Time course of uptake of oxidized LDL by macrophages

To assess the degree of modification of LDL during oxidation, the uptake of ^{125}I -labelled LDL by macrophages resulting in labelled soluble degradation products was monitored [29]. LDL was radioiodinated with Na^{125}I using a modification of the iodine monochloride method of McFarlane [30,31]. This produced biologically active ^{125}I -LDL with less than 1 ^{125}I atom per

100000 M_r , more than 97 % of ^{125}I radioactivity precipitated by 10 % (v/v) trichloroacetic acid, and less than 6 % of ^{125}I radioactivity extracted into chloroform/methanol (2:1, v/v). SDS/PAGE showed no visible degradation of apoB as the result of iodination. Resident peritoneal macrophages were isolated and cultured from CD1 mice weighing about 20–30 g by lavage with Dulbecco's PBS without Ca^{2+} or Mg^{2+} . Cells were spun down at 1000 rev./min for 15 min in an IEC Centra-4X bench-top centrifuge, and resuspended in Dulbecco's modified Eagle's medium (Gibco) with 0.1 g/l sodium pyruvate and 1.0 g/l glucose supplemented with 10 % (v/v) foetal calf serum (Gibco) and 50 $\mu\text{g}/\text{ml}$ gentamicin. Cells were then seeded in 22.6 mm-diameter wells in 12-well cluster plate (Costar) at 2 million peritoneal cells/ml per well. Plates of cells were incubated at 37 °C in a humidified atmosphere of 95 % air/5 % CO_2 for a minimum of 4 h, after which time the cells were washed with culture medium to remove any unattached cells; they were then ready for treatment with oxidized ^{125}I -LDL. At time points during the 24 h after Cu^{2+} -induced initiation of LDL oxidation, 2 μl of 100 μM EDTA was added to 32.5 μl (65 μg of apoB protein) of oxidized radioiodinated LDL to halt oxidation. These samples were stored at 4 °C until the oxidation series was complete. The oxidized LDL samples were diluted to 10 $\mu\text{g}/\text{ml}$ by the addition of Dulbecco's modified Eagle's medium containing 10 % (v/v) foetal calf serum and 50 $\mu\text{g}/\text{ml}$ gentamicin. This medium prevents modification of LDL by macrophages [32]. Oxidized LDL samples were then incubated (1 ml per well) for 24 h with the target macrophages and cell-free wells as controls. The medium was then removed for determination of trichloroacetic acid-soluble radioactivity after removal of free iodide with AgNO_3 . The macrophages were dissolved in 200 mM NaOH and assayed for total protein by a modified Lowry method [33]. This enabled the degradation of LDL by macrophages to be normalized for the amount of protein in each well.

RESULTS AND DISCUSSION

Neutron Guinier analysis of the time course of LDL oxidation

Guinier analysis of the scattering curves $I(Q)$ at the lowest Q values characterizes the overall size and M_r of the LDL samples during oxidation. Structural changes that occur during Cu^{2+} -induced oxidation of LDL were studied with samples from eight different donors at 2 mg of apoB/ml in Tris buffer and Cu^{2+} concentrations between 3.2 and 51.2 $\mu\text{mol}/\text{g}$ of apoB protein. A concentration of 6.4 μmol of Cu^{2+}/g of apoB corresponds to 0.64 μM Cu^{2+} at an LDL concentration of 100 μg of protein/ml, where a concentration of 1 mg of apoB/ml corresponds to 1.9 μM apoB. Most workers use a higher ratio of Cu^{2+} to LDL to oxidize LDL [3]. Neutron scattering of native LDL in $^2\text{H}_2\text{O}$ buffers readily yields the Guinier R_g and $I(0)$ parameters in an accessible Q range (Figure 1b of the preceding paper [19]). Here, changes in the Guinier plots of LDL during oxidation with 25.6 μmol of Cu^{2+}/g of apoB are shown in Figure 1(a). The slope of the Guinier plot is visibly increased over 50 h. As native LDL are polydisperse in nature [19], the R_g and $I(0)/c$ values [where c is the apoB concentration used to normalize the $I(0)$ values] are averaged values for the population of LDL particles present in the sample. Curvature in the Guinier plots became apparent during oxidation, and this indicated that sample heterogeneity had increased. A fixed Q range of 0.08–0.11 nm^{-1} was used to monitor the course of oxidation using the apparent R_g and $I(0)$ parameters. This is of necessity smaller than the Q range of 0.08–0.19 nm^{-1} used for native LDL as the R_g values increase during oxidation. The resulting R_g and $I(0)$ parameters were validated on the basis that this fit was a good linear representation

of the Guinier region, and agreed with the R_g and $I(0)$ values derived from the $P(r)$ analyses below.

