Oxidation of low-density lipoprotein with hypochlorite causes transformation of the lipoprotein into a high-uptake form for macrophages

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Oxidation of low-density lipoprotein (LDL) lipid is thought to represent the initial step in a series of oxidative modification reactions that ultimately transform this lipoprotein into an atherogenic high-uptake form that can cause lipid accumulation in cells. We have studied the effects of hypochlorite, a powerful oxidant released by activated monocytes and neutrophils, on isolated LDL. Exposure of LDL to reagent hypochlorite (NaOCl) at 4 °C resulted in immediate and preferential oxidation of amino acid residues of apoprotein B-100, the single protein associated with LDL. Neither lipoprotein lipid nor LDL-associated antioxidants, except ubiquinol-10, represented major targets for this oxidant. Even when high concentrations of NaOCl were used, only low levels of lipid hydroperoxides could be detected with the highly sensitive h.p.l.c. post-column chemiluminescence detection method. Lysine residues of apoprotein B-100 quantitatively represented the major target, scavenging some 68 % of the NaOCl added, with tryptophan and cysteine together accounting for an additional 10 % of the oxidant. Concomitant with the loss of LDL's amino groups, chloramines were formed and the anionic surface charge of the lipoprotein particle increased, indicated by a 3–4-fold increase in electrophoretic mobility above that of native LDL on agarose gels. While both these changes could be initially reversed by physiological reductants such as ascorbic acid and methionine, incubation of the NaOCl-modified LDL at 37 °C resulted in increasing resistance of the modified lysine residues against reductive reversal. Exposure of mouse peritoneal macrophages to NaOCl-oxidized LDL resulted in increased intracellular concentrations of cholesterol and cholesteryl esters. These findings suggest that lipid-soluble antioxidants associated with LDL do not efficiently protect the lipoprotein against oxidative damage mediated by hypochlorite, and that extensive lipid oxidation is not a necessary requirement for oxidative LDL modification that leads to a high-uptake form of the lipoprotein.

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 [1–4]. 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 [2]. However, after certain types of modification, a high-uptake form of LDL is formed that is no longer recognized by the LDL receptor and that can lead to foam cell formation.

A number of different modifications have been reported to lead to a high-uptake form of LDL. In 1979, Goldstein et al. reported that acetylation of LDL resulted in its rapid uptake by macrophages, with concomitant formation of foam cells [5]. Other modifications have since been shown to cause the same effect. Among these are treatment of LDL with other lysine- or arginine-modifying reagents [6,7], phospholipase C [8], lipoxygenase plus phospholipase A_2 [9], arterial proteoglycans and chondroitin 6-sulphate [10], breakdown products of lipid peroxidation [11], or products derived from platelets [12] or mast cells [13]. In addition, cells relevant to the microvasculature, such as endothelial cells [14] and smooth muscle cells [15], or monocytes/macrophages themselves [16], have been shown to modify LDL in such a way that it causes lipid accumulation within macrophages. A common feature of these cell-mediated LDL modification systems is that they require the presence of transition metals in the medium [15,17] and that the modification can at least partly be inhibited by antioxidant active compounds [18]. The implied involvement of oxidants in this cell-mediated transformation of LDL into a high-uptake form is supported further by the fact that the same effect can be achieved in cell-free systems by copper and iron ions [14,19], or by lipoxygenase together with phospholipase A_2 [9].

There is increasing biochemical, clinical and epidemiological evidence that oxidative LDL modification also contributes to the formation of foam cells *in vivo* (reviewed in [18,20]). While the precise nature, origin and site of generation of the responsible oxidant(s) remain unknown, it is generally assumed that the formation of oxidized lipids (e.g. lipid hydroperoxides) represents the initial step and is a prerequisite for oxidative modification of LDL *in vivo* and *in vitro*. Hydroperoxides of cholesteryl esters (CEOOH) are the major class of lipid hydroperoxides formed upon exposure of LDL to various oxidizing conditions [21], and their formation is attentuated by antioxidants surrounding and associated with the lipoprotein [21,22]. However, once formed, CEOOH can degrade (particularly in the presence of transition metals) to highly reactive moieties that can modify amino acid residues of apoprotein (apo) B-100, the single protein associated with LDL. These modifications of apo B-100 are thought to finally result in formation of a new 'epitope' that is specifically recognized by scavenger receptors ([23], and reviewed in [20]).

During our survey of the effects of different physiological oxidants on the early stages of LDL oxidation, we observed that sodium hypochlorite (NaOCl)-mediated modification of the lipoprotein apparently did not follow the initial sequence of

Abbreviations used: apo B-100, apoprotein B-100; $CoQ_{10}H_2$, ubiquinol-10; CEOOH, cholesteryl ester hydroperoxides; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); LDL, low-density lipoproteins; TNB, thionitrobenzoic acid.

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reactions described above for oxidative LDL modification. The results of the present study show that exposure of LDL to reagent NaOCl resulted in immediate and almost exclusive modification of the protein, causing transformation of the lipoprotein into a high-uptake form. Lipid-soluble antioxidants afforded little protection against this type of oxidative LDL modification, and only low levels of CEOOH were detected. From these findings we conclude that formation of large amounts of CEOOH is not a necessary requirement for *in vitro* oxidative LDL modification that leads to a high-uptake form of the lipoprotein.

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EXPERIMENTAL

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 solution, fluorescamine, 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) and methionine from Sigma; and reagent NaOCl (5% available chlorine minimum) from Aldrich. Three types of PBS were used: for LDL isolation, 50 mM PO_4^{3-}, pH 7.4, containing 150 mM NaCl and 0.1% (w/v) EDTA; for gel filtration and resuspension of LDL after isolation, 50 mM PO_4^{3-}, pH 7.4, containing 150 mM NaCl; and for macrophage preparation, Ca^{2+}- and Mg^{2+}-free PBS (Sigma). Organic solvents were of h.p.l.c. quality (Mallinckrodt) or the highest grade available. Hexane was washed with nanopure water prior to its use.

