Vitamin C protects against and reverses specific hypochlorous acid- and chloramine-dependent modifications of low-density lipoprotein

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Activated phagocytes produce the highly reactive oxidant hypochlorous acid (HOCl) via the myeloperoxidase-catalysed reaction of hydrogen peroxide with chloride ions. HOCl reacts readily with a number of susceptible targets on apolipoprotein B-100 of low-density lipoprotein (LDL), resulting in uncontrolled uptake of HOCl-modified LDL by macrophages. We have investigated the effects of vitamin C (ascorbate), an effective water-soluble antioxidant, on the HOCl- and chloramine-dependent modification of LDL. Co-incubation of vitamin C (25–200 μ M) with LDL resulted in concentration-dependent protection against HOCl (25–200 μ M)-mediated oxidation of tryptophan and lysine residues, formation of chloramines and increases in the relative electrophoretic mobility of LDL. Vitamin C also partially protected against oxidation of cysteine residues by HOCl, and fully protected against oxidation of these residues by the low-molecular-mass chloramines, *N*^ε-acetyl-lysine chloramine and taurine chloramine, and to a lesser extent monochloramine (each

at 25–200 μ M). Further, we found that HOCl (25–200 μ M)-dependent formation of chloramines on apolipoprotein B-100 was fully reversed by 200 μ M vitamin C; however, the loss of lysine residues and increase in relative electrophoretic mobility of LDL were only partially reversed, and the loss of tryptophan and cysteine residues was not reversed. Time-course experiments showed that the reversal by vitamin C of HOCl-dependent modifications became less efficient as the LDL was incubated for up to 4 h at 37 °C. These data show that vitamin C not only protects against, but also reverses, specific HOCl- and chloramine-dependent modifications of LDL. As HOCl-mediated LDL modifications have been strongly implicated in the pathogenesis of atherosclerosis, our data indicate that vitamin C could contribute to the anti-atherogenic defence against HOCl.

Key words: antioxidant, apolipoprotein B-100, ascorbate, atherosclerosis, oxidized LDL.

INTRODUCTION

Oxidatively modified low-density lipoprotein (LDL) has been strongly implicated in the pathogenesis of atherosclerosis [1]. Historically, metal-ion-mediated mechanisms have been the best studied models of LDL oxidation, although the pathophysiological relevance of these mechanisms *in vivo* is uncertain. Peripheral blood leucocytes, such as neutrophils and monocytes, produce the reactive oxidant hypochlorous acid (HOCl) following activation by respiratory burst stimulants [2]. The enzyme myeloperoxidase (MPO), which is released from activated leucocytes, catalyses the reaction of hydrogen peroxide with chloride ions to form HOCl [2]. Several studies have shown that MPO-derived HOCl may be a physiologically relevant oxidant in the development of atherosclerosis. Active MPO and epitopes recognized by antibodies against HOCl-modified proteins have been detected in human atherosclerotic lesions [3,4]. In addition, the specific MPO- and HOCl-modified protein biomarkers di-tyrosine and 3-chlorotyrosine have been identified in atherosclerotic plaques [5,6].

Unlike the metal-ion-dependent modification of LDL, which occurs primarily through lipid peroxidation and subsequent derivatization of apolipoprotein B-100 (apoB) lysine residues by reactive, lipid-hydroperoxide-derived aldehydes [7], HOCl reacts directly with lysine residues, with very little modification of the lipids in LDL [8,9]. Reaction of HOCl with the ϵ -amino groups of lysine residues results in the formation of mono- and dichloramines (reactions 1 and 2 respectively):

Chloramines on LDL have been implicated in the altered electrophoretic migration, aggregation and subsequent uncontrolled uptake of HOCl-modified LDL by macrophages in culture [8–10]. Other amino acid residues of apoB, such as cysteine, methionine, tryptophan and tyrosine, are also susceptible to oxidation by HOCl, although tyrosine is only a minor target [6,8,9]. Interestingly, a recent report indicated that LDL-associated chloramines break down over time to produce radicals that can initiate lipid peroxidation [11]. This finding may explain why a number of investigators have reported lipid peroxidation in isolated LDL exposed to reagent- or MPO-derived HOCl [9,12,13]. Formation of chlorinated lipids has also been reported [14], although these are only minor products, requiring large amounts of HOCl.

