Ability of Hypochlorous Acid and *N*-Chloramines to Chlorinate DNA and Its Constituents

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Myeloperoxidase is a heme enzyme released by activated phagocytes that is responsible for the generation of the strong oxidant hypochlorous acid (HOCl). Although HOCl has potent bactericidal properties and plays an important role in the human immune system, this oxidant also causes damage to tissues, particularly under inflammatory conditions. There is a strong link between chronic inflammation and the incidence of many cancers, which may be associated with the ability of HOCl and related oxidants such as *N*-chloramines to damage DNA. However, in contrast to HOCl, little is known about the reactivity of *N*-chloramines with DNA and its constituents. In this study, we examine the ability of HOCl and various *N*-chloramines to form chlorinated base products on nucleosides, nucleotides, DNA, and in cellular systems. Experiments were performed with *N*-chloramines formed on *N*α-acetyl-histidine (His-C), *N*α-acetyl-lysine (Lys-C), glycine (Gly-C), taurine (Tau-C), and ammonia (Mono-C). Treatment of DNA and related materials with HOCl and His-C resulted in the formation of 5-chloro-2′-deoxycytidine (5CldC), 8-chloro-2′-deoxyadenosine (8CldA) and 8-chloro-2′-deoxyguanosine (8CldG). With the nucleosides, 8CldG was the favored product in each case, and HOCl was the most efficient chlorinating agent. 5Cl(d)C was the most abundant product on exposure of the nucleotides and DNA to HOCl and His-C, with only low levels of chlorinated products observed with Lys-C, Gly-C, Tau-C, and Mono-C. 5CldC was also formed on exposure of smooth muscle cells to either HOCl or His-C. Cellular RNA was also a target for HOCl and His-C, with evidence for the formation of 5-chloro-cytidine (5ClC). This study shows that HOCl and the model *N*-chloramine, His-C, are able to chlorinate cellular genetic material, which may play a role in the development of various inflammatory cancers.

Introduction

Myeloperoxidase (MPO[1]) is released by activated phagocytes during inflammation, as part of the innate immune system. In the presence of physiological concentrations of chloride ions (100−150 mM) (*1*) and hydrogen peroxide (H_2O_2), MPO generates the potent antibacterial agent hypochlorous acid (HOCl), whose formation accounts for 20−70% of the H_2O_2 liberated during inflammation (*2*). At sites of inflammation *in vivo*, the concentration of HOCl has been estimated to reach 25−50 mM (*3*), while kinetic modeling studies of the phagosome suggest that concentrations as high as 100 mM are feasible (*4*). The high concentrations of HOCl observed under inflammatory conditions are postulated to result in damage to host tissue and contribute to disease progression (reviewed in refs *5 and 6*). MPO has been linked to many inflammatory diseases, such as systemic vasculitides (*7*), neurogenerative disease (*8*), cancer (*9*), kidney disease (*10*), and atherosclerosis (*11*).

HOCl is a highly reactive compound that reacts readily with biomolecules, including proteins, lipids, and DNA (reviewed in refs *6, 12, and 13*). Proteins are important major targets for HOCl, owing to their abundance and high reactivity with this oxidant (*14*). Protein-derived *N*-chloramines are formed readily on reaction of HOCl with Lys and His side-chains (*12*). These species are reactive and can mediate further protein damage (e.g., ref *15*) and induce the oxidation of other biological molecules, including lipids (*16*) and DNA (*17*).

Less is known about the consequences of the reaction of HOCl with RNA and DNA. The reactivity of HOCl with nucleosides is primarily determined by the location of the NH groups, with the heterocyclic (ring) NH groups generally more reactive than the exocyclic (free) NH_2 groups (e.g., $k_2 = 2.1 \times 10^4$ M^{-1} s^{-1}, cf. 2.4 M^{-1} s^{-1} for the reaction with N-1 and NH_2 at C-2 on GMP, respectively) (*18*). The majority of HOCl consumed in the reaction with DNA results in the formation of unstable, nucleoside-derived, *N*-chloramines (*17, 19*). These intermediates decompose readily, and may be involved in the formation of stable chlorinated products (*19−21*). A range of stable products are produced from the reaction of HOCl with DNA, including thymine glycol, 5-hydroxy-cytosine, 5-hydroxy-uracil, and hypoxanthine (*22, 23*). Similarly, there is evidence for the formation of a number of chlorinated bases including 5-chlorouracil (5ClU) (*22−25*), 8-chloro(2′-deoxy)adenosine (8Cl(d)A) (*20, 23, 26, 27*), 5-chloro(2′-deoxy)cytidine (5Cl(d)C) (*20, 23, 24, 27−29*), and 8-chloro(2′-deoxy)guanosine (8Cl(d)G) (*20, 27*) on reaction of HOCl with DNA. These products are specific for MPO-derived chlorinating oxidants and, in some cases, have been used as biomarkers for HOCl-induced damage (e.g., refs *30−32*).

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[1] Abbreviations: BCASMC, bovine coronary aortic smooth muscle cells; 8Cl(d)A, 8-chloro(2′-deoxy)adenosine; 5Cl(d)C, 5-chloro(2′-deoxy)cytidine; 8Cl(d)G, 8-chloro(2′-deoxy)guanosine; 5Cl(d)U, 5-chloro(deoxy)uridine; dA, 2′-deoxyadenosine; dC, 2′-deoxycytidine; dG, 2′-deoxyguanosine; dT, 2′-deoxythymidine; dU, 2′-deoxyuridine; DMEM, Dulbecco's modified Eagle's medium; DTNB, 5,5′-dithio-2-nitrobenzoic acid; Gly-C, glycine chloramine; HBSS, Hank's buffered salt solution; His-C, *N*α-acetyl-histidine chloramine; Lys-C, *N*α-acetyl-lysine chloramine; Mono-C, monochloramine; MPO, myeloperoxidase; SRM, selective reaction monitoring; Tau-C, taurine chloramine; TNB, 5-thio-2-nitrobenzoic acid.

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The presence of increased levels of chlorinated nucleosides in the inflammatory exudate of humans (*30*) and rats (*31*), and elevated levels of 5ClU in atherosclerotic plaques compared to healthy arteries (*32*) suggest that the chlorination of nucleic acids may be relevant under pathological conditions. Indeed, 8CldG has recently been reported to be a biomarker of early inflammation, with elevated levels of this chlorinated nucleoside detected in the urine of lipopolysaccaride-treated rats and diabetic patients (*33*). However, the mechanism of MPO oxidant-induced DNA chlorination *in vivo* is not well understood. It is likely that direct reaction of HOCl with cellular DNA may be a minor pathway, owing to the extracellular production of HOCl and its high reactivity with biological substrates. Under physiological conditions, *N*-chloramines are formed readily on ammonia (NH_3), taurine, free amino acids, as well as peptides and proteins (*2, 34, 35*). *In vitro*, various *N*-chloramines convert uracil to 5ClU (*32*). The role of taurine chloramine (Tau-C) in the chlorination of uracil is unclear, as it can both prevent (*30*) and promote (*32*) 5ClU formation. There is a lack of data regarding the ability of *N*-chloramines to mediate the chlorination of RNA and DNA, though tertiary amines, including nicotine and trimethylamine, enhance the chlorination of nucleosides and RNA by HOCl (*20*).

