Oxidation of low-density lipoprotein by hypochlorite causes aggregation that is mediated by modification of lysine residues rather than lipid oxidation

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Peroxidation of low-density lipoprotein (LDL) lipid is generally thought to represent the initial step in a series of modification reactions that ultimately transform the protein moiety of the lipoprotein into a form recognized by receptors different from those that bind native LDL. Uptake of LDL via these alternative receptors can lead to the formation of lipid-laden cells, which are typical for the early stages of atherogenesis. We have studied the oxidative modification of LDL by hypochlorite (^-OCl), a powerful oxidant produced from H_2O_2 and chloride via the action of myeloperoxidase which is released from activated neutrophils and monocytes. Exposure of LDL to reagent or enzymically generated ^-OCl at 4 or 37 °C resulted in immediate and preferential oxidation of amino acid residues of apolipoprotein B-100, the single protein associated with LDL. Lysine residues quantitatively represented the major target and, like tryptophan, were oxidized to approximately the same extent with reagent or enzymically generated ^-OCl . In contrast, LDL lipid oxidation was less favoured than protein oxidation, as judged by the

amounts of lipid hydroperoxides, chlorohydrins, cholesterol or fatty acid oxidation products formed. Treatment with ^-OCl caused aggregation of LDL, as shown by an increased turbidity of the oxidized LDL solution and elution from a size-exclusion h.p.l.c. column of high-molecular-mass LDL complexes. Chemical modification of lysine residues before oxidation with ^-OCl prevented aggregation, while it enhanced the extent of lipid peroxidation. Treatment of LDL with ^-OCl also caused the formation of carbonyl groups and release of ammonia; both these modifications were inhibited by lysine-residue modification before oxidation. These results demonstrate that aggregation reactions are dependent on initial lysine oxidation by ^-OCl , followed by deamination and carbonyl formation, but do not involve lipid (per)oxidation. We propose that the observed ^-OCl -mediated aggregation of LDL is caused, at least in part, by cross-linking of apoproteins by Schiff-base formation independently of lipid peroxidation.

INTRODUCTION

An elevated level of low-density lipoproteins (LDL) is a known risk factor for atherosclerosis, as most of the intracellular lipid accumulating in macrophages beneath the endothelium is thought to be derived from LDL, and formation of lipid-laden macrophages (or foam cells) is an important and early event in atherogenesis [1,2]. Cellular uptake of LDL particles normally occurs via the LDL-receptor pathway, which is tightly controlled in a way that does not allow formation of foam cells [1]. However, after certain types of modification, high-uptake forms of LDL are generated that can lead to foam-cell formation [3]. Despite much research, the precise nature, origin and site of production of the responsible modifying agent(s) remain unknown.

Monocytes/macrophages are thought to be important in the development of atherosclerosis, because they are present in large numbers in fatty streaks and early and late lesions, and may modify LDL to a high-uptake form *in vivo*. Formation of lipid hydroperoxides is generally assumed to represent the initial step and to be a prerequisite for oxidative modification of LDL *in vivo* and *in vitro* [3]. In fact, many groups use lipid (per)oxidation almost exclusively as the index of oxidative LDL modification. It has been shown by studies *in vitro* that various cells, including monocytes and macrophages, are capable of (per)oxidizing LDL lipids via radical reactions. These reactions appear to be dependent on transition metals, whether present in the media [4] or added by other means [5], although it is by no means certain that

such high concentrations of metal would be available *in vivo*. Modifications independent of lipid peroxidation have received little attention, although enzymes such as lipases [6], sphingomyelinase [7] and macrophage proteases [8], as well as physical aggregation [9], can modify LDL in a way that it causes lipid uptake by macrophages.

Upon appropriate stimulation, monocytes can release large amounts of myeloperoxidase (MPO, EC 1.11.1.7) into the extracellular space [10,11], although they lose this ability as they differentiate into macrophages [11]. MPO reacts with H_2O_2 to form, initially and transiently, compound I, which itself can react with Cl^- ions to form hypochlorite (^-OCl) [12] or, depending on the availability of substrates, participate in one-electron reactions [13,14]. Thus MPO plus H_2O_2 can oxidize many different compounds, including proteins, lipids, nucleotides and anti-oxidants.

