Secondary radicals derived from chloramines of apolipoprotein B-100 contribute to HOCl-induced lipid peroxidation of low-density lipoproteins

Linda J. HAZELL*, Michael J. DAVIES† and Roland STOCKER*[1]

*Biochemistry Group, The Heart Research Institute, 145 Missenden Road, Camperdown, NSW, 2050, Australia, and †EPR Group, The Heart Research Institute, 145 Missenden Road, Camperdown, NSW, 2050, Australia

Oxidation of low-density lipoproteins (LDL) is thought to contribute to atherogenesis. Although there is increasing evidence for a role of myeloperoxidase-derived oxidants such as hypochlorite (HOCl), the mechanism by which HOCl modifies LDL remains controversial. Some studies report the protein component to be the major site of attack, whereas others describe extensive lipid peroxidation. The present study addresses this controversy. The results obtained are consistent with the hypothesis that radical-induced oxidation of LDL's lipids by HOCl is a secondary reaction, with most HOCl consumed via rapid, non-radical reaction with apolipoprotein B-100. Subsequent incubation of HOCl-treated LDL gives rise to lipid peroxidation and antioxidant consumption in a time-dependent manner. Similarly, with myeloperoxidase/H_2O_2/Cl^- (the source of HOCl *in vivo*), protein oxidation is rapid and followed by an extended period of lipid peroxidation during which further protein oxidation does not occur. The secondary lipid peroxidation process involves EPR-detectable radicals, is attenuated by a radical trap or treatment of HOCl-oxidized LDL with methionine, and occurs less rapidly when the lipoprotein was depleted of α-tocopherol. The initial reaction of low concentrations of HOCl (400-fold or 800-fold molar excess) with LDL therefore seems to occur primarily by two-electron reactions with side-chain sites on apolipoprotein B-100. Some of the initial reaction products, identified as lysine-residue-derived chloramines, subsequently undergo homolytic (one-electron) reactions to give radicals that initiate antioxidant consumption and lipid oxidation via tocopherol-mediated peroxidation. The identification of these chloramines, and the radicals derived from them, as initiating agents in LDL lipid peroxidation offers potential new targets for antioxidative therapy in atherogenesis.

Key words: antioxidants, atherosclerosis, EPR, protein oxidation, α-tocopherol.

INTRODUCTION

The oxidative modification of low-density lipoproteins (LDL) is thought to contribute to atherogenesis [1–3]. Several different oxidants can oxidize LDL *in vitro*, although direct evidence for the participation of particular oxidants in the oxidative modification of the lipoprotein *in vivo* in the sub-endothelial space is presently limited to lipoxygenase [4,5], peroxynitrite [6], oxygen-centred radicals [7] and hypochlorite/hypochlorous acid (HOCl). Knowledge of the participation of HOCl is based on the presence of active myeloperoxidase [8], HOCl-oxidized proteins [9], 3-chlorotyrosine [10] and dityrosine [7] in human lesions. Indeed, HOCl can oxidize LDL *in vitro* [11,12]. Such oxidatively modified LDL has several atherogenic and/or pro-inflammatory features: it causes foam cell formation [12], stimulates the adherence of neutrophils to endothelial cells and enhances the production of reactive oxygen species by neutrophils [13] *in vitro*. In addition, HOCl-modified LDL cause endothelial leakage and stimulate leucocyte adherence to, and emigration into, the subendothelial space *in vivo* [14].

Although there is increasing evidence for a role of myeloperoxidase and HOCl in atherogenesis, the mechanism by which this oxidant modifies LDL (and other proteins) remains controversial. On the one hand, exposure of LDL to reagent HOCl (or HOCl formed by myeloperoxidase in the presence of H_2O_2 and Cl^- ions) has been reported to cause primarily the oxidation of amino acid residues of apolipoprotein B-100 (apoB), with little lipid peroxidation (measured as the formation of thiobarbituric acid-reactive material [11,15], hydroxides and hydroperoxides of cholesteryl esters [referred to in combination as CE-O(O)H] [12] or loss of cholesteryl esters [12,16]), or consumption of antioxidants [12]. Lipid peroxidation in LDL occurs as a radical chain process [17] that can be mediated by α-tocopherol (α-TOH) [18]. The fact that α-TOH largely fails to affect the HOCl-induced oxidation of apoB [19] further supports the idea that the primary reactions of this oxidant with LDL are non-radical. This is fully consistent with HOCl's being generally regarded as a nucleophilic oxidant [20].

In apparent contrast with a primarily nucleophilic action, some laboratories consistently report extensive peroxidation of LDL's lipids on treatment with reagent HOCl [13,21–23]. In addition, HOCl has been shown to cause the peroxidation of liposomal lipids [21,24], although the extent of this peroxidation was rather low. A common feature of these studies is that LDL (or liposomes) were incubated with HOCl at 37 °C for prolonged periods. We have observed previously that the concentrations of CE-O(O)H increase time-dependently when LDL, pretreated with HOCl at 4 °C, are incubated subsequently at 37 °C [16]. This, together with the fact that HOCl reacts virtually instantaneously with LDL even when added at 4 °C [12], suggests

Abbreviations used: AAPH, 2,2′-azobis-(2-amidinopropane) hydrochloride; apoB, apolipoprotein B-100; CE-O(O)H, hydroperoxides and hydroxides of cholesteryl esters; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; HOCl, physiological mixture of hypochlorite and hypochlorous acid present at pH 7.4; LDL, low-density lipoproteins; MPO, myeloperoxidase; α-TOH, α-tocopherol; TMP, tocopherol-mediated peroxidation.

[1] To whom correspondence should be addressed (e-mail r.stocker@hri.org.au).

that the observed lipid peroxidation represents a secondary rather than a primary reaction of HOCl. It is well established that in addition to cysteine and methionine, lysine residues are a primary target for HOCl [25,26]. In LDL treated at 4 °C with reagent HOCl at a molar HOCl-to-apoB ratio of 400:1, Lys residues are a major target and are converted into chloramines [12]. The latter are semi-stable species [16,26] and the degradation of LDL-derived chloramines to final products has not been investigated in detail.