In this study, we investigated the ability of five biologically relevant *N*-chloramines to chlorinate nucleosides, nucleotides, isolated DNA, and cellular RNA and DNA. Experiments were performed with *N*-chloramines generated on Gly (Gly-C), to model the *N*-terminal amino group, and Nα-acetyl-Lys (Lys-C) and Nα-acetyl-His (His-C) to model *N*-chloramines formed on protein side chains. Mono-C is known to be reactive with cells and is also believed to be formed under physiological conditions (*4*). Tau-C was also studied as taurine is present in high concentrations in neutrophils (*36*), and its role in mediating nucleoside chlorination is unclear.

Experimental Procedures

Materials. Water was filtered through a four-stage Milli-Q system (Millipore-Waters, Lane Cove, NSW, Australia). DNA (calf thymus, > 98% purity), free nucleosides and nucleotides, alkaline phosphatase, and ammonium formate were obtained from Sigma-Aldrich (St. Louis, MO). DNA solutions were prepared by stirring gently overnight at 4 °C in the presence of Chelex resin (Bio-Rad, Hercules, CA; 0.2 g mL^{-1}) to remove contaminating trace metal ions. Chlorinated nucleosides (8ClA; 8CldA; 5ClC; 5CldC; 8ClG; 8CldG) were obtained from BioLog Life Sciences Institute (Bremen, Germany). HOCl solutions were prepared immediately before use by dilution of a concentrated stock solution [ca. 0.5 M in 0.1 M NaOH (BDH, Poole, U.K.)] into water. HOCl concentrations were determined from the absorbance of $^{-}$OCl at 292 nm at pH 12 using a molar extinction coefficient of 350 M^{-1} cm^{-1} (*37*). All other chemicals were of analytical reagent grade.

***N*-Chloramine Formation and Quantification.** Mono-C, Gly-C, Lys-C, and Tau-C were generated by the reaction of HOCl with a 5-fold molar excess of the substrates ammonium sulfate, glycine, *N*α-acetyl-Lys and taurine, respectively (*38*). His-C was generated using a 2-fold molar excess of *N*α-acetyl-histidine, as previously described (*38*). Under these conditions, complete conversion of HOCl to the respective *N*-chloramine is observed (*38*). *N*-Chloramine concentrations were determined by the reaction with 5-thio-2-nitrobenzoic acid (TNB) at 412 nm as described previously (*39*) using an extinction coefficient of 13 600 M^{-1} cm^{-1} (*40*).

Oxidant Consumption on Reaction with DNA. Calf thymus DNA (50 μg in 200 μL H_2O) was incubated with an equal volume of HOCl or *N*-chloramine (500 μM) at 20 °C. The final pH of the reaction mixture was 7−8. Samples were filtered through 3 kDa molecular-weight cutoff filters (Millipore) for 5 min at 16000*g* to remove the DNA before quantifying the remaining oxidant using TNB.

Quantification of Chlorinated Products on Nucleosides and Nucleotides. The nucleosides deoxyadenosine (dA), deoxycytidine (dC), and deoxyguanosine (dG), and equimolar mixtures of dA, dC, dG, and deoxythymidine (dT) (500 μM) were incubated with equal volumes of HOCl (250 μM) or *N*-chloramines at 20 °C before quenching the reaction with Met (10 mM), after the required incubation time. The nucleotides ATP, CTP, and GTP, and an equimolar mixture of ATP, CTP, GTP, and TTP (500 μM) were treated as described above and the reaction quenched with 2.5 mM Met. The nucleotide samples were subsequently dephosphorylated for 1 h at 37 °C with alkaline phosphatase (25 U) in the presence of phosphatase buffer (80 mM Tris-HOCl and 160 μM EDTA, pH 8). Nucleotide samples were filtered through 10 kDa molecular-weight cutoff filters for 5 min at 16000*g* to remove alkaline phosphatase prior to analysis.

Quantification of base chlorination was carried out on a Shimadzu HPLC system using a Beckman Coulter Ultrasphere 5 μm ODS column (4.6 mm ×25 cm) fitted with a Pelliguard C-18 guard column (Supelco) at 30 °C. Samples were separated using a gradient elution system at a flow rate of 1 mL min^{-1} with 5 mM ammonium formate (Buffer A) and 5 mM ammonium formate in 50% (v/v) acetonitrile (Buffer B) as follows: 100% Buffer A for 10 min, followed by a linear increase of Buffer B to 20% over 30 min, 50% over the next 10 min, and 100% over a further 5 min. After isocratic 100% Buffer B for 3 min, Buffer A was returned to 100% over 3 min, followed by a 4 min re-equilibration. A modified gradient was used for nucleotide samples: 100% Buffer A for 5 min, followed by a linear increase of Buffer B to 30% over 10 min, 50% over the next 20 min, and 100% over a further 5 min. After isocratic 100% Buffer B for 5 min, Buffer A was returned to 100% over 5 min, followed by a 5 min re-equilibration. Bases were quantified by UV absorbance at 260 nm.

Chlorinated Base Production on Isolated DNA. Fifty micrograms of DNA (200 μL) was treated with an equal volume of HOCl or *N*-chloramines (25 μM−1 mM) at 20 °C for 5 min and 4 h (HOCl and His-C only) and 1 week (all). The samples were hydrolyzed and dephosphorylated as described previously (*41*), prior to LC-MS analysis.

Quantification of Chlorinated Products by LC-MS. Detection of chlorinated bases was performed in the positive ion mode with a Thermo Finnigan LCQ Deca XP Max ion trap mass spectrometer coupled to a Thermo Finnigan Surveyor HPLC system (Thermo Electron Corporation, Australia). Bases were separated on a reverse phase C-18 Alltima HP column (5 μM, 2.1 mm × 25 cm) equipped with a Pelliguard C-18 guard column, using 5 mM ammonium formate (Buffer A) and 5 mM ammonium formate in 50% (v/v) acetonitrile (Buffer B) at a flow rate of 200 μL min^{-1}. Samples were eluted by the following gradient: 100% Buffer A for 10 min, followed by a linear increase of Buffer B to 20% over 30 min, 50% over a further 10 min and 100% over the next 5 min. This was maintained for 5 min before returning to 100% A over 5 min with re-equilibration for 5 min. Parent bases were detected using an in-line UV detector (Perkin-Elmer -785A) at 260 nm. The chlorinated bases were subsequently detected by LC-MS-MS with the following conditions: capillary voltage, 4500 V; capillary temperature, 275 °C; capillary and sweep gases (both N_2), 42 and 24 units, respectively; and collision gas, helium.