During our survey of the effect of different physiological oxidants on the early stages of LDL oxidation, we observed that exposure to reagent ^-OCl transformed the lipoprotein into a form which caused extensive lipid accumulation in macrophages [15]. We observed immediate and almost exclusive modification of the protein, whereas only small amounts of hydroperoxides of cholesteryl ester (CEOOH) and phospholipids were formed, suggesting that ^-OCl reacted with protein in preference to lipid. The results of the present study demonstrate that, in addition to CEOOH, neither cholesterol oxidation products nor chlorohydrins were major products of LDL oxidation by ^-OCl . We also observed that treatment of LDL with ^-OCl resulted in lipoprotein

Abbreviations used: apo, apolipoprotein; CEOOH, cholesteryl ester hydroperoxides; DNPH, 2,4-dinitrophenylhydrazine; LDL, low-density lipoproteins; MPO, myeloperoxidase; ^-OCl , sodium hypochlorite or hypochlorous acid, since a mixture of these two species is present at pH 7.4.

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aggregation, carbonyl formation and release of ammonia. Because all of these processes were inhibited by modification of the lysine residues before oxidation, aggregation of LDL by $\cdot\text{OCl}$ requires lysine-residue modification, and this is likely to involve Schiff-base formation, potentially giving rise to apoprotein cross-links.

MATERIALS AND METHODS

Materials

Nanopure water was used for all buffers and aqueous solutions, which were subsequently treated with Chelex-100 (Bio-Rad) to remove contaminating transition metals. Heparinized Vacutainers were obtained from Becton-Dickson; bicinchoninic acid, fluorescamine, 5,5'-dithiobis-(2-nitrobenzoic acid), methionine, 2,4-dinitrophenylhydrazine (DNPH), phospholipase A_2 (from pig pancreas), cholesterol esterase from Sigma, and reagent $\cdot\text{OCl}$ (5% available chlorine minimum) from Aldrich. Phosphate (50 mM)-buffered saline (PBS) was used, with EDTA (0.1%, w/v) for LDL isolation and without EDTA for gel filtration and resuspension of the lipoprotein. Organic solvents were of h.p.l.c. quality (Mallinckrodt) or the highest grade available. MPO [purity index = 0.75 (A_{430}/A_{280})] was kindly provided by Dr. Anthony Kettle, Christchurch School of Medicine, Christchurch, New Zealand. The concentration of H_2O_2 was determined by its absorbance ($\epsilon_{240} = 43.6 \text{ M}^{-1} \cdot \text{cm}^{-1}$) [16].

LDL preparation and oxidation

Blood was obtained freshly from non-fasted healthy normolipidaemic male or female subjects (23–35 years old) and drawn into heparinized Vacutainers. LDL was isolated from plasma by ultracentrifugation by a rapid analytical method (2 h, 15 °C) [17] and gel-filtered (PD-10 column, Pharmacia), and its protein concentration was determined [18] with BSA (Sigma Diagnostics) as standard.

'Bolus oxidation' with reagent $\cdot\text{OCl}$ was carried out on ice and at final pH 7.4 by addition of 1 vol. of reagent $\cdot\text{OCl}$, freshly diluted in phosphate buffer (50 mM), to 4 vol. of LDL solution (final concn. 0.5–2.0 mg of protein/ml). The concentration of the total amount of oxidizing equivalents present in the diluted commercial $\cdot\text{OCl}$ solution was determined as described previously [15]. After addition of $\cdot\text{OCl}$, the reaction mixture was mixed briefly (< 1 s) and left on ice for 15 min before further treatment, unless otherwise indicated.

'Aliquot oxidation' was carried out at 37 °C with LDL in PBS at a final concentration of 40 nM apolipoprotein (apo) B-100. The LDL was preincubated at 37 °C for 10 min before MPO (final concn. 0.3 mg/l) or an equivalent volume of water (for H_2O_2 - and $\cdot\text{OCl}$ -oxidized samples) was added. Oxidation was initiated by addition of small and equimolar amounts of H_2O_2 (for H_2O_2 - and MPO-oxidized samples) or $\cdot\text{OCl}$ at 1.5 min intervals with brief mixing after each addition. Under these conditions, H_2O_2 was consumed by MPO within 1 min, and the maximum H_2O_2 concentration in MPO-containing reaction mixtures was 2.5 μM . Samples were protected from light between additions of oxidants. The total incubation time after the start of oxidation was 15 min, after which samples were placed in ice. Results shown in the Figures and Tables are derived from the bolus-oxidation method unless indicated otherwise.

Measurement of antioxidants and lipid content of LDL

LDL's content of cholesterol, unoxidized cholesteryl esters, CE OOH and lipid-soluble antioxidants (ubiquinol-10, α -toco-

pherol, lycopene, α - and β -carotene) were determined as described previously [17]. To minimize loss of ubiquinol-10 due to autooxidation, extractions were carried out as rapidly as possible and extracts were stored at -80°C until used.

