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8-Chloroadenine: a novel product formed from hypochlorous acid-induced damage to calf thymus DNA

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Hypochlorous acid (HOCl) is formed by the action of the enzyme myeloperoxidase on hydrogen peroxide and chloride ions. It has been shown to be highly bactericidal and cytotoxic by a variety of mechanisms, one of which, may be the modification of DNA. Previously we have demonstrated by GC–MS analysis that exposure of calf thymus DNA to HOCl causes extensive pyrimidine modification, including 5-chlorocytosine formation. Using GC–MS analysis, we now demonstrate the formation of an additional chlorinated base product, 8-Cl adenine. The addition of 50 μM HOCl was sufficient to produce a significant increase in this product. The reaction of HOCl with adenine in calf thymus DNA was shown to be rapid with the reaction complete after 1 min. pH-dependence studies suggest HOCl rather than its conjugate base (OCl^-) to be responsible for 8-Cl adenine formation. Other commercially available chlorinated base products, 6-Cl guanine or 2-Cl adenine were not detected. Therefore, 8-Cl adenine might prove a useful biomarker for studying the role of reactive chlorine species (RCS) during inflammatory processes.

Keywords: hypochlorous acid, reactive chlorine species, 8-chloroadenine, inflammation, oxidative DNA damage.

Abbreviations: GC–MS, gas chromatography–mass spectrometry; HOCl, hypochlorous acid; OCl^-, hypochlorite; 8-Cl adenine, 8-chloroadenine; 5-Cl uracil, 5-chlorouracil; 5-Cl cytosine, 5-chlorocytosine; 6-Cl guanine, 6-chloroguanine; 2-Cl adenine, 2-chloroadenine; TMCS, trimethylchorosilane; TMS, trimethylsilyl.

Introduction

The enzyme myeloperoxidase (MPO) is released by phagocytic cells at sites of inflammation and catalyses the formation of hypochlorous acid (HOCl) from hydrogen peroxide (H_2O_2) and chloride ions (Cl^-) (equation (1)). It has been claimed that a minimum of 25–40 % of the H_2O_2 generated by activated neutrophils is used to form HOCl (Foote *et al.* 1983, Weiss 1992).

$$H_2O_2 + Cl^- + MPO \longrightarrow HOCl + OH^- \quad (1)$$

The pK_a of HOCl is 7.46 and so it is approximately 50 % ionized to OCl^- at pH 7.4 (Morris 1966). In this paper hypochlorous acid is used to refer to the mixture of HOCl and OCl^- species. Thermodynamic calculations have shown HOCl to be both a one and two electron oxidant (Koppenol 1994). It is strongly microbicidal

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and exposure of many bacteria to HOCl leads to cell death within seconds (Albrich *et al.* 1981) via mechanisms involving inhibition of membrane transporters, respiratory enzymes and ATP depletion (Barette *et al.* 1989). In mammalian systems, HOCl is capable of oxidizing many important biological molecules such as sulphydryl and thioether moieties (Aruoma *et al.* 1989, Schraufstatter *et al.* 1990), plasma membrane ATPases, collagen, ascorbate, proteins including α_1-antiproteinase, nucleotides, and DNA repair enzymes (Halliwell *et al.* 1987, Aruoma *et al.* 1989, Shraufstatter *et al.* 1990, Van Rensberg *et al.* 1991, Weiss 1992, Van Rensberg *et al.* 1992, Folkes *et al.* 1995, Pero *et al.* 1996, Prutz 1996) and it is also capable of chlorinating fatty acid residues and cholesterol in cell membranes (Carr *et al.* 1996). Recently HOCl has been suggested to contribute to the development of atherosclerosis (Hazen and Heinicke 1997).

Previous studies on the reaction of HOCl with DNA using GC–MS analysis showed a pattern of oxidative damage largely specific for the pyrimidine constituents with minimal oxidative damage to purines (Whiteman *et al.* 1997). In addition, chlorinated cytosine residues, measured by GC–MS after conversion to 5-chlorouracil during acidic hydrolysis of the DNA, were detected. In this study, we identify a novel chlorinated purine base, 8-chloroadenine, which could conceivably be a useful bio-marker of HOCl damage to DNA *in vivo*.