Tandem MS (MS-MS) experiments were performed to detect chlorinated RNA and DNA products. The normalized collision energy was set at 30% for all bases. For DNA samples, the MS was programmed to undergo four scan events throughout the run, such that every fourth data point in the chromatogram was (a) a full scan MS, *m/z* 100−400; (b) SRM of *m/z* 263 (±2) → 146 (±1) $[5CldC + H]^+$ to monitor 5CldC; (c) SRM of *m/z* 302 (±1) → 186 (±1) $[8CldG + H]^+$ to monitor 8CldG; and (d) SRM of *m/z* 287 (±2) → 170 (±1) $[8CldA + H]^+$ to monitor 8CldA, as described previously (*27*). For RNA samples, every fourth data point in the chromatogram was (a) a full scan MS, *m/z* 100−400; (b) SRM of

m/z 279 (±2) → 146 (±1) $[5ClC + H]^+$ to monitor 5ClC; (c) SRM of *m/z* 319 (±2) → 186 (±1) $[8ClG + H]^+$ to monitor 8ClG; and (d) SRM of *m/z* 302 (±1) → 170 (±1) $[8ClA + H]^+$ to monitor 8ClA (*27*).

Cell Culture. A7r5 (rat aortic smooth muscle; ATCC # 1444) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat inactivated fetal bovine serum, 4 mM glutamine, 3 g L^{-1} D-glucose, 1 mM sodium pyruvate, 100 U mL^{-1} penicillin, and 0.1 mg mL^{-1} streptomycin (referred to as complete DMEM). Bovine coronary aortic smooth muscle cells (BCASMC) were cultured in BCASMC growth medium (Cell Applications). Both cell types were cultured in an atmosphere of 5% CO_2 at 37 °C and harvested with trypsin/EDTA (1:250) and centrifugation at 800*g* for 5 min.

Ethidium Bromide DNA Release Assay. A7r5 cells or BCASMC (0.5×10^6) were allowed to adhere to 12-well tissue culture plates for 2 h in the complete DMEM or BCASMC growth media at 37 °C, respectively. The cells were washed twice with Hank's buffered salt solution (HBSS), before being treated with HOCl or *N*-chloramines (0.25−4 μmol/10^6 cells) in HBSS for 2 or 4 h at 37 °C. The extent of cell lysis was determined by the addition of ethidium bromide (250 μM) and measuring the change in fluorescence at λ_{EX} 360 nm and λ_{EM} 580 nm (*42, 43*).

Chlorinated Base Production in Cells. A7r5 cells or BCASMC (4×10^6) were plated down overnight in complete DMEM or BCASMC growth media. The cells were washed twice with PBS, prior to treatment with HOCl (0.125−0.5 μmol/10^6 cells) or His-C (0.25−1 μmol/10^6 cells) in PBS for 4 h at 37 °C to prevent confounding reactions of the oxidants with cell media components. Cells were then harvested with trypsin/EDTA after a further wash with PBS and centrifuged at 16000*g* for 5 min before discarding the supernatant. Samples were resuspended in 200 μL of PBS and the DNA and RNA extracted concurrently using the QIAamp DNA mini kit (Qiagen). The DNA and RNA were then hydrolyzed and dephosphorylated and nucleoside chlorination quantified by LC-MS, as described above.

Quantification of Cellular ATP. A7r5 cells (50×10^3) were plated down overnight in white 96-well plates to adhere. The cells were washed twice with HBSS, before being treated with HOCl or *N*-chloramine (0.05−2 μmol/10^6 cells) in HBSS for 2 h at 37 °C. The ATP concentration was determined by ATPlite Luminescence ATP Detection System (Perkin-Elmer), with the luminescence measured by 10 s scans on a Fluoroskan Ascent plate reader (Thermo Electron Corp., Australia). ATP-free tips were used for the dispensing of cells and reagents.

Statistics. All statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software, San Diego, USA, www.graphpad.com), with $p < 0.05$ taken as significant. Details of specific tests are given in the text and figure legends.

Results

HOCl and *N*-Chloramine Treatment of Isolated Nucleosides and Nucleotides. Initially, the ability of HOCl to chlorinate free nucleosides and nucleotides, both individually or when present as an equimolar mixture, was studied. Reaction of HOCl (250 μM, 6.25 nmol) with isolated nucleosides (dA, dC, dG; 500 μM, 12.5 nmol) resulted in the loss of parent nucleoside (with dG) and formation of chlorinated base products (Figure 1A). 8CldA, 5CldC, and 8CldG were all generated within 5 min of incubation of HOCl with the respective individual parent nucleosides. With the exception of 5CldC, no further increase in base chlorination was observed on further incubation of HOCl with the nucleoside. In the case of 5CldC, an increase in product formation was observed on incubation for 4 h compared to 5 min, suggesting that secondary products such as dC-derived chloramines may be important in the chlorination observed (Figure 1A). In experiments with HOCl, dG was the most readily chlorinated base, followed by dC and dA. On reaction of HOCl with the equimolar mixture of bases (dA, dC, dG, and dT; each 500 μM, 12.5 nmol), 8CldG was the only chlorinated base detected (Figure 2).

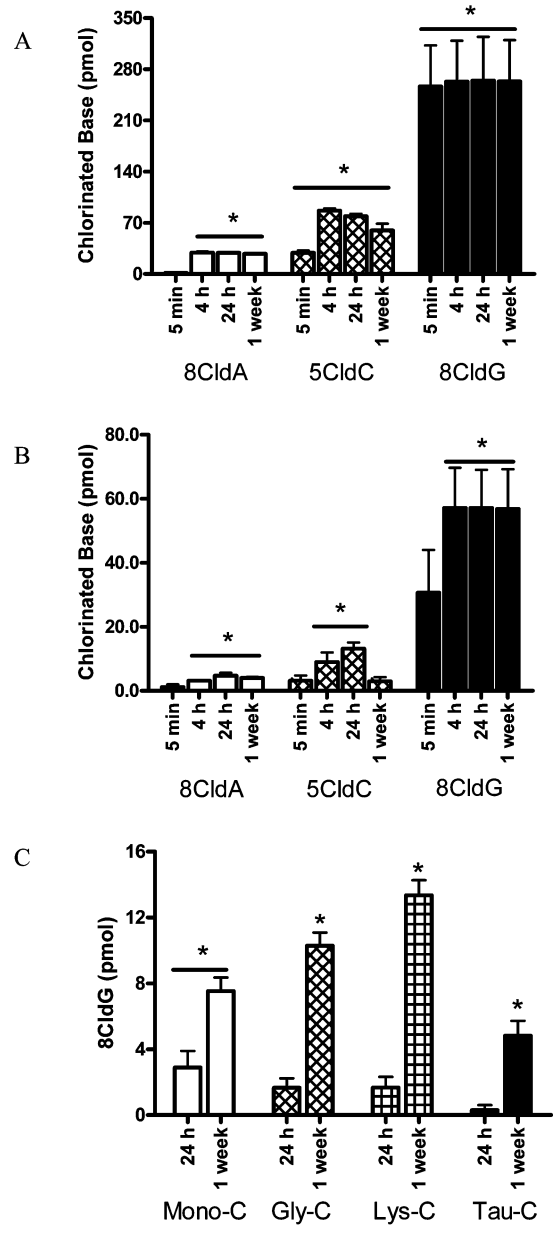

Figure 1. Formation of chlorinated bases on treatment of nucleosides with HOCl and *N*-chloramines. Nucleosides (dA, dC, dG; 500 μM, 12.5 nmol) were treated with (A) HOCl (B) His-C, or (C) Mono-C, Gly-C, Lys-C, or Tau-C (250 μM, 6.25 nmol) for the time indicated before quenching the reaction with methionine (10 mM) and quantification of chlorinated bases by UV analysis at 260 nm, after separation by HPLC. With HOCl and His-C, significant amounts of 8CldA (white); 5CldC (hatched); and 8CldG (black) were observed. With Mono-C (white), Gly-C (hatched), Lys-C (checked), or Tau-C (Black), only 8CldG was detected. * represents a significant increase in chlorinated base formation, compared to that in the non-treated control, determined by one-way ANOVA, with the Bonferroni post-test. Data represent the mean ± SEM of $n = 3$ experiments.