The extent of cholesterol oxidation and fatty acid chlorohydrin formation was determined by g.l.c./m.s. CaCl_2 (final concn. 2 mM) and phospholipase A_2 (38 units or 50.4 μg) were added to 10 ml of 40 nM LDL (for the aliquot-oxidation method) or 500 μl of 1.4 mM LDL (for the bolus-oxidation method) and the mixture incubated at 37 °C for 30 min. Taurocholic acid (final concn. 6 mM) and cholesterol esterase (1 unit or 1.06 mg) were then added, and the samples were incubated for a further 30 min at 37 °C and then acidified to pH 2.0 with HCl and extracted with 4 $\times$ 2 ml of dichloromethane. The combined dichloromethane (8 ml) containing fatty acids and cholesterol was washed with 0.1 M HCl (2 ml) and then evaporated, redissolved in methanol, sealed under N_2 and kept at -80°C until derivative formation. Production of derivatives of the samples and g.l.c./m.s. analyses of cholesterol oxidation products, fatty acid chlorohydrins and other fatty acid oxidation products were carried out as described previously [19].

Quantification and modification of amino acid residues

Unmodified lysine and tryptophan residues in LDL were quantified as described in [15]. Reductive methylation was carried out in 50 mM phosphate buffer, pH 9.0, as described previously [20]. Briefly, isolated LDL (1–2 mg/ml) was transferred to this buffer by gel-filtration and divided into three portions: control (no additions), reduced (NaBH_4 only), and reduced and methylated (additions of NaBH_4 and formaldehyde). NaBH_4 (final concn. approx. 1 mg/mg of LDL) was added in two or three aliquots before and part way through the addition of a large number of small aliquots of formaldehyde (6 M, total amount $\sim 1 \times 10^5$ mol of formaldehyde/mol of LDL) with 5 min between additions. Samples were acidified to pH 5.0 and then separately gel-filtered into PBS, pH 7.4. This treatment resulted in modification of 70–90% of apo B's lysine residues. After protein determination, the variously modified samples were adjusted to the same protein concentration and divided into smaller aliquots before being oxidized with bolus $\cdot\text{OCl}$.

To determine carbonyl groups, control, reduced and reduced plus methylated LDL samples were oxidized (final concn. 1.6 μM apo B-100) before an equal volume of DNPH (1 mM in 1 M HCl) was added. After incubation for 30 min at 50 °C, 1.5 vol. of 1 M NaOH was added and the A_{450} was measured. An average molar absorption coefficient of $22000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was used to estimate the carbonyl content [21]. For ammonia determination, the treated and oxidized samples were incubated at 37 °C for 2 h before an excess of methionine was added to each sample. Free ammonia concentrations were then measured with a RAICHEM kit (Baxter Diagnostics).

Alterations to LDL particles

Changes in surface charge of LDL were measured by agarose-gel electrophoresis [15]. LDL aggregation was determined by either h.p.l.c. gel filtration or turbidity measurements. H.p.l.c. was carried out on a LKB Ultropac TSK G4000SW column (60 cm $\times$ 0.75 cm) with a guard column (0.7 cm $\times$ 7.5 cm; SW Progrel-TSK) eluted with PBS, pH 7.4, at 0.5 ml/min [22] and monitored at 210 or 280 nm. For turbidity, the A_{680} of the treated and oxidized samples was measured.

Figure 1 Increased concentration of CE00H in reagent OCl^- -oxidized LDL incubated at 37 °C rather than 4 °C

(a) Samples were oxidized with the amounts of OCl^- indicated and incubated for 15 min at 37 °C or on ice before extraction and lipid analysis. (b) Samples were oxidized with reagent OCl^- /LDL (ratio 480–530) and incubated at 37 °C for the indicated length of time before extraction and analysis. The data shown are means $\pm$ S.D. of three separate experiments (for both a and b) using LDL isolated from different donors.

Table 1 Comparison of amounts of CE00H produced in LDL oxidized (by the aliquot method) with reagent OCl^- , MPO/ H_2O_2 / Cl^- , or treatment with H_2O_2 alone as a control

CE00H were measured by h.p.l.c. with post-column chemiluminescence detection (see the Materials and methods section). The three experiments shown were carried out independently with LDL from different donors.

