Hypochlorite-induced oxidation of proteins in plasma: formation of chloramines and nitrogen-centred radicals and their role in protein fragmentation

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Activated phagocyte cells generate hypochlorite (HOCl) via the release of H_2O_2 and the enzyme myeloperoxidase. Plasma proteins are major targets for HOCl, although little information is available about the mechanism(s) of oxidation. In this study the reaction of HOCl (at least 50 μM) with diluted fresh human plasma has been shown to generate material that oxidizes 5-thio-2-nitrobenzoic acid; these oxidants are believed to be chloramines formed from the reaction of HOCl with protein amine groups. Chloramines have also been detected with isolated plasma proteins treated with HOCl. In both cases chloramine formation accounts for approx. 20–30% of the added HOCl. These chloramines decompose in a time-dependent manner when incubated at 20 or 37 °C but not at 4 °C. Ascorbate and urate remove these chloramines in a time- and concentration-dependent manner, with the former being more efficient. The reaction of fresh diluted plasma with HOCl also gives rise to protein-derived nitrogen-centred radicals in a time- and HOCl-concentration-dependent manner; these have been detected by EPR spin trapping. Identical radicals have been detected with isolated HOCl-treated plasma proteins. Radical formation was inhibited by excess methionine, implicating protein-derived chloramines (probably from lysine side chains) as the radical source. Plasma protein fragmentation occurs in a time- and HOCl-concentration-dependent manner, as evidenced by the increased mobility of the EPR spin adducts, the detection of further radical species believed to be intermediates in protein degradation and the loss of the parent protein bands on SDS/PAGE. Fragmentation can be inhibited by methionine and other agents (ascorbate, urate, Trolox C or GSH) capable of removing chloramines and reactive radicals. These results are consistent with protein-derived chloramines, and the radicals derived from them, as contributing agents in HOCl-induced plasma protein oxidation.

Key words: EPR, free radicals, myeloperoxidase, spin trapping.

INTRODUCTION

Activation of phagocyte cells both *in vitro* and *in vivo* can result in the generation of $O_2^{-\bullet}$ and H_2O_2 and the release of the haem enzyme myeloperoxidase [1]. This enzyme catalyses the reaction of H_2O_2 with Cl^-, resulting in the formation of the potent oxidant HOCl [2]. HOCl has an important role in bacterial cell killing [3] but the excessive production of HOCl causes tissue damage. This is believed to be important in certain diseases such as atherosclerosis and inflammatory conditions [4]; for example, HOCl-modified proteins and active myeloperoxidase have been found in human atherosclerotic plaque tissue [5–7].

Studies *in vitro* have shown that proteins are major targets for HOCl [8–18]. The treatment of isolated proteins with HOCl is known to result in the alteration of amino acid side chains [19–24], protein fragmentation [18,25,26] and cross-linking/aggregation [27]. In some cases these reactions can render the protein more susceptible to degradation by intracellular protease enzymes. It has been shown, for example, that HOCl-treated fibronectin is more susceptible to degradation by elastase due to changes to both the primary and tertiary structures of the protein [25].

Various amino acid side chains are known to be particularly susceptible to modification by HOCl. Thus cysteine and methionine residues react rapidly with HOCl to give oxyacids and cystine (from Cys [28,29]) and sulphoxides (from Met [20]). The latter type of reaction is believed to be responsible for the inactivation of α_1-proteinase inhibitor [30,31]. Tyrosine undergoes ring chlorination to give 3-chlorotyrosine; this product has been employed as a marker of HOCl-induced damage [23,24]. The free amino groups of lysine residues are known to form chloramine (RNHCl) intermediates readily on reaction with HOCl [32,33]. This is believed to be of particular importance for two reasons. First, lysine residues are often present in proteins in higher amounts than other reactive residues. Secondly, the long lifetime of chloramines compared with those of HOCl or H_2O_2 means that it is likely that these materials, which retain the oxidizing equivalents of HOCl, diffuse a considerable distance and hence might cause damage at remote sites.

In more complex systems such as plasma, proteins are also believed to be important targets for HOCl. Thus it has been shown that initial reaction of HOCl with plasma results in the rapid depletion of the protein thiol groups and methionine residues [13,15]. Attack on lysine amino groups has been reported to occur on consumption of these groups [13]. Ascorbate reacts rapidly with HOCl [10,34,35] but is probably not a major HOCl scavenger in plasma [15], as this molecule is present at only low concentrations (approx. 50 μM) compared with plasma protein thiol groups [36]. Plasma urate also does not seem to be an effective HOCl scavenger [13,15].

Although the products of the reaction of HOCl with isolated amino acids, peptides and proteins are reasonably well characterized [18,27,37–40], the mechanism of HOCl-mediated plasma oxidation and particularly plasma protein damage is poorly understood. Radicals have not generally been implicated as intermediates in these reactions, although evidence has been presented for the involvement of radicals in the HOCl-mediated inactivation of α_1-anti-proteinase inhibitor [31] and the frag-

Abbreviations used: BCA, bicinchoninic acid; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; Nbs, 5-thio-2-nitrobenzoic acid; Nbs_2, 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent); HSA; human serum albumin; HTAC, hexadecyltrimethylammonium chloride.

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mentation of isolated proteins, peptides and free amino acids [18,40]. In this study the formation and decay of protein chloramines and the generation of radical intermediates in the reactions of HOCl with fresh human plasma have been investigated. The consequences of these reactions (protein fragmentation/aggregation) and the effects of various putative protective agents have also been studied.

MATERIALS AND METHODS

The water used was filtered through a four-stage Milli Q system (Millipore–Waters, Lane Cove, NSW, Australia) equipped with a 0.2 μm pore-size final filter. pH control was achieved by the use of 50 mM phosphate buffer, pH 7.4. Ascorbate oxidase (1000–3000 units/mg of protein) and uricase (33 units/g of solid) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO; Aldrich, Castle Hill, NSW, Australia) was purified before use by treatment with activated charcoal. All other chemicals were of analytical reagent grade. HOCl solutions were prepared immediately before use by dilution from a concentrated stock solution [approx. 1 M in 0.1 M NaOH (BDH, Poole, Dorset, U.K.)] into 50 mM phosphate buffer, pH 7.4. HOCl concentrations were determined from the absorbance of the chloramine adduct generated on reaction with taurine (0.1 M) at 252 nm on the basis of a molar absorption coefficient of 429 $M^{-1} \cdot cm^{-1}$ [41]. Protein concentrations were determined with the bicinchoninic acid (BCA) assay [42]. Treatment of solutions with chelex had no significant effect on the results obtained.

Stock solutions of α-tocopherol (Henkel, Sydney, Australia) were prepared in ethanol (approx. 0.1 M) with the concentration determined from the absorbance at 294 nm using a molar absorption coefficient of 3058 $M^{-1} \cdot cm^{-1}$. Micelles were prepared by adding the stock α-tocopherol solution (final concentration 500 μM) to either hexadecyltrimethylammonium chloride (HTAC) or SDS (100 mM) at 50 °C followed by sonication for 15 min in a sonicator bath [43].

Plasma was obtained from heparinized blood donated by healthy consenting adult volunteers (males and females) by centrifugation at 1000 ***g*** for 20 min at 4 °C. This was kept on ice in the dark, and used within 8 h. The total plasma protein concentration (approx. 875 μM), and the concentrations of individual proteins, were calculated from published values [36]. The thiol content of plasma was assayed by using 5,5′-dithiobis-(2-nitrobenzoic acid) (Nbs_2) and the mean ± S.D. was determined as 430 ± 50 μM.

Chloramine concentrations were determined by the use of 5-thio-2-nitrobenzoic acid (Nbs) as described previously [18,33]. Nbs was prepared from Nbs_2 (1 mM) by exposure to NaOH (50 mM) for 5 min before dilution into 50 mM phosphate buffer, pH 7.4. The concentration of Nbs remaining after reaction with plasma, or isolated plasma protein chloramines, was determined at 412 nm by using a molar absorption coefficient of 13600 $M^{-1} \cdot cm^{-1}$ [33], after subtraction of the absorbance from control samples to which neither plasma/plasma proteins nor HOCl had been added. Control samples to which untreated plasma or plasma proteins had been added gave slightly higher absorbances at 412 nm than the non-protein-containing samples, presumably as a result of reaction of protein thiols with contaminating unreacted Nbs_2 [33], a reaction that generates further Nbs. The consumption of Nbs by HOCl-treated plasma or HOCl-treated plasma proteins is therefore likely to be a slight underestimate of the true consumption of Nbs.