It has been demonstrated recently that on incubation at room temperature or 37 °C, chloramines formed on either Lys side-chain amino groups or at the N-terminus decay to give nitrogen-centred radicals, which in turn can give can give rise to other secondary radicals [26,27]. In view of these findings, we speculated that these chloramine-derived radicals might contribute to LDL lipid peroxidation induced by HOCl. The results of the present study are in accord with this hypothesis and hence represent a simple explanation for the previously conflicting reports on the importance of LDL lipid peroxidation induced by HOCl.

EXPERIMENTAL

Materials

Nanopure water was used for all buffers and aqueous solutions, which were subsequently treated with Chelex-100 (Bio-Rad, Hercules, CA, U.S.A.) to remove contaminating transition-metal ions. Heparinized vacutainers were obtained from Becton-Dickinson (Lincoln Park, NJ, U.S.A.), bicinchoninic acid assay reagents, GSH and fluorescamine were from Sigma Diagnostics (Castle Hill, NSW, Australia), α-TOH (analytically pure as determined by HPLC) was from Henkel (Sydney, Australia) and reagent HOCl (5% available chlorine minimum) from Aldrich (Castle Hill, NSW, Australia). The concentration of HOCl was determined spectrophotometrically at pH 13.0 by taking ϵ_{292} as 350 $M^{-1} \cdot cm^{-1}$ [28]. 2,2′-Azobis-(2-amidinopropane) hydrochloride (AAPH) was purchased from Polysciences (Warrington, PA, U.S.A.). Ebselen (2-phenyl-1,2-benzoisoselenazol) was a gift from Daiichi Pharmaceuticals (Tokyo, Japan). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO; Sigma Chemical Co., St. Louis, MO, U.S.A.) was purified before use by treatment with activated charcoal. PBS contained 150 mM NaCl and 50 mM sodium phosphate at pH 7.4 and was used for gel filtration and re-suspension of LDL after isolation. For the isolation of LDL, 0.1% EDTA was added to the PBS. Organic solvents were of HPLC quality (EM Science, Gibbstown, NJ, U.S.A.) or the highest grade available. Hexane was washed with nanopure water before use. Collection of blood for this work was approved by the local human ethics committee.

Preparation of LDL

Fresh blood was obtained from non-fasted, healthy male or female subjects (20–40 years old) and drawn into heparin vacutainers. The blood was centrifuged at 4 °C (1000 ***g*** for 15 min) to separate cells from plasma. LDL were isolated from plasma by ultracentrifugation by a rapid analytical method (417000 ***g***, 2 h, 15 °C) with the use of 5.1 ml tubes and a TL100.4 rotor [29]. All subsequent handling of the samples was performed at 4 °C. Where LDL were required at concentrations greater than 1 mg protein/ml, they were concentrated with Centriprep 30 microconcentrators (Amicon, Danvers, MA, U.S.A.). Low-molecular-mass compounds were removed by gel filtration through a PD-10 column (Pharmacia, Uppsala, Sweden), and the LDL were resuspended in PBS. For supplementation and replenishment procedures (see below), lipoprotein-depleted plasma was obtained by removing 1.7 ml of the bottom fraction of the plasma after its density ultracentrifugation described above.

Supplementation, depletion or replenishment of α-TOH in LDL

Freshly isolated LDL were depleted of α-TOH by rapid oxidation with AAPH at 37 °C, as described in detail previously [30]. The reaction was stopped by cooling to 4 °C on approx. 90% TOH depletion (as assessed by HPLC analysis; see below) and the AAPH was removed by gel filtration. Oxidation of LDL under these conditions caused a rapid consumption of α-TOH with little lipid peroxidation [18,30] and no significant loss of amino acid residues [19] except thiols [18]. Any small amounts of lipid hydroperoxides that formed were reduced to the unreactive hydroxides by treatment of the LDL with 2-phenyl-1,2-benzo-isoselenazol and GSH, followed by gel filtration [31]. LDL referred to as 'native' or 'supplemented' were treated the same way except that AAPH was replaced with PBS for the 37 °C incubation.

Subsequently, one aliquot of native LDL and of α-TOH-depleted LDL were each supplemented and replenished with α-TOH respectively by incubation in lipoprotein-deficient plasma (2:1, v/v) with 20 mM α-TOH in DMSO [30] at 37 °C for 4 h [32]. The other aliquots of native LDL and depleted LDL were each incubated with lipoprotein-deficient plasma treated with DMSO alone under the same conditions as those used for supplementation. The final concentration of DMSO in all samples was 3% (v/v). After supplementation or replenishment, LDL was re-isolated by ultracentrifugation as described for native LDL and then gel-filtered. Replenished LDL, with α-TOH at a comparable concentration to that found in native LDL, have been shown to behave similarly to native LDL on oxidation with a range of different oxidants [30], indicating that the above manipulations *in vitro* do not affect LDL 'oxidizability'. LDL were oxidized with HOCl within 2 days of collection of fresh blood. The concentration of LDL in the resulting solutions was standardized with the bicinchoninic acid assay [33]. The protocol described in the manufacturer's instructions was modified by the addition of SDS [34]; BSA (Sigma Diagnostics) was used as a standard.

HOCl-mediated oxidation of LDL

Oxidation of LDL with reagent HOCl was performed on ice by the addition of 1 vol. of HOCl, freshly diluted in PBS, to 4 vol. of LDL solution (final concentration 0.2–5 mg/ml protein). After the addition of HOCl, the reaction mixture was mixed briefly (less than 1 s) and incubated at 4 °C, at room temperature or at 37 °C for the indicated durations. When included in the oxidation reaction, DMPO (final concentration 167 mM) was added to the LDL before oxidation. Control experiments indicated that the extent of radical formation and protein oxidation induced by HOCl were not different whether DMPO was added before or after oxidation (results not shown). In contrast, Met (40 mM final concentration) was always added immediately after oxidation, before subsequent incubation.

Myeloperoxidase (MPO)-mediated oxidation of LDL

The MPO-mediated oxidation of LDL was performed at 37 °C in PBS. The reaction mixture consisted of LDL (0.11 mg/ml protein), MPO (5 nM, 2.2 units/ml) and H_2O_2 (final concentration 18 μM). The reaction mixture was preincubated at 37 °C for 10 min before the addition of H_2O_2, which was added as nine aliquots at 2 min intervals with brief mixing after each addition.

