Myeloperoxidase Generates 5-Chlorouracil in Human Atherosclerotic Tissue

A POTENTIAL PATHWAY FOR SOMATIC MUTAGENESIS BY MACROPHAGES*

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Somatic mutations induced by oxidative damage of DNA might play important roles in atherosclerosis. However, the underlying mechanisms remain poorly understood. Myeloperoxidase, a heme protein expressed by select populations of artery wall macrophages, initiates one potentially mutagenic pathway by generating hypochlorous acid. This potent chlorinating agent reacts rapidly with primary amines to yield long-lived, selectively reactive N-chloramines. In the current studies, we demonstrate that myeloperoxidase produced by human macrophages differentiated in the presence of granulocyte macrophage colony-stimulating factor generates 5-chlorouracil, a mutagenic thymine analog. The primary amine taurine fails to block the reaction, suggesting that N-haloamines produced by macrophages might oxidize uracil. Model system studies demonstrated that N-chloramines convert uracil to 5-chlorouracil. Interestingly, the tertiary amine nicotine dramatically enhances uracil chlorination, suggesting that cigarette smoke might promote nucleobase oxidation by N-chloramines. To look for evidence that myeloperoxidase promotes uracil oxidation in vivo, we measured 5-chlorouracil levels in human aortic tissue, using isotope dilution gas chromatography-mass spectrometry. The level of 5-chlorouracil was 10-fold higher in atherosclerotic aortic tissue obtained during vascular surgery than in normal aortic tissue, suggesting that halogenated nucleobases produced by macrophages might contribute to atherosclerosis. Because 5-chlorouracil can be incorporated into nuclear DNA, our observations raise the possibility that halogenation reactions initiated by phagocytes provide one pathway for mutagenesis, phenotypic modulation, and cytotoxicity during atherogenesis.

Oxidative damage is implicated in the pathogenesis of atherosclerosis, a chronic inflammatory disease (1, 2). Moreover, phagocytes, which congregate at sites of inflammation, might be important sources of the oxidants that create such damage (3). One pathway involves myeloperoxidase, a heme protein present at high concentrations in circulating neutrophils and monocytes (3, 4). Myeloperoxidase secreted by phagocytes uses hydrogen peroxide (H2O2) and chloride (Cl−) to generate the powerful oxidant hypochlorous acid (HOCl)3 (5) (Reaction 1).

\[
\text{H}_2\text{O}_2 + \text{Cl}^- + \text{H}^+ \rightarrow \text{HOCl} + \text{H}_2\text{O}
\]

REACTION 1

The importance of this reaction is underscored by the presence of enzymatically active myeloperoxidase and its characteristic products in human atherosclerotic lesions (6–9).

Resident mouse peritoneal macrophages and cultured human macrophages lack myeloperoxidase, suggesting that the enzyme disappears as monocytes differentiate into macrophages (3, 4). However, myeloperoxidase colocalizes in human atherosclerotic lesions with macrophages and is also found in the necrotic core of lesions prone to plaque rupture (10). Moreover, human monocytes exposed to granulocyte macrophage colony-stimulating factor (GM-CSF) continue to express active myeloperoxidase as they differentiate into macrophages in vitro (11). Immunohistochemical studies indicate that GM-CSF associates closely with macrophages expressing myeloperoxidase in human atherosclerotic tissue (11), suggesting that this growth factor might support myeloperoxidase expression in the developing atheroma.

Myeloperoxidase has been proposed to promote one of the earliest cellular events in atherosclerosis (10): conversion of intimal macrophages into lipid-laden foam cells (2, 12). Oxidized low density lipoprotein (LDL) may be involved in this process, because macrophage scavenger receptors take up LDL that has been modified by myeloperoxidase in vitro (13). Moreover, mass spectrometry has detected protein and lipid oxidation products characteristic of myeloperoxidase in LDL isolated from human atherosclerotic lesions (6–9), providing strong evidence that LDL is one of the enzyme’s targets. Myeloperoxidase has also been proposed to play a number of other roles in vascular disease, including the promotion of endothelial dysfunction and reversal of the cardioprotective effects of high density lipoprotein (14–16). In subjects with established coronary artery disease, blood levels of myeloperoxidase are elevated (17), and they predict the risk of myocardial infarction in subjects with unstable angina (18). A promoter polymorphism that lowers myeloperoxidase expression in vitro is associated with a decreased risk of clinical events in patients with coronary artery disease (19). Moreover, myeloperoxidase is taken up by cultured endothelial cells (14) and has been detected in endothelial cells in vivo, suggesting that it could contribute to endothelial dysfunction (20). It might also

