Reaction of protein chloramines with DNA and nucleosides: evidence for the formation of radicals, protein–DNA cross-links and DNA fragmentation

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Stimulated phagocyte cells produce the oxidant HOCl, via the release of the enzyme myeloperoxidase and hydrogen peroxide. HOCl is important in bacterial cell killing, but excessive or misplaced generation can damage the host tissue and may lead to the development of certain diseases such as cancer. The role of HOCl in the oxidation of isolated proteins, DNA and their components has been investigated extensively, but little work has been performed on the protein–DNA (nucleosome) complexes present in eukaryotic cell nuclei. Neither the selectivity of damage in such complexes nor the possibility of transfer of damage from the protein to DNA or vice versa, has been studied. In the present study, kinetic modelling has been employed to predict that reaction occurs predominantly with the protein and not with the DNA in the nucleosome, using molar HOCl excesses of up to 200-fold. With 50–200-fold excesses, 50–80 % of the HOCl is predicted to react with histone lysine and histidine residues to yield chloramines. The yield and stability of such chloramines predicted by these modelling studies agrees well with experimental data. Decomposition of these species gives protein-derived, nitrogen-centred radicals, probably on the lysine side

chains, as characterized by the EPR and spin-trapping experiments. It is shown that isolated lysine, histidine, peptide and protein chloramines can react with plasmid DNA to cause strand breaks. The protection against such damage afforded by the radical scavengers Trolox (a water-soluble α -tocopherol derivative) and 5,5-dimethyl-1-pyrroline-*N*-oxide suggests a radical-mediated process. The EPR experiments and product analyses have also provided evidence for the rapid addition of protein radicals, formed on chloramine decomposition, to pyrimidine nucleosides to give nucleobase radicals. Further evidence for the formation of such covalent cross-links has been obtained from experiments performed using ³H-lysine and ¹⁴C-histidine chloramines. These results are consistent with the predictions of the kinetic model and suggest that histones are major targets for HOCl in the nucleosome. Furthermore, the resulting protein chloramines and the radicals derived from them may act as contributing agents in HOCl-mediated DNA oxidation.

Key words: DNA damage, free radicals, hypochlorite, myeloperoxidase, spin trapping.

INTRODUCTION

Activation of phagocyte cells both *in vivo* and *in vitro* is known to result in the generation of O₂• and hydrogen peroxide (H₂O₂) and the release of the haem enzyme myeloperoxidase [1]. This enzyme catalyses the reaction of H₂O₂ with physiological concentrations of Cl[−] ions to give the powerful oxidant HOCl [2]. The pK_a of HOCl is 7.59 [3], thus at physiological pH a mixture of both HOCl and [−]OCl is present; HOCl is used throughout to designate this mixture. HOCl is a potent bactericidal agent [4], but excessive or misplaced generation of HOCl is known to cause tissue damage [5,6]. This is believed to be important in certain diseases and considerable evidence is available to support a link between chronic inflammation and certain cancers [6,7].

HOCl is known to react with a number of biological targets including proteins, DNA, lipids and cholesterol [5,8–16]. Proteins are likely to be major targets for reaction with HOCl within a cell due to their abundance and their high reactivity with HOCl [17]. The treatment of isolated proteins with HOCl is known to result mainly in the alteration of the amino acid side chains [18–21], with some protein fragmentation [10,22] and cross-linking/aggregation [23]. These reactions may render the protein more susceptible to degradation by intracellular protease enzymes [22]. HOCl reacts selectively with certain amino acid side chains [17]. Cys and Met residues are the most reactive ($k_2 > 3.0 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$); then His ($k_2 = 1.6 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$), Cysteine ($k_2 = 1.6 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$), the N-terminal amine group ($k_2 = 1.0 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$), Trp ($k_2 = 1.0 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$) and Lys

($k_2 = 5.0 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$) [17]. Reaction of HOCl with protein NH₂ groups results in the formation of short-lived chloramines (RNHCl species) [24,25]. These intermediates have been shown to decompose to give protein-derived, nitrogen-centred radicals [10,26].

Reaction of HOCl with DNA can result in both structural changes and chemical modifications, with the heterocyclic (ring) NH groups of guanosine and thymidine more reactive than the exocyclic NH₂ groups of guanosine, adenosine and cytidine [27–30]. The reaction of HOCl with these groups also results in the formation of chloramines [31–35]. The generation of these species can lead to the dissociation of double-stranded DNA due to the disruption of hydrogen bonding [28] and their decomposition can result in the formation of nitrogen-centred radicals [35–37]. The heterocyclic chloramines formed with thymidine and guanosine have been shown to be repaired rapidly by thiols and primary amines via chlorine transfer [27–29]. A number of stable chlorinated products have been detected from the reaction of HOCl with free bases, nucleosides, RNA and DNA [8,38,39]. These include 5-chlorocytosine [32,34,39], 5-chlorouracil [33,40], 8-chloroadenine [38] and 8-chloroguanosine [41]. Some of these chlorinated species exert potent biological effects, e.g. 8-chloroadenine is reported to induce apoptosis [42].

Nuclear DNA is known to be closely associated with Lys- and Arg-rich histone proteins via non-covalent ionic interactions [43]; this reversible bonding is important for normal cellular

Abbreviations used: DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DTBN, di-*t*-butylnitroxide; MNP, 2-methyl-2-nitrosopropane; NAH, *N*- α -acetyl-histidine; TNB, 5-thio-2-nitrobenzoic acid.

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function. In contrast, the formation and accumulation of covalent protein–DNA cross-links can be damaging for the cell, particularly as such linkages are known to be repaired inadequately [44]. The generation of HOCl-mediated protein–DNA cross-links have been observed previously [45]. It was postulated in this study that long-lived intermediates generated by HOCl, such as chloramines or radicals derived from them, were responsible for the cross-link formation observed between the single-stranded DNA-binding protein and DNA [45].

The Arg- and particularly Lys-rich nature of the histone proteins suggests that they may be the more favourable targets for reaction with HOCl compared with DNA in a cell nucleus. Thus we hypothesized that HOCl-induced damage to DNA within the nuclei may be mediated by initial reaction of HOCl with the histone proteins (rather than the DNA) to give, amongst other products, protein chloramines which subsequently mediate DNA damage. In the present study, kinetic data have been used to predict the reactivity of HOCl with the nucleosome. The results of these computer modelling studies support the above hypothesis and indicate that histone (particularly Lys- and His-derived) chloramines are the key intermediates. We have therefore investigated the role of Lys, His, peptide and protein chloramines, and species derived from these intermediates, in the formation of protein–DNA cross-links and DNA-strand cleavage.

EXPERIMENTAL

Materials

Water was filtered in a four-stage Milli-Q system (Millipore-Waters, Lane Cove, NSW, Australia). pH control was achieved using 0.1 M phosphate buffer (pH 7.4), pretreated with Chelex resin (BioRad, Hercules, CA, U.S.A.) to remove contaminating traces of metal ions. Amino acids, free nucleosides, poly-nucleotides, DNA (calf thymus) and RNA (transfer, type X from baker's yeast) were obtained from Sigma. DNA solutions were prepared by stirring gently overnight at 4 °C. Treatment of the RNA and DNA solutions with Chelex resin (50 mg · ml⁻¹) before use had no significant effect on the results obtained. The nucleohistone was obtained from Sigma (N8627) and it contained approx. 56 % of protein. The peptides Gly-Lys-Gly and Gly-His-Gly were obtained from Bachem (Bubendorf, Switzerland), 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO; ICN, Seven Hills, NSW, Australia) was purified before use by treatment with activated charcoal. Stock solutions of 0.1 M 2-methyl-2-nitrosopropane (MNP; Aldrich, Castle Hill, NSW, Australia) were generated by overnight dissolution of the solid dimer in acetonitrile at –20 °C; these solutions were diluted into the incubations such that the final acetonitrile concentration was ≤ 5 % (v/v). HOCl solutions were prepared immediately before use by the dilution of a concentrated stock solution [approx. 0.5 M in 0.1 M NaOH (BDH, Poole, U.K.)] in 0.1 M phosphate buffer (pH 7.4). HOCl concentrations were determined from the absorbance of ⁻OCl at 292 nm (pH 12) using a molar absorbance coefficient ϵ of 350 M⁻¹ · cm⁻¹ [3]. All other chemicals were of analytical reagent grade.