Vitamin E (α -tocopherol), the major lipid-soluble antioxidant in LDL, is unable to protect the lipoprotein against HOCl-mediated modification [15,16], and may actually enhance oxidation via ‘tocopherol-mediated peroxidation’ [11]. One potential defence against HOCl is vitamin C (ascorbate), an important water-soluble antioxidant in biological fluids [17]. Vitamin C scavenges HOCl in a stoichiometric manner (reaction 3) [18], and can reverse some HOCl-mediated damage (reaction 4) [8]:

Abbreviations used: ABD-F, 7-fluorobenz-2-oxa-1,3-diazole-4-sulphonamide; apoB, apolipoprotein B-100; CEOOH, cholesteryl ester hydroperoxides; DTPA, diethylenetriaminepenta-acetic acid; LDL, low-density lipoprotein; LysCA, *N*^ε-acetyl-lysine chloramine; MonoCA, monochloramine; MPO, myeloperoxidase; REM, relative electrophoretic mobility; TaurCA, taurine chloramine.

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where AH^- is ascorbate and A is dehydroascorbic acid. In addition, numerous studies have shown that supplementation with vitamin C decreases lipid and protein oxidation *in vivo*, inhibits leucocyte-endothelial-cell adhesion and increases endothelium-dependent vasodilation [19]. Epidemiological studies also have shown an inverse association between vitamin C intake, or status, and the risk from cardiovascular diseases [19]. Therefore, if HOCl is involved in atherogenesis through the oxidative modification of LDL particles, then vitamin C may be an important protective agent.

We have investigated the effects of vitamin C on the HOCl-dependent modification of LDL. Physiological levels of vitamin C were added to LDL during or after exposure to HOCl to determine whether there was, respectively, protection against or reversal of HOCl-mediated LDL damage. The protective effect of vitamin C against oxidation of LDL by model HOCl-derived chloramines was also investigated. Three chloramines were used: *N*-acetyl-lysine chloramine (LysCA), as a model for protein-associated chloramines; taurine chloramine (TaurCA), the primary chloramine produced by neutrophils; and monochloramine (MonoCA), as an example of a lipophilic chloramine.

EXPERIMENTAL

Isolation of LDL

Peripheral blood was obtained fresh from healthy male and female volunteers. The blood was centrifuged at 1125 *g* for 20 min at 4 °C to separate cells from plasma. LDL was isolated from plasma by a modified sequential centrifugation method [20], using a Beckman Optima TL benchtop ultracentrifuge (Beckman Instruments, Fullerton, CA, U.S.A.) with a TL-100.4 rotor and quick-seal 5 ml tubes. Briefly, the density of fresh plasma was increased to 1.019 mg/ml by addition of NaBr, and the tubes were filled, sealed and subsequently centrifuged at 540 000 *g* for 3.5 h at 5 °C. Following centrifugation, the top 1.0–1.5 ml (containing very-low-density lipoprotein/intermediate-density lipoprotein) was sliced from the tube. The liquid at the bottom of the tube (containing LDL and high-density lipoprotein) was collected and raised to a density of 1.067 g/ml by addition of NaBr. Following centrifugation at 540 000 *g* for 5 h at 5 °C, the top 1.0–1.5 ml, containing LDL, was sliced from the tube and was desalted by two sequential passages through PD-10 gel-filtration columns (Pharmacia Biotech, Uppsala, Sweden). Total protein was estimated using a modification [21] of the Lowry method, with BSA as a standard. For all experiments, LDL was diluted to a concentration of 0.5 mg of protein/ml (approx. 1 μM LDL) in PBS (10 mM phosphate, 140 mM NaCl, pH 7.4) containing the metal chelator diethylenetriaminepenta-acetic acid (DTPA; 100 μM).

Oxidation of LDL by HOCl and chloramines

HOCl (Aldrich, Milwaukee, WI, U.S.A.) was standardized at 292 nm (ϵ 350 $\text{M}^{-1}\cdot\text{cm}^{-1}$) following dilution into pH 12 buffer [22]. Standardized HOCl was freshly diluted in PBS; the pK_a of HOCl is 7.5, therefore the solution will contain both HOCl and OCl^- . HOCl (25–200 μM) was added with rapid, gentle mixing to the LDL, which was subsequently incubated for 30 min at 37 °C.

Ascorbate (Sigma, St. Louis, MO, U.S.A.) was freshly diluted in PBS containing 100 μM DTPA and standardized at 265 nm (ϵ 15 000 $\text{M}^{-1}\cdot\text{cm}^{-1}$) [18]. Ascorbate (25–200 μM) was added to LDL during (co-incubation) or after (post-incubation; 15 min at 37 °C) exposure to HOCl.