Treatment	OCl^- /LDL	CE00H/LDL particle (mol/mol)		
		Expt. 1	Expt. 2	Expt. 3
OCl^-	0	0.26	0.11	0.20
	250	0.69	0.91	0.74
	500	1.24	1.70	2.13
H_2O_2	0	0.31	0.14	0.50
	250	0.07	0.17	0.15
	500	0.10	0.42	0.23
MPO/ H_2O_2 / Cl^-	0	0.40	0.27	0.45
	250	4.03	3.78	6.95
	500	16.2	4.30	8.40

RESULTS

We have shown previously [15] that oxidation of LDL with reagent OCl^- at 4 °C results in formation of a 'high-uptake' lipoprotein, with only small amounts of lipid hydroperoxides detected. The results in Figure 1 confirmed and extended these findings. Thus, although the amounts of CE00H produced when LDL was exposed to reagent OCl^- were greater at 37 °C

Figure 2 Small amounts of cholesterol oxidation products are measurable by g.l.c./m.s. only at the highest concentrations of OCl^- investigated (1000 OCl^- /LDL)

LDL samples were oxidized with reagent OCl^- at 4 °C for 15 min before extraction, derivative formation and analysis as described in the Materials and methods section: (a) native LDL; (b) LDL oxidized with 1000 OCl^- /LDL. The identities of the peaks are as follows (relative amounts of product as a percentage of the total peak area of cholesterol plus products): 1, cholesterol (5 α -cholesten-3 β -ol); 2, 6 β -hydroxycholesterol (4-cholesten-3 β ,6 β -diol) (1%); 3, cholesterol 5 β ,6 β -epoxide (5 β ,6 β -epoxycholestan-3 β -ol) (4%); 4, cholesterol 5 α ,6 α -epoxide (5 α ,6 α -epoxycholestan-3 β -ol) (4%); 5, 4 β -hydroxycholesterol (5-cholesten-3 β ,4 β -diol) (3%); 6, 7, unrelated compounds.

than at 4 °C (Figure 1a), very little of the total oxidant added was consumed in such reactions, even when assuming that lipid peroxidation did not proceed via a chain reaction. The amounts of CE00H formed at 37 °C increased with time (Figure 1b), but reached a plateau after a length of time which depended on the initial OCl^- /LDL ratio. Thus LDL incubated with a ratio of 300 or 1000 OCl^- /LDL reached a plateau after 1 h or 4 h respectively (results not shown). There was no discernible difference in the extent of consumption of LDL's antioxidants (ubiquinol-10, α -tocopherol, lycopene, β -carotene and α -carotene) between oxidation at 4 and 37 °C (results not shown).

MPO produces OCl^- by a two-electron oxidation reaction. However, the enzyme is also able to cause one-electron oxidation reactions when appropriate substrates are available. MPO's one-electron reactions appear to be competitive with, or indifferent to, the presence of Cl^- , but not dependent on it [13,14]. Therefore it appeared possible that radical reactions could be more prevalent during oxidation of LDL by MPO/ H_2O_2 / Cl^- than by reagent OCl^- . Although the absolute values varied, the data in Table 1 show that the amounts of CE00H formed per LDL molecule were indeed larger when the lipoprotein was exposed to MPO/ H_2O_2 / Cl^- compared with reagent OCl^- . Reagent H_2O_2 alone resulted in very little peroxidation of the lipoprotein's lipids, consistent with previous reports by others [23]. However, although treatment of LDL with MPO/ H_2O_2 / Cl^- resulted in nearly 6-fold larger amounts of CE00H formed than with reagent OCl^- , cholesteryl ester peroxidation still accounted for only a minor proportion (3.2%) of the total oxidant consumed. The amounts of CE00H detected were unchanged in the absence or presence of Cl^- , and were similar to the amounts of phospholipid hydroperoxides formed (results not shown).

Our results show that formation of lipid hydroperoxides does not represent a major reaction of reagent or enzymically produced

Figure 3 $\text{MPO}/\text{Cl}^-/\text{H}_2\text{O}_2$ (continuous line) oxidizes amino acid residues of apo B-100 to a similar extent as reagent $^- \text{OCl}$ (broken line)

LDL samples were oxidized, as described in the Materials and methods section, by the bolus method for reagent $^- \text{OCl}$ -treated LDL and by the aliquot method for MPO-treated LDL. (a) Lysine was measured by the fluorescamine assay and the results were calculated assuming 356 lysine residues per LDL particle. (b) Tryptophan was measured by its fluorescence and the results were calculated assuming 37 tryptophan residues per LDL particle. The results shown represent the mean values $\pm$ S.D. from 4 independent experiments (for both a and b) with LDL isolated from different male and female donors.