Computational kinetic modelling of the reactivity of HOCl with the nucleosome

The amino acid and DNA-base composition of the nucleosome was obtained from the crystal structure for human nucleosome (comprising two DNA strands and duplicates of histones H2A, H2B, H3 and H4 (PDB 1AOI) [43]. The sequence for human histone H1 (P07305) was obtained from the SwissProt

Table 1 Parameters used for modelling the reactivity of HOCl with the nucleosome–histone H1 complex

All other protein side chains and the sugar phosphate backbone of DNA are assumed to undergo much slower, or no, reaction.

Component	No. per nucleosome	$k_2 = \text{M}^{-1} \cdot \text{s}^{-1}$
Protein		
Arg*	102	26
Asn*	27	0.03
Gln*	35	0.03
His*	20	1.6×10^5
Lys*	134	5.0×10^3
Cys*	2	3.0×10^7
Met*	11	3.8×10^7
Tyr*	33	44
Terminal amine*	9	1.0×10^5
Backbone amide*	1020	10†
DNA		
AMP	88	6.4 (exo)
CMP‡	58	66 (exo)
GMP‡	58	2.1×10^4 (hetero) 2.4 (exo)
TMP‡	88	4.3×10^3 (hetero)

* Rate constants reported in [17].

† Rate is the upper limit measured for backbone amides, giving a maximal estimate of direct backbone damage.

‡ Rate constants reported in [27].

database, and the total nucleosome–histone H1 composition was used for modelling. The rate constants for the reactions of HOCl with each amino acid side chain, the protein–backbone amides and DNA bases were taken from previous studies [17,27]. The nucleosome–histone H1 composition and the second-order rate constants were incorporated into a series of reactions that fully model the reactivity with HOCl (Table 1). Specfit software (version 3.0.15, Spectrum Software Associates) was allowed to undergo 100 iterations of the model to yield the predicted reactant and product concentrations after specified time intervals.

Agarose-gel electrophoresis

Plasmid DNA samples were prepared in a final volume of 25 μl , each containing 0.5 μg of plasmid pBR322 DNA (Roche, Castle Hill, NSW, Australia), with the respective chloramine solutions (10 μl , 5 mM chloramine, 25 mM of substrate). Samples were incubated at 37 °C and the reaction quenched, at the time points indicated, by the addition of methionine (10 μl , 0.1 M). Bromophenol Blue loading buffer [1 % in 50 % glycerol/TAE buffer: 40 mM Tris/20 mM sodium acetate/1 mM EDTA (pH 7.2)] was added and the samples were kept at 4 °C until DNA products were resolved by electrophoresis. Agarose-gel electrophoresis (approx. 20 h at 25 V) was performed using a 0.7 % agarose gel in TAE buffer [40 mM Tris/20 mM sodium acetate/1 mM EDTA (pH 7.2)] containing 0.5 $\mu\text{g} \cdot \text{ml}^{-1}$ ethidium bromide. Gels were scanned and analysed using a Gel Doc 1000 system (BioRad, Hercules, CA, U.S.A.) with a UV trans-illuminator.

Cross-linking experiments with radioactive chloramines

DNA (calf thymus, 100 $\mu\text{g} \cdot \text{ml}^{-1}$) was treated, at 37 °C, with ³H-Lys (1.25 μM) or ¹⁴C-His (50 μM) chloramines (generated by the addition of equal volumes of 2.5 and 100 μM HOCl to 3 μM ³H-Lys and 60 μM ¹⁴C-His respectively for 2 min at 21 °C). At the appropriate time, 3 M sodium acetate (0.1 vol.)

and ice-cold ethanol (2 vol.) were added and the samples were incubated overnight at -20°C to precipitate the DNA. The DNA was pelleted by centrifugation at 10000 g for 30 min at 4°C . The supernatant was removed, and the pellet washed with ice-cold ethanol (70% v/v) and allowed to dry in air before resuspending in $100\text{ }\mu\text{l}$ of water overnight at 4°C . This procedure was repeated, and the final $100\text{ }\mu\text{l}$ of the sample was added to 5 ml of Ultima GoldTM scintillant (Packard, Groningen, Netherlands) and counted for 5 min in a Packard tri-carb 2100TR liquid-scintillation counter.

Chloramine determination

Chloramine concentrations were determined by reaction with 5-thio-2-nitrobenzoic acid (TNB) as described previously [10,25]. TNB (typically $35\text{--}40\text{ }\mu\text{M}$) was prepared from the disulphide 5,5'-dithio-2-nitrobenzoic acid (1 mM) by exposure to NaOH (50 mM) for 5 min before dilution into 0.1 M phosphate buffer (pH 7.4). The concentration of TNB consumed after reaction with the various chloramines for 15 min was determined at 412 nm by using a molar absorbance coefficient ϵ of $13600\text{ M}^{-1}\cdot\text{cm}^{-1}$ [25].

EPR spectroscopy

EPR spectra were recorded at room temperature using a Bruker EMX X-band spectrometer with 100 kHz modulation and a cylindrical ER4103TM 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 addition of the spin trap to the reaction mixture unless stated otherwise. Hyperfine couplings were measured directly from the field scan and confirmed by simulation with the program WINSIM [46]. This software is freely available at the NIEHS website (<http://EPR.niehs.nih.gov>). Correlation coefficients between simulated and experimental spectra were > 0.95 . Typical EPR spectrometer settings were: gain, $1 \times 10^3\text{--}1 \times 10^6$; modulation amplitude, 0.01–0.05 mT; time constant, 0.16 s; scan time, 84 s; resolution, 1024 points; centre field, 348 mT; field scan, 8 mT (MNP experiments) or 10 mT (DMPO experiments); power, 25 mW; frequency, 9.76 GHz; with four scans averaged.

RESULTS

Computational kinetic modelling of the reactivity of HOCl with the nucleosome

The reactivity of the nucleosome–histone H1 complex with HOCl was modelled with a series of reactions derived from the amino acid and DNA-base compositions of the complex and the corresponding second-order rate constants for the reactions of the free amino acids/nucleosides (or simple derivatives) with HOCl. The rate constant used for the reaction with the backbone amides (peptide bond) was an upper limit, which allows the maximal amount of direct protein backbone attack to be estimated. The computational model (Table 1) was used to predict the initial product distribution on the nucleosome–histone H1 complex with increasing molar excesses of HOCl. The results of the modelling have been expressed as the proportion of HOCl reacting with each component of the nucleosome–histone H1 complex (Figure 1a) to provide information on which sites the majority of HOCl reacts with at each molar excess. This varies with the molar excess as the reaction with less-reactive residues becomes more prevalent once the more reactive residues are consumed. The partitioning of the HOCl reactivity between the protein (particularly His or Lys residues) and the DNA com-

Figure 1 Predicted reactivity of various molar excesses of HOCl with the nucleosome–histone H1 complex using the model described in Table 1

(a) The proportion of HOCl reactivity at each component. (b) The percentage of each residue remaining. Abbreviations: Term, terminal amino groups of the protein; GMP, TMP, reaction of HOCl with the heterocyclic nitrogen atoms of guanosine and thymidine respectively (with no contribution from the exocyclic amine in the case of GMP).

ponents, as determined by this model, is given in Table 2. The modelling results have also been expressed as a percentage of the initial components remaining (Figure 1b) to show the relative depletion rates for each residue.