Under these conditions each aliquot of H_2O_2 was consumed within 1.5 min and the maximum H_2O_2 concentration in the reaction mixture was 2.0 μM. MPO activity was measured with the guaicol assay [49 mM sodium phosphate/99 mM guaicol/421 μM H_2O_2/0.12 unit/ml MPO (pH 7.0 at 25 °C)] [35]. One unit of enzyme is defined as that producing an increase in A_{470} of 1.0 in 1 min.

Quantification of amino acid residues

Unmodified Lys residues in LDL were quantified by fluorescamine fluorescence (excitation at 390 nm, emission at 475 nm) with the manual procedure of Böhlen et al. [36] as described previously [12]. Trp fluorescence of LDL was measured in the absence of SDS with excitation at 280 nm and emission at 335 nm [37]. For both Lys and Trp residues, the fluorescence of HOCl-oxidized samples was expressed as a percentage of the fluorescence of the corresponding LDL sample not treated with HOCl.

Measurement of antioxidants and lipid content of LDL

To determine the content of LDL's antioxidants, unoxidized lipids and CE-O(O)H, a 100 μl aliquot of the reaction mixture was extracted with 1 ml of cold methanol containing 0.02% (v/v) acetic acid and 5 ml of hexane. The residue of the vacuum-dried hexane phase was redissolved in propan-2-ol for analysis. Cholesterol, cholesteryl esters and CE-O(O)H were separated by HPLC and quantified by UV and/or chemiluminescence detection as described [29]. α-TOH was determined by HPLC with electrochemical detection [29]. Lipids and α-TOH were quantified by area comparison with authentic standards, with cholesterol as internal standard.

EPR spectroscopy

EPR spectra were recorded at room temperature with a Bruker EMX X-band spectrometer with 100 kHz modulation and either a standard rectangular (ER 4102ST) or a cylindrical (ER 4103TM) cavity. Samples were contained in a standard, flattened, aqueous sample cell. Typical EPR spectrometer settings were: gain 3.1×10^6, modulation amplitude 0.1 mT, time constant 0.16 s, scan time 167 s, resolution 1024 points, centre field 348.0 mT, field scan 8 mT, power 31 mW, frequency 9.76 GHz, sum of five scans. Signal intensity was calculated from the peak-to-trough line heights of spectra recorded under identical conditions and is reported relative to that observed at the earliest time point measured (2–15 min).

RESULTS

The addition of HOCl to LDL at a molar ratio of oxidant to lipoprotein of 400:1 caused the rapid oxidation of Lys and Trp residues (Figure 1A). Lys oxidation, assessed with the fluorescamine assay, was complete 1 min after addition of HOCl (results not shown). The subsequent incubation of HOCl-oxidized LDL at 37 °C resulted in a gradual regeneration of a small proportion of Lys residues (Figure 1A), corresponding to 62 ± 74 (mean $\pm$ S.D., $n = 6$) residues per apoB molecule (of a total of 356 [38]) after 4 h of incubation. In contrast, the addition of HOCl to LDL gave rise to only small quantities of CE-O(O)H within the first 1 min, although the amount increased on further incubation at 37 °C (Figure 1B). The kinetics of these reactions suggest that the oxidation of Lys and Trp residues occurs by direct reaction with HOCl, which is preferred over the peroxidation of cholesteryl esters. Gel filtration of LDL immediately

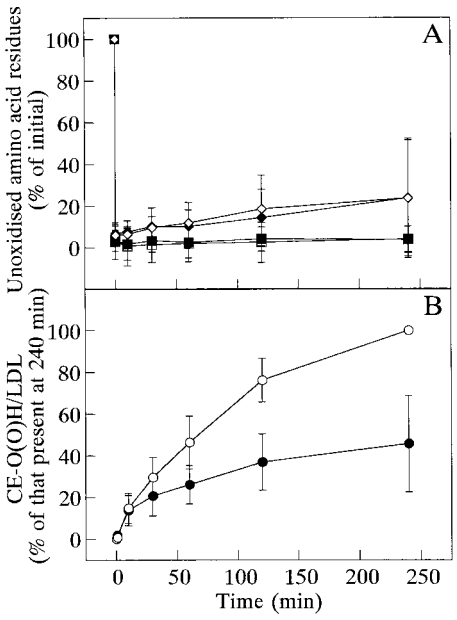

Figure 1 Lys and Trp are oxidized rapidly on treatment of LDL with HOCl, whereas CE-O(O)H accumulate slowly on incubation of the reaction mixture at 37 °C

LDL (5 mg/ml protein) in either the presence (◆, ■, ●) or absence (◇, □, ○) of DMPO were oxidized with a 400:1 ratio of HOCl to LDL particles at 4 °C, then incubated at 37 °C for the indicated durations. The initial time point was taken before the addition of HOCl. (**A**) Lys (◇, ◆) and Trp (□, ■) consumption were measured by decreases in fluorescamine and Trp fluorescence respectively. (**B**) CE-O(O)H (○, ●) were determined by HPLC with detection by UV and chemiluminescence. Results are means $\pm$ S.D. for three independent experiments with LDL obtained from three different donors. The concentration of CE-O(O)H at 4 h for samples lacking DMPO in each experiment varied [15.5, 6.9 and 9.9 molecules of CE-O(O)H per apoB molecule] and was set to 100% in each case; all other values were calculated as a percentage of this value.

after the addition of HOCl (and before incubation at 37 °C) did not substantially affect the extent of CE-O(O)H accumulation (results not shown), which excludes the possibility that subsequent lipid peroxidation occurred as a result of remaining HOCl.