The observed mean R_g and $I(0)/c$ values for an LDL sample during oxidation are summarized as a function of Cu^{2+} concentration in Figures 1(b) and 1(c). These values increase with time, which can be readily attributed to the presence of small but detectable increases in the amounts of aggregated LDL. These increases with time t approximately match a first-order chemical reaction, and could be fitted to exponential functions $f(t) = ae^{-kt} + b$, where b and $(a+b)$ are the starting and final R_g or $I(0)/c$ values, and k is the rate constant. Figures 1(b) and 1(c) show that, when the 6.4 μmol of Cu^{2+}/g of apoB oxidation curve for the first 10 h is compared with those for 25.6 and 51.2 μmol of Cu^{2+} , the rate of increase in k increases with Cu^{2+} concentration. For each LDL sample, the values of a and b for exponential curve fits were taken from the 51.2 μmol of Cu^{2+} data standardized at 50 h (which is equivalent to assuming that the same LDL aggregation state is attained after a sufficiently long oxidation) and the native LDL data at 0 h. The individual rates for each of the R_g and $I(0)/c$ data sets at a given $\text{Cu}^{2+}/\text{apoB}$ ratio were variable, but this could not be significantly correlated with the use of different LDL donors because of the limited accuracy of the analyses. Mean values for k were estimated to be 0.012 ± 0.008 , 0.04 ± 0.02 and $0.11 \pm 0.08 \text{ h}^{-1}$ for the 6.4, 25.6 and 51.2 μmol of Cu^{2+}/g of apoB data respectively for the R_g and $I(0)/c$ data for up to eight different LDL samples. The rates of aggregation thus clearly increase in proportion to the Cu^{2+} concentration.

The use of higher Cu^{2+} concentrations in LDL oxidation resulted in larger apparent R_g and $I(0)/c$ values when these are standardized at 10 and 50 h (Figure 2). Figures 1(b) and 1(c) show that these differences are statistically significant for a given LDL sample. Figure 2 summarizes the consistency of this result for up to eight different LDL samples with five different Cu^{2+} concentrations as well as the control without Cu^{2+} . In Figure 2(a), the increases in mean R_g values of the oxidized LDL samples (compared with native LDL) exhibited an excellent linear relationship with the Cu^{2+} concentration (correlation coefficients, r^2 , 0.99 and 0.85 respectively). The rates of increase are similar at 0.107 ± 0.005 and $0.122 \pm 0.026 \text{ nm}/\mu\text{mol}$ of Cu^{2+} , but the intercepts are significantly different at -0.2 ± 0.1 and $2.6 \pm 0.6 \text{ nm}$ after 10 and 50 h. This difference can be attributed to the spontaneous aggregation of native LDL during the incubation at 37 °C for 50 h. This is in agreement with the controls without Cu^{2+} present, for which the R_g values increased by an average of $1.7 \pm 1.3 \text{ nm}$ over 50 h (Figure 1b). In Figure 2(b), similar results were obtained for the increases in $I(0)/c$ after 10 h (compared with native LDL), where a good linear relationship was again observed ($r^2 = 0.97$), and the intercept at zero Cu^{2+} concentration was again zero within error at -4 ± 2 . After 50 h, the linear relationship for $I(0)/c$ data worsened slightly with $r^2 = 0.88$, although the intercept at zero Cu^{2+} concentration increased to 17 ± 5 . Increasing the apoB concentration to 4 mg/ml with 6.4 μmol of Cu^{2+}/g of apoB caused LDL to aggregate several times more rapidly than shown in Figure 2. Despite the semiquantitative nature of Guinier analysis, these linear relationships show that the size and M_r of LDL increase in proportion to the amount of Cu^{2+} present, both after 10 h and probably also after 50 h of oxidation. This is the expected result if the LDL particles self-aggregate continuously as a direct consequence of the addition of different amounts of Cu^{2+} . After 50 h at 37 °C, this picture of LDL aggregation is still substantially correct, although the poorer r^2 values show that the results have become more variable, as might be expected from this length of incubation. After 50 h, LDL are fully oxidized, and