LDL preparation and oxidation

Fresh blood was obtained from non-fasted, healthy, normolipidaemic male or female subjects (23–35 years old) and drawn into either heparin vacutainers (for rapid isolation of LDL) or into a syringe and then transferred to Falcon tubes containing EDTA (1 mg/ml of blood) (for large-scale LDL preparation). The blood was centrifuged at 4 °C (1000 *g* for 15 min; GH-3.7 rotor; Beckman) to separate cells from plasma. LDL was isolated from plasma by ultracentrifugation, either by the rapid analytical method described in Method 7 of ref. [24], with the modifications detailed in [21], or by the large-scale preparation method using two sequential centrifugation steps as described in [25]. All centrifugation steps and handling of the samples were carried out at 4 °C or on ice (2–4 °C). Potassium bromide and other low-molecular-mass compounds contaminating the LDL preparation (e.g. ascorbate and urate) were removed by gel filtration through a PD-10 column (Pharmacia), and the LDL was subsequently resuspended in PBS. The protein concentration of the resulting solution was determined by the bicinchoninic acid assay [26] using BSA (Sigma Diagnostics) as standard and the protocol described in the manufacturer's instructions. Oxidation was carried out on ice by the addition of 1 vol. of reagent NaOCl, freshly diluted in phosphate buffer (50 mM), to 4 vol. of LDL solution (0.5–2.0 mg/ml, final concentration). The concentration of the total amount of oxidizing equivalents present in the diluted commercial NaOCl solution was determined spectrophotometrically, measuring both hypochlorite and hypochlorous acid and using published molar absorption coefficients [27]. Special care was taken that the final pH of the diluted NaOCl solution, and hence the final reaction mixture, was pH 7.4 (unless stated otherwise). After addition of NaOCl the reaction mixture was mixed briefly (< 1 s) and left on ice for 15 min before further treatment, unless indicated otherwise.

Measurement of antioxidants and lipid content of LDL by h.p.l.c.

To determine the antioxidant and lipid content of LDL, a 200 μl aliquot of the reaction mixture was extracted with 2 ml of cold methanol and 10 ml of hexane. The biphasic extract was mixed vigorously and centrifuged at 200 *g* for 2 min, before 9 ml of the hexane phase was removed, dried under vacuum and redissolved in 180 μl of ethanol for analysis. Lipid-soluble antioxidants [ubiquinol-10 ($CoQ_{10}H_2$), α-tocopherol, lycopene, α- and β-carotene] were separated on an LC-18 column (Supelco; 25 cm × 0.46 cm with 5 cm guard column; 5 μm particle size) eluted with ethanol/methanol/propan-2-ol (73.6:22.5:3.9, by vol.) containing 20 mM lithium perchlorate and detected electrochemically at a voltage of +0.6 V (21,28]. To minimize loss of antioxidants, especially $CoQ_{10}H_2$, extraction and h.p.l.c. analysis were carried out as rapidly as possible.

Lipid hydroperoxides present in the aqueous/methanol and hexane extracts of LDL were determined by h.p.l.c. with post-column chemiluminescence detection as described previously [21,29]. Cholesterol, triacylglycerols and cholesteryl esters were separated on an LC-18 column (Supelco; 25 cm × 0.46 cm with 5 cm guard column; 5 μm particle size) eluted with t-butyl alcohol/methanol (1:1, v/v) at 1 ml/min and detected at 210 nm [29].

Quantification of amino acid residues

Unmodified lysine residues in LDL were quantified by fluorescamine fluorescence using the manual procedure of Böhlen [30]. In a typical assay, 20 μl of control or oxidized LDL (0.4–1.5 mg of protein/ml) was added to 730 μl of borate buffer (200 mM, pH 8.5) and mixed while 250 μl of fluorescamine (Sigma) (539 μM, in acetone) was added. Following incubation at room temperature for less than 120 min, fluorescence was measured at 475 nm using an excitation wavelength of 390 nm. All LDL samples were diluted appropriately in borate buffer to yield fluorescence between 10 and 600 arbitrary units, a range for which a linear relationship between fluorescence and amine concentrations was observed. The fluorescence of treated samples was expressed relative to that of the native sample, assuming that LDL has 356 lysine residues.

Chloramines were measured by the thionitrobenzoic acid (TNB) assay [31], with TNB being prepared from DTNB (Sigma) by basic hydrolysis [32]. Immediately prior to the assay, TNB was diluted in 50 mM phosphate buffer (pH 7.4), added to 50 μl of oxidized LDL (at approx. 1 mg of protein/ml), mixed and then incubated at room temperature for 10 min. To minimize the background TNB reading, the amount of TNB added was chosen on the basis that the highest chloramine concentrations present in any of the LDL samples resulted in near-complete oxidation of TNB. The content of chloramines in the LDL sample was determined spectrophotometrically (412 nm) using a standard curve obtained from reagent NaOCl and TNB, thereby reducing the contribution of potential side-reactions to the values calculated [33]. Cysteine residues in LDL were quantified by the DTNB assay [31] after treatment of the NaOCl-modified LDL with 2.4 mM methionine (to remove all TNB-oxidizing species), and solubilization of the lipoprotein with SDS (3%, w/v).

Tryptophan fluorescence of LDL (0.5 mg/ml) was measured at 335 nm using an excitation wavelength of 280 nm [34]. A linear relationship between loss of fluorescence and number of tryptophan residues oxidized was assumed.

Agarose gel electrophoresis

LDL (1–4 μg of protein) was loaded on agarose prepacked gels (obtained from Ciba Corning) and the gels were run in barbitone buffer (130 mM barbiturate/1 mM EDTA, pH 8.6) at 90 V for 45 min. After electrophoresis, the gels were fixed in methanol for 30 s, dried at 50 °C for 15 min, stained with Fat Red 7B stain, destained in 70% (v/v) methanol and dried overnight at 50 °C.