Plasma oxidations were performed by the addition of HOCl to fresh diluted plasma (of known protein concentration) at 4 °C, with the samples subsequently incubated as stated in the text. For the EPR experiments the spin trap was always added shortly after the HOCl to avoid the reaction of HOCl with the spin trap (a known source of spurious signals [44]). Experiments with isolated plasma protein chloramines were performed by the incubation of fresh diluted plasma with HOCl (5 mM) for 15 min at 4 °C before separation of the protein from excess HOCl on a PD-10 Sephadex column (Pharmacia, Uppsala, Sweden). Samples were then diluted to the required protein concentration, the spin trap (for the EPR experiments) or other additions were made (unless stated otherwise), and the samples were incubated as indicated in the text. Enzymic degradation of the HOCl-treated plasma proteins that had the spin trap DMPO added was performed at 20 °C under aerobic conditions for 0–30 min with Pronase (a mixture of proteases with different proteolytic activities; 7000 units/g; Boehringer Mannheim, Castle Hill, NSW, Australia).

EPR spectra were recorded at room temperature with a Bruker EMX X-band spectrometer with 100 kHz modulation and a cylindrical ER 4103TM cavity. Samples were contained in a flattened, aqueous-sample cell (WG-813-SQ; Wilmad, Buena, NJ, U.S.A.) and recording of the spectra was initiated within 2 min of the start of the reaction, except where specified otherwise. Hyperfine couplings were measured directly from the field scan and confirmed by spectral simulation with the program WINSIM [45]. The correlation coefficients between simulated and experimental data were more than 0.90. Typical EPR spectrometer settings were: gain 2×10^6, modulation amplitude 0.1 mT, time constant 0.16 s, scan time 84 s, resolution 1024 points, centre field 348 mT, field scan 10 mT, power 25 mW and frequency 9.76 GHz, with four scans averaged.

Plasma proteins were separated by the method of Laemmli [46] by using either 10 % or 4–12 % (w/v) gradient polyacrylamide gels. Protein samples were added to an equal volume of 60 mM Tris/HCl buffer, pH 6.8, containing glycerol (10 %, v/v), 2-mercaptoethanol (5 %, v/v), SDS (2 %, w/v) and Bromophenol Blue (0.01 %); these were then heated at 95 °C for 5 min before being cooled and loaded on the gel. Bands were detected with Coomassie Blue staining. Results presented are from single representative gels from experiments performed in triplicate (or greater) unless stated otherwise. Gels were scanned with a Bio-Rad GelDoc 1000 system (Bio-Rad, Hercules, CA, U.S.A.) and the staining intensity (density) of the parent protein bands was determined (over a linear range) with Bio-Rad molecular analysis software.

RESULTS

Detection and quantification of plasma-derived chloramines

The concentration and stability of chloramines formed on treatment of diluted plasma, and plasma proteins, with HOCl was investigated with the use of Nbs; it has been assumed that the yield of Nbs-reactive material (divided by two, to allow for the reaction stoichiometry [33]) is equivalent to the yield of chloramines. The concentration of chloramines formed in diluted fresh plasma was examined by the treatment of plasma (350 μM protein) with increasing concentrations of HOCl; the yield of chloramines was assayed after 15 min (to permit the complete consumption of HOCl) at 4 °C. Statistically significant levels ($P < 0.05$ compared with controls) of chloramines were detected at concentrations of HOCl of 50 μM or more, with these increasing in a dose-dependent manner with the quantity of HOCl added (Figure 1). The stoichiometry of chloramine formation with HOCl added was always less than 1: values were between 0.2 and 0.3.

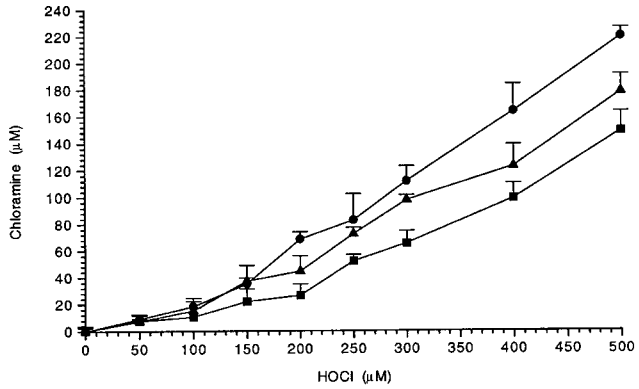

Figure 1 Formation of chloramines (Nbs-reactive material) on treatment of fresh human plasma with HOCl, and the effect of removal of ascorbate and low-molecular-mass material on the concentrations of chloramines

Fresh diluted plasma (■), plasma separated from the low-molecular-mass material with a Sephadex PD-10 column (●) or plasma treated with ascorbate oxidase (▲) (all 350 μM protein, containing approx. 175 μM thiol and 2 mM methionine residues) were treated with HOCl (0–500 μM) for 15 min at 4 °C before being assayed for chloramines with the Nbs assay as outlined in the Materials and methods section. Results are means ± S.D. for triplicate samples of plasma obtained from a single donor; similar results were obtained for other plasma donors. The differences in chloramine concentration between controls and all HOCl-treated samples ($\geqslant$ 50 μM) were statistically significant ($P < 0.05$) as calculated with a paired t test with the assumption of unequal variance.

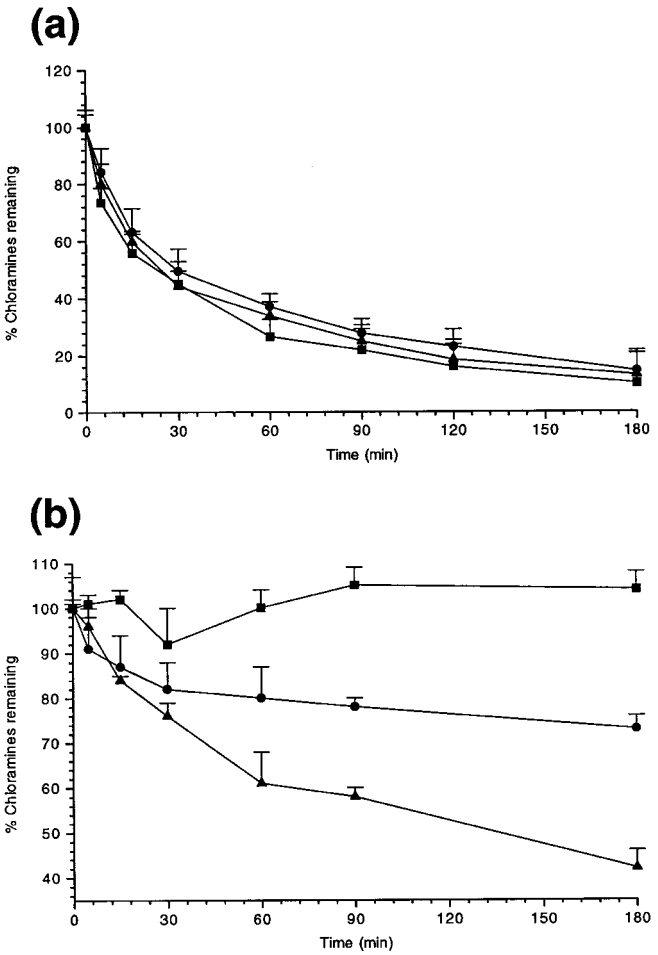

Figure 2 Time and temperature dependence of the decay of plasma and plasma protein chloramines generated on treatment of plasma with HOCl

Chloramine concentrations were measured with the Nbs assay as outlined in the Materials and methods section. (**a**) Decay of chloramines formed on the reaction of fresh diluted plasma (■), plasma separated from the low-molecular-mass material with a Sephadex PD-10 column (●) or plasma treated with ascorbate oxidase (▲) (all 350 μM protein) with HOCl (500 μM) for 15 min at 4 °C. Samples were subsequently incubated at 37 °C and the chloramine concentrations were assayed at the times indicated. (**b**) Decay of plasma-protein-derived chloramines formed on the treatment of plasma (500 μM protein) with HOCl (5 mM), with subsequent separation of the plasma proteins from excess oxidant on a Sephadex PD-10 column. Samples were incubated at 4 °C (■), 20 °C (●) and 37 °C (▲) and the chloramine concentrations were assayed at the times indicated. Results are means ± S.D. for triplicate samples of plasma obtained from a single donor; similar results were obtained for other plasma donors.

The contribution of plasma proteins to the overall yield of chloramines detected with intact fresh plasma was investigated by separation of the proteins from the low-molecular-mass material present in plasma by using two successive PD-10 Sephadex columns; this material is referred to below as isolated plasma proteins. The protein-containing fractions [determined with the BCA assay with human serum albumin (HSA) as standard; 350 μM final protein concentration] were subsequently treated with HOCl (0–500 μM) for 15 min at 4 °C before assay with Nbs (Figure 1). In both systems the addition of excess methionine (0.1 M) to the HOCl-treated plasma, before the determination of chloramine levels, resulted in a complete loss of the Nbs-reactive material, which was consistent with this being due to chloramines.