Three model chloramines, LysCA, TaurCA and MonoCA, were freshly prepared by addition of HOCl to a 2.5–5.0-fold

excess of the respective amine. Ammonia was first neutralized with HCl before preparation of MonoCA. Formation of the chloramines was monitored by their absorption maxima (A_{max} values of 252 nm for LysCA and TaurCA, and 242 nm for MonoCA; ϵ 429 $\text{M}^{-1}\cdot\text{cm}^{-1}$) [23]. The chloramines (25–200 μM) were added to the isolated LDL as described for HOCl.

Quantification of amino acid modifications

Tryptophan residues were measured directly by fluorescence [8]. LDL (10 μl) was diluted into 800 μl of PBS and the fluorescence was determined (excitation wavelength 280 nm; emission wavelength 335 nm) using a Hitachi F-2500 Fluorescence Spectrophotometer (Hitachi Instruments, San Jose, CA, U.S.A.).

Lysine residues were measured using fluorescamine [24]. LDL (10 μl) was diluted with 600 μl of pH 8.5 phosphate buffer, and 200 μl of fluorescamine (500 μM in acetonitrile) was added while vortexing. Fluorescence was determined at an excitation wavelength of 390 nm and an emission wavelength of 475 nm.

Chloramines were determined using thionitrobenzoic acid [25]. LDL (100 μl) was added to 300 μl of PBS plus 10 μl of thionitrobenzoic acid (10 mM), and the loss of colour was determined at 412 nm (ϵ 14 100 $\text{M}^{-1}\cdot\text{cm}^{-1}$) using a Beckman DU-640 spectrophotometer. Thionitrobenzoic acid was prepared from 5,5'-dithiobis-(2-nitrobenzoic acid) (Sigma) by alkaline hydrolysis with subsequent neutralization [25].

Cysteine residues were measured using the sensitive fluorescent probe 7-fluorobenz-2-oxa-1,3-diazole-4-sulphonamide (ABD-F; Molecular Probes, Eugene, OR, U.S.A.) [26]. LDL (100 μl) was added to 300 μl of pH 8.0 phosphate buffer containing 1 mM DTPA and incubated for 20 min at 60 °C following the addition of 10 μl ABD-F (10 mM). Fluorescence was determined at an excitation wavelength of 365 nm and an emission wavelength of 490 nm. Total thiols were determined, as above, following reduction of disulphides with tris-(2-carboxyethyl)phosphine (Molecular Probes) [27] in the presence of 1 % SDS.

The change in the relative electrophoretic mobility (REM) of LDL was determined by agarose-gel electrophoresis using a Paragon Lipoprotein Electrophoresis Kit (Beckman). Briefly, LDL (5 μl ; approx. 2.5 μg of protein) was loaded on to the gel, which was run at 100 V for 30 min. The bands were stained with Sudan Black.

Quantification of antioxidants and oxidized lipids

Ascorbate was measured using paired-ion, reverse-phase HPLC with electrochemical detection [17]. Briefly, LDL samples (20 μl) were precipitated with an equal volume of 5 % metaphosphoric acid in PBS containing 1 mM DTPA and centrifuged (13 600 *g* for 5 min). The supernatant was removed and neutralized with 2.58 M K_2HPO_4 (pH 9.8) before dilution in the mobile phase. Separation was carried out on an LC-18 column using a Hitachi L-6000 pump and a BAS LC-4B amperometric detector (BAS, West Lafayette, IN, U.S.A.).

α -Tocopherol was measured using HPLC with electrochemical detection [28]. Briefly, LDL (100 μl) was diluted to 250 μl with PBS containing DTPA and extracted with 500 μl of methanol and 5 ml of hexane with vortexing. After centrifugation (1125 *g* for 5 min), 4 ml of the upper hexane phase was dried under N_2 and dissolved in 100 μl of mobile phase for separation on an LC-18 column using a Hitachi L-6200 pump with a BAS LC-4C amperometric detector.

Cholesteryl ester hydroperoxides (CEOOH) were measured using HPLC with post-column chemiluminescence detection [29]. Samples were extracted as described for α -tocopherol and

separated on an LC-18 column using a Hitachi L-6200 pump with a Hewlett Packard 1050 UV detector (Hewlett Packard, Avondale, PA, U.S.A.). The post-column reagent containing microperoxidase (5 mg/l; Sigma) and isoluminol (1 mM; Sigma) was delivered using a Hitachi L-6000 pump with a Soma S-3400 Chemo Lumi Detector (Soma Optics, Tokyo, Japan).