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3 The abbreviations used are: HOCl, hypochlorous acid; BrdUrd, 5-bromodeoxyuridine; CldU, 5-chlorodeoxyuridine; DTPA, diethylenetriaminepentaacetic acid; GC, gas chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry; m/z, mass-to-charge ratio; GM-CSF, granulocyte macrophage colony-stimulating factor; LDL, low density lipoprotein; PMA, phorbol 12-myristate 13-acetate.
reverse the cardioprotective effects of high density lipoprotein, because recent evidence suggests that it oxidizes high density lipoprotein in humans (15, 21).

Mouse models have provided important insights into the pathogenesis of atherosclerosis. However, in contrast to human atherosclerotic tissue, macrophages in murine atherosclerotic tissue do not express immunoreactive myeloperoxidase on Western blotting (22). Additionally, only low levels of 3-chlorotyrosine, a characteristic product of myeloperoxidase, are detectable by mass spectrometry in mouse atherosclerotic tissue (22). Importantly, recent studies demonstrate that expression of human myeloperoxidase in macrophages promotes atherosclerosis in mice, providing direct evidence of the enzyme’s causal role in atherogenesis (23).

Conversion of macrophage-rich fatty streaks into clinically significant atherosclerotic lesions might also involve myeloperoxidase. The majority of smooth muscle cells in such lesions appear to be monoclonal, which led Benditt and Benditt (24) to propose that a mutation or viral hit transforms a single, isolated smooth muscle cell into the progenitor of a proliferative clone in the atherosclerotic intima. It is conceivable that DNA damage produced in the chronic inflammatory environment may provide the mutational hit required for such a transformation (25). Indeed, oxidized nucleobases have been detected in human atherosclerotic tissue (26). Moreover, somatic mutations may play other roles in atherogenesis as recent studies have linked chromosomal abnormalities, loss of heterozygosity, and microsatellite instability to human atherosclerosis (reviewed in Ref. 27).

Although it is clear that the potential role of DNA damage in atherosclerosis is an important issue, the sources and molecular mechanisms of such damage are poorly understood. For example, little is known regarding the pathways that promote the oxidation of nucleobases, nucleosides, and nucleic acids in the human artery wall. However, hypochlorous acid (HOCl), a product of myeloperoxidase in vivo, converts nucleobases into a variety of oxygenated and chlorinated species in vitro (28–32). Moreover, 5-chlorouracil, a specific product of the myeloperoxidase system in vitro, has been detected in inflammatory exudates and tissue (28, 33). By damaging DNA and other constituents of host cells, generation of halogenating oxidants by myeloperoxidase might contribute to the association between chronic inflammation and cancer. Indeed, a polymorphism in the myeloperoxidase promoter that leads to the association between chronic inflammation and cancer is strongly associated with 5-chlorouracil in the setting of atherosclerosis.