Materials and methods

Materials

Chemicals were of the highest quality available. Calf thymus DNA (Sigma type I), methionine, sodium hypochlorite, 2,6-diaminopurine, 6-chloroguanine and 2-chloroadenosine were purchased from Sigma Chemical Co. (Poole, Dorset, UK). 8-Chloroadenine was purchased from BioLog (Bremen, Germany).

Cellu.Sep dialysis membranes with a relative molecular mass cut off of 3500, silylation grade acetonitrile, and bis(trimethylsilyl)trifluoracetamide (BSTFA) (containing 1 % trimethylchlorosilane, TMCS) were obtained from Pierce Chemical Co. (Rockford, IL, USA). Distilled water passed through a purification system (Elga, High Wycombe, Bucks, UK) was used for all purposes.

DNA damage induced by hypochlorous acid

All buffer and DNA solutions were treated with Chelex-100 resin before use. Hypochlorite (OCl^-) concentration was quantified immediately before use spectrophotometrically at 290 nm (pH 12, $\varepsilon = 350\ M^{-1}\ cm^{-1}$) (Morris 1966). HOCl at various concentrations was added to a reaction mixture (final volume 2.0 ml) containing calf thymus DNA (0.5 mg ml^{-1}) in 250 mM phosphate buffer (K_2HPO_4–KH_2PO_4) at the pH stated. Addition of HOCl did not significantly alter pH. After incubation at 37 °C for up to 1 h, samples were dialysed against water at room temperature for 24 h.

Formation of 8-Cl adenine: time course study

DNA was treated with HOCl (500 μM) as described above and the reaction was quenched at various times with 10 mM of ice cold methionine, a powerful HOCl scavenger (Winterbourn 1985). The samples were stored on ice for no longer than 1 h until dialysis against water at room temperature for 24 h.

Analysis of 8-chloroadenine and other chlorinated bases by gas chromatography–mass spectrometry

Preparation, hydrolysis, derivatization and analysis of samples were essentially performed as described previously (Spencer *et al.* 1994, Whiteman *et al.* 1997). Briefly, DNA concentration was measured spectrophotometrically (E_{260} 1.0 = 50 μg ml^{-1}) after overnight dialysis. Aliquots of 100 μg DNA were then freeze-dried under reduced pressure after addition of internal standard (0.5 nmol 2,6-diaminopurine). Samples and authentic commercial standards were subsequently hydrolysed by addition of 0.5 ml of 60 % (v/v) formic acid and heating at 140 °C for 45 min in evacuated glass hydrolysis tubes. Samples were cooled, lyophilized and then derivatized under a nitrogen atmosphere in poly(tetrafluoroethylene)-capped glass vials (Pierce) by adding 100 μl of a BSTFA (+ 1 %

TMCS)/acetonitrile (4:1 v/v) mixture. Samples were derivatized at 90 °C for 45 min and then analysed by GC–MS (Hewlett-Packard 5890II interfaced with a Hewlett-Packard 5971A mass selective detector). The injection port and the GC–MS interface were kept at 250 °C and 290 °C, respectively. Separations were carried out on a fused silica capillary column (12 m × 0.2 mm i.d.) coated with cross-linked 5 % phenylmethylsiloxane (film thickness 0.33 μm) (Hewlett-Packard). Helium was the carrier gas with a flow of 0.93 ml min^{-1}. Derivatized samples (2.0 μl) were injected into the GC port using a split ratio of 6:1. Column temperature was increased from 125 °C to 175 °C at 8 °C min^{-1} after 2 min at 125 °C, then from 175 °C to 220 °C at 30 °C min^{-1} and held at 220 °C for 1 min, and finally from 220 °C to 290 °C at 40 °C min^{-1} and held at 290 °C for 2 min. Selected-ion monitoring was performed *m/z* at 313 and 298 (abundant and characteristic ions from the mass spectrum of derivatized 8-chloroadenine (figure 1(C)) using the electron-ionization mode at 70eV with the ion source maintained at 189 °C. Under these conditions 8-chloroadenine had a retention time of 10.20 min with a sensitivity limit of 67 fmol on column (defined as the lowest amount of compound with a peak area:noise ratio ≥ 5). This amount corresponds to approximately 0.2 nmoles mg^{-1} DNA in DNA samples. 6-Cl guanine and 2-Cl adenine had retention times of 10.47 min and 10.61 min and on column limits of sensitivity of 5 fmol and 50 fmol respectively.