Figure 1 Neutron Guinier analysis of the time course of LDL oxidation

(a) Oxidation was initiated with 25.6 μmol of Cu^{2+} /g of apoB. Guinier plots for an LDL sample at 2 mg of apoB/ml are shown at 0, 5, 13, 23, 31, 36 and 45 h after the initiation of oxidation. Guinier plots were fitted in the Q range 0.08–0.11 nm⁻¹; the QR_G range of the fits is indicated. Successive curves starting from zero time are displaced upwards by 1 log unit on the vertical axis for clarity. (b) and (c) Typical time-dependence of the apparent R_g and $I(0)/c$ parameters respectively of LDL during the time course of Cu^{2+} -initiated oxidation. LDL (2 mg of apoB/ml) was incubated with 6.4 (●), 25.6 (■) or 51.2 (▲) μmol of Cu^{2+} /g of apoB or 0 μmol of Cu^{2+} as a control (○). Error bars correspond to standard deviations of the Guinier analyses.

Figure 2 Increase in the apparent R_g and $I(0)/c$ values of LDL samples as a function of the amount of Cu^{2+} used to initiate LDL oxidation

Data are shown for 10 (○) and 50 h (●) after the addition of Cu^{2+} at concentrations of 0, 3.2, 6.4, 12.8, 25.6 and 51.2 μmol /g of apoB to LDL solutions at 2 mg of apoB/ml. The total data points for different LDL samples averaged at each Cu^{2+} concentration are eight, two, six, one, eight and six respectively. Results are means $\pm$ S.D. The R_g data are shown in (a), and the $I(0)/c$ data in (b).

it might be expected that both the R_g and $I(0)/c$ values would attain similar final values in Figure 2. That this is not clearly observed in Figure 2 suggests that LDL aggregation has reached different final states with different Cu^{2+} concentrations.

Neutron distance-distribution function $P(r)$ analysis of LDL oxidation

Calculation of the distance-distribution function $P(r)$ of LDL from $I(Q)$ provides information on the structure of LDL and its oxidized forms in real space. In the original analyses for native LDL [19], the transformation of $I(Q)$ to $P(r)$ was achieved by assuming a maximum dimension of 30 nm for LDL. This is significantly larger than the diameter of 22.8–25.2 nm determined for native LDL, as required for the transformation. For oxidized

LDL, Guinier analysis showed that aggregation occurred. Consequently the maximum dimension used to calculate $P(r)$ has to be increased. Values between 30 and 90 nm were tested for both native and oxidized LDL and a value of 60 nm was shown to be optimal giving well-behaved calculated $P(r)$ curves for native and oxidized data.

Figure 3 presents representative $I(Q)$ and $P(r)$ curves during the time course of LDL oxidation initiated with 25.6 μmol of Cu^{2+} /g of apoB. Figure 3(a) shows how the characteristic features of the scattering curve of native LDL changed during oxidation to a less-well-defined scattering curve. In particular, the sub-maximum at 0.55 nm⁻¹ (arrowed), which indicates a spherical structure in LDL, disappeared over the time course. In Figure 3(b), the corresponding $P(r)$ curves show that a range of macromolecular structures are produced during oxidation. This

Figure 3 Full scattering curves $I(Q)$ and the corresponding distance-distribution functions $P(r)$ for the time course oxidation of LDL

LDL was at 2 mg/ml protein concentration and Cu^{2+} oxidation was initiated with 25.6 μmol of Cu^{2+} /g of apoB. Starting from the zero-time $I(Q)$ curve, successive curves are displaced downwards by 3 log units for clarity, and the time after initiation of oxidation is shown to the right. Statistical errors (S.D.s) are shown when they are large enough to be seen. (a) $I(Q)$ curves, in which the submaximum at Q 0.55 nm⁻¹ is indicated; (b) ITP to give $P(r)$, in which the position of the maximum is denoted by M and the maximum dimension is denoted by L.

is best described in terms of the formation of a significant proportion of dimers and smaller proportions of trimer and higher oligomers. This is shown by the growth of intensity in the $P(r)$ curve beyond 25 nm, such that the averaged maximum dimension L of native LDL is rapidly increased from 22.8–25.2 nm [19] to approximately double that at about 53 nm shortly after oxidation had started. At the same time as L is doubled, a second peak maximum at r close to 26 nm becomes apparent, and grows in intensity during oxidation. As this peak is at half the value of L (53 nm), it is in the expected position if LDL dimers and trimers were to be formed. The changes shown in Figure 3 are similar when lower and higher Cu^{2+} concentrations are used, but occur at slower and faster rates respectively (results not shown). This is consistent with the outcome of the Guinier analyses above. Calculation of the Guinier R_g and $I(0)$ parameters from the $P(r)$ curves agree well with the values derived from Guinier plots (Figure 1), i.e. the ratio of each of the pairs of R_g and $I(0)$ values from the Guinier and $P(r)$ analyses is unity within error.