8CldG formation was also observed in analogous experiments with His-C, Lys-C, Gly-C, Tau-C, and Mono-C (Figure 1B and C). The concentration of 8CldG increased with longer incubation time (Figure 1B and C), consistent with slow base chlorination by the added *N*-chloramine. In each case, the extent of 8CldG observed was significantly lower than that observed with HOCl. With His-C, significant amounts of 8CldA or 5CldC were also observed, whereas no evidence was obtained for the formation

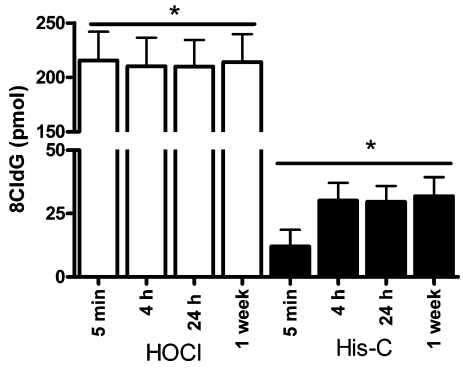

Figure 2. Formation of 8CldG on treatment of an equimolar mixture of nucleosides with HOCl or His-C. Samples containing dA, dC, dG, and dT (each 500 μM, 12.5 nmol) were treated with 250 μM (6.25 nmol) HOCl (white) or His-C (black) for the incubation times indicated before quenching the reaction with methionine (10 mM). 8CldG was quantified by UV analysis at 260 nm, after separation by HPLC. * represents significant 8CldG formation, compared to that in non-treated controls, determined by one-way ANOVA with the Bonferroni post-test. Data represent the mean ± SEM of $n = 3$ experiments.

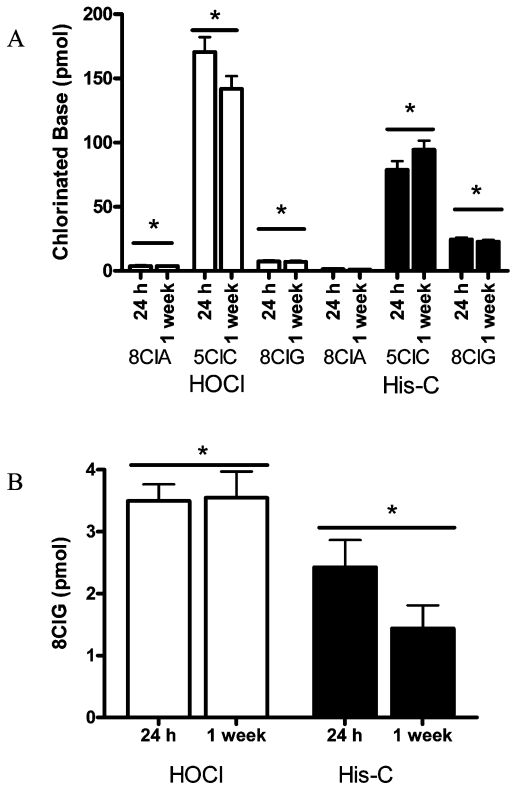

Figure 3. Formation of chlorinated bases on treatment of nucleotides with HOCl or His-C. Individual nucleotides ATP, CTP, and GTP (500 μM, 12.5 nmol; A) or an equimolar mixture of these nucleotides and TTP (each 125 μM, 3.125 nmol; B) were treated with 250 μM (6.25 nmol) HOCl (white) or His-C (black) for the incubation times indicated, before quenching the reaction with methionine (2.5 mM) and dephosphorylating the samples by incubation with alkaline phosphatase (25 U) for 1 h at 37 °C. Chlorinated base formation was quantified by UV analysis at 260 nm, after separation by HPLC. * represents a significant increase in chlorinated base formation, compared to that in the non-treated control, determined by one-way ANOVA, with the Bonferroni post-test. Data represent the mean ± SEM of $n = 3$ experiments.

of these products with Mono-C, Gly-C, Lys-C, or Tau-C, even after prolonged incubation (1 week). As with HOCl, 8CldG was the only product detected on treating an equimolar mixture of nucleosides with the *N*-chloramines (His-C shown in Figure 2).

Experiments with the isolated nucleotides ATP, CTP, or GTP (500 μM, 12.5 nmol) and HOCl or His-C (250 μM, 6.25 nmol)

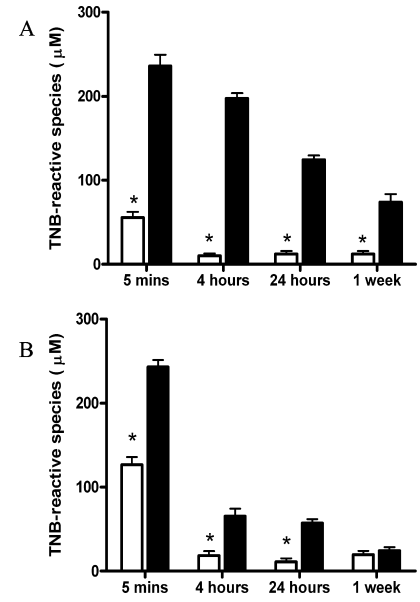

Figure 4. Consumption of oxidant on treating DNA with HOCl or His-C. Five hundred micromolar HOCl (A) and His-C (B) were incubated in the presence (white bars) and absence (black bars) of DNA (50 μg; from calf thymus) for the indicated incubation times before the removal of DNA by filtration through 3 kDa cutoff filters and quantification of the remaining oxidant by TNB assay. * represents a significant decrease in oxidant concentration, compared to that in the control incubated in the absence of DNA, determined by one-way ANOVA, with the Bonferroni post-test. Data represent the mean ± SEM of $n = 3$ experiments.

resulted in the formation of 8ClA, 5ClC, and 8ClG respectively (Figure 3A). However, with the nucleotides, 5ClC rather than 8ClG, was the most prominent chlorinated base formed, with only low levels of 8ClA detected. Reaction of HOCl and His-C with an equimolar mixture of ATP, CTP, GTP, and TTP gave only 8ClG, in accord with the nucleoside experiments. However, the yield of 8ClG in the experiments with an equimolar mixture of nucleotides was very low, compared to the experiments with the individual nucleotides (Figure 3B). Experiments were not performed with Mono-C, Gly-C, Lys-C, and Tau-C due to the slow reaction of these oxidants with nucleotides and the corresponding low yield of chlorinated bases observed (Figure 1C).