The stability of the plasma-derived chloramines was investigated by incubating the HOCl-treated samples at 37 °C for various periods before assay with Nbs. With intact diluted plasma (350 μM protein) treated with HOCl (500 μM), a time-dependent loss of the chloramines was observed (Figure 2a). An identical time course of chloramine decay was observed with isolated plasma proteins (separated as above; 350 μM protein) treated with HOCl (500 μM HOCl) and subsequently incubated at 37 °C (Figure 2a). The temperature dependence of the decay of the protein-bound chloramines was investigated by using isolated plasma protein fractions (500 μM final protein concentration) treated with HOCl (5 mM) and subsequently incubated at 4, 20 or 37 °C. No significant decrease in the concentration of chloramines was detected at 4 °C over a period of 3 h, whereas there was a 25% and a 60% decrease in the chloramine concentration in samples incubated at 20 and 37 °C respectively over 3 h (Figure 2b).

The concentration of chloramines formed with intact plasma was compared with that generated in plasma that had been depleted of ascorbate (plasma concentration approx. 50 μM [36]) by pretreatment with ascorbate oxidase (12.5 units/ml of plasma for 1 h at 20 °C). The treated plasma (350 μM protein) was subseqently reacted with low concentrations of HOCl (0–500 μM) for 15 min at 4 °C before assay with Nbs (Figure 1). This treatment resulted in a small increase in the levels of chloramines detected; the effect was more marked at high HOCl concentrations. A trend towards higher chloramine levels was also observed at lower HOCl concentrations. The increase induced by the removal of ascorbate was much smaller than that observed on the removal of all the low-molecular-mass material. Analogous experiments were also attempted with the enzyme uricase, which removes urate (plasma concentration approx. 300 μM; [36]). However, the low activity of this enzyme, and hence the large quantity of protein that needed to be added and long incubation times that had to be employed (see the time-dependent

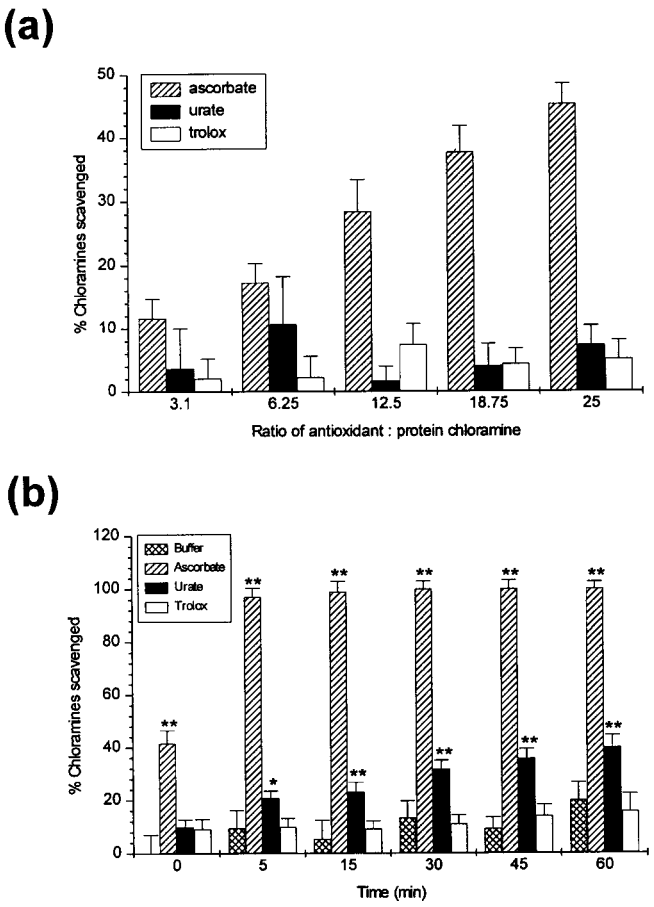

Figure 3 Effect of ascorbate, urate and Trolox C on the concentration of plasma-protein-derived chloramines generated on the reaction of plasma with HOCl

Chloramines were measured with the Nbs assay (see the Materials and methods section). Fresh diluted plasma (500 μM protein) was treated with HOCl (5 mM) for 15 min at 4 °C before separation of the plasma proteins from excess HOCl on a Sephadex PD-10 column. (**a**) Increasing concentrations of ascorbate (light grey bar), urate (dark grey bar) or Trolox (open bar) (625 μM–5 mM) were added to the separated plasma protein chloramines (200 μM) before immediate assay of the chloramine concentrations with Nbs. (**b**) Ascorbate (2.5 mM, light grey bar), urate (2.5 mM, dark grey bar), Trolox (2.5 mM, open bar) or buffer (50 mM, hatched bar) was added to the separated plasma protein chloramines (approx. 200 μM) and the samples were subsequently incubated for 0, 5, 15, 30, 45 or 60 min at 20 °C before assay of chloramine concentrations with Nbs. Results are means $\pm$ S.D. for triplicate samples. The statistical differences between chloramine concentrations between control (buffer added) and treated samples in (**b**) were assessed using a paired *t* test assuming unequal variances: $^{**}P \leqslant 0.01$; $^{*}P \leqslant 0.05$.

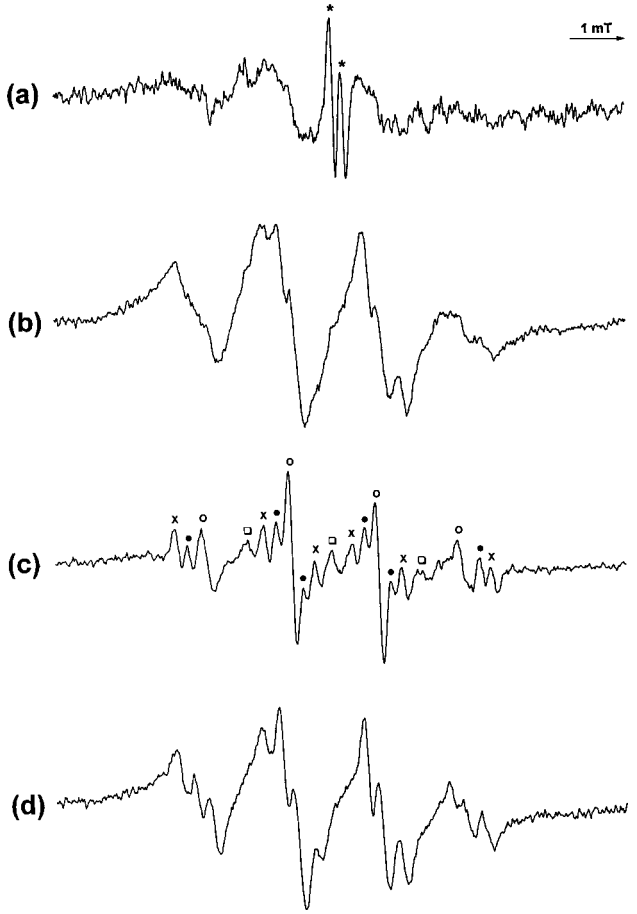

Figure 4 EPR spectra observed on reaction of plasma with HOCl in the presence of the spin trap DMPO

(**a**) Fresh diluted plasma samples (500 μM protein) were treated with HOCl (500 μM); the spin trap DMPO (125 mM) was added immediately after the HOCl. (**b**) As (**a**) except with 5 mM HOCl. (**c**) As (**a**) except with plasma (250 μM protein) and HOCl (10 mM). (**d**) As (**b**) except that the sample was incubated for 15 min with Pronase (0.2 mg/ml) at 20 °C before the spectrum was recorded. Signals in (**a**), (**b**) and (**d**) are attributed to the formation of nitrogen-centred protein-derived radical adducts with the exception of the features marked with an asterisk in (**a**), which are assigned to the ascorbyl radical. The signals marked × and ● in (**c**) are assigned to two different carbon-centred radical adducts, those marked ○ to DMPO-OH, and those marked □ to a DMPO degradation product; for further details see the text.

decay results reported above), made the results obtained difficult to interpret (results not shown).

The effect of addition of above-physiological levels of ascorbate, urate and Trolox C to HOCl-treated plasma proteins was also investigated. Plasma (500 μM protein) was treated with HOCl (5 mM) for 15 min at 4 °C and the HOCl-treated plasma proteins subsequently were separated from excess HOCl with a PD-10 Sephadex column. Increasing concentrations of ascorbate or urate (0.5–5 mM) were then added to the samples (which contained approx. 200 μM chloramines), which were assayed immediately (within 2 min) with Nbs. Treatment with ascorbate resulted in a concentration-dependent increase in the percentage of chloramines scavenged with increasing levels of ascorbate (Figure 3a). No significant differences were detected with urate or Trolox C. The effect of extended incubation times on these reactions was subsequently investigated with fixed concentrations (2.5 mM) of added ascorbate, urate or Trolox C (Figure 3b). Under these conditions, complete loss of the plasma protein chloramines (200 μM initial concentration) was observed with ascorbate after 5 mins. With urate a slow, inefficient, time-dependent scavenging was observed with approx. 60% of the initial chloramines still present after 60 min. Trolox C had no significant effect. The effect of GSH could not be examined because this interferes with the Nbs assay [47].