Statistical analysis

Statistical analyses were carried out by analysis of variance (ANOVA) with Fisher's *post hoc* analysis using StatView software (SAS Institute, Cary, NC, U.S.A.). Statistical significance was set at $P < 0.05$.

RESULTS

HOCl-mediated modifications of LDL

Exposure of LDL (0.5 mg of protein/ml; $\approx 1 \mu\text{M}$) to increasing concentrations of HOCl (25–200 μM) resulted in a number of modifications to apoB (Figure 1). The most sensitive target in LDL was the cysteine residues [30]. It is known that, of the 25 cysteine residues in LDL, only between three and five are in the reduced, thiol form [30,31]. Using a sensitive fluorescent assay we found that $> 90\%$ of the thiols were lost from 1 μM LDL incubated with 75 μM HOCl (Figure 1). This result compares favourably with that of Hazell and Stocker [8], who reported the complete loss of thiols from 1 μM LDL incubated with 50–75 μM HOCl. In addition, reduction of HOCl-treated LDL revealed a net 20% loss of total LDL cysteine residues following incubation with $\geq 50 \mu\text{M}$ HOCl (results not shown). This suggests that HOCl-mediated oxidation of the five free cysteine residues does not result in disulphide formation, but possibly in higher oxidation states, such as sulphinic or sulphonic acids [10]. Loss of lysine and tryptophan residues in LDL occurred in a HOCl-concentration-dependent manner with, respectively, 26 and 37% of the residues lost following incubation with 200 μM HOCl (Figure 1). Since apoB contains 356 lysine residues and 37

tryptophan residues [7], the former are quantitatively the major target of HOCl. The loss of lysine residues was mirrored by the formation of chloramines, which accounted for one-third of the added HOCl (Figure 1). A significant dose-dependent increase in REM was also observed with increasing concentrations of HOCl (Figure 1).

Since HOCl has been shown to react with methionine and tyrosine residues in LDL [6,10], we also investigated the relative importance of these reactions, using an LDL-free system. HOCl reacted rapidly and in a stoichiometric manner with *N*-acetyl-methionine (a model of protein methionine residues): with concentrations of 100 μM of both HOCl and *N*-acetylmethionine, $91 \pm 9\%$ ($n = 3$) of the HOCl was lost (results not shown). In contrast, 100 μM HOCl showed only low reactivity with 100 μM *N*-acetyltyrosine: $13 \pm 7\%$ ($n = 3$) of the HOCl was lost (results not shown). Since apoB contains 78 methionine residues [7], and HOCl is equally reactive with the sulphur-containing amino acids cysteine and methionine [32], it is likely that the methionine residues of apoB were oxidized at a similar rate as the cysteine residues, and therefore accounted for the concentration of $\geq 75 \mu\text{M}$ HOCl required to oxidize the thiol groups in LDL (Figure 1).

A small dose-dependent increase in CEOOH was observed following exposure of LDL to HOCl (results not shown): $0.35 \pm 0.08 \mu\text{M}$ CEOOH were detected following incubation with 200 μM HOCl ($n = 4$), which accounts for $< 0.2\%$ of the added HOCl. Hazell and Stocker [8] reported similar results: $\leq 0.2 \mu\text{M}$ CEOOH were detected with 240 μM HOCl. Similarly, α -tocopherol was not a significant target for HOCl (results not shown), as has been observed previously [15].

Ascorbate protects against specific HOCl-dependent LDL modifications

Since HOCl reacts with ascorbate in a stoichiometric manner (reaction 3), ascorbate may be able to protect LDL from HOCl-dependent modifications. LDL was exposed to increasing concentrations of HOCl (25–200 μM) in the presence of 200 μM ascorbate (co-incubation), and specific modifications to apoB were determined (Figure 2). Ascorbate strongly protected against the loss of tryptophan residues (Figure 2a) and partially protected against the loss of cysteine residues (Figure 2c). In the presence of 200 μM each of ascorbate and HOCl, approximately two-thirds of the cysteine residues of LDL became oxidized. Ascorbate also completely prevented the formation of chloramines (Figure 2b) and abolished the increase in REM (Figure 2d). Protection by ascorbate against the loss of lysine residues was only statistically significant at the highest HOCl concentration (Figure 2b), probably due to the variability of the fluorescamine assay. Analysis of ascorbate by HPLC showed a dose-dependent loss (Table 1): at 200 μM of both ascorbate and HOCl, approx. 150 μM of the ascorbate was oxidized, indicating that approx. 50 μM of the added HOCl had reacted with more reactive targets on LDL, most likely cysteine and methionine residues.