EXPERIMENTAL PROCEDURES

Materials—Myeloperoxidase (EC 1.11.1.7) was isolated from human neutrophils by sequential lectin affinity and size-exclusion chromatography (40, 41). Enzyme concentration ([A430/A280 > 0.8] was determined spectrophotometrically (ε430 = 178 mM$^{-1}$ cm$^{-1}$) (42). Sodium hypochlorite (NaOCl) was purchased from Fisher, and its concentration was determined spectrophotometrically (ε292 = 350 mM$^{-1}$ cm$^{-1}$) (43). 5-[[13C4,15N2]Bromouracil was synthesized as described previously (44). To prepare isotope-labeled 5-chlorouracil, [13C4,15N2]uracil (1 mM, Cambridge Isotope Laboratories, Inc., Andover, MA) was exposed to 1 mM HOCl for 15 min at 25 °C in 50 mM phosphoric acid and 1 mM NaCl. The reaction was quenched by adding 6 mM L-methionine. 5-[[13C4,15N2]Chlorouracil (isotope enrichment >98%) was isolated by HPLC (28). The synthetic compound had the same HPLC and GC retention times as authentic 5-chlorouracil; its trimethylsilyl derivative exhibited an identical electron ionization mass spectrum, with major fragment masses increased by 6 atomic mass units. All other chemicals were obtained from Sigma, Aldrich, or Fluka unless otherwise noted.

Chloramines Synthesis—The N-chloramines of taurine, glycine, and N4-acetyltyrosine were prepared by dropwise addition of 10 mM HOCl to 5 mM amine at 4 °C (final concentration of 0.91 mol/mol, HOCl/chloramine) at pH 5. Chloramine concentrations were determined with 5-thio-2-nitrobenzoic acid as described (45). Monochloramine and dichloramine concentrations were also determined spectrophotometrically (monochloramine ε352 = 429 mM$^{-1}$ cm$^{-1}$, dichloramine ε300 = 370 mM$^{-1}$ cm$^{-1}$ (36)) and were estimated to represent ~80 and ~20%, respectively, of the oxidizing equivalents initially present at pH 5 in the chloramine mixture. Unless otherwise indicated, all oxidation reactions were carried out at neutral pH by adding known amounts of chloramines to the reaction mixture.

Reaction of Uracil with Chloramines—Reactions were performed in buffer A (100 mM sodium chloride, 100 μM DTPA, and 50 mM sodium phosphate, pH 7.4) supplemented with 1 mM uracil. Reactions were initiated by adding 100 μM N-chloramine and terminated by adding 6 mM L-methionine. The pH of buffer A was varied using phosphoric acid and dibasic sodium phosphate.

Oxidation of Uracil with Myeloperoxidase-derived N4-Chloro-N4-acetyllysine—Reactions were performed in buffer A. N-Chloramine formation was initiated by adding 100 μM H2O2 to 20 μM myeloperoxidase and 200 μM N4-acetyllysine and incubated for 1 h at 37 °C. The concentration of H2O2 was determined spectrophotometrically (ε340 = 39.4 mM$^{-1}$ cm$^{-1}$) (46). Any HOCl remaining after 1 h was quenched by the addition of 200 μM N4-acetyltyrosine. The uracil chlorination reactions were then initiated by adding 1 mM uracil and incubated for 5 h at 37 °C. The reactions were terminated by adding 6 mM L-methionine.

Human Macrophages—The Human Studies Committees at the University of Washington School of Medicine approved all protocols involving human material. Human monocytes were isolated from the blood of fasting donors (47) by density gradient centrifugation, purified by adherence, and cultured in 35-mm plastic dishes (2 × 106 cells/dish, BD Biosciences) in RPMI Medium 1640 (Invitrogen) supplemented with 2 mM l-glutamine (BioWhittaker). Macrophages were cultured for 24 h in Medium 199 (Invitrogen) containing 20% autologous serum or in serum-free medium supplemented with 50 μg/ml recombinant human GM-CSF (R&D Systems).

Oxidation of Uracil by Human Macrophages—Cells were washed twice with phosphate-buffered saline, incubated in buffer B (10 mM sodium phosphate, 100 mM NaCl, 1.4 mM CaCl2, 1.4 mM MgSO4, 2 mM dextrose, and 100 μM DTPA, pH 7) supplemented with 1 mM uracil and, where indicated, activated with 300 nM phorbol 12-myristate 13-acetate...
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for 2 h at 37 °C. DTPA was included in the incubation buffer to inhibit metal-catalyzed reactions (48, 49). The cell medium was then collected and incubated for 18 h at 37 °C. Reactions were terminated by adding 6 mM 1-methionine. Isotope-labeled internal standards (13C4,15N2-labeled uracil, 5-chlorouracil, and 5-bromouracil, and [13C5,15N]uracil for monitoring artifactual halogenation) were added to the medium. Uracils were extracted from the medium with a 4:1 v/v ratio of 2-propanol/diethyl ether (22:78, v/v) (50) to medium, back-extracted with 0.2 M ammonium hydroxide, and concentrated to dryness under nitrogen.