Formation of radical species

Diluted fresh plasma (500 μM protein) treated with HOCl (300 μM to 5 mM) at 20 °C in the presence of DMPO (125 mM) gave rise, within 2 min of the initiation of the reaction, to broad anisotropic EPR signals with some partly resolved fine structure (see, for example, Figures 4a and 4b). These signals were observed

only in the presence of the spin trap; no signals were observed in the absence of HOCl. In the absence of plasma, a chlorimine species arising from the direct reaction of HOCl with the spin trap was detected; the spectrum of this radical was very different from those of the above species [44] (results not shown). The signals observed with the complete reaction system are assigned to large, slowly tumbling, protein-derived radical adducts (studies with isolated proteins are described in [18]). These signals decayed over approx. 60 min and no further species were detected (results not shown). At 37 °C, similar adducts were detected, though these decayed more rapidly. The intensity of the adduct signals increased with increasing HOCl concentration (compare Figures 4a and 4b). With 300 μM HOCl a further doublet signal [Figure 4a; a(H) 0.176 mT] assigned to the ascorbyl radical was also observed [48]; this signal was also detected in the absence of DMPO. Ascorbyl radical signals were also detected in the absence of HOCl, although at lower intensity (0.34 μM in the presence of 300 μM HOCl, 0.20 μM in its absence). This radical might be formed at higher HOCl concentrations but not detected, owing to overlap with the spin adduct signals.

The concentration of the protein-derived spin adducts obtained with diluted fresh plasma (500 μM protein) treated with 500 μM HOCl at 20 °C was assessed by using a stable nitroxide (TEMPO) as a standard. Under these conditions, approx. 150 μM chloramines were generated (see Figure 1) and approx. 15 μM of these chloramines decayed over the period required to acquire the EPR spectra (see Figure 2a); this resulted in the formation of approx. 0.55 μM spin adduct (i.e. approx. 4 % conversion of chloramine to radical adduct). This represents a lower limit and is likely to be a significant underestimate (see the Discussion section).

The nature of the plasma-derived radical adducts was investigated by fractionation of the plasma on a PD-10 Sephadex column. Reaction of HOCl (1.5 mM) with protein-containing fractions (BCA-assay-positive), in the presence of DMPO (125 mM) at 20 °C, gave broad EPR signals with partly resolved fine structure, as observed with intact plasma. Analogous experiments with later-eluting, non-protein, fractions did not give substrate-derived signals. Experiments were also performed in which the adducts generated from intact plasma were separated by filtration through a 30 kDa cut-off filter. In this case, signals were observed only from the material retained by the filter. These results are consistent with the observed signals' being due to protein-derived plasma spin adducts.

Further information about the plasma-protein-derived radical adducts was obtained by incubating the preformed adducts with Pronase (0.2 mg/ml, 20 °C, 15 min). This treatment resulted in the loss of the broad features and the detection of sharper, more isotropic signals consistent with the release of mobile, low-molecular-mass, spin adducts from the initial protein-derived adducts (Figure 4d; previous use of this technique is described in [49]). These sharp signals have been assigned to DMPO-OH [a(N) = a(H) 1.49 mT] and a second species with hyperfine coupling constants a(N) 1.50 mT, a(H) 1.79 mT, a(N) 0.29 mT. The latter is assigned to a protein-derived nitrogen-centred radical adduct on the basis of the small additional nitrogen coupling.

With fresh diluted plasma and high concentrations of HOCl, a marked sharpening of the spin adduct signals was detected, together with signals from additional spin adducts; this was particularly pronounced with very high levels of HOCl (Figure 4c). The sharpening of the broad spin adduct signals with increasing HOCl concentrations is ascribed to an increased extent of fragmentation of the plasma proteins under these conditions (see the SDS/PAGE experiments described below). The detection of the additional spin adducts is interpreted in terms of a change in the type of protein-derived radicals generated; similar behaviour has been observed with BSA, HSA and other purified proteins [18]. Spectral analysis confirmed the presence of at least two carbon-centred radicals [a(N) 1.55, a(H) 2.43 mT, and a(N) 1.55, a(H) 2.01 mT respectively] as well as DMPO-OH. The formation of these species is discussed further below. The signals detected in all these experiments were significantly inhibited by the addition of excess methionine (0.1 M) to the HOCl-treated plasma before the addition of the spin trap, suggesting that chloramines are precursors of these radicals.

Further evidence for a role for chloramines as radical precursors was obtained from time-course experiments (see Figure 2a). HOCl-treated plasma was incubated at 20 °C for varying periods before the addition of the spin trap DMPO; EPR signals from protein-derived nitrogen-centred radical adducts were detected even when DMPO was added 3 h after the initial addition of HOCl. The signals were, however, somewhat sharper in nature, and lower in intensity, at longer time points. The lower signal intensities at long time points are consistent with a slower rate of radical formation (presumably as a result of chloramine depletion; see Figure 2) and the sharper signals are consistent with an increased extent of protein fragmentation (and hence increased spin adduct mobility). A more rapid decrease in the intensity of the plasma-derived radical adducts was observed when HOCl-treated plasma was incubated at 37 °C for various periods before the addition of DMPO.

Reaction of ascorbate-depleted plasma (prepared as outlined above; 350 μM protein) with HOCl (2.1 mM) in the presence of DMPO (125 mM) resulted in the detection of signals that were qualitatively identical with, but more intense (approx. 130 % of controls) than, the signals observed with native plasma. Similar behaviour was observed with plasma proteins separated from all low-molecular-mass material (as above) and then reacted with HOCl (122 % of control). The increased protein radical yield observed when these materials were removed from plasma suggests that both ascorbate and other low-molecular-mass plasma components protect plasma proteins against HOCl-mediated damage.

The effect of the addition (or supplementation) of various antioxidants on radical formation from preformed plasma-derived chloramines was investigated. Diluted fresh plasma (500 μM protein) was pretreated with HOCl (5 mM) for 15 min at 4 °C; the plasma proteins were subsequently separated by using PD-10 columns. The plasma protein chloramines were then treated with ascorbate (0.15–1.5 mM) and DMPO (125 mM) in that order. No significant differences from untreated samples were observed with ascorbate concentrations less than 150 μM. Between 150 μM and 3.5 mM ascorbate, a stimulation of radical adduct formation was detected (e.g. by 240 % at 1.4 mM); at 3.6 mM ascorbate and higher, a decrease in intensity of the plasma-derived radical adducts was detected and additional signals from the ascorbyl radical were observed. No plasma-derived signals were observed with ascorbate concentrations above 8 mM. The enhancement of the protein-derived radical adduct signals with low concentrations of ascorbate is believed to be due to the ascorbate-dependent stimulation of chloramine decomposition, resulting in more rapid protein radical formation (see Figure 3, and above). At high ascorbate levels a second process, probably spin adduct reduction with concomitant ascorbyl radical formation, dominates.

Analogous experiments with GSH (0.15–8 mM) resulted in a concentration-dependent decrease in the intensity of the plasma-derived spin adducts, although significant inhibition was observed only with relatively high GSH concentrations (25 % at

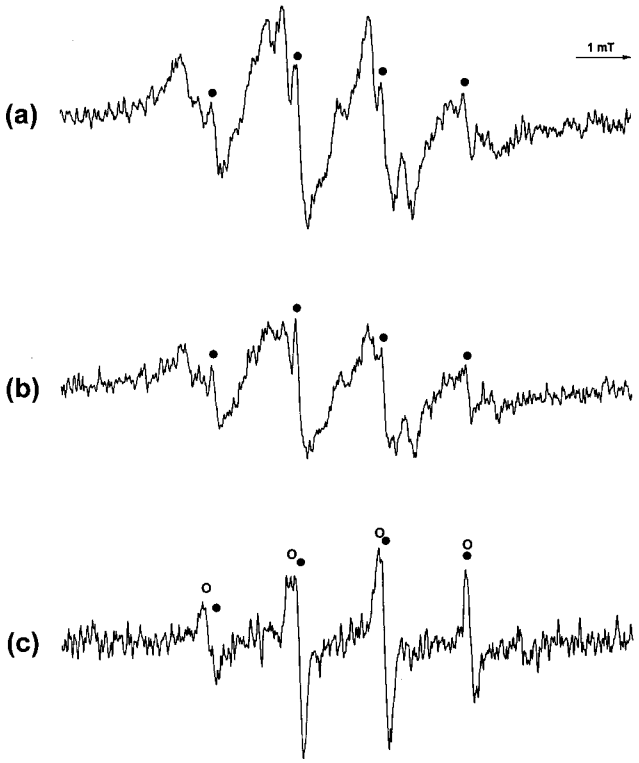

Figure 5 EPR spectra observed on the addition of GSH to HOCl-treated plasma in the presence of the spin trap DMPO

Fresh diluted plasma (500 μM protein) was reacted with HOCl (5 mM) followed by the addition of 50 mM phosphate buffer, pH 7.4 (**a**), 3.6 mM GSH (**b**) or 14.4 mM GSH (**c**), and then DMPO (125 mM). The broad features in (**a**) and (**b**) are assigned to nitrogen-centred protein-derived radical adducts. The features marked ● in all spectra are assigned to DMPO-OH; those marked ○ in (**c**) are assigned to the GSH-derived thiyl ($GS^{\bullet}$) adduct to DMPO.