HOCl and *N*-Chloramine Treatment of Isolated DNA. Initial studies examined the extent of oxidant consumption on reaction of HOCl and the *N*-chloramines with isolated DNA. The DNA was removed by filtration prior to oxidant quantification to prevent confounding results due to the presence of DNA-derived chloramines. Both HOCl and His-C were rapidly consumed by the DNA (Figure 4A and B). This loss in oxidant occurred much more rapidly than the thermal decay of the oxidant observed in the absence of DNA (Figure 4A and B). In contrast, only a minor difference in the rate of Mono-C, Gly-C, Lys-C, and Tau-C decay was observed in the presence or absence of DNA, suggesting that they react very slowly with DNA, in accord with the nucleoside experiments described above (data not shown).

Reaction of isolated DNA (50 μg) with increasing concentrations of HOCl (25 μM−1 mM, 0.625−25 nmol) for 5 min resulted in a concentration-dependent loss of parent bases (Figure 5A), with a corresponding increase in 8CldA and 5CldC formation (Figure 5B and C). Evidence was also obtained for the formation of low amounts of 8CldG (Figure 5D). The percentage conversion of parent base lost to chlorinated product formed was ca. 0.3, 5.6, and 0.01% for 8CldA, 5CldC, and

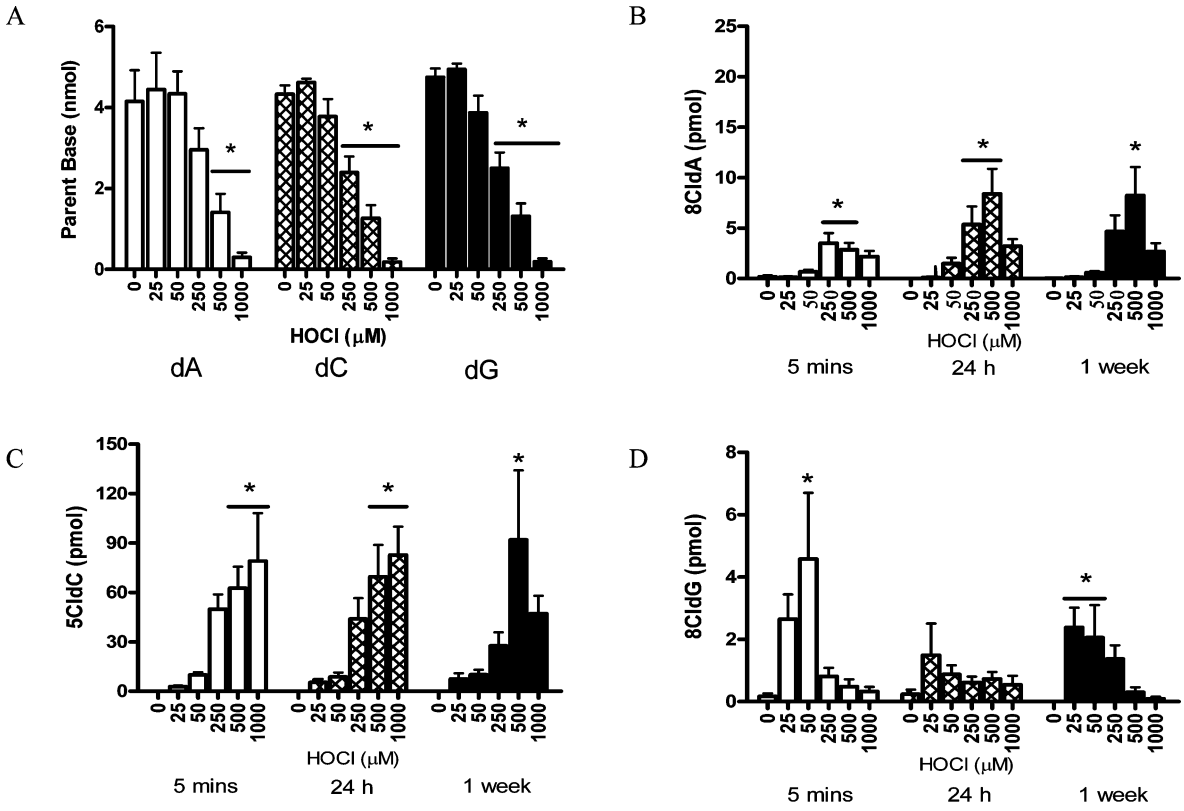

Figure 5. Formation of chlorinated products on treatment of DNA with HOCl. Calf-thymus DNA (50 μg) was treated with increasing concentrations of HOCl (25 μM−1 mM, 0.625−25 nmol) for varying times before precipitation, hydrolysis (with Nuclease P1; 10 U, 2 h at 37 °C), and dephosphorylation (with alkaline phosphatase; 12 U, 1 h at 37 °C). Graph A represents the concentration of parent dA (white bars), dC (hatched bars), and dG (black bars) remaining after the reaction with HOCl for 5 min. The concentration of chlorinated base products 8CldA (B), 5CldC (C), and 8CldG (D) present after treatment for 5 min (white), 24 h (hatched), or 1 week (black) was quantified by selective reaction monitoring by LC-MS-MS. * represents significant loss of parent base or chlorinated base formation, compared to that in the non-treated control, determined by one-way ANOVA, with Dunnett's multiple comparison test. Data represent the mean ± SEM of $n = 3$ experiments.

8CldG, respectively with 500 μM (12.5 nmol) HOCl. The concentration of 8CldG decreased with increasing HOCl, suggesting that this product is susceptible to further oxidation (Figure 5D). This was confirmed by the incubation of 8CldG with an equimolar concentration or 5-fold molar excess of HOCl for 24 h at 20 °C, which resulted in a 40% or complete loss of 8CldG, respectively (data not shown). A similar loss of 8CldA and 5CldC was also observed on treating DNA with high (1 mM, 25 nmol) concentrations of HOCl, suggesting that these products may also be susceptible to further oxidation in the presence of a large excess of oxidant. In each case, there was no significant increase in chlorinated base generation on reaction of HOCl with DNA for increasing time periods, suggesting that in general, the chlorination reactions occur within 5 min (Figure 5).

Reaction of DNA with His-C also resulted in the loss of parent bases and the generation of 8CldA, 5CldC, and 8CldG (Figure 6). In this case, higher yields of 8CldA and 8CldG were observed compared to HOCl, particularly on longer incubation of His-C with DNA (Figure 6). In this case, the percentage conversion of parent base lost to chlorinated product formed with 500 μM (12.5 nmol) His-C was ca. 1.3, 5.8, and 0.06% for 8CldA, 5CldC, and 8CldG, respectively. In contrast, only low levels of 8CldG were observed in experiments with Mono-C, Gly-C, Lys-C, and Tau-C, even on treatment for 1 week, consistent with the very slow rate of oxidant consumption (data not shown).

Reactivity of HOCl and *N*-chloramines with Cells. The extent of cell lysis observed on treating A7r5 cells with HOCl and *N*-chloramines for 2 and 4 h was determined by quantifying the release of DNA from the cells using ethidium bromide. With the exception of Mono-C, treatment of the cells with up to 2 μmol HOCl or various *N*-chloramines (per 10^6 cells) for 2 or 4 h resulted in the lysis of ≤25% of the cells (Figure 7). The A7r5 cells lysed more readily in the presence Mono-C, with ca. 13% and 28% lysis observed with ≤0.2 μmol/10^6 cells on incubation for 2 or 4 h, respectively. Experiments were not performed with higher concentrations of Mono-C owing to the formation of autofluorescent product(s). Lys-C and Tau-C induced very little cell lysis (<5%), even at very high oxidant concentrations (1−4 μmol/10^6 cells).