These modelling studies predict that the majority of HOCl ($> 99\%$) is consumed by the Cys and Met residues of the histone proteins when low molar excesses of HOCl ($\leq 10:1$) are present (Figure 1a, Table 2). Once the molar excess of HOCl exceeds the total number of Cys and Met residues, any additional HOCl is predicted to react with the His and Lys residues and the terminal amino groups of the proteins, and the heterocyclic nitrogen atoms of guanosine and thymidine bases. The reactivity has been modelled with molar HOCl excesses of up to 200-fold over the nucleosome–histone H1 complex and the proportion of HOCl reacting with the protein is predicted to be higher than that

Table 2 Proportion of HOCl predicted to react with the protein and DNA components of the nucleosome-histone H1 complex at varying molar excesses of HOCl

The percentage of the protein consumption of HOCl that is predicted to occur at the Lys and His residues is shown.

Molar excess of HOCl	Protein (%)	DNA (%)	Protein consumption of HOCl (%)	
			Lys	His
0.1:1	99.7	0.3	0.1	0.4
1:1	99.7	0.3	0.1	0.4
5:1	99.6	0.4	0.2	0.5
10:1	99.5	0.5	0.3	0.8
20:1	88.9	11.1	5.3	14.8
50:1	72.9	27.1	16.4	32.8
100:1	60.7	39.3	32.2	31.5
200:1	53.8	46.2	59.3	18.6

with the DNA bases in all cases. The main protein targets at higher molar excesses of HOCl (> 50:1) are predicted to be the His and Lys residues, which account for between 50 and 80 % of the total HOCl reacting with the protein at these molar excesses.

Formation and decay of chloramines generated on reaction of HOCl with nucleohistone

Initial experiments were performed with nucleohistone ($0.5 \text{ mg} \cdot \text{ml}^{-1}$, approx. $2.5 \mu\text{M}$) treated with HOCl ($250 \mu\text{M}$) and incubated over 6 h at 4, 21 and 37°C . This treatment resulted in the formation of chloramines, which were quantified using the TNB assay in accordance with previous studies [10,25]. The chloramine concentration observed initially on reaction of nucleohistone with a 100-fold molar excess of HOCl ($206 \pm 9 \mu\text{M}$) was in good agreement with the theoretical yield predicted by the kinetic modelling studies ($217 \mu\text{M}$). Similar agreement was observed in experiments with nucleohistone treated with a 50-fold molar excess of HOCl (106 ± 10 and $93 \mu\text{M}$ for the experimental and predicted values respectively).

The nucleohistone chloramines were significantly less stable at 37°C compared with incubation at lower temperatures. Thus 85 % of the chloramines decomposed at 37°C compared with losses of 52 and 19 % at 21 and 4°C respectively after 6 h incubation (results not shown). Similar effects have been observed in previous studies with protein chloramines [10,11]. The effect of the molar excess of HOCl, compared with nucleohistone, on the stability of the resulting chloramines was also investigated. However, no significant difference in the rate of chloramine decomposition was observed on reaction of nucleohistone ($0.5 \text{ mg} \cdot \text{ml}^{-1}$, approx. $2.5 \mu\text{M}$) with a 25-fold ($62.5 \mu\text{M}$), 50-fold ($125 \mu\text{M}$) or 100-fold ($250 \mu\text{M}$) molar excess of HOCl.

Formation of radical species on reaction of HOCl with nucleohistone

The involvement of radical intermediates in nucleosome damage induced by HOCl was investigated using EPR spectroscopy and spin trapping. Reaction of nucleohistone ($5 \text{ mg} \cdot \text{ml}^{-1}$ suspension, approx. $25 \mu\text{M}$) with HOCl ($625 \mu\text{M}$) for 2 min at 21°C followed by the addition of DMPO (125 mM) resulted in the detection of an EPR signal consisting of a triplet of doublets of triplets ($a_N = 1.45 \text{ mT}$, $a_H = 1.80 \text{ mT}$, $a_N = 0.31 \text{ mT}$) (Figure 2). This signal was only observed in the presence of all of the components of the reaction mixture. Reaction of HOCl with

Figure 2 Radicals formed on reaction of nucleohistone with HOCl

EPR spectrum observed on reaction of nucleohistone ($5 \text{ mg} \cdot \text{ml}^{-1}$, approx. $25 \mu\text{M}$) with HOCl ($625 \mu\text{M}$) for 2 min at 21°C followed by the addition of the spin-trap DMPO (125 mM). The signals are assigned to histone protein, nitrogen-centred radicals formed on the side chain of Lys residues.

DMPO alone gave a markedly different EPR signal consistent with the formation of the previously characterized *N*-chloroimine DMPO derivative [47]. The parameters of the nucleohistone adduct are consistent with the formation of nitrogen-centred, protein-derived radicals, generated from the Lys side-chain chloramines as observed with isolated proteins and plasma [10,26].

No change was detected in the nature of the nucleohistone-derived radical adducts on varying the concentration of HOCl ($125 \mu\text{M}$ – 2.5 mM , i.e. 5–100-fold molar excess), although the intensity of the EPR signals was greater in the presence of higher concentrations of HOCl. No evidence was obtained for the formation of DNA-derived radicals, even with the highest molar excess (> 100 fold) of HOCl examined, even though such species are readily detected with isolated DNA under identical conditions [36]. Experiments with the spin-trap MNP (10 mM) gave weak and poorly resolved EPR signals, which are consistent with the formation of a mixture of carbon-centred radical adducts and broad features characteristic of large, polymer-derived radical species (results not shown). Again, no change in the nature of the EPR signals was observed on varying the concentration of HOCl ($125 \mu\text{M}$ – 2.5 mM). The nature of the species which gives rise to the broad features was investigated further by incubation of the nucleohistone-derived radical adducts with either Pronase or DNase 1 (2 and 1250 units respectively for 30 min at 21°C). No significant changes in the EPR signals were observed after this treatment.

The carbon-centred radicals detected in these experiments may be due to rearrangement reactions of the initial protein-derived, nitrogen-centred radicals [26]. However, it has not been possible to determine whether these carbon-centred radicals are present on either the histone protein or the DNA moiety of the nucleohistone. In order to investigate the possibility that these carbon-centred species arise via damage transfer from reaction of the initial protein radicals with DNA, further studies were undertaken with pre-formed protein chloramines and DNA.

Effect of chloramines on the structural integrity of plasmid DNA

Initial studies investigated the reaction of chloramines formed on Lys and His residues with plasmid DNA as these amino acids were predicted by kinetic modelling studies to be favourable sites of attack on the reaction of HOCl with the nucleosome. Incubation of Lys chloramines (25 mM Lys, 5 mM chloramines)

Figure 3 The effect of chloramines on the structural integrity of plasmid DNA

Reaction of plasmid pBR322 DNA (0.5 μ g) with Lys chloramines (25 mM Lys, 5 mM chloramine) over 8 h at 37 $^{\circ}$ C. Lanes 1–7, control samples containing non-HOCl-treated Lys (25 mM) incubated for 0, 0.5, 1, 2, 4, 6 and 8 h respectively at 37 $^{\circ}$ C before the addition of Met (50 mM) to quench the reaction. Lanes 8–14, Lys-chloramine samples incubated for 0, 0.5, 1, 2, 4, 6 and 8 h respectively at 37 $^{\circ}$ C before the addition of Met (50 mM) to quench the reaction.

and pBR322 DNA (0.5 μ g) at 37 $^{\circ}$ C for the indicated times resulted in a progressive loss of the native (type 1) form of the plasmid and an increase in the concentration of the relaxed (type 2) form (Figure 3). This change in structure is consistent with the generation of single-strand breaks in the plasmid. These changes were neither observed in plasmid samples incubated under identical conditions in the absence of HOCl, nor on addition of Met (50 mM), a potent chloramine scavenger, to the Lys chloramines before incubation with the plasmid at 37 $^{\circ}$ C. This suggests that the damage observed under these conditions is mediated by chloramines. An additional DNA band near the top of the gel becomes more apparent in the plasmid samples incubated with Lys chloramines at long incubation times (Figure 3). The density of this band becomes greater on increasing the incubation time ($6.6 \pm 1.5\%$ initially, $12.3 \pm 0.7\%$ after 6 h), whereas no significant difference in the band density is observed in the non-chloramine-treated control samples ($5.9 \pm 1.2\%$ initially, $5.6 \pm 1.4\%$ after 6 h). This band may be due to the formation of aggregated or cross-linked material.