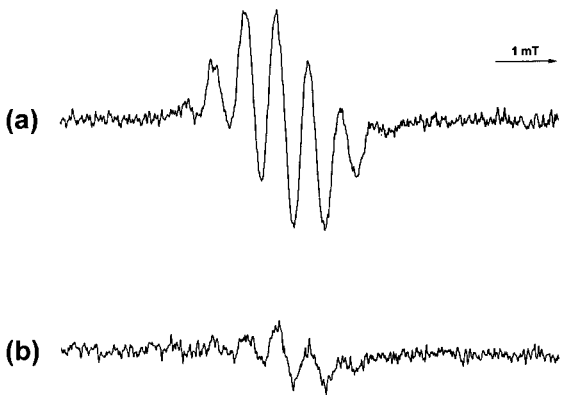

Figure 6 EPR spectra observed on the addition of α-tocopherol-containing micelles to HOCl-treated plasma

Fresh diluted plasma (500 μM protein) was treated with HOCl (5 mM); α-tocopherol-containing HTAC micelles (50 mM HTAC/250 μM α-tocopherol) or SDS micelles (50 mM SDS/250 μM α-tocopherol) were added subsequently. (**a**) HTAC micelles, (**b**) SDS micelles (50 mM). Signals were assigned to the α-tocopheroxyl radical present within the micelles.

3.5 mM, 55% at 7 mM and 94% at 14 mM GSH). A further EPR signal [a(N) 1.54 mT, a(H) 1.62 mT] was observed with GSH concentrations of 3.6 mM and higher (Figure 5); this signal is assigned to the glutathione thiyl radical ($GS^{\bullet}$) [50]. This radical

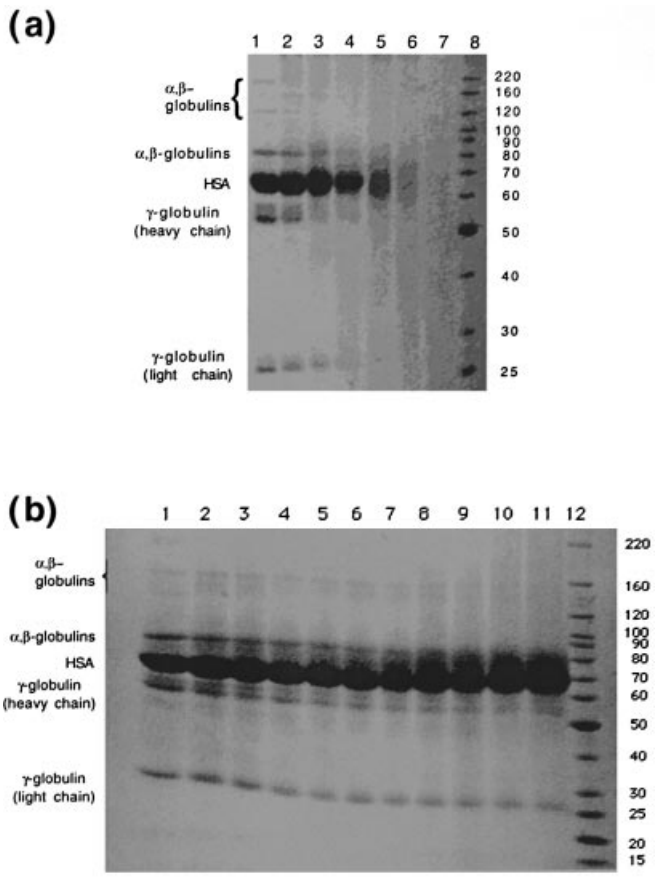

Figure 7 SDS/PAGE gels of plasma treated with HOCl

Fresh diluted plasma (25 μM protein) was treated with the stated concentrations of HOCl and incubated for the indicated durations before being quenched with 0.1 M methionine (to remove any excess HOCl and unreacted chloramines) and subsequent electrophoresis on 10% (w/v) (**a**) or 4–12% (w/v) (**b**) polyacrylamide gradient gels and stained with Coomassie Blue. (**a**) Fresh diluted plasma treated with various concentrations of HOCl: lane 1, plasma alone; lanes 2–7, plasma treated with 25-, 50-, 75-, 100-, 150- or 200-fold molar excess of HOCl respectively for 15 min at 37 °C before being quenched; lane 8, molecular mass markers (the molecular masses are indicated in kDa at the right). (**b**) Fresh diluted plasma treated with HOCl (1.25 mM, 50-fold molar excess) and incubated for various periods at 37 °C before being quenched with 0.1 M methionine and subsequent electrophoresis: lanes 1–11, reaction time 0, 5, 15, 30, 45, 60, 90, 120, 150 and 180 min respectively; lane 12, molecular mass markers (the molecular masses are indicated in kDa at the right).

is probably also present at lower GSH concentrations but obscured by the protein–radical-adduct signal. A less marked decrease in plasma-protein-derived radical adduct concentrations was observed with high concentrations of urate (approx. 1.5 mM final concentration); no additional signals were detected in this case, and no decrease in signal intensity was detected with lower concentrations.

The addition of Trolox C (5 mM) to isolated plasma protein chloramines (prepared as described above) resulted in the detection of the Trolox C-derived phenoxyl radical [18]; no DMPO was added in these experiments. This radical was not observed on the treatment of Trolox C with HOCl alone. Identical signals were observed when fresh diluted plasma (500 μM protein) was treated with HOCl (5 mM) and Trolox C, without separation of the plasma protein chloramines. The intensity of the Trolox C-derived phenoxyl radical signals observed with both systems was significantly decreased when excess methionine (50 mM) was

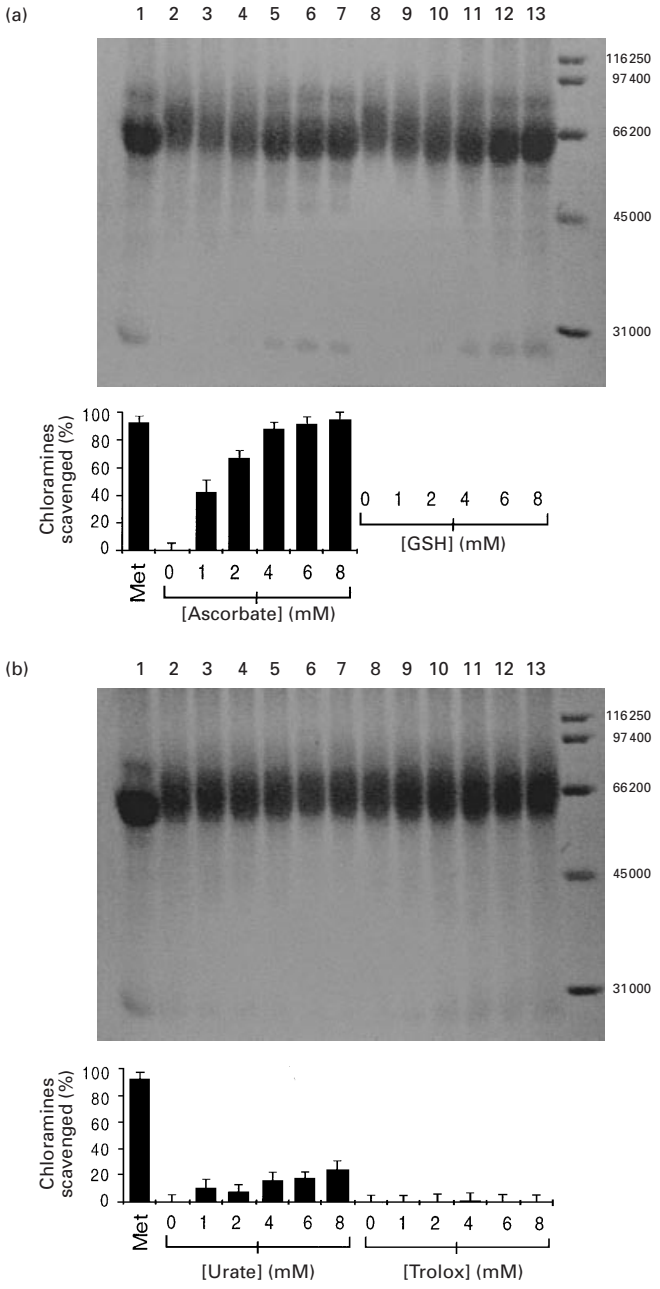

Figure 8 Effect of the addition to plasma of ascorbate, GSH, urate or Trolox C on the extent of HOCl-induced plasma protein fragmentation as assayed by SDS/PAGE