When LDL was co-incubated with increasing concentrations of ascorbate (25–200 μM) and either 100 or 200 μM HOCl (Figures 3a and 3b respectively), dose-dependent protection was observed for all the parameters examined, although protection was again incomplete for thiols. In most cases there was a stoichiometric association between the amount of ascorbate required to fully protect against an equivalent amount of HOCl, e.g. 100 μM ascorbate fully protected against the loss of tryptophan residues, the formation of chloramines and the increase in REM mediated by 100 μM HOCl (Figure 3a).

Figure 1 HOCl-mediated modifications of LDL

Freshly isolated LDL (0.5 mg of protein/ml) in PBS containing 100 μM DTPA was incubated for 30 min at 37 °C with increasing concentrations of HOCl (25–200 μM). ApoB cysteine (■), tryptophan (●) and lysine (▽) residues, chloramines (◇), and the REM of LDL (▲) were determined as described in the Experimental section. Percentage chloramines was estimated using a value of 356 lysine residues per LDL [7]. Results represent means $\pm$ S.D. of four different LDL preparations.

Figure 2 Protection by ascorbate from HOCl-mediated LDL modifications

Incubation conditions were as for Figure 1, with (○, ▽) or without (●, ▼) co-incubation with 200 μ M ascorbate. ApoB tryptophan (a), lysine (b; ○, ●) and cysteine (c) residues, chloramines (b; ▼, ▽) and REM (d) were determined as described in the Experimental section. Results represent means $\pm$ S.D. of four different LDL preparations. * P < 0.05 as determined by ANOVA with Fisher's *post hoc* analysis; # P < 0.05 for all concentrations of HOCl.

Table 1 Ascorbate levels following co-incubation and post-incubation with HOCl-modified LDL

LDL (0.5 mg of protein/ml) was exposed to increasing concentrations of HOCl in the presence of 200 μ M ascorbate (co-incubation), or ascorbate was added following exposure of LDL to HOCl (post-incubation). Ascorbate levels were determined by HPLC with electrochemical detection. Results represent means $\pm$ S.D. of four different LDL preparations.

HOCl (μ M)	Ascorbate (μ M)	
	Co-incubation	Post-incubation
0	199 $\pm$ 9	201 $\pm$ 9
25	178 $\pm$ 2	194 $\pm$ 2
50	160 $\pm$ 4	184 $\pm$ 2
75	138 $\pm$ 6	168 $\pm$ 4
100	116 $\pm$ 16	170 $\pm$ 4
150	86 $\pm$ 10	128 $\pm$ 18
200	48 $\pm$ 16	108 $\pm$ 10

Ascorbate reverses specific HOCl-dependent LDL modifications

Since apoB-associated chloramines are quantitatively the major product of HOCl on LDL [8], and since ascorbate can regenerate amino groups from chloramines (reaction 4), we investigated the

potential reversal of specific HOCl-dependent modifications by ascorbate. LDL-free experiments showed that LysCA, a model of a protein-associated chloramine, reacted with ascorbate to completion within 15 min at ambient temperature, in contrast with the immediate loss of ascorbate in the presence of HOCl (Figure 4a). The loss of ascorbate in the presence of LysCA was stoichiometric (Figure 4b) and identical to the loss of ascorbate in the presence of equivalent amounts of HOCl (results not shown). Concomitantly, stoichiometric regeneration of amino groups was observed following incubation of LysCA with ascorbate (Figure 4b), and no detectable chloramines remained, except at the highest concentration of LysCA used (Figure 4b).

Similarly, addition of ascorbate (200 μ M) to HOCl (25–200 μ M)-treated LDL (post-incubation) fully eliminated pre-formed chloramines on apoB and partially regenerated the lysine residues (Figure 5b). The change in REM was also only partially reversed (approx. 50 %) (Figure 5d), and the loss of tryptophan and cysteine residues was not reversed by ascorbate (Figures 5a and 5c respectively). Ascorbate levels following incubation with HOCl-treated LDL fell in a concentration-dependent manner (Table 1). Since approximately one-third of the added HOCl was converted into chloramines (see Figure 1), the loss of ascorbate during post-incubation was lower than the loss during co-incubation, but was sufficient to account for the total elimination of the chloramines.