The $P(r)$ curves are able to resolve separately the relative amounts of LDL monomers and oligomers during the oxidation. The time course of the changes in $P(r)$ are summarized in Figure 4. Similar data were obtained in all eight LDL oxidation experiments. As for the Guinier analyses above, no significant differences in rates could be detected within error for LDL obtained from different donors. The position r of the maximum M in the $P(r)$ curve (Figure 3b) increased by 1 to 4 nm in 50 h from a starting value of 10 nm for native LDL. The change in r

is largest at the highest Cu^{2+} concentration, and no change in r was seen within error in the controls without Cu^{2+} . Figure 4 shows the changes in intensity of $P(r)$ during an oxidation series for one LDL sample. The intensity of $P(r)$ at $r = 10$ –12 nm (which corresponds to unaggregated native LDL) decreased by small amounts as oxidation proceeded, and the controls without Cu^{2+} showed similar intensity changes (Figure 4a). This is as expected as native LDL would disappear during aggregation, and any decrease would provide a measure of the proportion of native LDL that has formed oligomers. The decrease in peak intensity at $r = 10$ –12 nm (up to 10%) is largest at the highest Cu^{2+} concentration. The growth in intensity of $P(r)$ at $r = 26$ nm (Figure 4b) mirrored closely the time course of the change in R_g data in Figures 1(b) and 1(c). Fits of the curves in Figure 4(b) to exponential functions showed that the rate constants k were the same within error as those derived from the Guinier analyses. The growth of aggregated forms of LDL thus accounts fully for the increases in the apparent Guinier R_g and $I(0)/c$ parameters during oxidation.

The $P(r)$ curves show that, as oxidation proceeded, small amounts of larger particles of at least twice the diameter of native LDL were rapidly formed. This suggests LDL dimerization and trimerization in the initial stages of oxidation. The growth of oligomers caused the amount of monomeric LDL to decrease. As oxidation progressed, this LDL structure became more complex with the development of more polydisperse larger oxidation products, as shown by the loss of detail in the $P(r)$ curve at 49 h in Figure 3(b).

Figure 4 Time-dependence of intensities in the $P(r)$ curves of LDL during oxidation

Oxidation was carried out at the four Cu^{2+} concentrations, namely the control with no Cu^{2+} ($\circ$), and with 6.4 ($\bullet$), 25.6 ($\blacksquare$) and 51.2 ($\blacktriangle$) μmol of Cu^{2+} /g of apoB. (a) Change in intensity of $P(r)$ at M during oxidation (r between 10–12 nm); (b) corresponding changes in $P(r)$ at $r = 25.8$ nm.

Neutron polydispersity $D_v(r)$ analyses of LDL oxidation

The full neutron-scattering curves also provide information on the polydispersity of the LDL sample in terms of the population distribution of LDL radii about a mean value [19]. This is obtained from reapplication of ITP to calculate the size-distribution function $D_v(r)$ on the assumption that LDL can be structurally represented by a sphere of uniform scattering density. Strictly speaking, neutron-scattering data on native LDL in $^2\text{H}_2\text{O}$ buffers are best described in terms of a sphere with an almost uniform high scattering density at the lipid core which is reduced near the surface. The approximation of a sphere with uniform scattering density is nonetheless satisfactory for present purposes.

Calculation of the $D_v(r)$ curves for an LDL sample with 51.2 μmol of Cu^{2+} /g of apoB exhibited changes with length of oxidation. Curve analyses were based on 30 splines from which the innermost three in the range $0 < r < 3$ nm were removed. Although a strong peak at $r = 10$ nm corresponding to the average LDL radius of 10 nm was initially observed (Figure 6d of the preceding paper, [19]), the intensity of this peak steadily diminished by 80% after oxidation for 48 h, together with the development of strong termination effects at $r < 7$ nm and $r > 13$ nm. The full width of the main peak at $r = 10$ nm at half-maximum intensity was almost unchanged between 3.5 and 3.8 nm for the first 30 h of oxidation, after which it rapidly increased to double its value and beyond. These calculations show that the assumptions required for the success of a reasonable $D_v(r)$ calculation were valid only for the first 30 h of oxidation, when large amounts of LDL monomers were present. After 30 h, it can no longer be said that LDL are well represented by a polydisperse assembly of spheres, and it ceases to be a spherical structure.