When MPO/H_2O_2/Cl^- was used to oxidize LDL, Lys and Trp residues were oxidized to the extent expected if the conversion of H_2O_2 into HOCl were stoichiometric, which is consistent with previous experiments comparing the enzymic system with reagent HOCl [16]. In these experiments, lower concentrations of HOCl were employed than in those with reagent HOCl owing to limitations in the availability of the enzyme and its limited turnover [25]. Small and defined aliquots of H_2O_2 were added to limit enzyme inactivation and to decrease the probability of the participation of H_2O_2 in (non-enzymic) oxidation. As seen with reagent HOCl, a rapid consumption of Lys and Trp residues was observed during the period of generation of HOCl. After the exhaustion of H_2O_2, no further oxidation of these amino acids occurred (Figure 2A). In contrast, CE-O(O)H accumulated during both the initial and subsequent periods (Figure 2B and inset). These results are consistent with the occurrence of Lys and Trp oxidation via primary reactions with enzymically generated HOCl, whereas CE-O(O)H accumulation presumably reflected secondary reactions. We cannot dissociate primary from secondary reactions occurring during the first 18 min of the incubation during which HOCl was generated. This might explain why a relatively (to reagent HOCl) large proportion of CE-

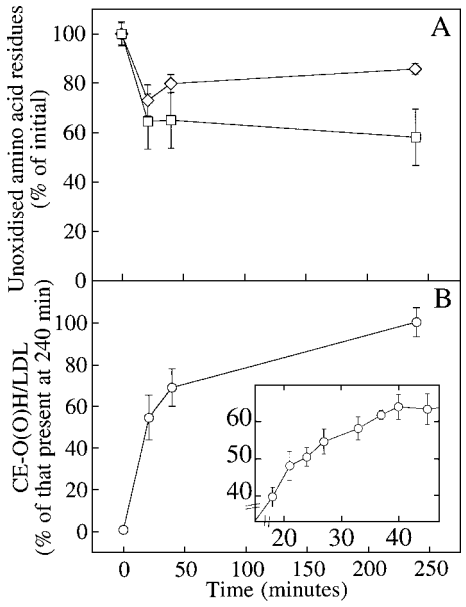

Figure 2 Lys and Trp oxidation by MPO/H_2O_2/Cl^- coincides with cessation of HOCl generation, whereas CE-O(O)H continue to accumulate

Oxidation by H_2O_2 aliquots was performed as described in the Materials and methods section. The initial time point was taken before any additions. Lys and Trp (**A**) and CE-O(O)H (**B**) were determined; symbols are the same as in the legend to Figure 1. There was no significant difference between the amounts of Lys and Trp at 20 and 240 min, whereas CE-O(O)H increased significantly over the same period ($P = 0.02$, with Student's t test). Results are means ± S.D. for three experiments, each performed in duplicate with LDL obtained from a single donor. The results (mean ± range) shown in the inset are from two experiments, each performed in duplicate. The concentration of CE-O(O)H at 4 h in each experiment varied [1.9, 3.0 and 3.0 molecules of CE-O(O)H per apoB molecule] and was set to 100%, with all other values calculated as a percentage of this. Control incubations excluding either MPO or H_2O_2 yielded no detectable protein oxidation; MPO or H_2O_2 marginally increased the CE-O(O)H detected after 4 h from 0.04 to 0.08 or 0.25 molecules of CE-O(O)H per LDL particle respectively.

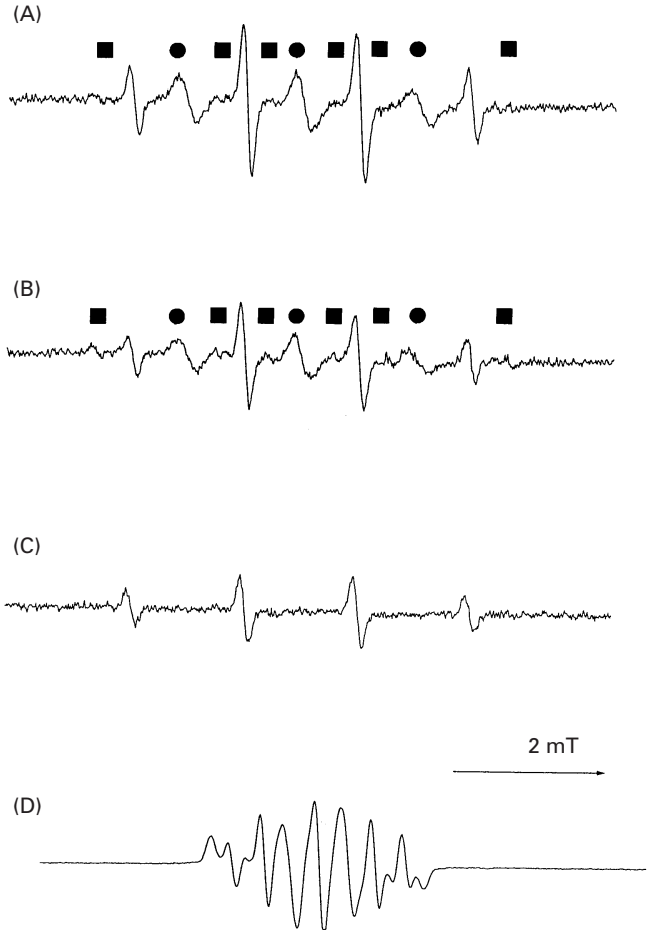

Figure 3 EPR spectra observed on reaction of HOCl with LDL in the presence and absence of Met

(**A**) EPR spectra observed on the reaction of LDL (4.7 mg/ml protein) and the spin trap DMPO (0.166 M) with HOCl (3.75 mM) in PBS, pH 7.4, with HOCl added last. Spectra were accumulated between 2 and 17 min after initiation of the reaction. (**B**) As (**A**) but with 40 mM Met added after the addition of HOCl. (**C**) As (**B**) but with Met added before the spin trap (i.e. the order of addition was LDL, HOCl, Met, DMPO). (**D**) As (**A**) but in the absence of LDL. The major signal in (**A**–**C**) was assigned to the spin adduct DMPO-OH^o [a(N) 1.49, a(H) 1.49 mT]. The signal marked (●) in (**A**) and (**B**) was due to a breakdown product of the spin trap. Weak additional features (■) present in (**A**) and (**B**) were assigned to carbon-centred radical adducts (see the text). The signal in (**D**) was assigned to the 2-(*N*-chloroimino)-5,5-dimethylpyrrolidine-1-oxyl radical formed via the direct reaction of HOCl with DMPO (see [39] for additional information). Spectra in (**A**–**C**) were recorded under identical spectrometer conditions; the spectrum in (**D**) was recorded with gain 20% of that in (**A**–**C**).

O(O)H accumulated during this time of presence/formation of HOCl rather during than the subsequent period of incubation.