Figure 3 Dose-dependent protection by ascorbate from HOCl-mediated LDL modifications

Incubation conditions were as for Figure 1, with (a) 100 μM or (b) 200 μM HOCl. ApoB tryptophan (●), lysine (▽) and cysteine (■) residues, chloramines (◇) and REM (▲) were determined as described in the Experimental section. Results represent means ± S.D. of four different LDL preparations.

The effects of ascorbate on time-dependent changes were examined in HOCl-modified LDL incubated for up to 4 h at 37 °C before addition of ascorbate (Figure 6). The regeneration of lysine residues and the reversal of the change in REM (Figures 6a and 6b respectively) by ascorbate became less efficient over time, suggesting that irreversible secondary reactions had occurred. The latter is also suggested by the time-dependent loss of chloramines and the slight rise in REM of LDL incubated with HOCl for up to 4 h without subsequent post-incubation with ascorbate (Figures 6a and 6b respectively).

Chloramine-mediated modification of LDL and the role of ascorbate

Like HOCl itself, HOCl-derived chloramines retain two oxidizing equivalents, and as such may be able to oxidize LDL. The reactivities of the HOCl-derived chloramines LysCA, TaurCA and MonoCA towards specific amino acids in solution were determined in order to assess relevant targets in LDL. LysCA reacted with both *N*-acetylcysteine and *N*-acetylmethionine in close to stoichiometric amounts, but showed little reactivity

Figure 4 Regeneration by ascorbate of amines from LysCA

(a) Time course of loss of ascorbate (100 μM) incubated alone (Cont), with LysCA or with HOCl (70 μM each) for up to 15 min at room temperature. (b) Concentration-dependence with regard to LysCA (25–100 μM) on the loss of ascorbate and the formation of amines following incubation for 15 min at ambient temperature. Ascorbate (●), amines (▽) and chloramines (■) were determined as described in the Experimental section. Results represent means ± S.D. of three independent experiments.

towards *N*-acetyltryptophan and none towards *N*-acetyltyrosine (Figure 7a). Similar results were observed with TaurCA and MonoCA (results not shown), with the exception that the TaurCA/*N*-acetylcysteine ratio was 1:2. The three chloramines also oxidized ascorbate, with LysCA being the most reactive, followed by MonoCA and TaurCA (Figure 7b).

The three model chloramines were added to LDL and the loss of thiols was determined (Figure 8a). Incubation with MonoCA resulted in an equivalent loss of thiols as that seen with HOCl, while LysCA and TaurCA were less reactive. Whereas HOCl reacted fully with LDL at the concentrations used (25–200 μM), between 50% (for MonoCA) and 65% (for LysCA and TaurCA) of the added chloramines (25–200 μM) were detected following the incubation period (results not shown). The chloramines were shown to be free and not associated with LDL, as determined following precipitation of the lipoprotein [33]. The amount of chloramine unaccounted for, i.e. the difference between that which had reacted with thiols and that remaining in the supernatant, had most probably reacted with LDL methionine residues (cf. Figure 7a).

Figure 5 Reversal by ascorbate of specific HOCI-mediated LDL modifications

Incubation conditions were as for Figure 1, with (○, ▽) or without (●, ▴) post-incubation with 200 μM ascorbate for 15 min at 37 °C. ApoB tryptophan (a), lysine (b; ○, ●) and cysteine (c) residues, chloramines (b; ▽, ▴) and REM (d) were determined as described in the Experimental section. Results represent means ± S.D. of four different LDL preparations. **P* < 0.05 as determined by ANOVA with Fisher's *post hoc* analysis; #*P* < 0.05 for all concentrations of HOCI.

Co-incubation of 200 μM ascorbate with LDL protected against thiol loss caused by LysCA, but was less protective against that caused by TaurCA, MonoCA and HOCI (Figure 8b). Ascorbate was lost in a close to stoichiometric manner by reaction with LysCA (results not shown), but sequentially less so for TaurCA and MonoCA, the latter of which more closely resembled HOCI (cf. Table 1). Following co-incubation with ascorbate, no unreacted chloramines were detected in the LDL samples (results not shown). The less efficient protection by ascorbate towards TaurCA- and MonoCA-mediated modification of apoB thiols could be explained in part by the lower rates of reaction of these chloramines with ascorbate, compared with that of LysCA (cf. Figure 7b).