Experiments with HOCl and His-C were also performed with primary BCASMC (0.25−2 μmol oxidant/10^6 cells) for 4 h. At the lowest concentration of HOCl (0.25 μmol/10^6 cells), there was no difference in the extent of cell lysis observed with the two cell types (Figure 7C). However, at higher concentrations of HOCl, significantly greater cell lysis was observed with BCASMC, compared to that with A7r5 cells (67% and 78%, respectively). Similarly, BCASMC were also more susceptible to lysis induced by His-C compared to A7r5 cells, under similar conditions (Figure 7C).

The ability of HOCl and His-C to chlorinate cellular RNA and DNA was investigated under conditions where <20% cell lysis was observed to minimize the loss of this material from the cell. Reaction of A7r5 cells with HOCl (0.125−0.5 μmol/10^6 cells) resulted in a loss of parent RNA and DNA bases, and the formation of 5CldC (Figure 8A). No evidence for the formation of 8CldA, 8CldG, or RNA base chlorination was obtained in this case. Low levels of 5CldC were observed on

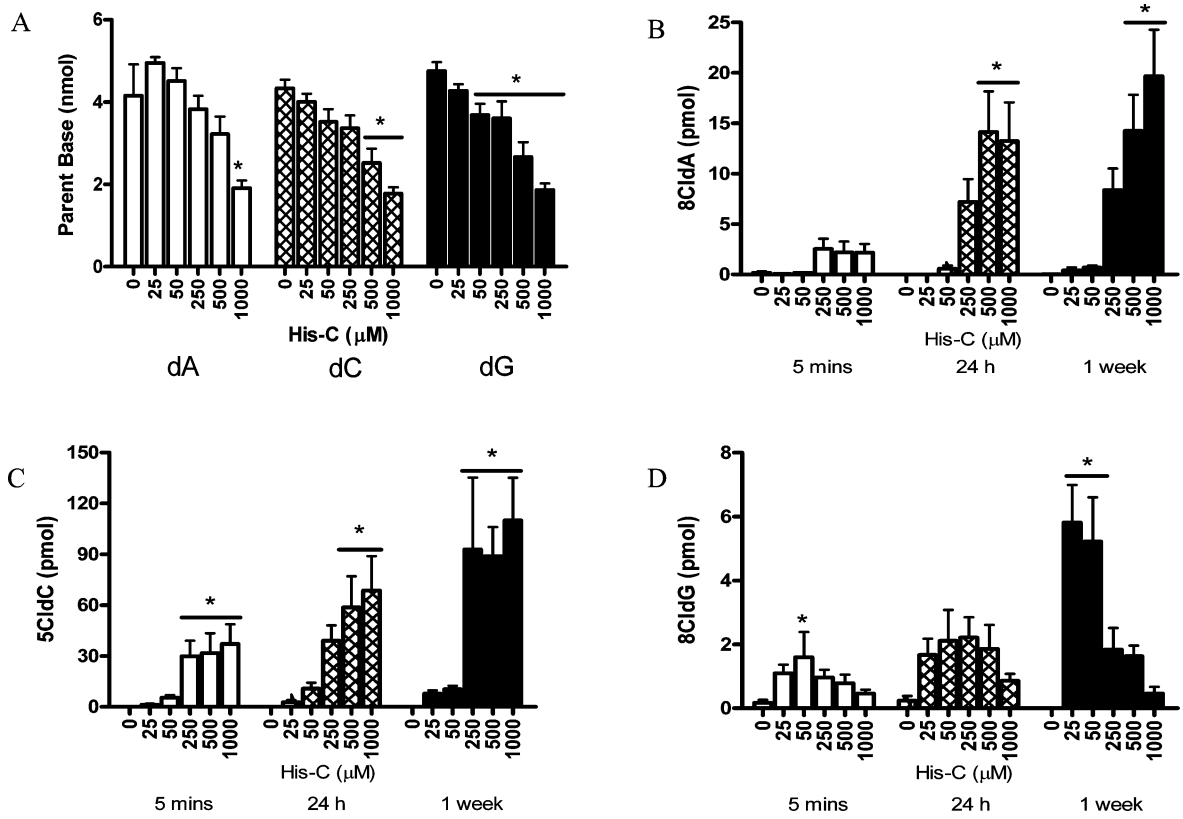

Figure 6. Formation of chlorinated products on treatment of DNA with His-C. Calf-thymus DNA (50 μg) was treated with increasing concentrations of His-C (25 μM−1 mM, 0.625−25 nmol) for varying times before precipitation, hydrolysis (with Nuclease P1; 10 U, 2 h at 37 °C), and dephosphorylation (with alkaline phosphatase; 12 U, 1 h at 37 °C). Graph A represents the concentration of parent dA (white bars), dC (hatched bars), and dG (black bars) remaining after the reaction with His-C for 5 min. The concentration of chlorinated base products 8CldA (B), 5CldC (C), and 8CldG (D) after treatment for 5 min (white), 24 h (hatched), or 1 week (black) was quantified by selective reaction monitoring by LC-MS-MS. * represents significant loss of parent base or chlorinated base formation, compared to that in the non-treated control, determined by one-way ANOVA, with Dunnett's multiple comparison test. Data represent the mean ± SEM of $n = 3$ experiments.

treatment of A7r5 cells with His-C (0.25−1 μmol/10^6 cells) for 4 h (Figure 8A).

The formation of 5CldC was also observed on treating BCASMC with HOCl (0.125−0.5 μmol/10^6 cells) or His-C (0.25−1 μmol/10^6 cells) (Figure 8B).With HOCl, the level of 5CldC observed with BCASMC was somewhat lower than that with the A7r5 cells, whereas the extent of 5CldC formation by His-C was similar for both cell types (Figure 8B). With BCASMC, experiments with HOCl and His-C also resulted in the formation of the chlorinated RNA bases, 5ClC (Figure 8C) and 8ClA (4−50 lesions/10^6 Ado). The formation of chlorinated RNA and DNA bases on treating cells with Mono-C, Gly-C, Lys-C, and Tau-C was not investigated, owing to the lack of reactivity observed in the experiments with isolated DNA.

The potential reactivity of HOCl and *N*-chloramines with cellular ATP was investigated in A7r5 cells by quantifying the concentration of cellular ATP, under conditions where minimal cell lysis was observed. The analogous experiments were not performed with BCASMC owing to the increased susceptibility of these cells to HOCl and His-C mediated lysis (Figure 7C). Treatment of A7r5 cells (50×10^3) with HOCl and *N*-chloramines (0.05−2.0 μmol/10^6 cells) over 2 h resulted in a significant decrease in the concentration of cellular ATP (Figure 9). In each case, the loss of cellular ATP was more significant than that observed in the control experiments with PBS or the respective parent amine (Figure 9). The most rapid loss of ATP was observed on treating the cells with Mono-C, with almost complete loss of the ATP observed on addition of ≥0.2 μmol Mono-C/10^6 cells (Figure 9A). Rapid loss of ATP was also observed with HOCl, Gly-C, and His-C (Figure 9). Interestingly, Lys-C and Tau-C both induced a significant loss of cellular ATP, whereas no evidence for cell lysis was obtained.