Neutron analyses of vortexed LDL samples

If native LDL is vortexed for short times, self-aggregation is induced [21,22]. Self-aggregated LDL are rapidly taken up by macrophages [21]. A comparison of the scattering curves $I(Q)$ and distance-distribution curves $P(r)$ of vortexed LDL with those for oxidized LDL will indicate the degree of structural similarity between the two aggregation processes.

Guinier plots from an LDL preparation of 2 mg of apoB/ml which had been vortexed for between 0 and 5 min are shown in Figure 5(a). The Q range used was based on that used for Cu^{2+} oxidation, and the results were within error of those derived from $P(r)$ curves. With increased times of vortexing, the Guinier region of the $I(Q)$ scattering curves became increasingly steep. This was paralleled by the increasing turbidity of the LDL samples, where a clear phase separation occurred on standing for several hours. The resulting changes in the apparent R_g and $I(0)/c$ parameters are summarized in Figure 5(b), and are very similar for a second LDL preparation from a different donor. Both the apparent R_g and $I(0)/c$ parameters increased with increasing vortexing time, indicating increasing amounts of aggregated LDL. The changes in the R_g and $I(0)/c$ parameters were more dramatic than the changes seen during oxidation of LDL initiated with Cu^{2+} . The averaged R_g of the vortexed samples rapidly increased from 8.0 to 21.8 nm in 5 min (Figure 5b), in distinction to an increase from 8.1 to 16.0 nm in 45 h for the oxidation with 51.2 μmol of Cu^{2+} /g of apoB (Figure 1b). The averaged $I(0)/c$ values increased by 460% during vortexing, compared with an increase of 220% during the same oxidation. It has been shown by electron microscopy that even brief vortexing at 0.5 mg of apoB/ml for 5 s results in aggregation of LDL [22], which is consistent with the neutron-scattering data. As LDL are also reported to aggregate on standing at 4 °C for 2–3 months [22], this would account for the observed small increases in the R_g and $I(0)$ values in the control LDL samples incubated at 37 °C without Cu^{2+} in the oxidation experiments (Figure 2).

Figure 6 shows the full $I(Q)$ and $P(r)$ curves for vortexed LDL. The transformations from $I(Q)$ to $P(r)$ were achieved by using a maximum dimension of 60 nm as for Cu^{2+} -oxidized LDL (Figure 3). Figure 6(a) illustrates the structural disintegration of the scattering curve after various periods of vortexing. The characteristic shape of the $I(Q)$ curve for native LDL became less well defined after only brief vortexing times. In particular, the submaximum at approximately $Q = 0.55 \text{ nm}^{-1}$, which is reflective of a spherical structure, weakened and disappeared after 3 min of vortexing. The corresponding $P(r)$ curves in Figure 6(b) show the growth of intensity at high r which indicates the development of larger aggregated structures. The observable maximum dimension L increased from 20 nm to the maximum of 60 nm after

Figure 5 Neutron Guinier analysis of vortexed LDL samples

(a) Guinier analyses for an LDL sample of 2 mg of apoB/ml are shown after vortexing for 0, 10 and 30 s, 1, 2, 4 and 5 min. Guinier plots were fitted in the Q range $0.08\text{--}0.11\text{ nm}^{-1}$; the QR_G range is indicated. Successive curves after the zero-time curve are displaced upwards by 1 log unit for clarity. (b) The dependence of the apparent R_G (●) and $I(Q)/c$ (○) values on the time of vortexing is summarized, together with the statistical error bars (S.D.) of the Guinier analyses.

Figure 6 Full scattering curves $I(Q)$ and the corresponding distance-distribution functions $P(r)$ for vortexed LDL samples

LDL was at 2 mg/ml protein concentration. Starting from the zero-time $I(Q)$ curve, successive curves are displaced downwards by 4 log units for clarity, and the time of vortexing is shown to the right. Statistical errors (S.D.s) are shown when they are large enough to be seen. (a) $I(Q)$ curves, in which the submaximum at $Q = 0.55\text{ nm}^{-1}$ is indicated; (b) ITP to give $P(r)$, in which the position of the maximum is denoted by M and the maximum dimension is denoted by L.