Isolation of macrophages and uptake of LDL

Peritoneal macrophages were obtained from Swiss TO mice by lavage with cold Dulbecco's modified Eagle's medium (Gibco) containing 0.38% (w/v) sodium citrate [35]. The cells were plated in 35 mm-diam. wells (Costar) at 5×10^6 cells/well, incubated for 2 h in the above medium containing 1 g of glucose/litre and then washed four times with PBS to remove non-adherent cells. For lipid loading, native or modified LDL was added to a final concentration of 0.1 mg of protein/ml and the cells were incubated at 37 °C in Hank's balanced salts solution for 4 or 20 h for microscopic analysis by Oil Red-O staining and lipid analysis. For the latter, cells were washed three times with cold PBS following incubation and then lysed by the addition of 0.6 ml of 0.2 M NaOH. An aliquot (400 μl) was taken for extraction with methanol/hexane as described above for LDL; the hexane extract was dried under vacuum and then redissolved in the mobile phase used for the h.p.l.c. analysis. Lipids were separated on an LC-18 column (Supelco; 25 cm × 4.6 cm with 5 cm guard column; 5 μm particle size) using acetonitrile/propan-2-ol (3:7, v/v) as the eluant and quantified at 210 nm.

RESULTS

Lipid-soluble antioxidants associated with LDL have been previously shown to attenuate the build-up of CEOOH when the lipoprotein is exposed to radical oxidants [21]. Figure 1 shows that exposure of freshly isolated LDL to increasing concentrations of the non-radical oxidant NaOCl under pH-controlled conditions (pH 7.4) resulted in an immediate and linear loss of all antioxidants. Among the LDL antioxidants measured, $CoQ_{10}H_2$ was consumed first, before α-tocopherol and carotenoids, as was observed when LDL was exposed to other oxidizing conditions [21]. Considering their initial concentration, and assuming that each molecule of antioxidant consumed scavenged one molecule of NaOCl, consumption of all antioxidants accounted for only approx. 1% of the reagent NaOCl added at a ratio of 300 mol of NaOCl/mol of LDL. In sharp contrast to the situation with radical oxidants [21], treatment of LDL with NaOCl resulted in formation of barely detectable CEOOH ($\leqslant$ 0.2 mol of CEOOH/mol of LDL), even though more than 200 molecules of NaOCl per LDL particle were present and the highly sensitive h.p.l.c. post-column chemiluminescence method for the detection of CEOOH were used (Figure 1). Similarly, only submicromolar concentrations of phospholipid hydroperoxides were detected (results not shown). In one experiment where excessive amounts of NaOCl were used (700–1200 molecules of oxidant/LDL particle), CEOOH were not detected at all (results not shown). When LDL was incubated at 37 °C for 2 h after NaOCl addition, slightly larger amounts of CEOOH were found, i.e. 3.3 mol of CEOOH/mol of LDL at a ratio of 200 molecules of NaOCl/lipoprotein particle. Treatment of LDL with NaOCl did not result in a concentration-dependent loss of either cholesterol or

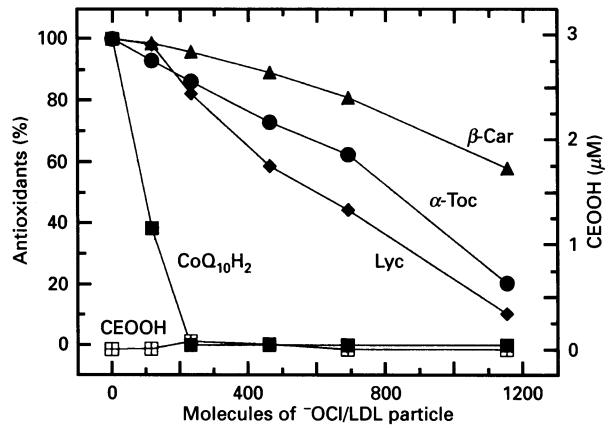

Figure 1 NaOCl-mediated oxidation of LDL causes oxidation of lipid-soluble antioxidants but the formation of only barely detectable amounts of CEOOH

Isolated LDL (0.59 μM) was treated on ice at pH 7.4 with various concentrations of reagent NaOCl, and left on ice for 15 min before extraction and analysis of antioxidants and CEOOH (⊞) (see Experimental section). Initial concentrations of the LDL-associated antioxidants were: ■, $CoQ_{10}H_2$ (0.25 μM); ●, α-tocopherol (α-Toc, 8.7 μM); ◆, lycopene (Lyc, 0.24 μM); ▲, β-carotene (β-Car, 0.50 μM). The results shown are typical of four independent experiments carried out with LDL obtained from one female and three male donors. The rates of loss of the antioxidants, calculated from the slopes of the lines of best fit for each antioxidant from each individual experiment, were: $CoQ_{10}H_2$, 0.51 ± 0.12, α-tocopherol, 0.064 ± 0.058; lycopene, 0.074 ± 0.008; β-carotene, 0.03 ± 0.02 (means ± S.D.). The maximal concentration of phospholipid hydroperoxides detected was 0.2 μM (at 240 molecules of NaOCl/LDL particle).

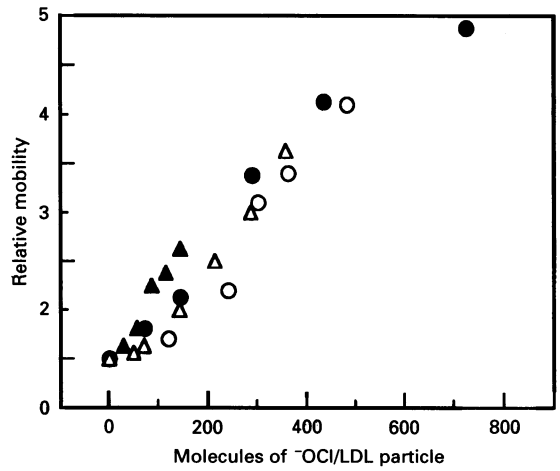

Figure 2 NaOCl-mediated increase in the net negative surface charge of LDL

Isolated LDL was oxidized on ice at pH 7.4 with various concentrations of NaOCl, incubated for 15 min and its mobility examined by agarose gel electrophoresis as described in the Experimental section. The relative mobility of the various LDL samples was calculated as the ratio of the distances that the treated and untreated (native) LDL migrated on the gel. The results shown were obtained from four independent experiments (presented as different symbols) using LDL from male and female donors which was exposed to increasing amounts of NaOCl up to 800 molecules of oxidant/LDL particle. The correlation coefficient between relative mobility and number of oxidant molecules added/LDL particle is 0.967.