Isolation of Uracils from Medium—A C18 solid-phase extraction column (3 ml, Supelco) was conditioned with 1 volume of methanol followed by equilibration with 5 volumes of 0.1% acetic acid. Dried uracils extracted from conditioned human macrophage medium were dissolved in 0.5 ml of 0.1% acetic acid and applied to the column. The column was washed twice with 1 ml of 0.1% acetic acid to elute uracils. Halogenated uracils were then eluted with three 1-ml washes of 0.1% acetic acid in 10% methanol. Material eluted from the column was collected and dried under nitrogen for GC/MS analysis.

Extraction of Uracils from Tissue—Vascular tissue resected at surgery was immediately placed in ice-cold antioxidant buffer (100 μM DTPA, 1 mM t-butylated hydroxytoluene, 1% (v/v) ethanol, 140 mM NaCl, 10 mM sodium phosphate, pH 7.4), and frozen at −80 °C until analysis. The thawed tissue (−0.5–1.5 g of wet weight) was stripped of the adventitia, cut into small pieces, and homogenized at 4 °C with a mechanical tissue grinder in extraction buffer (10 ml/g tissue, 1 Complete Mini protease inhibitor mixture tablet (Roche Applied Science) per 10 ml, 10 mM azide, 15 mM L-methionine, 100 mM L-butyrolactone toluene, 1 mM phenylethylsulfonyl fluoride, 0.2 M Tris–HCl, pH 6.0). Prior to homogenization, extraction buffer was supplemented with isotope-labeled internal standards (13C4,15N2-labeled uracil, 5-chlorouracil, and 5-bromouracil and [13C5,15N]uracil to monitor ex vivo halogenation). Following the addition of a 4:1 v/v ratio of 2-propanol/diethyl ether (22:78, v/v) (50) to sample, uracils were back-extracted with 0.2 M ammonium hydroxide and fractionated by reversed-phase HPLC.

Reversed-phase HPLC—Chloramine reaction mixtures and uracils extracted from human tissue were eluted from a C18 reversed-phase column (Ultrasphere, 5-μm resin, 4.6×250 mm, Beckman Coulter) by HPLC at 1 ml/min with 95% solvent A (50 mM ammonium acetate) and 5% solvent B (50 mM ammonium acetate in 90% methanol) for 4 min followed by a linear gradient to 100% solvent B over 20 min (51). The yield of 5-chlorouracil from N-chloramine reactions was quantified by comparing integrated peak areas to standard curves generated with commercially available 5-chlorouracil. For mass spectrometric analysis and quantification of uracils extracted from human tissue, uracil, 5-chlorouracil, and 5-bromouracil fractions were collected and concentrated under nitrogen.

Gas Chromatography—Mass Spectrometry—Pentafluorobenzyl derivatives of uracil and halogenated uracil were quantified by isotope dilution GC/MS (52). To analyze the tissue samples, HPLC fractions were suspended in 0.1% acetic acid to elute uracils. Halogenated uracils were then eluted with three 1-ml washes of 0.1% acetic acid in 10% methanol. Material eluted from the column was collected and dried under nitrogen for GC/MS analysis.