3 min of vortexing. The effects seen in the $P(r)$ curves are similar to but more dramatic than those seen during Cu^{2+} -initiated oxidation of LDL. The $P(r)$ curve after 1 min of vortexing resembles that after 14 h of oxidation with $25.6\text{ }\mu\text{mol}$ of Cu^{2+} /g of apoB (Figure 3b). Closer inspection, however, shows that the

time course of structural change is not the same in Figures 3(b) and 6(b). After 3 min of vortexing, only a single peak M is visible in the $P(r)$ curve at $r = 23\text{ nm}$. After 49 h of oxidation using $25.6\text{ }\mu\text{mol}$ of Cu^{2+} /g of apoB, the form of the $P(r)$ curve is more diffuse (Figure 3b). The difference between the outcome of the

Figure 7 SDS/PAGE analysis of the time course of LDL oxidation

The gels in (a), (b) and (c) correspond to LDL (2 mg of apoB/ml) incubated with 6.4, 25.6 and 51.2 μmol of Cu^{2+} /g of apoB respectively. Lanes A–H, 2 μg of LDL protein 0, 2, 4, 6, 8, 12.5, 16.5 and 20.5 h after the initiation of oxidation; lane S, M_r markers with values marked to the right.

vortexing and oxidation experiments is reproducible in the two vortexing and 19 oxidation time courses based on 3.2–51.2 μmol of Cu^{2+} /g of apoB for 50–70 h. This difference can be attributed to the gradual development of dimers and other oligomers of LDL which coexist with monomers during Cu^{2+} oxidation, whereas larger oligomers of LDL and smaller amounts of dimers appear to be formed within a short time during vortexing.

SDS/PAGE analysis of the time course of LDL oxidation

In order to correlate the structural changes seen by neutron scattering with the time course of biochemical degradation of LDL, SDS/PAGE was performed to assess the integrity of the apoB protein of LDL during oxidation. Figure 7 shows Coomassie Blue-stained SDS/polyacrylamide gels after electrophoresis of 2 μg aliquots of apoB protein obtained from the time course of LDL oxidation initiated by 6.4, 25.6 and 51.2 μmol of Cu^{2+} /g of apoB. In agreement with the protein sequence and its glycosylation (glycosylated M_r 536 000), Figure 7(a) shows apoB as a single discrete band of M_r 550 000 up to 4 h after initiation of oxidation with 6.4 μmol of Cu^{2+} /g of apoB. From 4 h onwards, smaller M_r fragments became visible. The M_r values of these smaller bands are between 180 000 and 260 000, and are therefore in good agreement with the oxidation data of Fong et al. [10] using 5 μmol of Cu^{2+} with 0.1 mg of apoB/ml and those of Sattler et al. [12] using 16 μmol of Cu^{2+} with 0.25 mg of apoB/ml. After oxidation for 16.5 h, the intact apoB band at M_r 550 000 had disappeared, to be replaced by a broad band of lower M_r . Similar events took place on oxidations with 25.6 and 51.2 μmol of Cu^{2+} /g of apoB, but at faster rates (Figures 7b and 7c). Here, the native apoB band was replaced by a broad diffuse band of lower M_r after 6 h. Smaller fragments of M_r 180 000–260 000 were produced at all the Cu^{2+} concentrations. In Figures 7(b) and 7(c), high- M_r products can be seen at the top of the gel, which indicates that covalent aggregation of apoB protein may have occurred. The intensity of this aggregation is increased at a Cu^{2+} concentration of 51.2 compared with that at 25.6 μmol of Cu^{2+} /g of apoB, although this cannot be quantified as the aggregates do not appear to run completely into the gel matrix. In the non-

reducing gels, intact LDL ran as a broad band indicating heterogeneity. A small amount of material, which may represent aggregates in the relatively concentrated solutions of LDL, was observable at the top of the gel where it had not entered (not shown). The amount of this material increased with increasing oxidation and was also present when lower concentrations of Cu^{2+} were used (not shown).

Degradation and formation of high- M_r forms of apoB during Cu^{2+} oxidation in a different buffer system were also reported by Bellamy et al. [11]. SDS/PAGE data and those from the neutron-scattering experiments can be explained on the basis that LDL aggregation increases progressively with time. During the first 5 h, it appears to be non-covalent and involves the formation of LDL dimers at least. This is supported by the demonstration of high- M_r material which did not enter the non-reducing gels. Non-covalent aggregation may contribute more than covalent aggregation at lower concentrations of Cu^{2+} when the rates of oxidation are slower. After formation of these aggregates, degradation of LDL into smaller fragments after 10 h appears to coincide with the further association and fusion of LDL particles into larger particles. The observation of covalent high- M_r protein aggregates on SDS/PAGE after 10 h with 51.2 μmol of Cu^{2+} /g of apoB is also consistent with the formation of larger LDL aggregates during the neutron-scattering experiment. Solution scattering is sensitive to the presence of aggregates as the Guinier $I(0)$ parameter is proportional to M_r^2 . This, however, means that the neutron-scattering data are insensitive to the possible formation of small amounts of low- M_r fragments of LDL during oxidation, and no information on the presence or absence of these can be deduced, although the existence of large quantities of small LDL fragments can be ruled out.