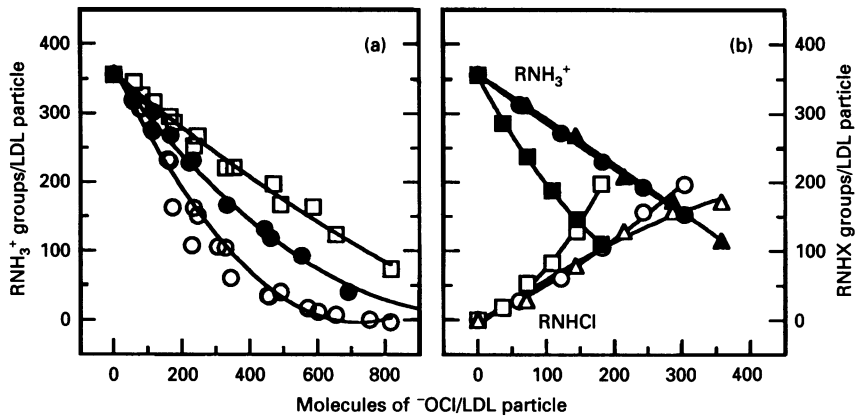

Figure 3 The NaOCl-mediated loss of amino groups from LDL is pH-dependent (a) and results in the stoichiometric formation of chloramines (b)

LDL was isolated, oxidized and incubated on ice for 15 min before free amino groups and chloramines were measured using the fluorescamine and TNB assays respectively (see Experimental section). The pH values used in (**a**) were: ○, 12.0; ■, 7.4; □, 6.0. (**b**) LDL was treated with NaOCl at pH 7.4. Amines (RNH_3^+; closed symbols) and chloramines (RHNCl; open symbols) were measured. The numbers shown were calculated on the basis that apo B-100 of native LDL contains 356 lysine residues [45]. The results in (**a**) show the data obtained from five independent experiments with LDL from four different male and female donors. For (**b**), results of three separate experiments (circles, squares and triangles) are shown.

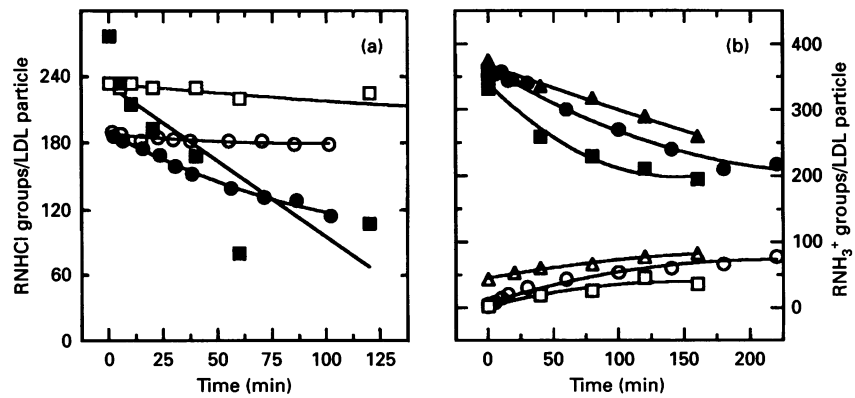

Figure 4 Chloramines formed by NaOCl treatment of LDL are lost gradually over time (a) and can be reduced back to amines by ascorbate (b)

(**a**) LDL previously oxidized with 193 (○, ●) or 309 (□, ■) molecules of NaOCl/lipoprotein particle was incubated either on ice (○, □) or at 37 °C (●, ■) and the remaining chloramines measured by TNB (see the Experimental section). (**b**) Time-dependent changes in the efficacy of ascorbate to regenerate amines from chloramines in LDL previously oxidized with 532 (○, ●), 461 (△, ▲) or 546 (□, ■) molecules of NaOCl per lipoprotein particle. The NaOCl-oxidized LDL was incubated at 37 °C for various lengths of time prior to the addition of either a 100-fold molar excess (over NaOCl) of ascorbate dissolved in phosphate buffer (●, ▲, ■), or the same volume of phosphate buffer (○, △, □). For (**a**), the results of two independent experiments are shown. A third separate experiment, with a similar result, is not included for clarity. For (**b**), the results are shown of three independent experiments carried out with LDL isolated from both males and females.

cholesteryl esters (results not shown), the latter being the major lipid component of LDL. Since our LDL preparations were free of reactive water-soluble antioxidants (see the Experimental section), these findings suggested to us that target(s) other than LDL lipids and antioxidants were primarily reacting with NaOCl.

Exposure of LDL to increasing amounts of NaOCl resulted in a massive increase in the net negative surface charge of the lipoprotein particle, as indicated by its increased electrophoretic mobility on agarose gels (Figure 2). Relative mobilities approx. 3–4-fold greater than that of control unoxidized LDL particles were obtained with a NaOCl/LDL particle ratio of 700. The increase in electrophoretic mobility occurred without delay, and initially was related linearly to the NaOCl/LDL ratio used. With increasing NaOCl concentrations the LDL band on the agarose gel appeared to be broadened (results not shown).

An increase in the anionic charge of LDL has been reported to result from modification of the ϵ-amino group of peptidyl lysine residues of apo B-100 [6]. Previous work on the action of NaOCl on proteins showed that amines, together with thioesters, thiols, tryptophan and tyrosine, are preferential targets of this oxidant [31,36]. Indeed, we also found that, upon addition to the lipoprotein, NaOCl reacted with and converted accessible amines of apo B-100 into monochloramines (Figure 3b). The extent of NaOCl-mediated modification of lysine residues of apo B was pH-dependent, the reaction being more efficient under alkaline conditions (Figure 3a). Chloramines were formed linearly down to low concentrations of NaOCl, suggesting that other potentially reactive moieties (e.g. antioxidants or methionine residues) did not prevent this type of modification.