Increasing amounts of ascorbate, GSH, urate or Trolox resulted in a decrease in the fragmentation of plasma proteins, as shown by the increase in the staining of the parent bands. Fresh diluted plasma (250 μM protein) was treated with HOCl (25 mM) for 15 min at 4 °C before separation from excess HOCl on a Sephadex PD-10 column. HOCl-treated plasma proteins (25 μM) were subsequently treated with the stated additions and incubated at 37 °C for 3 h. Samples were then loaded on 10% (w/v) polyacrylamide gels, run as described in the Materials and methods section, and stained with Coomassie Blue. (**a**) Lane 1, with methionine (0.1 M) added; lanes 2 and 8, with phosphate buffer (50 mM) added; lanes 3–7, with ascorbate (1, 2, 4, 6 or 8 mM respectively) added; lanes 9–13, with GSH (1, 2, 4, 6 or 8 mM respectively) added. The right-hand lane contained molecular mass markers (the molecular masses are indicated in kDa at the right). (**b**) Lane 1, with methionine (0.1 M) added; lanes 2 and 8, with phosphate buffer (50 mM) added; lanes 3–7, with urate (1, 2, 4, 6 or 8 mM respectively) added; lanes 9–13, with Trolox C (1, 2, 4, 6 or 8 mM respectively) added. The right-hand lane contained molecular mass markers (the molecular masses are indicated in kDa at the right). The histograms displayed below the gels indicate the extent of scavenging of the protein chloramines initially present in the sample after treatment with methionine, buffer, ascorbate, urate and Trolox C (concentrations as above) in samples identical with those in the gel lanes immediately above them after incubation for 15 min at 20 °C, measured with the Nbs assay (see the Materials and methods section). Results means ± S.D. for duplicate samples.

added before Trolox C, supporting the hypothesis that radicals derived from plasma-protein-derived chloramines are the damaging species. In experiments in which DMPO was added, the presence of Trolox C (5 mM) resulted in a significant decrease in the concentration of protein-derived radical adducts as expected on the basis of competition between DMPO and Trolox C for the available radicals.

The addition of HOCl-treated plasma (5 mM HOCl/500 μM plasma) or isolated plasma protein chloramines (prepared as described above) to α-tocopherol (250 μM) contained in HTAC or SDS micelles (50 mM), in the absence of DMPO, gave the α-tocopheroxyl radical [51]. The intensity of these signals was micelle-dependent, with more intense signals observed with (positively charged) HTAC than (negatively charged) SDS micelles; the latter was 12% of the former (Figure 6). This difference might be due to steric or electronic interactions between the HOCl-treated plasma proteins and the micelles. This suggestion is supported by the observation that the signals observed with the SDS micelles were significantly enhanced in intensity (approx. 2-fold) by pretreating either the plasma/HOCl mixture or the separated HOCl-treated plasma proteins with the proteolytic enzyme Pronase before addition to the α-tocopherol-containing micelles; this treatment presumably lessened the effects of such interactions by partly degrading the plasma proteins. No differences were observed when analogous experiments were performed with HTAC micelles. Treatment of either the HOCl-treated plasma or the isolated plasma protein chloramines with excess methionine (50 mM) before addition to the α-tocopherol-containing micelles resulted in a marked inhibition (approx. 70%) of the α-tocopheroxyl radical concentration.

Effect of HOCl on the structural integrity of plasma proteins

SDS/PAGE experiments were performed to investigate the integrity of plasma proteins treated by exposure to HOCl. Diluted fresh plasma (25 μM protein) was incubated with increasing concentrations of HOCl (0–12.5 mM) for 15 min at either 4 or 37 °C before the addition of excess methionine (0.1 M) to remove unreacted HOCl and chloramines. This treatment resulted in a decrease in the intensity of staining (with Coomassie Blue) of the parent plasma protein bands in both cases, although this was much more marked at 37 °C (Figure 7a) than at 4 °C (results not shown). No evidence was obtained for the presence of either discrete fragments or well-defined cross-linked/aggregated material, suggesting that the reaction of HOCl with plasma proteins results in the loss of the parent protein via the formation of a large number of poorly defined, possibly random, fragments. The extent of loss of each of the major plasma protein bands with increasing HOCl concentration was assessed by quantification of the extent of staining (see the Materials and methods section). Such analysis showed that there was a proportionally greater loss of some protein bands than of others, with the α- and β-globulins being more susceptible to degradation than the γ-globulins or HSA (results not shown). In all cases the addition of methionine (0.1 M) to the plasma immediately after that of HOCl resulted in a decrease in the HOCl-mediated destruction of the plasma proteins as evidenced by a less marked decrease in the staining intensity of the parent protein bands.

This implies that the removal of protein chloramines can protect against degradation of the parent protein.

A similar decrease in the intensity of the staining of the bands of parent proteins was observed on incubating samples of plasma (25 μM protein) pretreated with HOCl (50-fold molar excess over protein) at 37 °C for periods of up to 3 h before the addition of excess methionine (0.1 M) to scavenge any remaining chloramines (Figure 7b). Quantification of the degree of staining again showed that some proteins were more susceptible to degradation than others (results not shown). Overall, HOCl seems to be capable of inducing plasma protein degradation in both a concentration-dependent and a time-dependent manner; this process requires, at least in part, the formation and subsequent decomposition of protein chloramines.

The potential protective effects of added (or supplemented) antioxidants on HOCl-dependent plasma protein fragmentation was examined. Plasma treated with HOCl (100:1 molar ratio of HOCl to protein) was incubated at 4 °C for 15 min before the separation of excess HOCl on a PD-10 Sephadex column. Samples were then incubated at 37 °C for 3 h with ascorbate, GSH, urate or Trolox (0–8 mM) before the addition of excess methionine (0.1 M) to stop further reaction. The addition of increasing concentrations of ascorbate, GSH, urate or Trolox C to the HOCl-treated plasma protected the plasma proteins from HOCl-mediated fragmentation to differing extents (Figure 8). The concentration of plasma protein chloramines remaining 15 min after the addition of ascorbate, urate or Trolox C was also determined by using the Nbs assay on identical samples (Figure 8); analogous data could not be obtained with GSH because this interferes with the Nbs assay. Ascorbate and GSH were more effective at preventing fragmentation of the plasma proteins than was urate or Trolox C. The protective effect of ascorbate might be due to repair/removal of the protein chloramines by ascorbate and/or scavenging of the resulting chloramine-derived radicals. In contrast, urate, which has been shown to be less effective in removing/repairing protein chloramines, was not as effective. The slight protective effect observed with Trolox C, which has been shown (see above) to be unreactive with plasma-protein-derived chloramines, is attributed to scavenging of plasma-protein-derived radicals formed on decomposition of protein chloramines/chloramides.

DISCUSSION

In this study it has been shown that the reaction of HOCl (at least 50 μM) with either diluted fresh human plasma (350 μM protein) or isolated plasma proteins generates material that reacts with Nbs. This material is believed to be primarily chloramines formed from the reaction of HOCl with protein side-chain amine groups. The formation of this material could be inhibited by the addition of methionine, which removes such species. With both plasma and isolated plasma proteins the stoichiometry of chloramine formation is less than 1, with values typically in the range 0.2–0.3. These chloramines decompose when incubated for extended periods at either 20 or 37 °C but not at 4 °C. The addition of ascorbate and urate increased the rate of decay in a time- and concentration-dependent manner. The reaction of both fresh diluted plasma and isolated plasma proteins with HOCl has also been shown to give rise to protein-derived nitrogen-centred radicals. The yield of these materials is dependent on the incubation time and the concentration of HOCl; the proteins on which these species are formed remain to be identified. The loss of these signals on treatment with excess methionine is consistent with protein-derived chloramines (probably from the lysine side chain) being the radical source. Similar behaviour and analogous spin adducts have been detected previously with isolated purified proteins, peptides and free lysine [18,40]. Fragmentation of the plasma proteins occurs in both a time- and an HOCl concentration-dependent manner, as evidenced both by the mobility of the EPR spin adducts, by the detection of additional radicals and by SDS/PAGE experiments. This fragmentation could be inhibited by excess methionine as well as other agents capable of removing chloramines and radicals derived from them (e.g. ascorbate, urate, GSH and Trolox C). These results imply a role for protein-derived chloramines, and the radicals derived from them, in HOCl-induced plasma protein fragmentation.