Quantification of 8-Cl adenine was achieved by relating the peak area of the compound with the internal standard peak area (2,6-diaminopurine) and applying the following formula:

$$\text{Concentration (nmol mg}^{-1}\text{ DNA)} = A/A_{IST} \times [\text{IST}] \times (1/K)$$

where K = relative molar response factor for each damaged base, A = peak area of product, A_{IST} = the peak area of the internal standard, and [IST] = concentration of internal standard (5 nmol mg^{-1} DNA). K constants were calculated from the slopes of the calibration curves constructed using known concentrations of internal standards and authentic compounds.

Results

Identification of 8-chloroadenine

Selective ion monitoring (SIM) at *m/z* 298 of HOCl-treated calf thymus DNA led to the identification of a peak at 10.2 min, which was identified as 8-Cl adenine after comparison of retention time and mass spectra with authentic standard (figure 1). Abundant $M^{+\bullet}$ and $(M-15)^+$ at *m/z* 313 and 298 respectively, were observed corresponding to the bis-Me_3 Si derivative. Highly characteristic 'A + 2' isotopic distribution patterns for clusters 313 and 298 amu indicate these ions contain a single chlorine atom. Loss of Cl from $M^{+\bullet}$ probably accounted for the ion at *m/z* 278.

Action of HOCl on DNA

Preliminary studies showed 8-Cl adenine to be stable during formic acid (60 % v/v) hydrolysis of DNA and derivatization at 90 °C. The addition of 500 μM HOCl to DNA led to the formation of (mean ± SD) 2.63 ± 0.13 mmoles 8-Cl adenine $mole^{-1}$ of HOCl. This yield was slightly lower than the yield of other HOCl-modified DNA bases such as 5-Cl uracil (3.97 ± 0.36) mmoles $mole^{-1}$ HOCl), 5-OH uracil (4.98 ± 0.24) mmoles $mole^{-1}$ HOCl), 5-OH cytosine (6.21 ± 0.65) mmoles $mole^{-1}$ HOCl), *cis* thymine glycol (13.31 ± 1.10) mmoles $mole^{-1}$ HOCl) and *trans* thymine glycol (4.05 ± .26) mmoles $moles^{-1}$ HOCl) [yields calculated from Whiteman *et al.* (1997)]. Figure 2(A) shows the concentration-dependent formation of 8-Cl adenine. Addition of 50 μM HOCl was sufficient to produce a significant increase in formation of 8-Cl adenine. However, no formation of 6-Cl guanine or 2-Cl adenine was detected even when 500 μM HOCl was used (not shown). Formation of 8-Cl adenine was dependent on the pH of the reaction mixture; yields were greatest at pH 5.0 and decreased with increasing pH (7.4 to 9.0) (figure 2(B)).