Similar effects were observed in the corresponding experiments with His, *N*- α -acetyl-histidine (NAH) and the peptides Gly-Lys-Gly and Gly-His-Gly. The rate of loss of type 1 DNA observed with NAH and the peptides was slower than that detected with Lys and His chloramines. This may be associated with an increase in the stability of the chloramines in the former case. Thus, in general, a greater extent of DNA-strand cleavage is observed on incubation of the plasmid DNA with the less-stable chloramines.

DNA-plasmid strand cleavage was also detected in experiments with protein chloramines formed on ubiquitin and BSA. In this case, significant loss of the native type 1 plasmid was only observed on incubation of the protein chloramine with the DNA for greater than 8 h at 37 $^{\circ}$ C despite the short-lived nature of the protein chloramines. An extra band, consistent with the formation of the high-molecular-mass material, was also observed with the protein chloramine samples. No changes were observed on incubation with native ubiquitin and BSA (results not shown). This suggests that protein chloramines can mediate the formation of DNA cross-links in addition to strand breaks. Experiments with histone-protein chloramines were unsuccessful due to the strong, non-covalent, association of native histone

(a)

(b)

Figure 4 Effect of antioxidants on the Lys chloramines-induced DNA-strand cleavage

(a) The percentage of type 1, supercoiled plasmid DNA remaining after reacting the plasmid pBR322 DNA (0.5 μ g) with Lys chloramines (10 mM Lys, 2 mM chloramine) for 3 and 6 h at 37 $^{\circ}$ C in the presence of buffer [0.1 M Chelex-treated phosphate buffer (pH 7.4); black bars], Met (10 mM; diagonal line filled bars), GSH (10 mM; horizontal line filled bars), DMPO (10 mM; hatched bars) and Trolox (10 mM; white bars). (b) The stability of Lys chloramines (10 mM Lys, 2 mM chloramine) over a period time at 37 $^{\circ}$ C in the presence of buffer [●; 0.1 M Chelex-treated phosphate buffer (pH 7.4)], methionine (▲; 10 mM), DMPO (■; 10 mM) and Trolox (◆; 0 mM). Data are means $\pm$ S.D. for three separate experiments. *, statistical analysis performed using a one-way ANOVA with Dunnett's post-hoc testing which compares each condition with the control sample at each time point (buffer only black bars; $P < 0.001$).

proteins with DNA, which prevents the resolution of the plasmid products by agarose electrophoresis [48].

The effect of putative antioxidants on the reaction of Lys chloramines with plasmid DNA was investigated. Thus pBR322 plasmid (0.5 μ g) was treated with Lys chloramines (10 mM Lys, 2 mM chloramine) for 3 and 6 h at 37 $^{\circ}$ C in the presence of Met, GSH, Trolox (a water-soluble α -tocopherol derivative) and DMPO (all 10 mM) and the percentage of type 1 plasmid remaining was determined (Figure 4a). Both Met and GSH afforded complete protection against the damage mediated by Lys chloramines after 3 and 6 h incubations respectively. A significant

Figure 5 Cross-linking of chloramines to DNA

An increase in radioactive counts associated with DNA on reaction of (a) ^3H -Lys chloramines (1.5 μM Lys, 1.25 μM chloramine) and (b) ^{14}C -His chloramines (30 μM His, 50 μM chloramine) with DNA (calf thymus, 10 μg) for indicated times at 37 $^\circ\text{C}$ before the reaction was quenched by addition of methionine (5 mM). The black bars represent samples containing chloramines and the white bars represent the non-HOCl-treated control samples. Data are means $\pm$ S.D. for three separate experiments.

level of plasmid protection ($P < 0.001$) was also observed in the samples containing Trolox and DMPO. Trolox and DMPO react readily with radicals [11] and do not quench Lys chloramines under the conditions employed in these experiments (Figure 4b). This suggests that radicals mediate a significant amount of the plasmid DNA-strand cleavage, induced by Lys-derived chloramines. It was not possible to investigate the role of radicals in the His-chloramine-mediated plasmid strand cleavage due to direct (also rapid) reaction of His chloramines with Trolox and DMPO. The observation of an additional band, characteristic of high-molecular-mass DNA, on treatment of plasmid DNA with the chloramines, suggests that these species can mediate the formation of DNA cross-links in addition to strand cleavage. The generation of chloramine-induced DNA cross-links was investigated further in experiments with ^3H -Lys and ^{14}C -His chloramines.

Figure 6 Ability of nucleosides, nucleotides and RNA to scavenge BSA-derived, nitrogen-centred radicals

The intensity of the EPR signals (measured by double integration of the EPR signals) observed on incubation of BSA chloramines (250 μM BSA treated with 2.5 mM HOCl for 5 min at 21 $^\circ\text{C}$) with thymidine, uridine, cytidine, ATP, GTP (all 50 mM) and RNA (12.5 mg $\cdot$ ml $^{-1}$, approx. 50 mM) in the presence of DMPO (10 mM) for 5 min at 37 $^\circ\text{C}$. Data are means $\pm$ S.D. for three separate experiments. *, statistical analysis performed using a one-way ANOVA with Dunnett's post-hoc testing, which compares each condition with the control sample (buffer only; $P < 0.001$).

Generation of protein–DNA cross-links

DNA (calf thymus, 10 μg) was treated with chloramines (1.25 μM) formed on ^3H -Lys (1.5 μM , approx. 1.3×10^7 d.p.m.) at 37 $^\circ\text{C}$. The DNA was separated from any unreacted ^3H -Lys by precipitation, and the radioactive counts were measured. An increase in counts was observed with the increase in the incubation time of the DNA with the ^3H -Lys chloramines (Figure 5a), suggesting that the ^3H -Lys is bound to the DNA. This increase in radioactive counts was not detected in experiments with non-HOCl treated ^3H -Lys or ^3H -Lys chloramines quenched with Met before addition to DNA. Similar results were obtained in experiments with DNA (calf thymus, 10 μg) and ^{14}C -His chloramines (30 μM His/50 μM chloramine, approx. 5×10^5 d.p.m.) (Figure 5b). The increase in the radioactive counts associated with the purified DNA was dependent on the presence of ^{14}C -His chloramines, with low levels of radioactive counts observed in experiments with ^{14}C -His only, and Met-quenched ^{14}C -His chloramines. Thus both Lys and His cross-link formation is chloramine-dependent. In each case, the overall incorporation of the radiolabelled material is low (0.05% after 480 min and 0.2% after 240 min for ^3H -Lys and ^{14}C -His respectively) with the concentrations employed, although this may underestimate the true extent of this process as no effort was made to maximize this reaction. The role of radical intermediates in protein chloramine-mediated DNA damage and cross-linking was examined by EPR spectroscopy and spin-trapping experiments.