The time-dependent formation of CE-O(O)H in HOCl-oxidized LDL was inhibited by DMPO (Figure 1B), whereas the radical trap had no effect on the initial extent of oxidation and the subsequent concentrations of Lys and Trp residues determined (Figure 1A). The increase in CE-O(O)H accumulation during the incubation of HOCl-treated LDL at 37 °C, and the inhibition by DMPO, were observed in three separate experiments, although the absolute magnitude of lipid peroxidation varied between experiments (see the legend to Figure 1). Although we do not at present know the reason(s) for the observed variation in the extent of HOCl-induced lipid peroxidation, the effects of DMPO suggest that the HOCl-induced lipid peroxidation reactions are likely to be radical-mediated, whereas the oxidation of Lys and Trp residues does not involve radical intermediates.

The formation of radicals from HOCl-oxidized LDL was investigated directly with EPR spin trapping. When HOCl was added to LDL in the presence of DMPO, an EPR-detectable signal corresponding to the DMPO-OH adduct was detected as the major signal (Figure 3A) at the first time point that we were able to measure (2–15 min). Weak signals from additional carbon-centred species [a(N) 1.57, 1(H) 2.45 mT and a(N) 1.53, a(H) 2.03 mT] were also detected, particularly at early time points. These signals are assigned to protein side-chain-derived radicals analogous to those detected with other proteins [26,27] and were too weak for accurate quantification. Identical signals were observed if DMPO was added immediately after the addition of HOCl to LDL (results not shown). The intensity of these signals (i.e. radical adduct concentrations) was diminished when excess Met was added before the spin trap, but little difference was observed when the Met was included after the DMPO (Figures 3B and 3C). When HOCl was added to DMPO in the absence of LDL, the major signal observed was that of a DMPO-derived chloramine (Figure 3D) [39]. These findings indicate that the signals observed in the presence of LDL do not arise via a direct reaction of HOCl with DMPO [40]. During the incubation of the HOCl-oxidized LDL at room temperature, the EPR signal due to DMPO-OH increased 2–3-fold over a period of 180 min (Figure 4). These results suggest that there is both a rapid initial burst of formation of radicals from HOCl-treated LDL and a

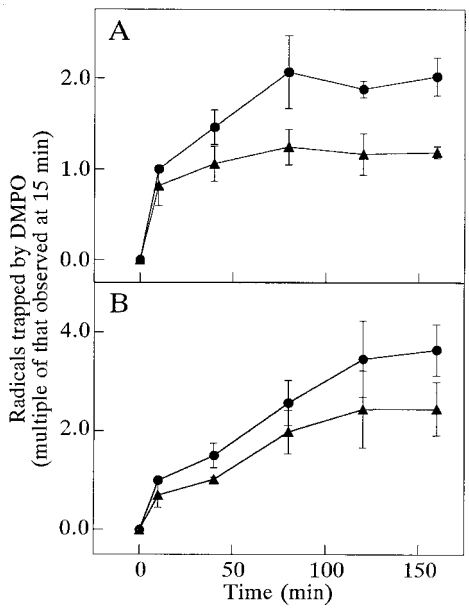

Figure 4 Time-dependent trapping by DMPO of radicals produced during incubation of HOCl-oxidized LDL at room temperature in the absence and presence of Met

LDL (2.5 or 5 mg/ml protein) were oxidized at 4 °C with 400:1 (**A**) or 800:1 (**B**) ratios of HOCl to LDL and then incubated at room temperature for the indicated durations in the presence (▲) or absence (●) of Met (40 mM), which was added after treatment with HOCl but before incubation. The formation of DMPO-OH was detected by EPR spectroscopy and the signal intensity is expressed as a fraction of that detected at 15 min in the absence of Met. Results are means ± S.D. for three independent experiments with LDL obtained from three different donors.

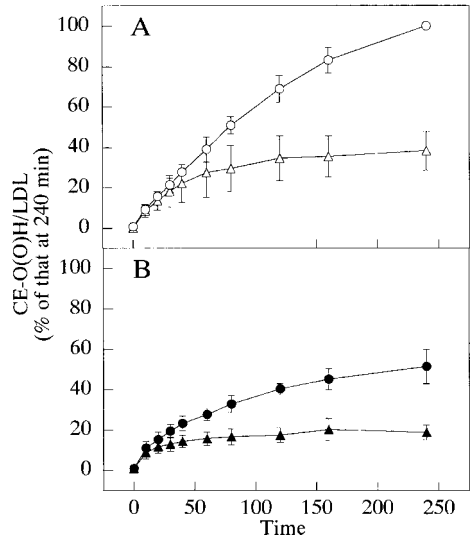

Figure 5 Methionine and DMPO inhibit the time-dependent accumulation of CE-O(O)H observed on incubation of HOCl-oxidized LDL at 37 °C

LDL (2.5 mg protein/ml) were oxidized with an 800:1 ratio of HOCl/LDL particles at 4 °C in the absence (**A**) or presence (**B**) of DMPO (167 mM) and then incubated at 37 °C for the indicated durations in the presence (△, ▲) or absence (○, ●) of Met (40 mM). CE-O(O)H were determined by HPLC with UV and chemiluminescence detection. Results are means ± S.D. for three independent experiments with LDL obtained from three different donors. The values of CE-O(O)H in LDL without DMPO and Met at 4 h in each experiment varied [8.5, 19.0 and 18.4 molecules of CE-O(O)H per apoB molecule] and were used to calculate percentage values for all other samples in that experiment.

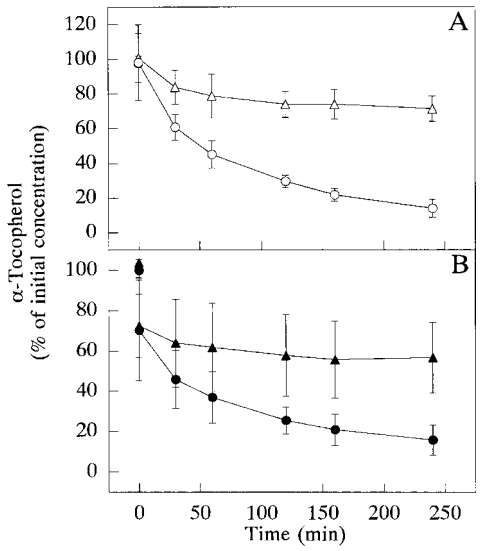

Figure 6 Time-dependent consumption of α-TOH during incubation of HOCl-oxidized LDL at 37 °C is inhibited by Met but not DMPO

Experimental conditions and symbols used were as described in the legend to Figure 5. α-TOH was determined as described in the Materials and methods section. Results are means ± S.D. for three independent experiments with LDL obtained from three different donors. Initial α-TOH values (100%) were 6.2, 7.2 and 5.3 molecules of α-TOH per LDL particle. Separate aliquots were removed immediately before and after the addition of HOCl.

slower, prolonged secondary phase. Both phases of radical formation are thought to arise via the decomposition of initial HOCl-induced oxidation products.