DISCUSSION

Although ascorbate can scavenge HOCI in a stoichiometric manner [18], the potential protective effects of ascorbate against HOCI-mediated modifications of LDL have not been investigated previously. Ascorbate is the most effective water-soluble antioxidant in human plasma [17], where it is present in concentrations ranging from 25 to 150 μM [34]. HOCI is the major strong oxidant produced by stimulated neutrophils [2] and is generated at levels of 100–200 nmol/5 × 10⁶ cells [35]. Therefore in the present study we used both ascorbate and HOCI at

physiologically relevant concentrations, i.e. 25–200 μM. Exposure of LDL to HOCI results in a number of modifications, which are almost exclusively on apoB. We found that ascorbate effectively protects LDL against most of the HOCI-mediated modifications in a close to stoichiometric manner. Cysteine residues, however, were only partially protected by ascorbate. These results can be explained by the relative reaction rates of HOCI with ascorbate and different amino acids, i.e. HOCI is approximately an order of magnitude less reactive with ascorbate ($k_2 \approx 6 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$) than with the sulphur-containing amino acids methionine and cysteine ($k_2 > 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$), but is at least an order of magnitude more reactive with ascorbate than with other amino acids ($k_2 \approx 5 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$) [32,36].

In the present study we also found that physiological concentrations of ascorbate reacted with HOCI-derived chloramines to regenerate the amines, i.e. reversing some of the HOCI-mediated LDL modifications. Interestingly, the HOCI-dependent increase in the anodic electrophoretic mobility of LDL was only partially reversed by ascorbate. This increase in REM is thought to be due to modification of the apoB lysine residues, with a concomitant increase in the net negative charge of the LDL particle [8]. Furthermore, the reversal of the lysine loss and of the change in REM by ascorbate became less efficient with increasing incubation time of LDL with HOCI. These data, and the time-dependent loss of chloramines in HOCI-modified LDL, suggest

Figure 6 Time-dependent reversal by ascorbate of specific HOCl-mediated LDL modifications

LDL (0.5 mg of protein/ml) was incubated for up to 4 h at 37 °C with 200 μM HOCl, followed by incubation with (○, ▽) or without (●, ▼) 200 μM ascorbate for 15 min at 37 °C (post-incubation). Measurement of lysine residues (a; ○, ●), chloramines (a; ▽, ▼) and REM (b) were carried out as described in the Experimental section. Results represent means ± S.D. of four different LDL preparations. **P* < 0.05 as determined by ANOVA with Fisher's *post hoc* analysis.

that irreversible secondary reactions occurred, such as the formation of carbonyls and subsequent LDL cross-linking and aggregation [9,16]. A recent report indicated that chloramines also break down upon incubation to produce radicals that can initiate oxidation of LDL lipids through 'tocopherol-mediated peroxidation' [11]. Since ascorbate not only reverses the formation of HOCl-derived chloramines, but also acts as an effective 'co-antioxidant' for tocopherol [37], ascorbate should protect LDL against chloramine-induced lipid peroxidation very effectively [11].

N-Chloramines are quantitatively the major product when LDL is exposed to HOCl [8]. Although chloramines are less reactive than HOCl, they still retain sufficient oxidizing capacity to oxidize the sulphur-containing amino acids cysteine and methionine [38], as well as ascorbate [18]. Chloramines have different stabilities and reactivities depending on their structures [23]. LysCA [CH₂-CO-NH-CH(COO⁻)-CH₂-CH₂-CH₂-CH₂-NHCl], a stable ϵ -chloramine, was used in the present study as a model for protein-associated chloramines [39]. The other model chloramines used were TaurCA (SO₃⁻-CH₂-CH₂-NHCl), a stable

Figure 7 Reaction of chloramines with selected amino acids and ascorbate

(a) LysCA (100 μM) was incubated with increasing concentrations (25–400 μM) of *N*-acetylcysteine (●), *N*-acetylmethionine (▽), *N*-acetyltryptophan (■) or *N*-acetyltyrosine (◇) for 30 min at ambient temperature. LysCA levels were determined as described in the Experimental section. Results represent means ± S.D. of three independent experiments. (b) Time-dependent loss of ascorbate (100 μM) incubated with LysCA, TaurCA or MonoCA (80 μM each) for up to 15 min at ambient temperature. Ascorbate levels were measured at 265 nm (ϵ 15000 M⁻¹·cm⁻¹).