Discussion

The aim of this study was to investigate the ability of HOCl and various *N*-chloramines to chlorinate nucleosides, nucleotides, and DNA, as *N*-chloramines are likely to be important mediators of cellular damage under conditions of inflammation, and little is known about their reactivity with DNA and related materials. With the individual nucleosides, 8CldG was the favored product, with reactivity in the order HOCl > His-C ≫ Lys-C ≈ Gly-C > Mono-C ≈ Tau-C. Formation of 5CldC and 8CldA was also observed in experiments with HOCl and His-C. A similar pattern of reactivity of HOCl with nucleosides has been reported previously (*20, 44*). Evidence for the formation of 5CldC, 8CldG, and 8CldA was also obtained on treatment of isolated DNA with HOCl and His-C but not Mono-C, Lys-C, Gly-C, and Tau-C, which reacted very slowly with DNA and gave low yields of 8CldG only.

The extent of conversion of each parent base to the respective chlorinated product in the experiments with HOCl and calf thymus DNA is similar to or greater than that observed previously (*20, 23, 26, 45*). In general, the formation of chlorinated products accounts for <10% of the HOCl consumed by the DNA. Interestingly, the level of chlorinated base generation observed on reaction of His-C with DNA is generally greater than that observed in experiments with HOCl, particularly with higher concentrations of oxidant. This is attributed to the induction of further oxidation of the chlorinated products

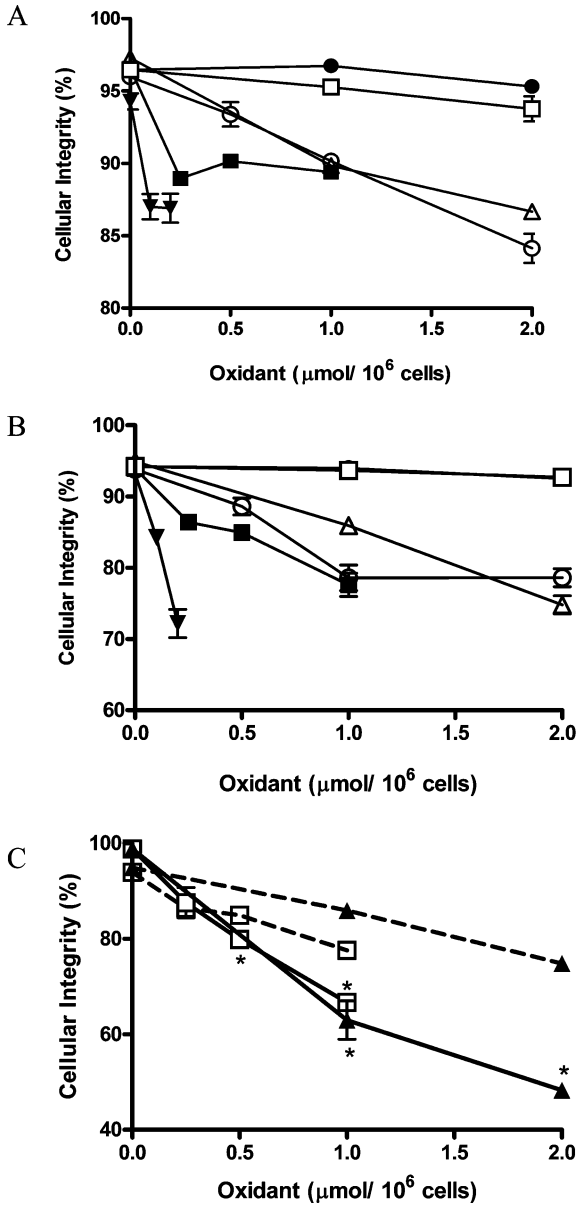

Figure 7. Cell lysis observed in smooth muscle cells treated with HOCl and *N*-chloramines. Graphs A and B show the results of A7r5 cells treated with increasing concentrations of HOCl (■), His-C (△), Mono-C (▲), Gly-C, (○), Lys-C (●), or Tau-C (□) for (A) 2 h or (B) 4 h at 37 °C, with the extent of cell lysis determined by the ethidium bromide DNA release assay. Graph C shows results from BCASMC treated with HOCl (□) and His-C (▲) for 4 h (solid line) and the resulting cell lysis compared to analogous treatment of A7r5 cells (dashed line). Data represent the mean ± SEM of $n \geq 3$ experiments. * represents a significant difference in cellular integrity between A7r5 and BCASMC, using two-way ANOVA with Bonferroni post-test.

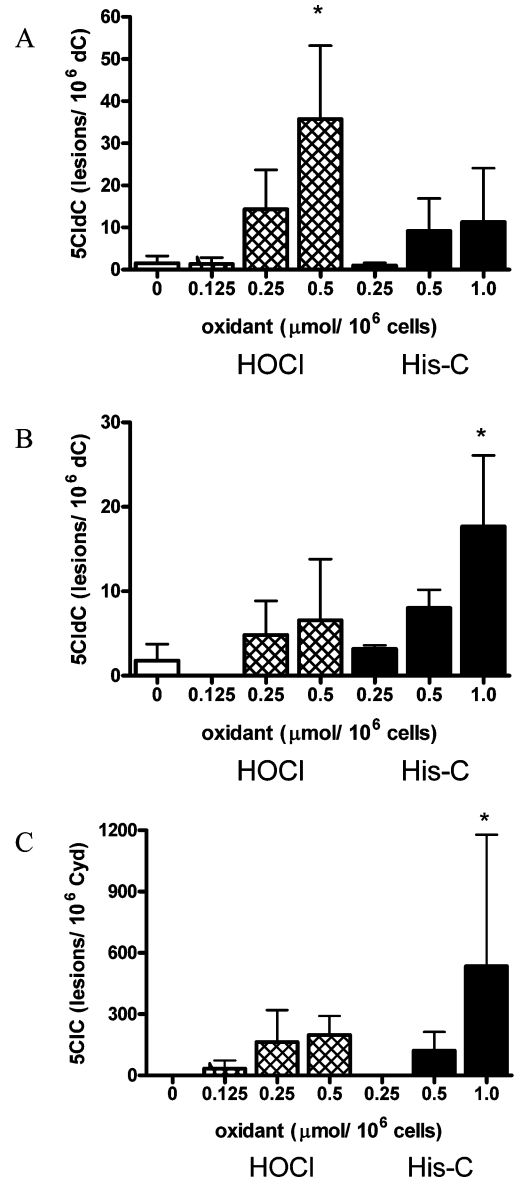

Figure 8. Formation of chlorinated bases on cellular DNA and RNA. Graph A represents A7r5 cells, and graphs B and C represent BCASMC treated with HOCl (0.125−0.5 μmol/10^6 cells; hatched bars) and His-C (0.25−1 μmol/10^6 cells; black bars) in HBSS for 4 h at 37 °C. The cellular DNA and RNA were extracted, hydrolyzed, and dephosphorylated to the individual nucleosides. The concentration of the chlorinated base products was quantified by selective reaction monitoring by LC-MS-MS. A and B show the formation of 5CldC from A7r5 cells and BCASMC, respectively; C shows the formation of 5ClC in BCASMC. * represents significant chlorinated base formation, compared to that in the non-treated control, determined by one-way ANOVA, with Dunnett's multiple comparison test. Data represent the mean ± SEM of $n = 3$ experiments.

with HOCl, as a similar loss of the parent bases is observed in each case. The sensitivity of chlorinated and other modified nucleosides to further oxidation resulting in the loss of these materials has been reported previously (*23, 46, 47*).