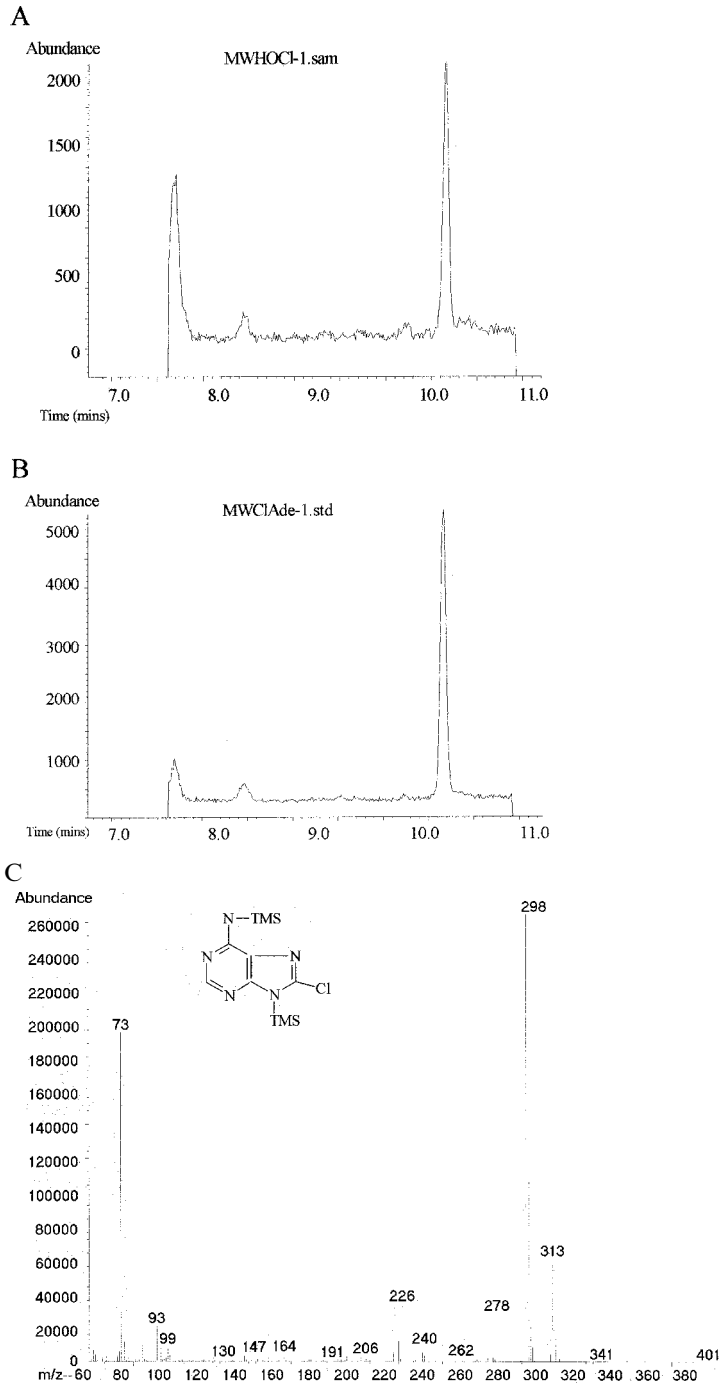

Figure 1. Comparison of the peak at 10.2 min from HOCl treated DNA with authentic 8-Cl adenine standard. A: Selective ion monitoring trace of HOCl (200 μM) treated, hydrolysed and derivatized DNA. B: Selective ion monitoring trace of authentic derivatized 8-Cl adenine (approx 0.4 pmoles on column). C: Mass spectrum for hydrolysed and derivatized 8-Cl adenine. Abundant $M^{+\bullet}$ and $(M-15)^{+}$ ions for *m/z* values for 298 and 313 respectively were observed corresponding to the bis-Me_3 Si derivative. Loss of Cl from $M^{+\bullet}$ probably accounted for the ion at *m/z* 278. Experiments were conducted as described in Materials and Methods.