Previous studies have suggested that the primary targets of HOCl in plasma are thiol groups and methionine residues on proteins [13,15]; these residues are present at approx. 430 μM (experimental value; see the Materials and methods section) and approx. 5 mM [36] respectively in fresh plasma. Though low-molecular-mass thiols and free methionine react extremely rapidly with HOCl [10], the contribution of such molecules to HOCl consumption in plasma would be expected to be small, owing to their low concentrations [36]. Ascorbate has also been suggested as a major target for HOCl, although the low concentration of ascorbate in plasma (approx. 50 μM), compared with other HOCl-reactive residues, suggests that this might have only a marginal role [10]. Lysine side-chain (plus N-terminal) amino groups react rapidly with HOCl, although with rate constants lower than for free thiols and methionine [10]. A previous study on the reaction of HOCl with various potential targets in plasma has reported a loss of amino groups only after depletion of thiols [13], implying that the reaction of HOCl is selective for thiol and methionine residues. In contrast, another study has concluded that sulphur-containing amino acids on proteins are unlikely to be the only target for HOCl [10]. The latter conclusion is supported by a consideration of the relative molar ratios of such groups in intact plasma, with lysine amino groups (plus N-terminal amino functions) estimated as being present at approx. 50 mM, which is greater than that of thiols (approx. 500 μM [13,15]) and methionine groups (approx. 5 mM). Furthermore, lysine residues would in general be expected to be present on the surface of proteins (because they are charged) and hence exposed to HOCl in solution. In contrast, the hydrophobic methionine residues would be expected to be buried within proteins. As a consequence it is perhaps not surprising that we observe chloramines (Nbs-reactive material) at HOCl concentrations (at least 50 μM) well below the level that could be consumed by all the thiol (approx. 175 μM) and methionine (approx. 2 mM) residues present in the diluted (approx. 350 μM protein) plasma studied. We have not quantified the loss of plasma thiol groups on treatment with HOCl in this study, with the use of Nbs_2, because such measurements cannot be readily made in the presence of chloramines. We have also not measured Met consumption because the extent of the loss would be only a small fraction of the total present and hence within the error limits of HPLC detection methods (typically approx. 5–10 %, see also below).

The chloramine yield is well below stoichiometric (20–30 % of the HOCl provided). The apparent discrepancy between chloramine detection in this work and a previous study that suggested that lysine residues are only consumed after the removal of all thiol (and methionine) residues [13] can be readily rationalized. First, lysine-derived chloramines are unstable; their decomposition can be exacerbated by the presence of thiols, methionine, ascorbate and (to a smaller extent) urate, potentially leading to the erroneous conclusion that this reaction does not occur. This hypothesis is supported by the observation that the stoichiometry

of chloramine formation increases with increasing amounts of HOCl, which can be readily explained by the depletion of materials that can regenerate amino groups from semi-stable chloramines. The observed yield of chloramines might therefore be an underestimate of the true yield, with the amount detected merely reflecting that which is less rapidly repaired by such processes. Second, the conversion of lysine residues to chloramines represents only a very small fraction of the total plasma lysine content [see Figure 1, in which the highest HOCl concentration tested (500 μM) gives less than 200 μM chloramines from 350 μM plasma protein with a total lysine content of approx. 15 mM, i.e. less than 1.5 % conversion], well within the error limits of most assays [13].

Treatment of plasma and isolated plasma proteins with a wide range of HOCl concentrations gives rise to nitrogen-centred protein-derived radicals, which have been detected as their corresponding DMPO adducts. The concentration of HOCl required to observe these signals is somewhat higher (300 μM) than that required to detect chloramine formation (approx. 50 μM). This presumably reflects the sensitivity limit of the EPR spectrometer, the low and slow conversion of chloramines to radicals, and/or potential removal of either chloramines or the radicals derived from them by plasma thiol groups. It is likely, although not proved, that these protein-derived radicals are present on most, if not all, plasma proteins. Whether the distribution of adducts reflects the target size (i.e. total amino acid composition) or the number of lysine (and/or other reactive residues) on each protein remains to be established. Previous results suggest that the protein lysine content might be an important factor [18]. The exact mechanism of formation of these protein-derived nitrogen-centred radicals from chloramines remains to be established (homolysis of the N–Cl bond to give $RNH^{\bullet}$ and $Cl^{\bullet}$, compared with one-electron reduction by trace metal ions to give $RNH^{\bullet}$ and Cl^{-} [18]). The increased rate of chloramine decay at 37 °C compared with 20 °C and the lack of decomposition at 4 °C suggest that thermal homolysis might be important. The absence of any effect of treatment with chelex suggests that metal-ion-mediated reactions are not significant. The DMPO-OH signals detected by EPR might arise from the rapid hydrolysis of the Cl° adduct to DMPO [18].

Treatments that affect the chloramine yield also alter the radical adduct concentrations. In some cases these effects parallel each other, e.g. excess methionine removes chloramines and also inhibits the formation of radical adducts. In contrast, ascorbate and GSH enhance chloramine decomposition but via processes that result, at least in part, in the formation of further radical species from both ascorbate/GSH and the chloramines. This difference might reflect the propensity of these species to undergo two-electron (non-radical) reactions for methionine, and one-electron (radical) reactions for ascorbate/GSH. Similar behaviour has been observed with some sulphur-containing compounds (C. L. Hawkins and M. J. Davies, unpublished work).

The yield of radical adducts detected, in comparison with chloramines lost (approx. 4 %), is low. This value is a lower limit at a particular time point and does not take into consideration two important factors. Firstly, the inefficiency of spin adduct formation, which would be expected to be low despite the spin trap concentration used, as a result of the high rate constants for reaction of the nitrogen-centred radicals with other targets via both intramolecular and intermolecular reactions [18,40] and the high concentration of such targets [the plasma samples employed contain more than 0.3 M total (free plus protein) amino acids as well as considerable concentrations of other targets], and secondly, the decay of the spin adduct, once formed, during the period required to acquire the EPR spectra. The efficiency of nitrogen-centred radical formation from chloramines is therefore likely to be higher than this.

At high HOCl concentrations, fragmentation of plasma protein occurs as judged by both EPR and SDS/PAGE experiments. The carbon-centred radicals detected by EPR are believed to arise from rearrangement/fragmentation of the initial lysine-derived nitrogen-centred radicals [18,40] and/or via the formation of alternative initial radicals. The latter might arise via the reaction of HOCl at backbone amide/peptide bonds [38,52] and the subsequent decomposition of the resulting chloramides to give nitrogen- and carbon-centred radicals [18,52]; a role for (non-radical) hydrolysis of such chloramides in the observed fragmentation cannot, however, be excluded. Both types of nitrogen-centred radical (lysine side-chain and putative backbone amide) might be responsible for backbone cleavage (studies with isolated proteins have been described previously [18]). The extent of fragmentation with a fixed concentration of HOCl is protein-dependent; the α- and β-globulins are more susceptible to fragmentation than HSA or γ-globulins. The reason for this selectivity is not known, although it might reflect the extent of initial reaction of HOCl with each protein (which is probably determined by the number of HOCl-reactive residues present) and/or the ratio of reactive side chain to backbone sites (i.e. the number of Cys, Met, Lys and Trp residues compared with the number of backbone amide sites). The lower concentration of Trolox C required to detect the Trolox C phenoxyl radical, compared with that required to observe protection against protein fragmentation, presumably reflects reaction with radicals formed at either side-chain or backbone sites in the former case, but primarily reaction at backbone sites in the latter.

Plasma from which low-molecular-mass material had been removed gave rise to increased levels of protein chloramines and an increased concentration of radicals. This suggests that low-molecular-mass plasma components have a role in decreasing HOCl-mediated damage to plasma proteins; this effect is, however, small. This protective effect might be due to direct reaction with HOCl or chloramine 'repair'. Evidence has been obtained for both processes.

The removal of ascorbate from plasma (by the use of ascorbate oxidase) resulted in a small increase in the concentration of both plasma-protein-derived radicals and protein chloramines. The removal of all low-molecular-mass material from the plasma had more marked effects, suggesting that other plasma components (e.g. free amino acids and other amine- and sulphur-containing compounds) also protect plasma proteins against damage by HOCl. No significant differences were observed with urate-depleted plasma (results not shown). Bilirubin, which protects α_1-anti-proteinase inhibitor from HOCl-mediated inactivation [53], is unlikely to have a significant role, because protein-bound bilirubin (e.g. to BSA) is unable to prevent HOCl-induced protein fragmentation ([53], C. L. Hawkins and M. J. Davies, unpublished work). Urea (approx. 4 mM in plasma [36]) also has minimal effects (C. L. Hawkins and M. J. Davies, unpublished work).

An alternative potential protective mechanism against HOCl-induced damage involves the removal of either protein-derived chloramines or radicals formed from these. This has been investigated by generating plasma-protein-derived chloramines and separating them from excess HOCl before reaction with ascorbate, GSH and urate. Ascorbate was a very effective scavenger of protein chloramines, thereby decreasing the concentration of radicals and the extent of protein fragmentation. However, low concentrations of ascorbate increased the concentration of protein radicals observed; this might have been due to enhanced chloramine decomposition and hence radical gen-

eration. GSH was also very effective at decreasing the concentration of protein-derived radicals detected and the extent of protein fragmentation; this was probably due to chloramine removal but could not be tested directly. The occurrence of such efficient repair/scavenging reactions might explain previous observations on the rapid oxidation of thiols in plasma and possibly in cells [13,15,28,54]. In contrast, urate had only minor effects.