Activated Human Macrophages Generate 5-Chlorouracil—To determine whether myeloperoxidase from macrophages chlorinates uracil in vitro, we isolated human monocytes from plasma by density centrifugation, purified by adhesion, and incubated for 24 h in medium supplemented with 20% autologous serum or 50 μg/ml GM-CSF. Macrophages incubated in buffer B supplemented with 1 mM uracil were activated with 300 nM PMA. After a 2-h incubation at 37 °C, the medium was collected and incubated for 18 h at 37 °C. The reaction was terminated by adding 6 mM L-methionine. Where indicated, nicotine (10 μM), colchicine (10 μM), L-methionine (6 mM), Met (10 mM), catalase (10 μg/ml), superoxide dismutase (10 μg/ml), or taurine (50 mM; Taur) was included in buffer B. 5-Chlorouracil was extracted from the medium, isolated using a solid-phase C18 column, converted to its pentafluorobenzyl derivative, and analyzed by isotope dilution negative-ion chemical ionization GC/MS with selected ion monitoring. Shown are selected ion chromatograms of endogenous 5-chlorouracil (4; m/z 325). Note the coelution of the ions of the analytes and internal standards. Results from quantification of 5-chlorouracil (B) are the means ± S.D. of two independent experiments with replicate incubations.

RESULTS

Activated Human Macrophages Generate 5-Chlorouracil—to determine whether myeloperoxidase from macrophages chlorinates uracil in vitro, we isolated human monocytes from plasma by density centrifugation and adhesion, incubated the monocytes for 24 h in medium supplemented with autologous serum or GM-CSF, and then exposed the cells to 1 mM uracil in a physiological buffer at neutral pH. The cells were stimulated with phorbol 12-myristate 13-acetate (PMA). PMA induces phagocytes to secrete myeloperoxidase and activates their membrane-bound NADPH oxidase (3), thus generating superoxide anion, which subsequently dismutates into H2O2, a substrate for myeloperoxidase.

Macrophages stimulated with PMA produced a peak of material that GC/MS identified as 5-chlorouracil by its GC retention time (Fig. 1A), mass spectrum, and isotope pattern of the derivative (data not shown). Formation of 5-chlorouracil required activation of the macrophages with PMA and was inhibited by azide and catalase, implicating a heme lyzer. Ions were monitored in the negative-ion electron capture ionization mode with methane as the reagent gas. Injector and interface temperatures were 280 °C and 290 °C, respectively. The initial GC oven temperature was 170 °C for 1 min, followed by a 10 °C/min increasing ramp to 250 °C. Toluene was injected between samples to ensure that traces of analyte were not being carried over during the analysis.
Because taurine reacts rapidly with HOCl to form N-chlorotaurine, this observation raises the possibility that N-haloamines might promote uracil oxidation by macrophages.

**Chloramines Convert Uracil to S-Chlorouracil**—To determine whether N-chloramines might act as secondary halogenating agents, we chose taurine, glycine, and N-acetyllysinine as model amino acids. Taurine was selected because it may act as a physiological trap for HOCl (38, 57). Glycine was used to mimic N-chloramines derived from α-amino acids, and N-acetyllysinine was used to mimic N-chloramines derived from the side chain of protein-bound lysine. In plasma, glycine is one of the most abundant free amino acids (~175 μM), and total primary amines are present at even higher concentrations (~50 mM, primarily protein-bound) (58), suggesting that reactions executed by any N-chloramine derived from the amino groups of these compounds might be physiologically relevant.

We exposed uracil to 100 μM taurine, glycine, or N-acetyllysinine N-chloramine in physiological buffer at pH 7.4 for 5 h at 37°C. HPLC analysis with monitoring of absorbance at 274 nm revealed a major peak of new material with a retention time and UV-visible absorption spectrum identical to those of authentic 5-chlorouracil (Fig. 2, A and B).

Uracil chlorination by the N-chloramines was linear for the first hour of incubation and then gradually slowed. The overall reaction rate was markedly slower (Fig. 3A) than that observed with reagent HOCl or the complete myeloperoxidase-H₂O₂-Cl⁻ system (28). Uracil chlorination increased with increasing N-chloramine concentration (Fig. 3B). Of the three N-chloramines studied, N¹-chloro-N¹-acetyllysinine produced the highest yield of 5-chlorouracil, followed by N-chloroglycine and N-chlorotaurine, respectively. This pattern agrees with previous studies of the relative reactivity of the N-chloramines with 5-thio-2-nitrobenzoic acid, ascorbate, GSH, and cysteine (59).