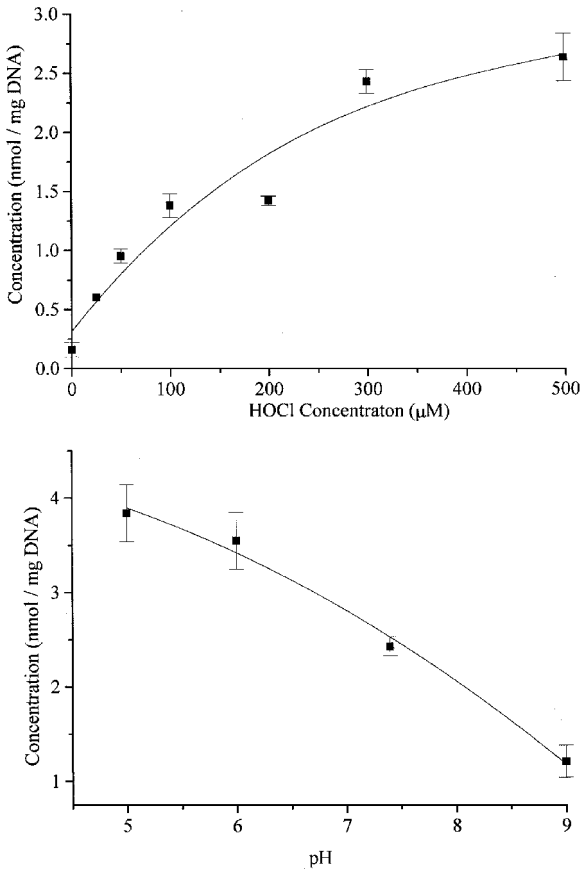

Figure 2. Effect of HOCl on the generation of 8-Cl adenine. A: Effect of increasing HOCl concentrations on the generation of 8-Cl adenine from calf thymus DNA, pH 7.4. B: Effect of pH on 8-Cl adenine formation. HOCl (500 μM) was added to calf thymus DNA at the pH stated. Buffers at all pH values contained 250 mM K_2HPO_4–KH_2PO_4. Experiments were conducted as described in Materials and Methods. Data points are means of three or more separate experiments plotted ± SD.

Time course of formation

Figure 3 shows the time course of 8-Cl adenine formation after HOCl (500 μM) addition to calf thymus DNA. The reaction was complete 60 s after HOCl addition.

Discussion

HOCl has been shown to attack nucleotides and individual DNA bases *in vitro* (Patton *et al.* 1972, Hoyano *et al.* 1973, Gould *et al.* 1984a, b, Itoh *et al.* 1987, Prutz 1996) and to inactivate various DNA repair enzymes (Van Rensberg *et al.* 1991, Pero *et al.* 1996). These effects could lead to mutation and contribute to the increased risk of carcinogenesis that is associated with chronic inflammation. 5-Cl Cytosine has previously been shown to be a major stable end product of cytosine chlorination by HOCl (Patton *et al.* 1972, Hoyano *et al.* 1973, Whiteman *et al.* 1997) but the formation of 8-Cl adenine from the reaction of HOCl with DNA has not previously been reported, to our knowledge. In this paper we report that 8-Cl

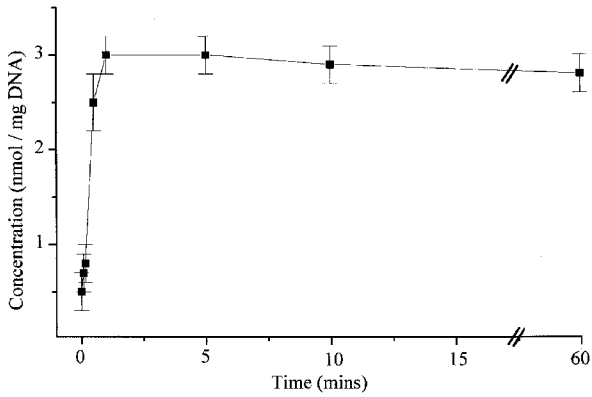

Figure 3. Time course for the formation of 8-Cl adenine. HOCl (500 μM) was added to calf thymus DNA (0.5 mg ml^{-1}, pH 7.4) for increasing periods of time. At the desired time 10 mM of ice cold methionine was added to quench any unreacted HOCl. Experiments were conducted as described in Materials and Methods. Data points are means of three or more separate experiments plotted ± SD.

adenine is also formed in isolated DNA, in amounts approaching those of 5-Cl cytosine. Although the yield of 8-Cl adenine was slightly less than that of 5-Cl cytosine, a previously identified chlorinated DNA adduct (2.63 mmol $mole^{-1}$ HOCl compared with 3.97 mmol $mole^{-1}$ HOCl) (Whiteman *et al.* 1997) the formation of 8-Cl adenine accounted for approximately 4 % of the total measurable DNA modification products, broadly comparable to other DNA bases modified by HOCl (Whiteman *et al.* 1997).