The major type of primary oxidation products in LDL treated with HOCl at an oxidant-to-apoB ratio of 400:1 are chloramines [12]. Protein-bound chloramines can give rise to radicals [26] and are reduced by reaction with Met [41]. Treatment of HOCl-oxidized LDL with Met inhibited the formation of some, but not all, radicals (Figures 3B, 3C and 4A), and the extent of this inhibition decreased as the initial HOCl-to-apoB ratio increased (Figure 4B). These results imply that chloramines are a major (but not necessarily the only) source of radicals generated during the incubation of HOCl-oxidized LDL; other HOCl-modified components of LDL might also have an important role, particularly with more extensively oxidized LDL.

Consistent with the less intense EPR signals detected when Met was added before the spin trap, the addition of Met also decreased the amount of CE-O(O)H accumulating during the incubation of HOCl-oxidized LDL at room temperature (results not shown) or at 37 °C for 4 h (Figure 5). The decreased accumulation of CE-O(O)H further suggests that chloramine-derived radicals are the most likely initiating agents for the peroxidation of core lipids. The absolute amount of CE-O(O)H that accumulated varied between experiments (see the legend to Figure 5), although the trends were similar between experiments. We attempted to exclude the possibility that transition metals were causing the variation between experiments by routinely chelex-treating all buffers, because iron is known to enhance chloramine decomposition [42,43]. The effect of DMPO on lipid peroxidation varied with the incubation temperature. At 37 °C, DMPO clearly inhibited lipid peroxidation (Figure 5), whereas at room temperature the effect was less clear (results not shown). The incubation of HOCl-treated LDL at 37 °C resulted in a time-dependent consumption of α-TOH (Figure 6), the kinetics of

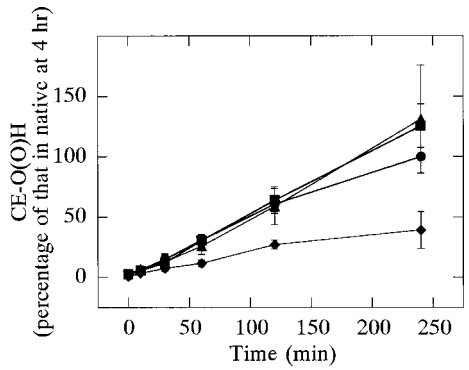

Figure 7 α-TOH is required but is not limiting for time-dependent peroxidation of core lipids in HOCl-oxidized LDL

Native LDL (●), LDL supplemented with α-TOH (■), LDL depleted of α-TOH (◆) and depleted LDL replenished with α-TOH (▲) were prepared as described in the Materials and methods section. Lipoproteins (each 0.2 mg/ml protein) were then oxidized (400:1 ratio of HOCl to LDL) at 4 °C and then incubated at 37 °C for the indicated durations. CE-O(O)H were determined by HPLC with chemiluminescence detection. Results are means ± S.D. for three independent experiments with LDL obtained from three different donors. The α-TOH contents of native, supplemented, depleted and replenished LDL were in the ranges 6.0–17.0, 32–141, 1.0–3.3 and 25–142 molecules of α-TOH per apoB molecule respectively. The values of CE-O(O)H in native LDL at 4 h in each experiment varied [12.1, 10.1 and 9.6 molecules of CE-O(O)H per apoB molecule] and were used to calculate percentage values for all other samples.

which reflected that of CE-O(O)H accumulation. The extent of α-TOH loss was inhibited strongly in the presence of Met, whereas DMPO was without effect (Figure 6). This might reflect the difference between the removal of the precursor chloramine species and the efficiency of trapping of radicals once they have been formed. The accumulation of CE-O(O)H in LDL undergoing oxidation can vary without detectable changes in the rate of α-TOH consumption [18].

We have shown previously that radical-induced LDL lipid peroxidation can proceed via tocopherol-mediated peroxidation (TMP) [18,30,44]. A feature of TMP is that, at low fluxes, radicals require α-TOH for the initiation of lipid peroxidation, to an extent that increases with decreasing reactivity of the radical species involved [18,30]. To investigate the role of TMP in HOCl-induced LDL lipid peroxidation, we therefore compared the extent of CE-O(O)H accumulation in HOCl-treated control LDL with that in LDL depleted of α-TOH *in vitro* [30]. Depleting LDL of approx. 90% of its endogenous α-TOH significantly decreased the extent of lipid peroxidation induced by HOCl (Figure 7). Replenishing such α-TOH-depleted LDL with α-TOH restored the 'oxidizability' of the lipoprotein particle (Figure 7). Increasing LDL's content of α-TOH from 6 to 142 molecules of α-TOH per lipoprotein did not affect the extent of CE-O(O)H accumulation (Figure 7). These results suggest that the peroxidation of core lipids in HOCl-oxidized LDL requires α-TOH, although the amount of α-TOH in native LDL does not seem to limit such lipid peroxidation.

DISCUSSION

The results obtained in this study suggest that the oxidation of LDL's lipids by both reagent and enzymically generated HOCl is a secondary reaction, with most of the oxidant being consumed in initial reactions with the protein. This is in accord with previous work that showed that Lys, Trp, Met and Cys residues of apoB rather than lipids or antioxidants are the major targets for HOCl at 800:1 or lower molar excess over apoB [12,16]. The subsequent incubation of such samples at 37 °C does, however, result in the oxidation of both lipids [measured as CE-O(O)H] and antioxidants (loss of α-TOH).

This secondary oxidation process involves radical formation, as evidenced by EPR spin trapping. Both the concentration of the EPR spin adduct and the extent of lipid peroxidation were attenuated by the treatment of HOCl-oxidized LDL with Met before incubation at 37 °C. Lipid peroxidation was also decreased in the presence of the EPR spin trap. These inhibitory effects are interpreted by the removal of chloramines by Met [41] and trapping of the chloramine-derived radicals (which would otherwise initiate lipid oxidation) by DMPO respectively. Indeed, isolated proteins, peptides and free amino acids treated with small excesses of HOCl give rise to Lys-derived chloramines that decompose to nitrogen- and carbon-centred radicals [26,27].