Chlorination of DNA (and RNA) was also observed on treatment of A7r5 and BCASMC with HOCl and His-C, with evidence for the formation of 5CldC but not 8CldA or 8CldG, in each case. With the A7r5 cells, the level of 5CldC observed was greater in experiments with HOCl, whereas similar levels of this chlorinated product were observed on exposure of BCASMC to either HOCl or His-C. The reason for this difference is not certain but may be associated with a greater ability of His-C to permeate BCASMC, as this cell type is also more susceptible to His-C induced lysis. The greater oxidation of dC compared to that of dA and dG on exposure of the cells to HOCl agrees with previous data, though the lack of detectable levels of 8CldA and 8CldG was unexpected (*22, 27*). This may be associated with the loss of these materials via further oxidation reactions, owing to the longer incubation conditions and higher oxidant concentrations employed in the current study.

Evidence was also obtained for the formation of 5ClC and 8ClA on exposure of BCASMC to HOCl and His-C. In this case, the concentration of chlorinated RNA products was somewhat higher than the analogous DNA products (ca. 20-fold, with 0.5 μmol HOCl/10^6 cells). This is consistent with previous reports that RNA is more sensitive to modification by

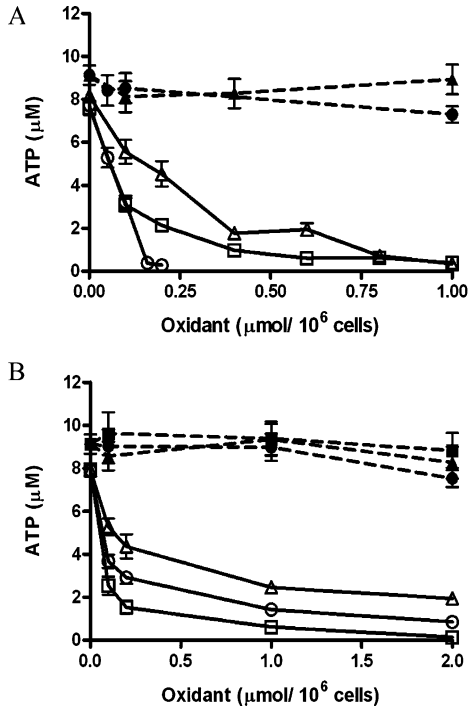

Figure 9. Depletion of ATP in A7r5 cells treated with HOCl and *N*-chloramines. A7r5 cells were treated with (A) increasing concentrations of HOCl (□), His-C (△), and Mono-C (○), and (B) Gly-C (□), Lys-C (△), or Tau-C (○), or the respective parent amine (solid symbols) for 2 h at 37 °C in black 96-well plates. ATP concentration was determined by ATPlite Luminescence ATP Detection Assay System. Data represent the mean ± SEM of $n = 3$ experiments.

oxidants compared to DNA, on account of the RNA being less compartmentalized and mainly cytoplasmic in nature (*27, 48*). Evidence for RNA, but not DNA, chlorination was also observed on exposure of *E. coli* to HOCl generated via the MPO/H_2O_2/Cl^- system (*28*).

These data show that His-C can chlorinate both isolated and cellular DNA to a similar or greater extent than HOCl, suggesting that it may be an important secondary oxidant in MPO oxidant-mediated damage. This supports previous studies demonstrating that His-C and related imidzole-derived *N*-chloramines react readily with other protein components (*49, 50*) and low-molecular mass antioxidants including ascorbate and GSH (*51*). In contrast, Mono-C, Lys-C, Gly-C, and Tau-C are poor chlorinating agents on exposure to both nucleosides and DNA, though there is evidence that Lys-C and Tau-C can mediate the formation of 5ClU (*32*). However, these *N*-chloramines are able to induce cellular damage, as evidenced by their ability to rapidly deplete intracellular ATP. This loss in ATP may be due to the direct reaction of the oxidants with ATP or by inhibition of cellular ATP generation and/or regeneration capability, via the reaction with other targets. The fact that significant ATP depletion and chlorination was only observed on treatment of the isolated material with HOCl and His-C suggests the latter is more likely. Indeed, treatment of *E. coli* with HOCl resulted in the loss of ATPase activity of the membrane-bound F_1 complex of the proton-translocating ATP synthase, the enzyme responsible for reversible energy-linked cellular ATP formation (*52*). Similarly, depletion of ATP is observed on exposure of cells to the non-cell permeable Tau-C and Lys-C, which again may be associated with reactions involving membrane-bound ATPases (*53*).

The consequences of DNA and RNA chlorination on cellular function are not well established. Various cultured mammalian cells can incorporate 5CldC into genomic DNA (*54*). Similarly, 5ClU can be readily converted to 5CldU by thymidine phosphorylase, before incorporation into DNA via the action of DNA polymerase (*55, 56*). 5CldU is also formed from 5CldC via deamination mediated by cellular enzymes (*54*). This is significant because 5CldU is a well-established thymidine analogue mutagen that mispairs with guanosine, causing G•C → A•T and A•T → G•C transitions (*57*), and can induce sister chromatid exchanges (*55, 56*). 5CldC also perturbs epigenetic signals by mimicking 5-methyl-cytosine and enhancing the binding of methyl-CpG binding proteins (*58*). This is significant as methylation of cytosine occurs predominantly in CpG dinucleotides (where a cytosine nucleotide occurs next to a guanine nucleotide in a linear sequence) in islands of concentrated CpG sequences located in promoter regions. Methyl-CpG binding proteins facilitate the recruitment of histone modifying enzymes, which triggers a cascade of events resulting in gene silencing (e.g., of tumor suppressor genes) (*59, 60*). HOCl can preferentially chlorinate cytosine at CpG dinucleotides (*61*). In addition, treatment of cells with 8ClA results in the accumulation of 8-chloro-ATP and incorporation of this modified base into the cellular mRNA (*62*). This leads to the inhibition of mRNA synthesis and eventual cell death via apoptosis (*62*).

In summary, we show that HOCl and His-C are capable of chlorinating DNA and related materials, both in isolation and in a cellular environment. His-C chlorinates DNA to a similar or greater extent than HOCl, supporting a role of imidazole-derived, *N*-chloramines as secondary oxidants. This is significant as His residues are one of the most reactive amino acid side chains and hence are likely to be favored sites for reaction of HOCl under physiological conditions, owing to the abundance of proteins (*14*). The formation of imidazole-derived chloramines on histamine, a low-molecular mass species released by inflammatory mediators (*51*), and/or intracellular proteins, for example, may play a role in the chlorination of genetic material observed under chronic inflammatory conditions.

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