8-Cl adenine was formed from the reaction of HOCl with calf thymus DNA in a concentration and pH-dependent manner (figure 2). It is estimated that at sites of chronic inflammation extracellular HOCl concentrations of at least 80 μM are achievable (Kalyanaraman *et al.* 1985). The optimal formation of 8-Cl adenine at lower pH suggests that the acid (HOCl) rather than the base (OCl^-) was responsible for its formation, since the pK_a of HOCl is ~7.4 (Morris 1966). Indeed, during chronic inflammation and especially during repeated cycles of hypoxia reperfusion injury, the pH has been reported to become acidic (Blake *et al.* 1989), whereby HOCl would predominate, potentially promoting 8-Cl adenine formation. Even at pH 7.4, 50 % exists in the HOCl form. However, the complex nature of the DNA macromolecule means that competing reactions with sugar and phosphate moieties may also consume HOCl and OCl^-. Additionally, HOCl may be consumed by its reaction with oxidized DNA base lesions, most notably 8-OH guanine and FAPy guanine which have been demonstrated to be depleted in isolated DNA treated with HOCl (Whiteman *et al.* 1997). It is also possible that HOCl reacts with DNA to form chloramines and unstable secondary intermediates (Patton *et al.* 1972, Hoyano *et al.* 1973, Gould *et al.* 1984b, Itoh *et al.* 1987) that we did not measure. Therefore only a small percentage of HOCl is accounted for when measuring modified DNA bases alone. Our laboratory is currently attempting to identify novel biomolecules of HOCl attack on DNA.

Our studies show that DNA is rapidly attacked by HOCl and that low concentrations of HOCl can lead to the formation of chlorinated base lesions. A significant amount of 8-Cl adenine was generated at HOCl concentrations less than

50 μM. The extent to which HOCl can penetrate to the nucleus of intact cells and damage DNA is yet to be determined, but HOCl has been demonstrated by several researchers to traverse cell membranes and liposomes (Barette *et al.* 1989, Schraufstatter *et al.* 1990, Van Rensberg *et al.* 1991, 1992, Pero *et al.* 1996, Carr *et al.* 1997). In addition it has recently been reported that during phagocytosis, neutrophils evolve chlorine gas (Cl_2), in equilibrium with HOCl (Hazen *et al.* 1996) which may be more freely available to traverse cellular membranes to attack and produce chlorinated DNA lesions including 8-Cl adenine. Formation of Cl_2 gas is more favourable under acidic pH and therefore at sites of chronic inflammation (Hazen *et al.* 1996).

There is a need for further bio-markers of HOCl-induced damage because at present the only bio-marker for the *in vivo* formation of RCS (HOCl, Cl_2, NO_2Cl) at sites of chronic inflammation, is 3-chlorotyrosine. This bio-marker was found to be elevated in some atherosclerotic aortae (Hazen and Heinicke 1997) but has yet to be investigated in other diseases/pathological states where RCS may be involved. Using the GC–MS technique described here, 8-Cl adenine can be detected in calf thymus DNA samples with relatively good sensitivity (< 0.2 nmoles mg^{-1} DNA) and when measured in conjunction with 5-Cl cytosine, may help elucidate the role of HOCl in chronic inflammatory conditions, especially in the progression towards carcinogenesis. This may be apparent in chronic inflammatory conditions such as rheumatoid arthritis where the pool of antioxidant defence molecules is depleted (reviewed in Halliwell 1995).

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