These results rationalize the discrepancy between our previous results [12,16] and those of other workers [13,21–23]. The latter examined solely the extent of lipid oxidation after the incubation of LDL with HOCl at 37 °C for considerable periods, without examining early time points of lipid oxidation or alteration of the protein component of LDL. Thus these studies 'selected' for the secondary, rather than the initial, HOCl-induced reactions. The current results might also explain why the exposure of high-density lipoproteins to HOCl *in vitro* was found to cause more extensive oxidation of the lipid than of the protein moieties [45]; in that study, HOCl-treated samples were incubated initially for 1 h at 37 °C and then for 2–24 h at 4 °C before analysis.

The present results have important implications for the development of antioxidant therapies against HOCl-mediated injury. Thus, in addition to inhibiting HOCl formation, compounds that rapidly remove chloramines (i.e. the radical source) would be expected to prevent lipid peroxidation, regardless of whether the compounds directly inhibit radical reactions. Two-electron reduction of the chloramine to regenerate the parent amine might be a potentially useful strategy, because excess Met (which acts in this manner with concomitant oxidation to the sulphoxide) affords protection against LDL lipid peroxidation (the present study) or protein fragmentation [26]. Removal of the radical precursors is likely to be advantageous over scavenging of radicals once formed, as chloramines have much longer lifetimes than most radicals, thereby offering a longer time-frame for encounter and elimination.

The major endogenous antioxidant in LDL, α-TOH, is unable to protect LDL's apoB from HOCl-induced oxidation [19]. Furthermore, α-TOH might act as a pro-oxidant for lipids, as α-TOH-depleted LDL are more resistant to secondary lipid peroxidation (Figure 7). This is fully consistent with the TMP model of lipoprotein lipid peroxidation [18,44,46], in which α-TOH is an antioxidant only under high radical flux conditions (i.e. under which radical–radical termination reactions become significant as a result of the presence of more than one radical per particle) or in the presence of co-antioxidants that remove radicals out of the particle [47]. In our system *in vitro*, α-TOH is unable to act in the latter manner because co-antioxidants are no longer present (in the case of ubiquinol-10 [12]) or have not been added.

In the present study, α-TOH could be acting as a radical transfer agent by reacting with radicals generated from chloramines. Alternatively, it might act as a chain-carrying agent after the initiation of lipid oxidation by a chloramine-derived radical. The latter might indeed take place because lipid peroxidation occurs even in α-TOH-depleted LDL, although we cannot exclude the possibility that the low level of oxidation seen in these depleted particles arises from residual α-TOH. Supplementation of LDL (or reconstitution of depleted particles) to give

supranormal concentrations of α-TOH does not, however, enhance the rate of lipid peroxidation above that of controls, suggesting that α-TOH is only rate-limiting when present at very low concentrations. The rate-limiting factor(s) under other conditions could be the rate of chloramine decomposition.

The exact mechanism(s) by which the initial protein-derived species initiate secondary lipid peroxidation and antioxidant consumption remain to be fully elucidated. Reaction with LDL's Lys, Trp, Cys and Met residues [25] accounts for a high percentage of the HOCl added, particularly when small excesses of HOCl are employed [12]. However, these residues do not consume all HOCl, so alternative targets such as other amino acid side chains, amide groups and phosphatidylcholine might have a role. Amides are > 10-fold less reactive towards HOCl as free amines [27], and seem to be major targets only after the depletion of more reactive sites. Whether products formed at sites other than Lys amino groups on proteins are capable of generating radicals is unknown. Studies with a range of isolated proteins and peptides and small excesses of HOCl have, however, detected radical formation only from Lys side-chains and free N-terminal amine groups [26,27]. This suggests that chloramines formed from apoB's Lys residues are the major source of radicals detected in this study. This is consistent with the inhibitory effect of added Met, though this treatment did not completely eliminate the spin adduct signals and lipid peroxidation. This incomplete inhibition might be due to a limited accessibility of Met to the chloramines, although it is also consistent with other species being involved in the initiation of peroxidation. However, the current results are consistent with initial two-electron reaction of HOCl with the ϵ-amino group of Lys residues on apoB, giving chloramines that subsequently decompose to radicals, which are the major, if not the only, initiators of lipid peroxidation.

Blocking the ϵ-amino group of Lys residues in LDL or BSA enhances (rather than inhibits) LDL lipid peroxidation [16] or albumin fragmentation [26] respectively. This suggests that in the absence of Lys, HOCl reacts with other targets that can give rise to radicals, so that the reaction of HOCl with Lys residues can be regarded as a protective (antioxidant) action [26].

Overall this study offers a rational explanation for previous contradictory reports on the occurrence of lipid peroxidation in HOCl-treated LDL. Thus the initial reaction of low excesses of HOCl with LDL occurs primarily by two-electron (nucleophilic) reactions with the side-chain sites of apoB. Lys-derived chloramines are among the products of these initial reactions and subsequently undergo homolytic (one-electron) reactions to give radicals that can initiate lipid peroxidation and antioxidant consumption. The extent of this lipid peroxidation is inversely dependent on the α-TOH concentration, with tocopherol depletion resulting in a lower yield of CE-O(O)H than in controls. This is consistent with apoB chloramine-derived radicals initiating lipid oxidation via TMP.

We thank Dr. W. Jessup for reading the manuscript critically and Dr. Clare Hawkins for helpful discussions. This study was supported by the National Heart Foundation (grant G9554338 to R. S. and L. J. H.) and the Australian Research Council (M. J. D.).