$^- \text{OCl}$ with LDL. These results do not exclude the possibility that lipid-oxidation products other than lipid hydroperoxides are formed. Of particular interest are fatty acid chlorohydrins, which are known to be formed when isolated fatty acids or phospholipids are treated with hypochlorite [19,24]. However, g.l.c./m.s. analysis of the lipids from $^- \text{OCl}$ -treated LDL showed no detectable chlorohydrins, even when a high ratio of 1000 $^- \text{OCl}$ /LDL was used (Figure 2). If more than 0.5% of the individual fatty acids were present as chlorohydrins, they would have been detected. G.l.c./m.s. analysis further showed that significant amounts of cholesterol-oxidation products were also not observed except at the highest $^- \text{OCl}$ concentration used (1000 $^- \text{OCl}$ /LDL). In this case, $\sim 12\%$ of LDL's cholesterol had reacted, on the basis of comparison of peak areas on g.l.c./m.s. The cholesterol oxidation products observed were 6 β -hydroxycholesterol, cholesterol 5 β ,6 β -epoxide, cholesterol 5 α ,6 α -epoxide and 4 β -hydroxycholesterol, as was the case with $^- \text{OCl}$ -oxidized cholesterol-containing model membranes [19,24]. Although g.l.c./m.s. is able to detect a number of fatty acid oxidation products, including fatty acid epoxides, hydroxy and dihydroxy derivatives, at concentrations lower than 10 nM, none of these was present in $^- \text{OCl}$ -oxidized LDL.

We have shown previously that amino acid residues of apo B-100, particularly lysine, quantitatively represent LDL's major target for oxidation by reagent $^- \text{OCl}$ [15]. Figure 3 shows the comparison between modification of lysine and tryptophan in LDL oxidized either by reagent $^- \text{OCl}$ or by $\text{MPO}/\text{H}_2\text{O}_2/\text{Cl}^-$; the variations between different LDL preparations are larger than

Figure 4 Oxidation of LDL with reagent $^- \text{OCl}$ causes aggregation (continuous line) of the lipoprotein that can be reversed by addition of methionine 15 min after oxidation (broken line)

Samples were oxidized by the bolus method, separated by h.p.l.c. gel filtration (see the Materials and methods section) and the eluate was monitored at 210 nm. Qualitatively similar results were obtained when the eluate was monitored at 280 nm. The peak eluted at ~ 55 min (c) represents methionine, present in the low-molecular-mass fraction. The aggregation with $^- \text{OCl}$ was repeated four times, and the de-aggregation with methionine was repeated twice. All repetitions gave similar results with LDL from different donors.

the variation between the reagent and enzyme treatments. Also, no lag phase in the loss of lysine and tryptophan residues was observed, indicating no antioxidant protection or preferential oxidation of another target. Thus the lysine residues of apo B-100 represent the major single target in LDL for $^- \text{OCl}$, independent of whether reagent or enzymically produced oxidant is used. We concluded that reagent $^- \text{OCl}$ was a suitable 'model' for $\text{MPO}/\text{H}_2\text{O}_2/\text{Cl}^-$ oxidation of LDL, in agreement with a previous report [25], and used the former for all subsequent experiments.

Oxidation of LDL with $^- \text{OCl}$ results in formation of a 'high-uptake' LDL [15], i.e. a form of LDL that, when exposed to macrophages, causes intracellular accumulation of lipids. Several types of modification of LDL can cause such an effect, including a wide variety of lysine- or arginine-modifying reagents [26,27] and lipoprotein aggregation [7,9]. Exposure of LDL to increasing amounts of $^- \text{OCl}$ gave rise to higher-molecular-mass aggregates of increasing size, absorbing at 210 nm (Figure 4). Thus at 600 $^- \text{OCl}$ /LDL most of the LDL was eluted with the void volume of the gel-filtration column (Figure 4c), whereas at 300 $^- \text{OCl}$ /LDL (Figure 4b) a considerable amount of material was eluted with a retention time between the void volume and that of native LDL (Figure 4a). Similar results (not shown) were obtained when the eluate was monitored at 280 nm, indicating that the lipid and protein moieties of the $^- \text{OCl}$ -treated LDL aggregates remained

Figure 5 Oxidation of LDL with reagent ^-OCI causes aggregation of the lipoprotein which can be prevented by the reductive methylation of LDL

For details, see the Materials and methods section. Turbidity was expressed as the difference in absorbance between oxidized and unoxidized samples. Symbols: control LDL (●), LDL that had been treated with $NaBH_4$ only (▲) or with formaldehyde and $NaBH_4$ (△). The results shown are means $\pm$ S.D. of four separate experiments carried out with LDL from different donors.

Table 2 Formation of CEOOH by treatment of LDL with reagent ^-OCI can be increased by the reductive methylation of LDL before oxidation

Samples were oxidized by the bolus method at 4 °C. CEOOH were measured by h.p.l.c. with post-column chemiluminescence detection (see the Materials and methods section). The three experiments shown were carried out independently with LDL from different donors; n.d., not determined.