Chloramines are oxidizing species that can react with certain molecules, e.g. with amines by exchange of the Cl^+ moiety [37,38]. We observed that, while the chloramines present in NaOCl-modified LDL were almost completely stable at 4 °C for at least 3 days, they were lost rapidly ($t_{\frac{1}{2}}$ approx. 100 min) upon

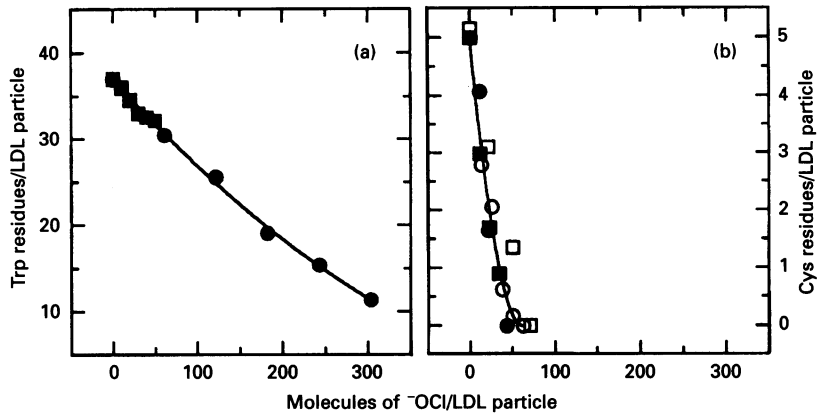

Figure 5 NaOCl-mediated loss of (a) tryptophan and (b) cysteine residues of apo B-100 in LDL

The lipoprotein was oxidized with various concentrations of reagent NaOCl and the unmodified tryptophan and cysteine were quantitated by fluorescence and DTNB reaction respectively (see the Experimental section). The numbers of unmodified amino acid residues shown assume that apo B-100 of native LDL contains 37 tryptophan [45] and five free cysteine residues [47]. The results of two and four separate experiments are shown in (**a**) and (**b**) respectively. Different symbols refer to different sets of experiments.

Table 1 Relative contributions of LDL components to the scavenging of reagent NaOCl

The values given for the initial number of moieties represent the number of different targets for NaOCl oxidation present in native LDL. In the case of lipids, the total number of double bond groups per LDL is shown, as calculated from [58]. The total number of antioxidant active molecules is calculated from data in [21,59], whereas those for the amino acids of apo B are derived from [45,47]. Number of moieties lost is the number of moieties oxidized after exposure of LDL to reagent NaOCl on ice for 15 min at a ratio of 300 molecules of oxidant/lipoprotein particle. The numbers in parentheses represent the relative contribution of each LDL component to the oxidant scavenging, expressed as a percentage of the total amount of NaOCl added. It was assumed that each component reacted with one molecule of NaOCl. n.d., not determined accurately (value was < 1% of total oxidant added). The total amount of oxidant accounted for by the components listed was 79.4%.

	Numbers of moieties per LDL particle	
LDL component	Initial	Lost
Lipids	1900	n.d.
Antioxidants	12	4 (1.3)
Amino acyl residues		
Lysine	356	203 (67.7)
Tryptophan	37	26 (8.7)
Cysteine	5	5 (1.7)

incubation of the modified lipoprotein in 50 mM phosphate buffer, pH 7.4, at 37 °C (Figure 4a). The rate of loss appeared to be proportional to the initial degree of oxidation. Some of the chloramines reverted to amines; in a representative example, approx. 25% of the initial chloramines reappeared as amines after 24 h of incubation of the NaOCl-treated LDL at room temperature (results not shown). Reversion of chloramines to amines was almost complete when a 100-fold molar excess of ascorbate (relative to NaOCl) was added to LDL immediately after its oxidative modification (Figure 4b). In parallel to this 'regaining' of amino groups, the increased electrophoretic mobility of the modified lipoprotein on agarose gels was also reversed from 4.0 times to 1.5 times the R_F value of control LDL, suggesting a link between these two parameters. Significant reversion of the surface charge of the LDL was also observed with other reducing substances or amino acids. Thus when cysteine, methionine, DTNB or tryptophan was added to the LDL immediately after its modification at a 5–30-fold molar excess relative to NaOCl, the lipoprotein's R_F values on agarose gels were significantly decreased (from R_F values of 4.0 to between 1.5 and 2.8 increased). Other amino acids were either less effective (tyrosine, lysine, histidine and glutamic acid) or without effect (all remaining amino acids). With increasing length of the incubation period of NaOCl-oxidized LDL at 37 °C, the modified amino groups became more resistant towards reductive reversal by ascorbate (Figure 4b). Similarly, while addition of excess methionine to LDL immediately after its oxidation resulted in an approx. 70% decrease in the R_F value, this effect was decreased to approx. 20% when LDL was incubated for 2 h at 37 °C prior to the addition of methionine.

Oxidation of LDL by reagent NaOCl also resulted in an immediate and initially linear loss of tryptophan and cysteine residues of apo B-100 (Figure 5). Oxidation of cysteine residues of LDL appeared to be very rapid, as only approx. 50 molecules of oxidant per LDL particle were required to oxidize all thiol groups accessible to DTNB in SDS-treated LDL (Figure 5b). Table 1 shows that, at a ratio of 300 molecules of NaOCl/LDL particle, almost 80% of the oxidant added could be accounted for by reactions examined in this study. Of the accounted NaOCl, 78% reacted with apo B, whereas only approx. 1% was scavenged by LDL-associated antioxidants and < 1% reacted to give CEOOH and phospholipid hydroperoxides (assuming that lipid oxidation had no chain character, i.e. chain length < 1.0; if oxidation proceeded via a chain [21], 1% would be an overestimation).

Lipid loading of macrophages is commonly used as a guide to determine the potential atherogenicity of a particular kind of LDL modification. We therefore assessed whether exposure of resident mouse peritoneal macrophages to NaOCl-modified LDL resulted in intracellular lipid accumulation. As at least some of the NaOCl-mediated modifications of LDL are potentially reversible (see above), cell culture studies were conducted in medium free of reductants and over relatively short incubation periods. LDL treated on ice with 408 molecules of NaOCl/lipoprotein particle and then incubated with macrophages at 37 °C for 4 h resulted in cellular lipid accumulation, as judged microscopically by Oil Red-O staining (results not shown) and

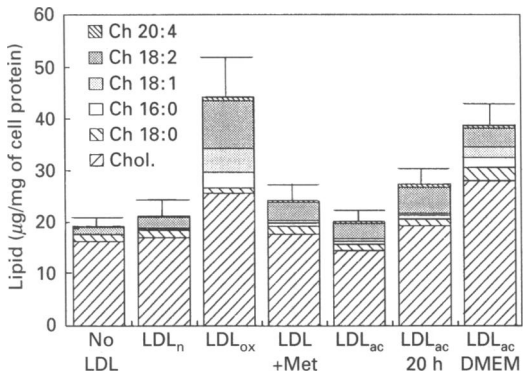

Figure 6 Exposure of macrophages to NaOCl-oxidized LDL causes them to accumulate lipid