REFERENCES

1 Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C. and Witztum, J. L. (1989) New Engl. J. Med. **320**, 915–924
2 Berliner, J. A. and Heinecke, J. W. (1996) Free Radicals Biol. Med. **20**, 707–727
3 Diaz, M. N., Frei, B., Vita, J. A. and Keaney, Jr., J. F. (1997) New Engl. J. Med. **337**, 408–416
4 Kuhn, H., Belkner, J., Zaiss, S., Fahrenklemper, T. and Wohlfeil, S. (1994) J. Exp. Med. **179**, 1903–1911
5 Folcik, V. A., Nivar-Aristy, R. A., Krajewski, L. P. and Cathcart, M. K. (1995) J. Clin. Invest. **96**, 504–510
6 Leeuwenburgh, C., Hardy, M. M., Hazen, S. L., Wagner, P., Oh-ishi, S., Steinbrecher, U. P. and Heinecke, J. W. (1997) J. Biol. Chem. **272**, 1433–1436
7 Fu, S., Davies, M. J., Stocker, R. and Dean, R. T. (1998) Biochem. J. **333**, 519–525
8 Daugherty, A., Dunn, J. L., Rateri, D. L. and Heinecke, J. W. (1994) J. Clin. Invest. **94**, 437–444
9 Hazell, L. J., Arnold, L., Flowers, D., Waeg, G., Malle, E. and Stocker, R. (1996) J. Clin. Invest. **97**, 1535–1544
10 Hazen, S. L. and Heinecke, J. W. (1997) J. Clin. Invest. **99**, 2075–2081
11 Arnhold, J., Wiegel, D., Richter, O., Hammerschmidt, S., Arnold, K. and Krumbiegel, M. (1991) Biomed. Biochim. Acta **50**, 967–973
12 Hazell, L. J. and Stocker, R. (1993) Biochem. J. **290**, 165–172
13 Kopprasch, S., Leonhardt, W., Pietzsch, J. and Kühne, H. (1998) Atherosclerosis **136**, 315–324
14 Liao, L., Aw, T. Y., Kvietys, P. R. and Granger, D. N. (1995) Arterioscler. Thromb. Vasc. Biol. **15**, 2305–2311
15 Yang, C. Y., Gu, Z. W., Yang, H. X., Yang, M., Gotto, Jr., A. M. and Smith, C. V. (1997) Free Radicals Biol. Med. **23**, 82–89
16 Hazell, L. J., van den Berg, J. J. M. and Stocker, R. (1994) Biochem. J. **302**, 297–304
17 Sato, K., Niki, E. and Shimasaki, H. (1990) Arch. Biochem. Biophys. **279**, 402–405
18 Bowry, V. W. and Stocker, R. (1993) J. Am. Chem. Soc. **115**, 6029–6044
19 Hazell, L. J. and Stocker, R. (1997) FEBS Lett. **414**, 541–544
20 March, J. (1977) Advanced Organic Chemistry. Reactions, Mechanisms, and Structure, 2nd edn., pp. 741–742, McGraw-Hill, New York
21 Stelmaszynska, T., Kukovetz, E., Egger, G. and Schaur, R. J. (1992) Int. J. Biochem. **24**, 121–128
22 Panasenko, O. M., Evgina, S. A., Aidyraliev, R. K., Sergienko, V. I. and Vladimirov, Y. A. (1994) Free Radicals Biol. Med. **16**, 143–148
23 Panasenko, O. M., Briviba, K., Klotz, L. O. and Sies, H. (1997) Arch. Biochem. Biophys. **343**, 254–259
24 Panasenko, O. M., Arnhold, J., Schiller, J., Arnold, K. and Sergienko, V. I. (1994) Biochim. Biophys. Acta **1215**, 259–266
25 Winterbourn, C. C. (1985) Biochim. Biophys. Acta **840**, 204–210
26 Hawkins, C. L. and Davies, M. J. (1998) Biochem. J. **322**, 617–625
27 Hawkins, C. L. and Davies, M. J. (1998) J. Chem. Soc. Perkin Trans. **2**, 1937–1945
28 Morris, J. C. (1966) J. Phys. Chem. **70**, 3798–3805
29 Sattler, W., Mohr, D. and Stocker, R. (1994) Methods Enzymol. **233**, 469–489
30 Neuzil, J., Thomas, S. R. and Stocker, R. (1997) Free Radicals Biol. Med. **22**, 57–71
31 Frei, B. and Gaziano, J. M. (1993) J. Lipid Res. **34**, 2135–2145
32 Esterbauer, H., Dieber-Rotheneder, M., Striegl, G. and Waeg, G. (1991) Am. J. Clin. Nutr. **53**, 314S–321S
33 Smith, P. K., Krohn, R. J., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985) Anal. Biochem. **150**, 76–85
34 Morton, R. E. and Evans, T. A. (1992) Anal. Biochem. **204**, 332–334
35 Desser, R. K., Himmelhoch, S. R., Evans, W. H., Januska, M., Mage, M. and Shelton, E. (1972) Arch. Biochem. Biophys. **148**, 452–465
36 Böhlen, P., Stein, S., Dairman, W. and Udenfriend, S. (1973) Arch. Biochem. Biophys. **155**, 213–220
37 Teale, F. W. J. (1960) Biochem. J. **76**, 381–388
38 Yang, C.-Y., Chan, L. and Gotto, A. M. (1987) in Plasma Lipoproteins (Gotto, Jr., A. M., ed.), pp. 77–93, Elsevier, Amsterdam
39 Bandara, B. M. R., Hinojosa, O. and Bernofsky, C. (1994) J. Org. Chem. **59**, 1642–1654
40 Bernofsky, C., Bandara, B. M., Hinojosa, O. and Strauss, S. L. (1990) Free Radicals Res. Commun. **9**, 303–315
41 Thomas, E. L., Jefferson, M. M. and Grisham, M. B. (1982) Biochemistry **21**, 6299–6308
42 Neale, R. S. (1971) Synthesis, 1–15
43 Sosnovsky, G. and Rawlinson, D. J. (1972) Adv. Free Radical Chem. **4**, 203–284
44 Bowry, V. W., Ingold, K. U. and Stocker, R. (1992) Biochem. J. **288**, 341–344
45 Panzenboeck, U., Raitmayer, S., Reicher, H., Lindner, H., Glatter, O., Malle, E. and Sattler, W. (1997) J. Biol. Chem. **272**, 29711–29720
46 Ingold, K. U., Bowry, V. W., Stocker, R. and Walling, C. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 45–49
47 Bowry, V. W., Mohr, D., Cleary, J. and Stocker, R. (1995) J. Biol. Chem. **270**, 5756–5763

Received 7 December 1998/29 January 1999; accepted 19 February 1999