Reaction of protein nitrogen-centred radicals with nucleosides and RNA

The relative reactivity of BSA-derived, nitrogen-centred radicals with the individual nucleosides and RNA was compared in experiments using DMPO as a spin trap. BSA was employed as a model as the nitrogen-centred radicals, formed on decomposition

Figure 7 Reaction of Lys chloramines with the pyrimidine bases

EPR spectra observed on reaction of Lys chloramines (10 mM Lys, 4 mM chloramine) with (a) thymidine, (b) uridine and (c) cytidine (all 40 mM) in the presence of MNP (10 mM) for 5 min at 37 °C. Signals are assigned to the formation of C5-yl radicals (●) and C6-yl radicals (not marked) formed by the addition of Lys-derived radicals to the C5–C6 pyrimidine double bond. Signals marked * are assigned to DTBN.

of the chloramine formed on this protein, have been characterized previously [10]. The reaction of BSA chloramines (generated on reaction of 250 μ M BSA with 2.5 mM HOCl for 5 min at 21 °C) with 50 mM thymidine and subsequent incubation at 37 °C for 5 min in the presence of 10 mM DMPO resulted in a significant loss ($P < 0.001$) in the intensity of the protein nitrogen-centred radical (relative to samples without added thymidine) as assessed by double integration of the EPR signals (Figure 6). A similar loss in the protein nitrogen-centred radical was observed in experiments with uridine, ATP and GTP (Figure 6). ATP and GTP were employed in the present study due to the low solubility of adenosine and guanosine. A small loss in the intensity of the protein nitrogen-centred radical was detected with cytidine (50 mM) and RNA (transfer, 12.5 mg $\cdot$ ml $^{-1}$, approx. 50 mM in terms of nucleotides) (Figure 6). Similar experiments were not performed with DNA. These results are consistent with the occurrence of competitive reactions of the initial protein nitrogen-centred radicals with nucleosides and DMPO. The reactivity of protein radicals with the pyrimidine nucleosides was investigated further in experiments with MNP.

Formation of radical species on the reaction of protein radicals with pyrimidine nucleosides

Initial studies investigated the reaction of chloramines formed on Lys and His residues with the pyrimidine nucleosides; these amino acids were predicted, by kinetic modelling studies, to

Table 3 Parameters of the radicals observed on reaction of Lys, His, peptide and protein chloramines with the pyrimidine nucleosides using MNP as a spin trap

Chloramine	Nucleoside	Hyperfine coupling constants (± 0.02) (mT)			Assignment
		a_N	a_H	a_{other}	
Lysine	None	1.71			DTBN
	Thymidine	1.59			C5-yl
		1.44	0.29	0.06 (N)	C6-yl
	Uridine	1.53	0.35		C5-yl
		1.48	0.15	0.24 (N)	C6-yl
	Cytidine	1.48	0.43		C5-yl
NAH		1.49	0.18	0.23 (N)	C6-yl
	None	1.41	0.51	0.24 (N)	Ring
				0.11 (H)	
	Thymidine	1.59			C5-yl
		1.45	0.29		C6-yl
	Uridine	1.52	0.35		C5-yl
Gly-Lys-Gly Gly-His-Gly		1.50	0.15	0.21 (N)	C6-yl
	Cytidine	1.71			DTBN
	None	1.48	0.29	0.29 (N)	Backbone
		1.04			Acyl
	Thymidine	1.58			C5-yl
		1.42	0.29		C6-yl
	Uridine	1.50	0.33		C5-yl
		1.47	0.14	0.23 (N)	C6-yl
	Cytidine	1.50	0.35		C5-yl
		1.46	0.15	0.24 (N)	C6-yl

be favourable sites of attack on reaction of HOCl with the nucleosome. It has been shown previously that thermal decomposition of Lys chloramines results in the formation of side chain-derived, nitrogen-centred, radicals [10,26]. The reaction of Lys chloramines (10 mM Lys, 4 mM chloramine) with thymidine (40 mM) in the presence of MNP (10 mM) for 5 min at 37 °C gave intense EPR signals consistent with the formation of two nucleoside-derived, carbon-centred, radical adducts (Figure 7a, Table 3). Different EPR signals, again attributed to the generation of two carbon-centred radicals, were detected with uridine and cytidine (Figures 8b and 8c respectively, Table 3). In all cases, signals were only observed in the presence of all of the components of the reaction mixture. Omission of either the pyrimidine base or the Lys chloramine gave a different triplet signal ($a_N = 1.71$ mT) attributed to the formation of di-*t*-butylnitroxide (DTBN) radical, a well-characterized spin-trap decomposition product [49].

These carbon-centred radicals are assigned, in each case, to adducts generated via addition of an attacking radical at either C6 (to form a C5-yl radical, major species), or C5 (to form a C6-yl radical, minor species) of the C5–C6 double bond of the pyrimidine ring to form the cross-linked material; this is in accordance with the previous studies [35,36,50,51]. The attacking radicals responsible for the generation of C5-yl and C6-yl pyrimidine-base radicals are believed to be the Lys side-chain-derived, nitrogen-centred radicals (Scheme 1).

Similar behaviour was observed in experiments with HOCl-treated NAH and thymidine or uridine. Experiments with cytidine gave only low-intensity EPR signals consistent with the formation of DTBN radical. Incubation of NAH chloramines with MNP in the absence of the pyrimidine base gave weak and complex EPR signals ($a_N = 1.41$ mT, $a_H = 0.51$ mT, $a_N = 0.24$ mT, $a_H = 0.11$ mT), tentatively assigned to a carbon-centred,

Figure 8 Reaction of protein chloramines with thymidine

EPR signals observed on reaction of (a) BSA chloramines (0.4 mM BSA treated with 4 mM HOCl for 5 min at 21 °C) with thymidine (20 mM) in the presence of MNP (10 mM) for 5 min at 37 °C; (b) same as (a) but with the addition of Pronase (2 units) and further incubation for 30 min at 21 °C; (c) same as (b) but in the absence of thymidine; (d) same as (a) but with histone H1 chloramines (0.4 mM histone H1 treated with 4 mM HOCl for 5 min at 21 °C). Signals in (a), (b) and (d) are assigned to thymidine C5-yl (●) and C6-yl (○) radicals formed on the addition of protein-attacking radicals to the C5–C6 pyrimidine double bond. Signals in (c) are attributed to the formation of BSA-derived, carbon-centred radical adducts.

NAH ring-derived radical. The parameters of this species are distinct from the hyperfine coupling constant of the pyrimidine nucleoside-derived radical adducts. The difference in the reactivity of NAH with thymidine and uridine compared with cytidine may be associated with the ability of the heterocyclic NAH chloramine to undergo chlorine-transfer reactions (C. L. Hawkins and M. J. Davies, unpublished work).

Similar experiments with Gly-Lys-Gly and Gly-His-Gly chloramines also resulted in the detection of carbon-centred, C5-yl and C6-yl radical adducts with each pyrimidine nucleoside (Table 3). In each case, additional low-intensity signals were observed which were also detected in experiments performed in the absence of the pyrimidine base; these have been assigned to peptide-derived, carbon-centred radicals (radical 1: $a_N = 1.48$ mT, $a_H = 0.29$ mT, $a_N = 0.29$ mT; radical 2: $a_N = 1.04$ mT). The parameters of these peptide radical adducts are consistent with the formation of a backbone radical with partial structure $\cdot\text{C}(\text{N})\text{HR}$ and an acyl radical respectively. No nucleoside-derived radical adducts were observed on reaction of Gly-Gly-Gly chloramines with the pyrimidine bases in the presence of MNP (results not shown). This suggests that the attacking radicals are formed on either the Lys- or the His-side chain and not the free N-terminal amino group.

Reaction of BSA chloramines (generated on reaction of 0.4 mM BSA treated with 4 mM HOCl for 5 min at 21 °C) with thymidine (20 mM) in the presence of MNP (10 mM) for 5 min at 37 °C gave intense and broad EPR signals. These signals are characteristic of large, slow-tumbling, protein-derived radicals (Figure 8a). Information about the nature of these radicals was obtained in experiments where the complete reaction mixture was incubated with the proteolytic enzyme Pronase (2 units, 30 min, 21 °C) to release small fragments from the protein-derived radical adducts; these give sharper, more isotropic, spectra as a result of their increased rate of tumbling [10]. This treatment resulted in the detection of sharper EPR signals consistent with the formation of the C5-yl and C6-yl species of thymidine (Figure 8b).