Unless indicated otherwise, mouse peritoneal macrophages were incubated at 37 °C for 4 h in Hanks' balanced salt solution in the absence (No LDL) or presence of native (LDL$_n$), NaOCl-oxidized (LDL$_{ox}$; 408 molecules of reagent NaOCl/lipoprotein particle), or acetylated (LDL$_{ac}$) LDL. Following incubation, the cells were washed, lysed and the lipids extracted and analysed as described in the Experimental section. The results presented are the means of a single experiment performed in triplicate, with the error given representing the S.D. of total lipid. Similar relative increases in the cellular lipid content were obtained with LDL$_{ox}$ in three independent experiments each performed in triplicate, while the absolute values varied approx. 5-fold. Abbreviations: LDL$_{ox}$ + Met, cells incubated with LDL$_{ox}$ to which an 18-fold molar excess of methionine (over NaOCl) had been added; LDL$_{ac}$ 20 h; acetylated LDL incubated with cells for 20 h; LDL$_{ac}$ DMEM, acetylated LDL incubated with cells for 4 h in Dulbecco's modified Eagle's medium. Chol. indicates free cholesterol; Ch 20:4 (etc.) indicates cholesteryl esters with the indicated numbers of carbonations and double bonds.

h.p.l.c. analysis of the extracted cell lipids (Figure 6). Increased levels of both free cholesterol and cholesteryl esters were detected in cells incubated with NaOCl-treated but not native LDL. LDL exposed to lower concentrations of oxidant was much less efficient in causing lipid accumulation (results not shown). Treatment of the oxidized LDL with a 10-fold molar excess of methionine (over NaOCl) before its addition to the macrophages strongly attenuated accumulation of lipids (Figure 6). Incubation of macrophages with acetylated LDL under these conditions resulted in poor lipid loading within 4 h, suggesting that NaOCl-modified LDL is more efficient in lipid loading of macrophages than is acetylated LDL. Significant lipid did accumulate when acetylated LDL was present for 20 h, or when the cells were incubated in Dulbecco's modified Eagle's medium rather than Hanks balanced salt solution (Figure 6).

DISCUSSION

The results of this study show that *in vitro* exposure of LDL to reagent NaOCl results in oxidation of the lipoprotein in such a way that it causes intracellular accumulation of cholesterol and cholesteryl esters when added to macrophages in culture. A novel feature of NaOCl-mediated oxidative formation of high-uptake LDL is that apo B-100 represents the major target for this oxidant. LDL-associated antioxidants and lipids reacted with only a minor proportion of the added oxidant (see Table 1). In particular, very small amounts of oxidized lipids were detected, substantial amounts of neutral lipids were not consumed, and lipid-soluble antioxidants failed to significantly inhibit apoprotein oxidation, as lysine, cysteine and tryptophan residues were lost without delay and initially at linear rates. As such, NaOCl-mediated lipoprotein oxidation does not follow the previously proposed and now commonly accepted scheme of events for oxidative LDL modification [20], where oxidation of lipids precedes that of apo B-100.

NaOCl is a very strong oxidant produced by activated neutrophils via the myeloperoxidase-catalysed reaction of hydrogen peroxide with Cl^-. The known chemical reactivity of the non-radical oxidant NaOCl includes its addition to double bonds, giving rise to chlorohydrins and the chlorination of amino groups [39]. Winterbourn and colleagues recently showed that reagent and myeloperoxidase-formed hypochlorite readily produced chlorohydrins from unsaturated fatty acids, and they suggested that formation of such oxidatively modified lipids could be of physiological importance, e.g. in neutrophil-mediated cellular cytotoxicity [40]. While we have not tested for the presence of chlorinated lipids, it seems unlikely that such a chlorohydrination reaction is of major quantitative importance to our studies on NaOCl-mediated LDL oxidation: we did not detect substantial consumption of cholesteryl linoleate (the major unsaturated lipid in LDL) or of other unsaturated neutral lipids, and only approx. 20 % of the reagent NaOCl was not accounted for by the measured reactions with either LDL-associated protein or antioxidants (Table 1). We also failed to detect substantial amounts of phospholipid hydroperoxides in HOCl-treated LDL.

A previous report has described the peroxidation of lipids by an iodide/H_2O_2/myeloperoxidase system, although no peroxides were detected (measured by thiobarbituric acid-reactive material) after addition of NaCl to this system [41]. We detected very small amounts of CEOOH and phospholipid hydroperoxides in NaOCl-treated LDL by the use of the highly sensitive h.p.l.c. post-column chemiluminescence detection method, and this agrees with our previous observation that exposure of LDL to stimulated neutrophils causes the formation of sub-micromolar levels of CEOOH [21]. While at present we cannot exclude the possibility that NaOCl actually degraded small amounts of CEOOH formed when very high ratios of NaOCl/LDL were used, together our results suggest that oxidation of lipids may not be of major importance in hypochlorite-mediated oxidative damage to LDL *in vitro*.

Lysine residues of the LDL apo B-100 represented quantitatively the most important target for reagent NaOCl. The pH-dependency of the degree of modification of lysine residues may be explained by a variation in the extent of the chlorination reaction depending on the ionization state of both the ϵ-amino group of lysine (pK_a 10.5) and NaOCl (pK_a 7.5). Alternatively, competing reactions such as dichlorination of amine groups or oxidation of non-lysine residues may be more favourable than monochlorination at low pH [42,43].