β -chloramine which is the major chloramine produced by neutrophils [40], and MonoCA (NH₂Cl), an α -chloramine, used as an example of a lipophilic chloramine [41]. The three chloramines exhibited differing reactivities towards LDL thiols in the order MonoCA = HOCl > LysCA > TaurCA. MonoCA, like HOCl, is lipophilic, and as such would be able to penetrate into the LDL particle and oxidize the buried thiol groups [31]. Both TaurCA and LysCA are larger, charged molecules and, therefore, less likely to penetrate into LDL. Ascorbate was able to protect LDL cysteine residues against the chloramines in the order LysCA > TaurCA > MonoCA > HOCl. These differences are likely to reflect the relative reactivities of the different chloramines with ascorbate compared with thiol groups.

Generation of HOCl by the MPO/H₂O₂/Cl⁻ system produces similar modifications to LDL as reagent HOCl [9]. However, the interaction of ascorbate with the enzymic system may be complex, due to the fact that ascorbate not only scavenges HOCl and chloramines, but also paradoxically enhances the chlorinating activity of MPO [42]. The latter mechanism involves regeneration

Figure 8 Protection by ascorbate from chloramine-dependent thiol loss in LDL

(a) Freshly isolated LDL (0.5 mg of protein/ml) in PBS containing 100 μM DTPA was incubated for 30 min at 37 °C with increasing concentrations (25–200 μM) of HOCl (●), LysCA (▽), TaurCA (■) or MonoCA (◇). ApoB cysteine residues were determined as described in the Experimental section. (b) LDL was incubated as above with HOCl (●), LysCA (▽), TaurCA (■) or MonoCA (◇) in the presence of 200 μM ascorbate. Results represent means ± S.D. of three different LDL preparations.

by ascorbate of the native enzyme from the compound II intermediate, which is catalytically inactive in the chlorination reaction. The predominant effect of ascorbate is likely to depend on its concentration, with catalytic (low micromolar) concentrations stimulating enzymic activity and higher concentrations scavenging HOCl and chloramines. In addition, ascorbate at high concentrations could act as a competitive inhibitor of HOCl formation by MPO, since the reaction rate of ascorbate with compound I (k_2 1.1×10^6 M⁻¹·s⁻¹) [43] is comparable with that of compound I with chloride (k_2 4.7×10^6 M⁻¹·s⁻¹) [44].

LDL can be oxidatively modified by a number of different cell types, including neutrophils, monocytes, macrophages and endothelial cells [1]. Ascorbate has been shown to protect against the neutrophil-mediated oxidation of LDL [28,45] by scavenging oxidants generated by the cells following stimulation of the respiratory burst [2]. An additional mechanism may be decreasing the production of oxidants by intracellular ascorbate. Stimulation of the respiratory burst of neutrophils results in oxidation of 30–40 % of the intracellular ascorbate to dehydroascorbic acid

[46]. Similarly, loading of human vascular endothelial cells with ascorbate decreases cellular levels of oxidants and inhibits modification of LDL by these cells [47].

Oxidative modification of LDL has been strongly implicated in the development of atherosclerosis [1]. Modification of LDL by HOCl results in enhanced macrophage uptake and subsequent formation of lipid-laden foam cells, the hallmark of atherosclerotic lesions [8]. In addition, several reports have shown that HOCl-modified LDL stimulates neutrophil oxidant production, degranulation, adherence to endothelial cells and migration [48–50]. HOCl-modified LDL can also enhance cytokine production by monocytes and degranulation of mast cells, and increase the permeability of vascular cells [49,50]. These pathophysiological properties of HOCl-modified LDL towards leucocytes and vascular cells could potentially contribute to atherogenesis. As such, prevention of HOCl-dependent modifications of LDL by ascorbate could be an important, specific antioxidant defence mechanism against atherogenesis.

In summary, the results presented here demonstrate that physiological concentrations of ascorbate protect against most HOCl- and chloramine-dependent modifications of LDL. Ascorbate also reverses specific HOCl-dependent modifications, such as chloramine formation, which have been strongly implicated in the uncontrolled uptake of HOCl-modified LDL by macrophages, an important early step in atherogenesis. Therefore our results indicate that ascorbate could contribute to the anti-atherogenic defence against HOCl.

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