Time course of uptake of oxidized LDL by macrophages

In order to correlate the structural changes seen by neutron scattering with the biological affinity of oxidized LDL, the uptake of oxidized LDL by macrophages and its degradation to soluble ^{125}I -labelled peptides was determined. Macrophages and monocytes have few high-affinity receptors for LDL, but readily

Figure 8 Time course of degradation of ^{125}I -LDL samples by mouse peritoneal macrophages

The amount of trichloroacetic acid-soluble LDL protein/mg of macrophage is shown as a function of the time after initiation of oxidation. LDL (2 mg of apoB/ml) was incubated with 6.4 (●), 25.6 (■) or 51.2 (▲) μmol of Cu^{2+} /g of apoB together with a 0 μmol of Cu^{2+} sample as a control (○), for periods between 0 and 24 h. Results are means $\pm$ S.D.

take up chemically modified LDL through the scavenger receptor [34,35]. Figure 8 shows the formation of ^{125}I -labelled trichloroacetic acid-soluble products from macrophage-degraded oxidized LDL treated at three different Cu^{2+} concentrations. A lag phase of 2 h was observed on oxidation with 6.4 or 25.6 μmol of Cu^{2+} /g of apoB, which decreased to 1 h for the oxidation with 51.2 μmol of Cu^{2+} /g of apoB. At the time points shown, the maximum uptake and degradation of LDL found with 6.4 and 25.6 μmol of Cu^{2+} /g of apoB occurred at 10 h, whereas for the oxidation initiated with 51.2 μmol of Cu^{2+} , maximum uptake and degradation occurred after 5 h. In all three experiments, degradation by macrophages decreased on prolonged oxidation with Cu^{2+} .

The changes occurred at a relatively early stage of oxidation, indicating that only partial modification of apoB was necessary before macrophage uptake and degradation of modified LDL was achieved. Comparing the aggregation observed by neutron scattering and the changes detected by SDS/PAGE, it can be seen that the initial association of oxidized LDL particles to form non-covalent aggregates coincides with the period of maximal uptake by the macrophage assay. The reduction in degradation may be due to one of two effects. Oxidized LDL are resistant to lysosomal proteolysis and may accumulate in the cells. Alternatively LDL uptake is diminished after prolonged incubation with Cu^{2+} ; however, there is no experimental evidence for this.

Conclusions

The time-course studies offer for the first time a continuous picture of the averaged overall structure of LDL during Cu^{2+} -initiated oxidation under conditions close to physiological. Because of improvement in the methodology of neutron-scattering-data collection, oxidation at an LDL concentration of 2 mg of apoB/ml could be studied. This is close enough to physiological for the experiments to be biologically relevant, and they were unaffected by concentration effects that readily influence scattering experiments on LDL [19]. Normally LDL are found at 0.69 ± 0.23 mg of apoB/ml in plasma [36], but this can double to 1.39 ± 0.34 mg of apoB/ml in the subendothelial (intimal) space which is where oxidation is thought to occur [37].

Data collection in $^2\text{H}_2\text{O}$ buffers was a necessary feature of the neutron-scattering experiments because this minimized the background level of incoherent scattering (thus permitting data collection times to be 15 min per run during the time course). This solvent showed LDL as a particle of almost uniform internal scattering density, i.e. these experiments investigate principally the overall shape of LDL without significant influence from the internal arrangement of protein and lipid within the LDL structure. A particular merit of the neutron-scattering data is that the development of aggregated forms of LDL could be separately determined from the amounts of LDL monomers by $P(r)$ analysis. The characteristic time course of the growth of LDL aggregates approximately follows first-order kinetics, and both the rate of this and the extent of structural change over 50 h is dependent on the Cu^{2+} concentration. By these means, during the 50–70 h of an oxidation experiment, neutron scattering showed that LDL underwent a continuous process of aggregation into dimers and higher oligomers. Complementary biochemical assays throughout the oxidation time course under conditions identical with those used for neutron-scattering-data collection (except that $^2\text{H}_2\text{O}$ solvents were used) enabled the structural changes to be correlated with biochemical events.