The lack of a detectable lag period in the NaOCl-mediated loss of lysine residues from LDL is surprising considering that cysteine and methionine are 100 times more reactive towards this oxidant than is lysine [44]. On oxidizing human serum albumin with reagent NaOCl, Arnhold et al. (1990) [36] observed a distinct break-point in the rate of amine loss, with an initial phase of low [NaOCl] during which thiols but no amines were lost. These authors calculated that the break-point was obtained at a [NaOCl] equivalent to the amount of oxidant required to oxidize the single cysteine and the six methionine residues present in human albumin. There are 78 methionine residues [45,46] and five cysteine residues [47] per LDL particle. Thus an equivalent lag period, if existing in LDL, would have been visible with the range of [NaOCl] used in our studies. Its absence suggests that the methionine residues are buried in the LDL particle and/or do not prevent reaction of lysine with NaOCl. All five cysteine residues in LDL appeared to be accessible to reagent NaOCl (Figure 5b), in contrast to a recent study using LDL and -SH group labels which varied in polarity [48]. With the large range

of NaOCl concentrations used, we would not have been able to readily detect whether or not the small number of cysteine residues in LDL preferentially reacted with NaOCl, thereby protecting lysine and other amino acid residues from modification. Methionine residues are not the preferential target in fibronectin exposed to either reagent or myeloperoxidase-formed NaOCl, as such treatment resulted in immediate and linear loss of tryptophan fluorescence [31]. Whether, and if so to what extent, methionine in LDL does react with NaOCl remains to be established, although the data presented in Table 1 are consistent with it scavenging at least some of the NaOCl. In addition, scavenging by tyrosine residues of apo B may also contribute to the as yet unaccounted for NaOCl.

Most of the NaOCl-mediated change in the surface charge of oxidized LDL appeared to result from the neutralization of the positive charge of the lysine residues by chlorination, as addition of a mild reducing agent (e.g. ascorbate, methionine) removed chloramines and almost completely reverted the mobility of oxidized LDL to that of native LDL. This, together with the observation that neutralization of lysine residues of apo-B is important in determining the ability of the scavenger receptor of macrophages to recognize LDL [6], suggest that formation of the high-uptake form of LDL by NaOCl may initially be 'repairable' by physiological reductants present in the circulation or extravascular space. However, we also observed that an increasing percentage of oxidatively modified lysine residues become resistant to reduction upon incubation of the oxidized LDL at 37 °C, indicating that secondary oxidation reactions do occur that may no longer be 'repaired'.

We have not as yet been able to identify the nature of these secondary modification(s). Our results on the loss of LDL chloramines suggest a half-life of approx. 100 min at 37 °C, and this is lower than that reported for endogenous chloramines produced from activated neutrophils ($t_{\frac{1}{2}}$ of several hours at 37 °C) [49,50]. This suggests that chloramines in LDL are reacting with other moieties in the particle, thereby either regenerating amino groups (see the Results section) or producing further modifications. As mentioned earlier, *N*-chloramines are semistable oxidants that can react with amines, thiols or thioesters [38]. It seems possible also that some of the chloramines formed decompose to imines, which are known to act as cross-linking agents, similar to aldehydes. The latter have been described to cause the formation of a high-uptake form of LDL [11,51]. We are presently investigating the importance of chloramines in formation of high-uptake LDL.

While our studies show that *in vitro* exposure to reagent NaOCl causes transformation of human LDL into a high-uptake form, the precise pathway and mechanism of uptake of NaOCl-modified LDL by macrophages, as well as the physiological relevance of this type of oxidative LDL modification, remain to be investigated. Hypochlorite can be produced from H_2O_2 and Cl^- either non-enzymically from copper ions [52] or by (myelo)-peroxidase(s) present in certain immune cells such as monocytes, neutrophils and eosinophils [49,53,54]. Reagent NaOCl has been shown to react in similar ways to the myeloperoxidase/H_2O_2/Cl^- system [44], and therefore results comparable with those reported here may be obtained by exposing LDL to either the isolated enzyme or, possibly, activated neutrophils. The amounts of hypochlorite used per LDL particle in our studies are within those attained by maximally activated neutrophils at circulating cell concentrations [54]. Obviously, when stimulated *in vivo*, appropriate cells release hypochlorite into a complex environment containing a multiplicity of oxidizable endogenous [55,56] and exogenous [37,57] substrates competing with LDL for the oxidant(s). However, the possibility that LDL reacts with at least some of the hypochlorite, perhaps under conditions of local depletion of aqueous antioxidants, cannot be excluded. Our studies show that, if it occurs, this oxidative LDL modification would not be readily detected by analysing the biological material for lipid hydroperoxides.