Similar results were obtained in analogous experiments with uridine and cytidine (results not shown). Incubation of BSA chloramines with MNP, in the absence of the pyrimidine bases, also gave a broad and an isotropic EPR signal. However, different carbon-centred radical adducts were detected on the addition of Pronase to the reaction mixture (Figure 8c). Thus the formation of the C5-yl and C6-yl radical adducts was dependent on the presence of the thymidine and the BSA chloramines.

Separation of reaction mixtures containing the BSA–thymidine radicals with a 10000 molecular-mass cut-off filter resulted in EPR signals being observed only in the high-molecular-mass fraction retained by the filter. No evidence was obtained for the generation of low-molecular-mass radical adducts. Further support for the assignment of the observed signals to covalently linked protein–base species was obtained in experiments where BSA chloramines were reacted with thymidine using MNP as a spin trap, in the presence of a high concentration of NaCl (1 M) to disrupt any non-covalent association of the thymidine with the protein; this treatment gave identical adducts. Similarly, addition of BSA (0.4 mM) to thymidine-derived radicals formed by the reaction of $\text{HO}\cdot$ [generated by H_2O_2 (2 mM) and $\text{Fe}(\text{II})$ (0.5 mM)] with thymidine (20 mM) using MNP (10 mM) had no effect on the nature of the nucleoside radicals confirming that the broad nature of the observed adducts is not due to non-specific binding of thymidine to DNA.

Reaction of histone H1 chloramines (0.4 mM treated with 4 mM HOCl for 2 min at 21 °C) with thymidine (20 mM) gave EPR signals with MNP (10 mM) consistent with the formation of the nucleoside C5-yl and C6-yl radicals (Figure 8d). Similar results were obtained in experiments with uridine and cytidine (results not shown). These signals are broader than the spectral features observed with the peptides and are attributed to the formation of protein–nucleoside adducts. These histone H1–nucleoside radical adducts are more mobile than those observed with BSA, possibly due to the smaller and more extended structure of the histone H1 protein.

Studies performed with the chloramines and the purine nucleosides using MNP as a spin trap were unsuccessful, as only DTBN radical was detected in each case. This may be due to the instability of the resulting spin adducts, and/or a slow rate of purine-derived radical trapping with MNP due to spin delocalization.

DISCUSSION

Excessive or misplaced generation of oxidants, such as HOCl, by activated phagocytes is believed to be important in the initiation and accumulation of the oxidative damage observed in the progression of a number of diseases including cancers arising from chronic inflammation [7]. Both HOCl and chloramines have been shown to be mutagenic in a number of cells, suggesting that these oxidants (or species derived from them) can both enter cells and interact with nuclear material [7,52,53]. However, the mechanisms and intermediates involved in these processes are poorly understood. Proteins are likely to be major targets for reaction with HOCl within a cell due to their abundance and high rate constants for reaction [17]. The present study was designed to investigate the role of protein chloramines in mediating DNA damage.

The kinetic modelling performed in the present study predicts that the majority of HOCl reacting with the nucleosome is consumed by the histone proteins when HOCl excesses of up to 200-fold are employed. The main targets on the protein are predicted to be Lys and His residues, which account for between 50 and 80 % of the HOCl added. This is in accord with previous experimental

Scheme 1 Proposed mechanism for the formation of C5-yl and C6-yl radicals on reaction of the pyrimidine bases (e.g. thymidine) with Lys-chloramine-derived radicals

observations [54]. The initial products of the reaction at these residues are chloramines. Although there are a number of factors, such as the assumption that all sites are equally accessible and that steric and electronic effects have no effect on HOCl selectivity, which cannot be incorporated into this kinetic model (see also [17]), there is very good agreement between the yield of nucleohistone chloramines predicted by the model and that observed experimentally ($217\ \mu\text{M}$ predicted and $206 \pm 9\ \mu\text{M}$ detected with a 100-fold molar excess of HOCl).

The reaction of chloramines formed on Lys and His residues (both free and protein-bound) with DNA has been shown to give rise to DNA-strand breaks and protein–DNA cross-links. In each case, this damage could be prevented by the removal of chloramines with excess Met before reaction with DNA. The rate and extent of DNA damage observed has been shown to depend on the nature of the chloramines, with strand cleavage occurring rapidly with free Lys and His chloramines, but at slower rates with NAH, peptide and protein chloramines. These differences correlate, for the free and peptide chloramines, with their stability and rate of radical formation. Thus Lys and His chloramines are less stable than the chloramines formed on NAH, Gly-Lys-Gly or Gly-His-Gly. However, evidence has also been obtained for a role of other factors, particularly steric bulk, in the rate and extent of plasmid damage, as the chloramines formed on ubiquitin and BSA give a smaller extent of damage

than that predicted solely on the basis of the rate of chloramine decomposition, which is relatively rapid. This may be associated with the size and the three-dimensional structure of these proteins, which prevent close association of the chloramine with the plasmid.

Lys, His and protein chloramines are known to decompose by two independent pathways. In the case of His chloramines, it has been shown that DNA damage can occur via the transfer of chlorine from the His side-chain chloramines to the nucleobase exocyclic NH_2 groups of the plasmid DNA. This reaction results in the generation of DNA chloramines. These species are known to both disrupt the structure of DNA [27], and to decay to give the DNA-derived radicals [36]. The latter can induce strand breaks [36]. Both Lys and His chloramines can also undergo thermal decomposition to give nitrogen-centred radicals [10,26]. Subsequent rearrangement or fragmentation of these species results in the generation of carbon-centred radicals (and presumably peroxy radicals in the presence of O_2). A role for such radicals in chloramine-mediated DNA-strand cleavage is supported, in the case of Lys chloramines, by the protection afforded by the radical scavengers Trolox and DMPO that do not directly remove chloramines on the time scales used in these experiments. These radicals may then abstract a hydrogen atom from the sugar–phosphate backbone to give strand cleavage (see data for other radicals, including peroxy species [55]). Thus

some of the observed DNA damage can be attributed to reactions of the Lys or His radicals, produced on thermal decomposition of the chloramines, with the sugar-phosphate backbone. The site selectivity (i.e. whether it occurs most readily at particular sites) of such damage has not been investigated. EPR experiments have provided evidence for the rapid addition of these radicals to pyrimidine nucleosides to give protein-base dimers. The carbon-centred radicals observed with pre-formed chloramines are assigned to species produced via the addition of a radical arising from the decomposition of the chloramines, to the C5–C6 double bond of the pyrimidine nucleoside, to generate the C6-yl and C5-yl radical adducts respectively (Scheme 1) [35]. The protein–DNA cross-links could not be disrupted by high concentrations of NaCl, confirming their covalent nature. Related dimers have been characterized on reaction of pyrimidine nitrogen-centred radicals with the C5–C6 double bond of another pyrimidine molecule [35,56]. The exact nature of the radicals, which react with the nucleosides, remains to be established. These may be the initial nitrogen-centred species observed with Lys and His, or the carbon-centred radicals (see the species detected with Gly-Lys-Gly, Gly-His-Gly and proteins) that result from rearrangement or fragmentation of these species [26].

Amino acid–DNA cross-links, as evidenced by an increase in the radioactive counts on isolated DNA, were also observed on incubation of ^3H -Lys and ^{14}C -His chloramines with DNA at 37 °C. It is likely that similar cross-links are also formed between protein chloramines and DNA although this has not been determined in the current studies. These results support the suggestion made previously that HOCl-mediated protein–DNA cross-link formation involves long-lived intermediates such as chloramines or radicals [45]. The cross-linking observed in an earlier study [45] was protein-dependent, and required the presence of single-stranded DNA-binding protein, which is capable of achieving a specific orientation with DNA before cross-link formation. Thus unlike the results obtained in the present study, cross-link formation was not observed in experiments with non-DNA-binding proteins such as BSA [45].