Treatment	OCI-/LDL	CEOOH/LDL particle		
		Expt. 1	Expt. 2	Expt. 3
Control	0	0.05	0.01	0.05
	103	n.d.	n.d.	0.08
	209	0.15	n.d.	n.d.
	416	0.35	0.17	0.13
	625	0.52	0.35	0.26
Reduced	0	0.03	0.00	0.01
	103	n.d.	n.d.	n.d.
	209	0.24	n.d.	n.d.
	416	0.32	0.21	0.11
	625	0.65	0.37	0.18
Reduced and methylated	0	0.06	0.00	0.02
	103	n.d.	n.d.	0.391
	209	1.92	n.d.	n.d.
	416	5.48	3.13	2.69
	625	18.9	9.10	4.44

combined throughout gel-filtration. Aggregation was almost completely reversed by addition of methionine 15 min after ^-OCI oxidation of LDL at 4 °C (Figure 4c). Addition of lysine had a similar, yet less pronounced, result (not shown) on LDL de-aggregation.

A serious problem associated with gel-filtration was that column performance increasingly deteriorated as a result of multiple injection of ^-OCI -treated LDL samples. Deterioration of the column performance was characterized by increasingly broader eluted peaks, materials remaining on the column, and increasing pressure. These problems were not overcome by any of the washing procedures suggested by the manufacturers (results not shown). Such deterioration was observed with two

Figure 6 Antioxidant consumption in reagent ^-OCI -treated LDL (1.6 mM) is increased by the reductive methylation of LDL before oxidation

For details, see the Materials and methods section. Symbols are as described in the legend to Figure 5. Antioxidants were analysed in the organic-phase extracts of LDL (see the Materials and methods section) and expressed as a percentage of their initial concentrations, which were: α -tocopherol, $1.05 \pm 0.33 \mu M$; lycopene, $18.5 \pm 6.3 nM$; β -carotene, $49.7 \pm 13.6 nM$. The results shown represent means $\pm$ S.D. of three independent experiments with LDL from different donors.

different columns of the same gel type, and was the result of ^-OCI treatment of LDL, as it was not observed after multiple (> 50) injections of native LDL, high-density lipoprotein or very-low-density lipoprotein (results not shown). Owing to the high costs associated with regular replacement of the h.p.l.c. columns, we therefore assessed LDL aggregation by turbidity measurement, as done previously by others [9].

Figure 5 shows that treatment of control LDL with ^-OCI resulted in a concentration-dependent increase in turbidity. In contrast, LDL whose lysine residues were reductively methylated before exposure to ^-OCI did not aggregate, as indicated by a lack of increase in turbidity. The lack of aggregation was not due to some conformational or chemical changes caused by the $NaBH_4$ reduction that precedes methylation, since samples treated with $NaBH_4$ but not formaldehyde aggregated in the same way upon ^-OCI oxidation as did untreated LDL. These results demonstrated that ^-OCI -mediated modification of apo B's lysine residues was required for the observed oxidant-induced aggregation of LDL.

Although reductive methylation of apo B's lysine residues strongly decreased the extent of ^-OCI -mediated aggregation of

Figure 7 Oxidation of LDL with reagent ^-OCl causes the production of carbonyl groups and ammonia, which can be prevented by the reductive methylation of LDL

For details, see the Materials and methods section. Symbols are as described in legend to Figure 5. (a) The amount of carbonyl groups was measured by using DNPH and calculated by using an absorption coefficient for the reaction product of an 'average carbonyl' (see the Materials and methods section). (b) Free ammonia was determined by the RAICHEM enzyme assay and measuring consumption of NADPH. The results shown represent means $\pm$ S.D. of three experiments with LDL from different donors.

LDL, such treatment increased (per)oxidation of lipid components of the lipoprotein. Thus, compared with native LDL, ^-OCl oxidation of reductively methylated LDL resulted in significantly higher rates of both formation of CEOOH (Table 2) and consumption of lipid-soluble antioxidants (Figure 6). This provided direct evidence that peroxidative modification of LDL's lipids was not involved in ^-OCl -mediated aggregation of the lipoprotein. Rather, the protein appeared to protect the lipid and lipophilic antioxidants from oxidation by ^-OCl . The cause of the variation between LDL samples (Table 2) is not known.