The decomposition of plasma-protein-derived chloramines can generate α-tocopheroxyl radicals from α-tocopherol present in detergent micelles. This occurs via a radical process involving chloramines, as evidenced by the protective effect of excess methionine, the lack of signals on direct treatment of α-tocopherol-containing micelles with HOCl (see also [55]), and the observed competition between DMPO and α-tocopherol when both potential targets were present. Thus protein chloramine-derived radicals can initiate the oxidation of molecules present in organic phases. Such reactions might have a role in the initiation of damage to lipoproteins and cell membranes via the formation of α-tocopheroxyl radicals and the occurrence of tocopherol-mediated peroxidation [56]. The transfer of oxidizing equivalents from HOCl to plasma proteins and hence to other susceptible targets via the formation and subsequent reactions of protein chloramines (and radicals derived from them) is an attractive potential mechanism for the observed oxidation of biological structures that might otherwise not initially be a major target for HOCl. This might explain why the oxidation of lipoproteins (and some other targets) occurs relatively readily even though such material would be predicted to be only marginally targeted by HOCl as a result of its low relative concentration (in terms of absolute, or protein, mass) in plasma.

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REFERENCES

1 Weiss, S. J. and LoBuglio, A. F. (1982) Lab. Invest. **47**, 5–18
2 Kettle, A. J. and Winterbourn, C. C. (1997) Redox Rep. **3**, 3–15
3 Thomas, E. L. (1979) Infect. Immun. **23**, 522–531
4 Jesaitis, A. J. and Dratz, E. A. (1992) The Molecular Basis of Oxidative Damage by Leukocytes, CRC Press, Boca Raton, FL
5 Daugherty, A., Dunn, J. L., Rateri, D. L. and Heinecke, J. W. (1994) J. Clin. Invest. **94**, 437–444
6 Hazell, L. J., Arnold, L., Flowers, D., Waeg, G., Malle, E. and Stocker, R. (1996) J. Clin. Invest. **97**, 1535–1544
7 Hazen, S. L. and Heinecke, J. W. (1997) J. Clin. Invest. **99**, 2075–2081
8 Wright, N. C. (1926) Biochem. J. **20**, 524–532
9 Baker, R. W. R. (1946) Biochem. **41**, 337–342
10 Winterbourn, C. C. (1985) Biochim. Biophys. Acta **840**, 204–210
11 Aruoma, O. I. and Halliwell, B. (1987) Biochem. J. **248**, 973–976
12 Arnhold, J., Hammerschmidt, S., Wagner, M., Mueller, S., Arnold, K. and Grimm, E. (1990) Biomed. Biochim. Acta **49**, 991–997
13 Arnhold, J., Hammerschmidt, S. and Arnold, K. (1991) Biochim. Biophys. Acta **1097**, 145–151
14 Arnhold, J., Wiegel, D., Richter, O., Hammerschmidt, S., Arnold, K. and Krumbiegel, M. (1991) Biomed. Biochim. Acta **50**, 967–973
15 Hu, M. L., Louie, S., Cross, C. E., Motchnik, P. and Halliwell, B. (1993) J. Lab. Clin. Med. **121**, 257–262
16 Yang, C., Gu, Z., Yang, H., Yang, M., Wiseman, W. S., Rogers, L. K., Welty, S. E., Katta, V., Rohde, M. F. and Smith, C. V. (1997) Free Radical Biol. Med. **22**, 1235–1240
17 Yang, C., Gu, Z., Yang, H., Yang, M., Gotto, A. M. and Smith, C. V. (1997) Free Radical Biol. Med. **23**, 82–89
18 Hawkins, C. L. and Davies, M. J. (1998) Biochem. J. **332**, 617–625
19 Clark, R. A., Szot, S., Williams, M. A. and Kagan, H. M. (1986) Biochem. Biophys. Res. Commun. **135**, 451–457
20 Beck-Speier, I., Leuschel, L., Luippold, G. and Maier, K. L. (1988) FEBS Lett. **227**, 1–4
21 Fliss, H. (1988) Mol. Cell. Biochem. **84**, 177–188
22 Heinecke, J. W., Li, W., Daehnke, H. L. and Goldstein, J. A. (1993) J. Biol. Chem. **268**, 4069–4077
23 Domigan, N. M., Charlton, T. S., Duncan, M. W., Winterbourn, C. C. and Kettle, A. J. (1995) J. Biol. Chem. **270**, 16542–16548
24 Hazen, S. L., Crowley, J. R., Mueller, D. M. and Heinecke, J. W. (1997) Free Radical Biol. Med. **23**, 909–916
25 Vissers, M. C. and Winterbourn, C. C. (1991) Arch. Biochem. Biophys. **285**, 53–59
26 Jolivalt, C., Leininger Muller, B., Drozdz, R., Naskalski, J. W. and Siest, G. (1996) Neurosci. Lett. **210**, 61–64
27 Hazell, L. J., van den Berg, J. J. M. and Stocker, R. (1994) Biochem. J. **302**, 297–304
28 Carr, A. C. and Winterbourn, C. C. (1997) Biochem. J. **327**, 275–281
29 Winterbourn, C. C. and Brennan, S. O. (1997) Biochem. J. **326**, 87–92
30 Clark, R. A., Stone, P. J., El Hag, A., Calore, J. D. and Franzblau, C. (1981) J. Biol. Chem. **256**, 3348–3353
31 Matheson, N. R. and Travis, J. (1985) Biochemistry **24**, 1941–1945
32 Weiss, S. J., Lampert, M. B. and Test, S. T. (1983) Science **222**, 625–628
33 Thomas, E. L., Grisham, M. B. and Jefferson, M. M. (1986) Methods Enzymol. **132**, 569–585
34 Halliwell, B., Wasil, M. and Grootveld, M. (1987) FEBS Lett. **213**, 15–18
35 Folkes, L. K., Candeias, L. P. and Wardman, P. (1995) Arch. Biochem. Biophys. **323**, 120–126
36 Lentner, C. (1984) Geigy Scientific Tables: Physical Chemistry, Composition of Blood, Hematology, Somatometric Data, Ciba-Geigy Ltd., Basle
37 Zgliczynski, J. M., Stelmaszynska, T., Domanski, J. and Ostrowski, W. (1971) Biochim. Biophys. Acta **235**, 419–424
38 Selvaraj, R. J., Paul, B. B., Strauss, R. R., Jacobs, A. A. and Sbarra, A. J. (1974) Infect. Immun. **9**, 255–260
39 Stelmaszynska, T. and Zgliczynski, J. M. (1978) Eur. J. Biochem. **92**, 301–308
40 Hawkins, C. L. and Davies, M. J. (1998) J. Chem. Soc. Perkin Trans. **2**, 1937–1945
41 Thomas, E. L., Bozeman, P. M., Jefferson, M. M. and King, C. C. (1995) J. Biol. Chem. **270**, 2906–2913
42 Bollag, D. M., Rozycki, M. D. and Edelstein, S. J. (1996) Protein Methods, Wiley–Liss, New York
43 Witting, P. K., Westerlund, C. and Stocker, R. (1996) J. Lipid Res. **37**, 853–867
44 Bandara, B. M. R., Hinojosa, O. and Bernofsky, C. (1994) J. Org. Chem. **59**, 1642–1654
45 Duling, D. R. (1994) J. Magn. Reson. **104B**, 105–110
46 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
47 Yan, L. J., Traber, M. G., Kobuchi, H., Matsugo, S., Tritschler, H. J. and Packer, L. (1996) Arch. Biochem. Biophys. **327**, 330–334
48 Yamazaki, I., Mason, H. and Piette, L. (1960) J. Biol. Chem. **235**, 2444–2449
49 Davies, M. J., Gilbert, B. C. and Haywood, R. M. (1991) Free Radical Res. Commun. **15**, 111–127
50 Davies, M. J., Forni, L. G. and Shuter, S. L. (1987) Chem.–Biol. Interact. **61**, 177–188
51 Burton, G. W., Doba, T., Gabe, E. J., Hughes, L., Lee, F. L., Prasad, L. and Ingold, K. U. (1985) J. Am. Chem. Soc. **107**, 7053–7065
52 Neale, R. S. (1971) Synthesis **1**, 1–15
53 Stocker, R. and Peterhans, E. (1989) Free Radical Res. Commun. **6**, 57–66
54 Vissers, M. C. and Winterbourn, C. C. (1995) Biochem. J. **307**, 57–62
55 Hazell, L. J. and Stocker, R. (1997) FEBS Lett. **414**, 541–544
56 Hazell, L. J., Davies, M. J. and Stocker, R. (1999) Biochem. J. **339**, 489–495

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