The vortexing experiments show that a different set of structural events occur during this type of LDL aggregation. By electron microscopy, Guyton et al. [22] show that vortexed LDL contain comparatively large droplets of neutral lipid up to 600 nm in size which are interspersed with lipid vesicles and intact LDL particles. These aggregates contain most of the apoB protein. Substantial fractions of phospholipid and free cholesterol from the original LDL remain in solution. Even though LDL that are aggregated by oxidative processes or by vortexing are readily taken up by macrophages, it cannot be concluded that the vortexing of LDL is representative of the structural changes that take place during Cu^{2+} -induced aggregation.

Aggregation of LDL is detectable by neutron scattering within the first few hours of oxidation. Consumption of α -tocopherol and β -carotene within LDL provides a marker for the relative appearance of aggregates, together with X-ray-scattering data on the internal lipid-protein structure of LDL ([22a]; D. F. Meyer, C. H. MacPhee, P. H. E. Groot, K. E. Suckling, K. R. Bruckdorfer and S. J. Perkins, unpublished work). These experiments show that different amounts of thiobarbituric acid-reacting substances and lipid hydroperoxide-oxidation products result from different Cu^{2+} concentrations, thereby showing that in the first 10 h the appearance of these products correlates with different amounts of aggregation as observed by neutron scattering and SDS/PAGE. Complementary SDS/PAGE analysis demonstrates that covalent aggregates occur at later stages of oxidation at higher Cu^{2+} concentrations (Figure 7). Neutron scattering thus detected the formation of non-covalent aggregates at a very early stage in Cu^{2+} -initiated oxidation before covalently linked apoB and degraded apoB protein are generated. It is known that apoB is modified at relatively early stages of oxidation. Radiolabelling studies [39] show that aldehydes from the breakdown of lipid hydroperoxides are located in the apoB protein of oxidized LDL. The covalent binding of aldehydes to positively charged lysine residues causes a net increase in negative charge, as seen by an increased relative electrophoretic mobility [35,40], and this mediates the formation of covalent aggregates through cross-linking. Fragmentation of apoB observed by SDS/PAGE could result from the direct oxidation of the apoB polypeptide chain by bound Cu^{2+} ions or by oxidative attack by lipid alkoxyl radicals [16,41].

In the macrophage assays, the higher the Cu^{2+} concentration, the greater the degradation of LDL to trichloroacetic acid-

soluble products in the first 10 h of oxidation (Figure 8). A common maximum value would have been expected, as variation of the Cu^{2+} concentration does not seem to have effects other than speeding up the oxidation process in the early stages (lag and propagation phase). Larger amounts of aggregates are detected by neutron-scattering analysis with increases in Cu^{2+} concentrations (Figures 1b and 1c). The initial process of non-covalent aggregation observed by neutron scattering is thus associated with the maximum uptake of LDL on the evidence of the macrophage-degradation assays. It is of interest that, after 24 h, all three Cu^{2+} concentrations led to similar lower amounts of LDL-degradation products (Figure 8), as this implies that the oxidized LDL samples are in a similar state after that time.

Other evidence shows that both biochemical modification of LDL and its aggregation are significant for its uptake by macrophages. It is known that modification of LDL with 4-hydroxynonenal, an oxidation product of lipid peroxidation, causes the formation of LDL aggregates. These aggregates are not taken up by scavenger receptors, but instead via phagocytosis [42]. The latter authors reported that lipoprotein aggregation is an essential condition for intracellular lipid accumulation, these processes showing a direct and strong correlation ($r = 0.86$). A tendency to aggregate is seen in LDL with an increased negative charge [43]. Such an increase in negative charge occurs as a result of covalent binding of breakdown products of lipid hydroperoxides to positively charged lysine residues. Whether aggregation of LDL occurs *in vivo* has not been demonstrated, but it is a possible explanation for uptake of oxidatively modified LDL by macrophage scavenger receptors. It has been shown [21] that aggregated LDL produced by vortexing are taken up by phagocytosis. It has also been shown that both oxidized monomeric LDL and oxidized aggregated LDL are taken up faster by macrophages [44]. Uptake may also be explained by the formation of immune complexes with antibodies against LDL aggregates, which are then recognized and taken up by the macrophage Fc receptor [45]. Non-specific endocytosis may also be important for macrophage uptake. The ultrastructural appearance of LDL aggregated by vortexing is remarkably similar to the appearance of extracellular lipid deposits in atherosclerosis [22].

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