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REFERENCES

1 Goldstein, J. L. and Brown, M. S. (1977) Annu. Rev. Biochem. **46**, 897–930
2 Brown, M. S. and Goldstein, J. L. (1986) Science **232**, 34–47
3 Schaffner, T., Taylor, K., Bartucci, E. J., Fischer-Dzoga, K., Beeson, J. H., Glagov, S. and Wissler, R. W. (1980) Am. J. Pathol. **100**, 57–80
4 Geer, J. C. (1965) Lab. Invest. **14**, 1764–1783
5 Goldstein, J. L., Ho, Y. K., Basu, S. K. and Brown, M. S. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 333–337
6 Haberland, M. E., Olch, C. L. and Fogelman, A. M. (1984) J. Biol. Chem. **259**, 11305–11311
7 Mahley, R. W., Innerarity, T. L., Pitas, R. E., Weisgraber, K. H., Brown, J. H. and Gross, E. (1977) J. Biol. Chem. **252**, 7279–7287
8 Suits, A. G., Chait, A., Aviram, M. and Heinecke, J. W. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 2713–2717
9 Sparrow, C. P., Parthasarathy, S. and Steinberg, D. (1988) J. Lipid. Res. **29**, 745–753
10 Camejo, G., Hurt, E., Wiklund, O., Rosengren, B., Lopez, F. and Bondjers, G. (1991) Biochim. Biophys. Acta **1096**, 253–261
11 Jürgens, G., Lang, J. and Esterbauer, H. (1986) Biochim. Biophys. Acta **875**, 103–114
12 Aviram, M., Fuhrman, B., Keidar, S., Maor, I., Rosenblat, M., Dankner, G. and Brook, G. (1989) J. Clin. Chem. Clin. Biochem. **27**, 3–12
13 Kokkonen, J. O. and Kovanen, P. T. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 2287–2291
14 Steinbrecher, U. P., Parthasarathy, S., Leake, D. S., Witztum, J. L. and Steinberg, D. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 3883–3887
15 Heinecke, J. W., Baker, L., Rosen, H. and Chait, A. (1986) J. Clin. Invest. **77**, 757–761
16 Parthasarathy, S., Printz, D. J., Boyd, D., Joy, L. and Steinberg, D. (1986) Arteriosclerosis **6**, 505–510
17 Leake, D. S. and Rankin, S. M. (1990) Biochem. J. **270**, 741–748
18 Steinbrecher, U. P., Zhang, H. and Lougheed, M. (1990) Free Radicals Biol. Med. **9**, 155–168
19 Heinecke, J. W., Rosen, H. and Chait, A. (1984) J. Clin. Invest. **74**, 1890–1894
20 Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C. and Witztum, J. L. (1989) N. Engl. J. Med. **320**, 915–924
21 Stocker, R., Bowry, V. W. and Frei, B. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 1646–1650
22 Frei, B., Stocker, R. and Ames, B. N. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 9748–9752
23 Sparrow, C. P., Parthasarathy, S. and Steinberg, D. (1989) J. Biol. Chem. **264**, 2599–2604
24 Chung, B. H., Segrest, J. P., Ray, M. J., Brunzell, J. D., Hokanson, J. E., Krauss, R. M., Beaudrie, K. and Cone, J. T. (1986) Methods Enzymol. **128**, 181–209
25 Jessup, W., Darley-Usmar, V., O'Leary, V. and Bedwell, S. (1991) Biochem. J. **278**, 163–169
26 Smith, P. K., Krohn, R. J., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olsen, B. J. and Klenk, D. C. (1985) Anal. Biochem. **150**, 76–85
27 Morris, J. C. (1966) J. Phys. Chem. **70**, 3798–3805
28 Lang, J. K., Gohil, K. and Packer, L. (1986) Anal. Biochem. **157**, 106–116
29 Yamamoto, Y., Brodsky, M. H., Baker, J. C. and Ames, B. N. (1987) Anal. Biochem. **160**, 7–13
30 Böhlen, P., Stein, S., Dairman, W. and Udenfriend, S. (1973) Arch. Biochem. Biophys. **155**, 213–220
31 Vissers, M. C. M. and Winterbourn, C. C. (1991) Arch. Biochem. Biophys. **285**, 53–59
32 Riddles, P. W., Blakeley, R. L. and Zerner, B. (1983) Methods Enzymol. **91**, 49–60
33 Silverstein, R. M. and Hager, L. P. (1974) Biochemistry **13**, 5069–5073

34 Teale, F. W. J. (1960) Biochem. J. **76**, 381–388
35 Jessup, W., Mohr, D., Gieseg, S. P., Dean, R. T. and Stocker, R. (1992) Biochim. Biophys. Acta **1180**, 73–82
36 Arnhold, J., Hammerschmidt, S., Wagner, M., Mueller, S., Arnold, K. and Grimm, E. (1990) Biomed. Biochim. Acta **49**, 991–997
37 Grisham, M. B., Jefferson, M. M., Melton, D. F. and Thomas, E. L. (1984) J. Biol. Chem. **259**, 10404–10413
38 Thomas, E. L. (1979) Infect. Immun. **23**, 522–531
39 March, J. (1977) Advanced Organic Chemistry: Reactions, Mechanisms and Structure, pp. 741–742, McGraw-Hill, New York
40 Winterbourn, C. C., van den Berg, J. J. M., Roitman, E. and Kuypers, F. A. (1992) Arch. Biochem. Biophys. **296**, 547–555
41 Carlin, G. and Djursäter, R. (1988) Free Radicals Res. Commun. **4**, 251–257
42 Morris, J. C. (1967) in Principles and Applications of Water Chemistry (Faust, S. D. and Hunter, J. V., eds.), pp. 23–51, John Wiley and Sons, New York
43 Stelmaszynska, T. and Zgliczynski, J. M. (1978) Eur. J. Biochem. **92**, 301–308
44 Winterbourn, C. C. (1985) Biochim. Biophys. Acta **840**, 204–210
45 Yang, C.-Y., Chan, L. and Gotto, A. M. (1987) in Plasma Lipoproteins (Gotto, A. M., Jr., ed.), pp. 77–93, Elsevier, Amsterdam
46 Chen, S. W., Yang, C. Y., Chen, P. F., Setzer, D., Tanimura, M., Li, W. S., Gotto, A. M. Jr. and Chan, L. (1986) J. Biol. Chem. **261**, 12918–12921
47 Yang, C.-Y., Kim, T. W., Weng, S.-A., Lee, B., Yang, M. and Gotto, A. M. Jr. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 5523–5527
48 Sommer, A., Gorges, R., Kostner, G. M., Paltauf, F. and Hermetter, A. (1991) Biochemistry **30**, 11245–11249
49 Test, S. T., Lampert, M. B., Ossanna, P. J., Thoene, J. G. and Weiss, S. J. (1984) J. Clin. Invest. **74**, 1341–1349
50 Weiss, S. J., Lampert, M. B. and Test, S. T. (1983) Science **222**, 625–628
51 Steinbrecher, U. P., Lougheed, M., Kwan, W.-C. and Dirks, M. (1989) J. Biol. Chem. **264**, 15216–15223
52 Frenkel, K., Blum, F. and Troll, W. (1986) J. Cell. Biochem. **30**, 181–193
53 Harrison, J. E. and Schultz, J. (1976) J. Biol. Chem. **251**, 1371–1374
54 Weiss, S. J., Test, S. T., Eckmann, C. M., Roos, D. and Regiani, S. (1986) Science **234**, 200–203
55 Wasil, M., Halliwell, B., Hutchinson, D. C. S. and Baum, H. (1987) Biochem. J. **243**, 219–223
56 Halliwell, B., Wasil, M. and Grootveld, M. (1987) FEBS Lett. **213**, 15–18
57 Fliss, H., Weissbach, H. and Brot, N. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 7160–7164
58 Sattler, W., Puhl, H., Hayn, M., Kostner, G. M. and Esterbauer, H. (1991) Anal. Biochem. **198**, 184–190
59 Dieber-Rotheneder, M., Puhl, H., Waeg, G., Stiegl, G. and Esterbauer, H. (1991) J. Lipid Res. **32**, 1325–1332

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