It is known that treatment of cells with HOCl results in rapid depletion of intracellular GSH and protein–thiol groups, and that this precedes the detection of significant levels of chloramines [57,58]. We have shown previously [59] that the reaction of HOCl with a number of different cell types gives radical adducts, which are assigned to the protein-derived, nitrogen-centred radicals. These species were observed with concentrations of HOCl that might be expected at sites of acute inflammation (e.g. the detection of 170 μM HOCl formed by 2×10^6 activated neutrophils/ml over a period of 1 h [2]). This suggests that the reaction of HOCl with proteins to give chloramines is a major process within cells after the depletion of cellular GSH. The formation of intracellular chloramines may be of particular significance as these species are sufficiently long-lived to be able to diffuse away from the site of generation and subsequently give rise to damage at remote sites, e.g. within the nucleus, via the induction of DNA fragmentation and protein–DNA cross-links.

In summary, we have used kinetic modelling to predict that most of the reaction of HOCl with the nucleosome will occur with the histone proteins rather than DNA. It is shown that Lys and His residues are the likely targets for reaction with HOCl, after depletion of reactive Cys and Met residues, and that this process gives rise to chloramines. These species can subsequently decompose to give strand breaks. This process is believed to involve the generation of reactive side-chain-derived radicals. These species also react with the C5–C6 double bond of pyrimidine bases to yield carbon-centred, nucleoside-derived radicals and specific covalent protein–nucleoside cross-links. Similar

cross-links have been detected on reaction of Lys and His chloramines with DNA. Thus in a cellular system, HOCl-induced damage to DNA may be mediated by reactions of pre-formed protein chloramines rather than, or in addition to, direct reaction with HOCl.

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REFERENCES

- Weiss, S. J. and LoBuglio, A. F. (1982) Phagocyte-generated oxygen metabolites and cellular injury. *Lab. Invest.* **47**, 5–18
- Kettle, A. J. and Winterbourn, C. C. (1997) Myeloperoxidase: a key regulator of neutrophil oxidant production. *Redox Rep.* **3**, 3–15
- Morris, J. C. (1966) The acid ionization constant of HOCl from 5 °C to 35 °C. *J. Phys. Chem.* **70**, 3798–3805
- Thomas, E. L. (1979) Myeloperoxidase, hydrogen peroxide, chloride antimicrobial system: nitrogen-chlorine derivatives of bacterial components in bactericidal action against *Escherichia coli*. *Infect. Immun.* **23**, 522–531
- Winterbourn, C. C. and Kettle, A. J. (2000) Biomarkers of myeloperoxidase-derived hypochlorous acid. *Free Radic. Biol. Med.* **29**, 403–409
- Jesaitis, A. J. and Dratz, E. A. (1992) *The Molecular Basis of Oxidative Damage by Leukocytes*, CRC Press, Boca Raton, FL
- Weitzman, S. A. and Gordon, L. I. (1990) Inflammation and cancer: role of phagocyte-generated oxidants in carcinogenesis. *Blood* **76**, 655–663
- Whiteman, M., Jenner, A. and Halliwell, B. (1997) Hypochlorous acid-induced base modifications in isolated calf thymus DNA. *Chem. Res. Toxicol.* **10**, 1240–1246
- Arnhold, J., Hammerschmidt, S., Wagner, M., Mueller, S., Arnold, K. and Grimm, E. (1990) On the action of hypochlorite on human serum albumin. *Biomed. Biochim. Acta* **49**, 991–997
- Hawkins, C. L. and Davies, M. J. (1998) Hypochlorite-induced damage to proteins: formation of nitrogen-centred radicals from lysine residues and their role in protein fragmentation. *Biochem. J.* **332**, 617–625
- Hawkins, C. L. and Davies, M. J. (1999) Hypochlorite-induced oxidation of proteins in plasma: formation of chloramines and nitrogen-centred radicals and their role in protein fragmentation. *Biochem. J.* **340**, 539–548
- Carr, A. C., Vissers, M. C., Domigan, N. M. and Winterbourn, C. C. (1997) Modification of red cell membrane lipids by hypochlorous acid and haemolysis by preformed lipid chlorohydrins. *Redox Rep.* **3**, 263–271
- Arnhold, J., Panasenkov, O. M., Schiller, J., Vladimirov Yu, A. and Arnold, K. (1995) The action of hypochlorous acid on phosphatidylcholine liposomes in dependence on the content of double bonds. Stoichiometry and NMR analysis. *Chem. Phys. Lipids* **78**, 55–64
- Carr, A. C., van den Berg, J. J. M. and Winterbourn, C. C. (1996) Chlorination of cholesterol in cell membranes by hypochlorous acid. *Arch. Biochem. Biophys.* **332**, 63–69
- Panasenko, O. M. and Arnhold, J. (1996) Mechanism of hypochlorite-induced peroxidation in phospholipid liposomes. *Membr. Cell Biol.* **10**, 93–104
- Winterbourn, C. C. (1985) Comparative reactivities of various biological compounds with myeloperoxidase-hydrogen peroxide-chloride, and similarity of the oxidant to hypochlorite. *Biochim. Biophys. Acta* **840**, 204–210
- Pattison, D. and Davies, M. J. (2001) Absolute rate constants for the reaction of hypochlorous acid with protein side-chains and peptide bonds. *Chem. Res. Toxicol.* **14**, 1453–1464
- Clark, R. A., Szot, S., Williams, M. A. and Kagan, H. M. (1986) Oxidation of lysine side-chains of elastin by the myeloperoxidase system and by stimulated human neutrophils. *Biochem. Biophys. Res. Commun.* **135**, 451–457
- Beck-Speier, I., Leuschel, L., Luippold, G. and Maier, K. L. (1988) Proteins released from stimulated neutrophils contain very high levels of oxidised methionine. *FEBS Lett.* **227**, 1–4
- Heinecke, J. W. (1999) Mass spectrometric quantification of amino acid oxidation products in proteins: insights into pathways that promote LDL oxidation in the human artery wall. *FASEB J.* **13**, 1113–1120
- Domigan, N. M., Charlton, T. S., Duncan, M. W., Winterbourn, C. C. and Kettle, A. J. (1995) Chlorination of tyrosyl residues in peptides by myeloperoxidase and human neutrophils. *J. Biol. Chem.* **270**, 16542–16548
- Vissers, M. C. and Winterbourn, C. C. (1991) Oxidative damage to fibronectin. I. The effects of the neutrophil myeloperoxidase system and HOCl. *Arch. Biochem. Biophys.* **285**, 53–59
- Hazell, L. J., van den Berg, J. J. M. and Stocker, R. (1994) Oxidation of low-density lipoprotein by hypochlorite causes aggregation that is mediated by modification of lysine residues rather than lipid oxidation. *Biochem. J.* **302**, 297–304