Oxidation of amines has been suggested to produce aldehydes that may cause cross-linking [28] through reaction with an ϵ -amino group (to form a Schiff base) or other residues (such as histidine). To assess aldehyde formation, we measured carbonyl groups in control and ^-OCl -treated LDL and found their content to increase with increasing oxidant concentration (Figure 7a). The amounts of carbonyl groups detected corresponded to $\sim 17\%$ of the chloramines formed. Carbonyl formation was inhibited partially by reductive methylation of LDL before oxidation, suggesting that some carbonyl groups of the reductively methylated LDL did not originate from lysine residues. Lipid peroxidation products could contribute to these non-lysine-derived carbonyl groups, as they are produced in increased amounts in reductively methylated LDL (Table 2) and can give rise to aldehydes.

Incubation of ^-OCl -oxidized LDL at $37^\circ C$ resulted in a concentration-dependent formation of ammonia (Figure 7b) that

was inhibited completely by reductive methylation of LDL before its oxidation. The absolute amount of carbonyls and ammonia measured differ quantitatively. This may be due to the lipophilic and volatile nature of ammonia or to the formation of additional carbonyl groups during the assay procedure.

DISCUSSION

In biological systems, ^-OCl is produced from H_2O_2 and Cl^- via the action of MPO with the initial formation of compound I. The latter can undergo one-electron redox reactions and hence could conceivably initiate lipid peroxidation. Consistent with this, we observed larger amounts of CEOOH when LDL was oxidized with MPO/ H_2O_2/Cl^- rather than reagent ^-OCl . Also, the formation of CEOOH by enzymically generated ^-OCl was not dependent on Cl^- , suggesting a direct reaction of LDL with compound I rather than an indirect effect mediated via ^-OCl . It has recently been shown that horseradish peroxidase treatment of LDL results in increased TBARS (thiobarbituric acid-reacting substances) values [29]. It is difficult to compare this with our results, because it is not possible to relate the TBARS value obtained to an actual CEOOH concentration, since little qualitative or quantitative relationship exists between TBARS values and CEOOH concentration [30].

CEOOH represent the major single class of lipid hydroperoxides in radical-oxidized human LDL [31]. The h.p.l.c.-chemiluminescence method that we used is both highly sensitive (detection limit in the low nM range) and very specific for CEOOH [17]. Although an increase in temperature or using MPO rather than reagent ^-OCl during oxidation both increased the production of CEOOH slightly, it is still a reaction of minor quantitative importance. ^-OCl and MPO can oxidize isolated non-esterified fatty acids and phospholipids to chlorohydrins [19,24]. However, even at high concentrations of this oxidant, chlorohydrins, fatty acid oxidation products or cholesterol oxidation products were not formed in substantial amounts in LDL (Figure 2). Together, these results demonstrate that LDL lipid oxidation in general does not appear to be important with this oxidant. In contrast with LDL's lipids, modification of the lipoprotein's lysine and tryptophan residues quantitatively represented major reactions ([15]; Figure 3 of the present paper). Since modification of these two residues was not different with the two ^-OCl systems, we conclude that oxidation of LDL by reagent ^-OCl is a good model for the oxidation mediated by MPO/ H_2O_2/Cl^- .

The apparent absence of substantial amounts of lipid hydroperoxides in ^-OCl -treated LDL is consistent with observations by Arnhold et al. [32]. Also, Carlin and Djursäter [33] found that while MPO could peroxidize phospholipids in the presence of bromide, physiological concentrations of NaCl completely inhibited this peroxidation. On the other hand, Stelmazynska et al. [34] reported a large degree of lipid peroxidation upon treatment of LDL with MPO/ H_2O_2/Cl^- , as assessed by the A_{234} . These authors carried out the oxidation at pH 6.0, in the presence of EDTA with large amounts of MPO and a high final concentration of oxidant produced (corresponding to ~ 1000 $^-OCl/LDL$). However, when using reagent ^-OCl at the same high ratio of oxidant to LDL and under the experimental conditions described above, we failed to observe a significant increase in A_{234} (L. J. Hazell and R. Stocker, unpublished work). MPO, used to produce half the mole ratio of oxidant to LDL used by Stelmazynska et al. [34], produced peroxidation of 1% of the total cholesteryl esters in LDL, compared with peroxidation of 60% of the lipids that those authors reported. We have not tried higher concentrations of MPO, which are needed

to produce higher concentrations of oxidant, because of limited availability of the enzyme. The reason for the apparent disparity in results remains uncertain, although our results using several independent and specific methods for the analysis of lipid-oxidation products argue strongly against their importance in the initial stages of $^{\circ}\text{OCl}$ oxidation of LDL. In fact, our results suggest that apo B-100 protects the lipids from oxidative attack by this oxidant. This is supported directly by the fact that reductive methylation of the lysine residues increases the extent of both lipid peroxidation (Table 2) and consumption of anti-oxidants (Figure 6).