- 24 Weiss, S. J., Lampert, M. B. and Test, S. T. (1983) Long-lived oxidants generated by human neutrophils: characterization and bioactivity. *Science* **222**, 625–628
- 25 Thomas, E. L., Grisham, M. B. and Jefferson, M. M. (1986) Preparation and characterization of chloramines. *Methods Enzymol.* **132**, 569–585
- 26 Hawkins, C. L. and Davies, M. J. (1998) Reaction of HOCl with amino acids and peptides: EPR evidence for rapid rearrangement and fragmentation reactions of nitrogen-centered radicals. *J. Chem. Soc. Perkin Trans.* **2**, 1937–1945
- 27 Prutz, W. A. (1996) Hypochlorous acid interactions with thiols, nucleotides, DNA, and other biological substrates. *Arch. Biochem. Biophys.* **332**, 110–120
- 28 Prutz, W. A. (1998) Interactions of hypochlorous acid with pyrimidine nucleotides, and secondary reactions of chlorinated pyrimidines with GSH, NADH, and other substrates. *Arch. Biochem. Biophys.* **349**, 183–191
- 29 Prutz, W. A. (1999) Consecutive halogen transfer between various functional groups induced by reaction of hypohalous acids: NADH oxidation by halogenated amide groups. *Arch. Biochem. Biophys.* **371**, 107–114
- 30 Prutz, W. A., Kissner, R., Koppenol, W. H. and Ruegger, H. (2000) On the irreversible destruction of reduced nicotinamide nucleotides by hypohalous acids. *Arch. Biochem. Biophys.* **380**, 181–191
- 31 Hayatsu, H., Pan, S.-K. and Ukita, T. (1971) Reaction of sodium hypochlorite with nucleic acids and their constituents. *Chem. Pharm. Bull.* **19**, 2189–2192
- 32 Patton, W., Bacon, V., Duffield, A. M., Halpern, B., Hoyano, Y., Pereira, W. and Lederberg, J. (1972) Chlorination studies. I. The reaction of aqueous hypochlorous acid with cytosine. *Biochem. Biophys. Res. Commun.* **48**, 880–884
- 33 Gould, J. P., Richards, J. T. and Miles, M. G. (1984) The kinetics and primary products of uracil chlorination. *Water Res.* **18**, 205–212
- 34 Gould, J. P., Richards, J. T. and Miles, M. G. (1984) The formation of stable organic chloramines during the aqueous chlorination of cytosine and 5-methylcytosine. *Water Res.* **18**, 991–999
- 35 Hawkins, C. L. and Davies, M. J. (2001) Hypochlorite-induced damage to nucleosides: formation of chloramines and nitrogen-centered radicals. *Chem. Res. Toxicol.* **14**, 1071–1081
- 36 Hawkins, C. L. and Davies, M. J. (2002) Hypochlorite-induced damage to DNA, RNA and polynucleotides: formation of chloramines and nitrogen-centered radicals. *Chem. Res. Toxicol.* **15**, 83–92
- 37 Bernofsky, C. (1991) Nucleotide chloramines and neutrophil-mediated cytotoxicity. *FASEB J.* **5**, 295–300
- 38 Whiteman, M., Jenner, A. and Halliwell, B. (1999) 8-chloroadenine: a novel product formed from hypochlorous acid-induced damage to calf thymus DNA. *Biomarkers* **4**, 303–310
- 39 Henderson, J. P., Byun, J. and Heinecke, J. W. (1999) Chlorination of nucleobases, RNA and DNA by myeloperoxidase: a pathway for cytotoxicity and mutagenesis by activated phagocytes. *Redox Rep.* **4**, 319–320
- 40 Hoyano, Y., Bacon, V., Summons, R. E., Pereira, W. E., Halpern, B. and Duffield, A. M. (1973) Chlorination studies. IV. The reaction of aqueous hypochlorous acid with pyrimidine and purine bases. *Biochem. Biophys. Res. Commun.* **53**, 1195–1199
- 41 Masuda, M., Suzuki, T., Friesen, M. D., Ravanat, J. L., Cadet, J., Pignatelli, B., Nishino, H. and Ohshima, H. (2001) Chlorination of guanosine and other nucleosides by hypochlorous acid and myeloperoxidase of activated human neutrophils. Catalysis by nicotine and trimethylamine. *J. Biol. Chem.* **276**, 40486–40496
- 42 Henderson, J. P., Byun, J., Williams, M. V., McCormick, M. L., Parks, W. C., Ridnour, L. A. and Heinecke, J. W. (2001) Bromination of deoxycytidine by eosinophil peroxidase: a mechanism for mutagenesis by oxidative damage of nucleotide precursors. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 1631–1636
- 43 Wolffe, A. (1998) *Chromatin: Structure and Function*, Academic Press, San Diego
- 44 Halliwell, B. and Gutteridge, J. M. C. (1999) *Free Radicals in Biology and Medicine*, Oxford University Press, Oxford
- 45 Kulcharyk, P. A. and Heinecke, J. W. (2001) Hypochlorous acid produced by the myeloperoxidase system of human phagocytes induces covalent cross-links between DNA and protein. *Biochemistry* **40**, 3648–3656
- 46 Duling, D. R. (1994) Simulation of multiple isotropic spin trap EPR spectra. *J. Magn. Reson.* **104B**, 105–110
- 47 Bernofsky, C., Bandara, B. M. and Hinojosa, O. (1990) Electron spin resonance studies of the reaction of hypochlorite with 5,5-dimethyl-1-pyrroline-n-oxide. *Free Radic. Biol. Med.* **8**, 231–239
- 48 Luxford, C., Dean, R. T. and Davies, M. J. (2000) Radicals derived from histone hydroperoxides damage nucleobases in RNA and DNA. *Chem. Res. Toxicol.* **13**, 665–672
- 49 Buettner, G. R. (1987) Spin trapping: ESR parameters of spin adducts. *Free Radic. Biol. Med.* **3**, 259–303
- 50 Catterall, H., Davies, M. J., Gilbert, B. C. and Polack, N. P. (1993) EPR spin-trapping studies of the reaction of the hydroxyl radical with pyrimidine nucleobases, nucleosides and nucleotides, polynucleotides and RNA. Direct evidence for sites of initial attack and strand breakage. *J. Chem. Soc. Perkin Trans.* **2**, 2039–2047
- 51 Ho, W. F., Gilbert, B. C. and Davies, M. J. (1997) EPR spin trapping studies of the reaction of the hydroxyl and other electrophilic radicals with uridine and related compounds. Isotopic substitution to refine analyses and aid quantification. *J. Chem. Soc. Perkin Trans.* **2**, 2533–2538
- 52 Thomas, E. L., Grisham, M. B. and Jefferson, M. M. (1986) Cytotoxicity of chloramines. *Methods Enzymol.* **132**, 585–593
- 53 Suzuki, H., Seto, K., Mori, M., Suzuki, M., Miura, S. and Ishii, H. (1998) Monochloramine induced DNA fragmentation in gastric cell line MKN45. *Am. J. Physiol.* **275**, G712–G716
- 54 Nightingale, Z. D., Lancha Jr, A. H., Handelman, S. K., Dolnikowski, G. G., Busse, S. C., Dratz, E. A., Blumberg, J. B. and Handelman, G. J. (2000) Relative reactivity of lysine and other peptide-bound amino acids to oxidation by hypochlorite. *Free Radic. Biol. Med.* **29**, 425–433
- 55 von Sonntag, C. (1987) *The Chemical Basis of Radiation Biology*, Taylor and Francis, London
- 56 Wagner, J. R., Cadet, J. and Fisher, G. J. (1984) Photo-oxidation of thymine sensitized by 2-methyl-1,4-naphthoquinone: analysis of products including three novel photo-dimers. *Photochem. Photobiol.* **40**, 589–597
- 57 Vissers, M. C. and Winterbourn, C. C. (1995) Oxidation of intracellular glutathione after exposure of human red blood cells to hypochlorous acid. *Biochem. J.* **307**, 57–62
- 58 Pullar, J. M., Winterbourn, C. C. and Vissers, M. C. M. (1999) Loss of GSH and thiol enzymes in endothelial cells exposed to sublethal concentrations of hypochlorous acid. *Am. J. Physiol.* **277**, H1505–H1512
- 59 Hawkins, C. L., Brown, B. E. and Davies, M. J. (2001) Hypochlorite- and hypobromite-mediated radical formation and its role in cell lysis. *Arch. Biochem. Biophys.* **395**, 137–145

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