Treatment of LDL with reagent $^{\circ}\text{OCl}$ caused the lipoprotein to aggregate (Figures 4 and 5). That aggregation can be reversed by methionine and, to a lesser extent, lysine, or inhibited by reductive methylation of LDL's lysine residues, suggests that chloramines are likely to be required for this process. This, together with our findings of $^{\circ}\text{OCl}$ -mediated formation of chloramines [15], aldehydes and ammonia (Figure 7), and that all of these processes were inhibited by reductive methylation of the apo B's lysine residues before oxidation, is consistent with the following sequence of reactions leading to LDL aggregates:

The primary product of $^{\circ}\text{OCl}$ -mediated oxidation of apo B's ϵ -amino groups are chloramines (Reaction 1). α -Chloramines can break down to aldehydes [35], most likely through hydrolysis of an intermediate imine, and with concomitant release of ammonia. An ϵ -amino group is likely to undergo the same reactions, although they are possibly less favourable (Reactions 2 and 3). The aldehyde formed may then react, in a reversible reaction [36], with another ϵ -amino group, from the same or a different apo B protein to give a Schiff base (Reaction 4). The latter may hydrolyse (Reaction — 4) or become reduced to a stable secondary amine (Reaction 5), with concomitant formation of inter- or intra-molecular cross-links [36]. It appears that only a small number of chloramines are involved in the aggregation of LDL as relatively small amounts of ammonia and aldehydes were formed (Figure 7), consistent with the reported extent of chloramine loss during storage of $^{\circ}\text{OCl}$ -oxidized LDL [15].

Our proposed scheme receives additional support from work published by others. Thus oxidation of human lipoprotein (a) and LDL by reagent $^{\circ}\text{OCl}$ has been shown to cause formation of non-disulphide cross-linked high-molecular-mass apo B species detected by SDS/PAGE [37]. Other proteins are also cross-linked by exposure to either reagent or enzymically produced $^{\circ}\text{OCl}$ [38,39], as are low-molecular-mass amines to proteins [40]. Furthermore, oxidation of elastin by $^{\circ}\text{OCl}$ caused loss of ^3H from the ϵ -carbon of lysine residues [28]. Together, these findings suggest that $^{\circ}\text{OCl}$ /MPO-mediated cross-linking of LDL represents a feasible mechanism by which this lipoprotein may be transformed into an atherogenic form. Other mechanisms are also possible. Thus, in the presence of free tyrosine [14,41] or catechol [41], MPO/ H_2O_2 may cross-link proteins via bityrosine formation, in the former, or via reactions involving *o*-quinone, in the latter.

Schiff-base-type modification has also been suggested to occur in LDL oxidized by transition-metal-dependent radical reactions, in which case aldehydes are formed from breakdown products of

lipid hydroperoxides. This raises the possibility that the material reported in human plaque or in the circulation of patients with atherosclerosis that cross-reacted with antibodies raised against aldehyde-modified LDL (see, e.g. [42,43]) may have in part derived from $^{\circ}\text{OCl}$ -modified LDL.

The formation of LDL aggregates by $^{\circ}\text{OCl}$ may have several effects relevant to atherosclerosis. Aggregates of LDL, in general, cause lipid loading in macrophages *in vitro* [6,7,9], and we have observed that $^{\circ}\text{OCl}$ -modified LDL also causes foam-cell formation [15]. Furthermore, cross-linked LDL (copper-oxidized, 4-hydroxynonenal- or myricetin-treated), once internalized by macrophages, is more resistant to degradation [44], therefore potentially amplifying its atherogenic properties.

The role, if any, of $^{\circ}\text{OCl}$ -modified LDL in atherosclerosis is not known at present. This oxidant is produced by MPO, which itself is found in high concentrations in monocytes and neutrophils and is released into the extracellular environment upon stimulation of these cells. Monocytes are thought to be important in the initiation of atherosclerosis, due to their presence in high numbers in very early atherogenesis, and so this oxidant should be considered as a possible cause of oxidative damage to LDL *in vivo*. Studies investigating lipid peroxidation of LDL by monocytes and neutrophils have either shown very little lipid peroxidation [45], or required large amounts of bound or free transition metals in the media [5,46] to obtain large amounts of oxidized lipids. Our studies show that, under conditions that resemble the physiological situation, $^{\circ}\text{OCl}$ oxidizes LDL to a high-uptake form for macrophages without extensive lipid oxidation. These studies warrant further investigation of the possible presence in human plaque of MPO- and $^{\circ}\text{OCl}$ -modified LDL.

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