To determine if the formation of N-chloramines by myeloperoxidase promotes the formation of 5-chlorouracil, we first exposed N¹-acetyllysinine to the complete myeloperoxidase-H₂O₂-Cl⁻ system in a physiological buffer at pH 7.4. Under these conditions, HOCl should react quantitatively with primary amines to form N-chloramines. Any residual HOCl was then quenched by adding N¹-acetyltyrosine, which reacts
slowly with N-chloramines. Next, 1 mM uracil was added to the
N*-chloro-N*-acetyllysine reaction mixture, and the reaction mixture
was subjected to HPLC following a 5-h incubation at 37 °C. Under these
conditions, we readily detected the generation of 5-chlorouracil (Fig.
4A). Subsequent studies were performed with 10 μM nicotine, a patho-
physiologically plausible concentration that fell in the middle of the
linear portion of the curve. At 10 μM, nicotine increased the yield of
5-chlorouracil produced by the three different N-chloramines from 9-
to 20-fold (Fig. 4A). Nicotine-enhanced chlorination increased with
increasing reaction time (Fig. 4B) and N-chloramine concentration (Fig.
4C). N-Chlorotaurine was, again, the least reactive of the three N-
chloramines, and N*-chlo-ro-N*-acetyllysine and N-chloroglycine were
more reactive. However, unlike the reactions with N-chloramines alone,
the yields of 5-chlorouracil produced by N-chloroglycine and
N*-chloro-N*-acetyllysine were similar to one another in the presence
of nicotine.

To examine the specificity of the action of nicotine, we investigated
the effects of primary (glycine, N*-acetyllysine), secondary (proline),
tertiary (cotine, caffeine, and imipramine), and quaternary (betaine)
amines on the formation of 5-chlorouracil. Histamine, a combination
primary and secondary amine, and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, a possible carcinogen found in cooked meats
and fish that contains a primary, secondary, and tertiary amine (60),
were also examined. Only the tertiary amines nicotine and imipramine (a
dicyclic antidepressant) significantly enhanced uracil chlorination by
N*-chloro-N*-acetyllysine (Fig. 5). N-Chlorotaurine, and N-chlorogly-
cine (data not shown). Importantly, the yield of 5-chlorouracil relative
to N-chloramine was nearly quantitative in the presence of nicotine at
a concentration that was 10-fold lower than that of the N-chloramine,
suggesting that nicotine may catalyze the reaction pathway.

Effects of Antioxidants and Nitrite on the Formation of 5-Chlorouracil by Chloramines and Nicotine—We also investigated the effects of vari-
ous potential scavengers of reactive intermediates on the formation
of 5-chlorouracil by N*-chloro-N*-acetyllysine. When present at the same
concentration as the N-chloramine, ascorbate, N*-acetylcysteine, or
GSH completely inhibited 5-chlorouracil formation as monitored by
HPLC analysis (Table 1). The same compounds also decreased the abso-
late amount of 5-chlorouracil produced in the presence of nicotine
(data not shown), but only GSH completely inhibited uracil chlorina-
tion. The one-electron scavengers trolox and phenol and the hydroxyl
radical scavenger mannitol had virtually no effect on 5-chlorouracil
production with or without nicotine. Interestingly, the N3
anion azide,
slightly enhanced uracil chlorination in the absence of nicotine but had
no effect in its presence. In contrast, nitrite had little effect on chlorina-
tion under either condition. Taurine modestly inhibited chlorination of
uracil by N*-chloro-N*-acetyllysine both with and without nicotine.

**FIGURE 4.** Effect of nicotine on production of 5-chlorouracil by N*-chlorotaurine,
N-chloroglycine, or N*-chloro-N*-acetyllysine. The reaction was initiated by adding
nicotine (10 nmol) and N-chloramine (100 nmol) to 1 ml of buffer A supplemented with
1 mM uracil. The reaction was incubated for 5 h at 37 °C and terminated by adding 6 mM
methionine. Where indicated, the nicotine concentration (A), reaction time (B), or
N-chloramine concentration (C) was varied. S-Clorouracil was quantified by HPLC anal-
ysis. Results represent the means ± S.D. of two independent experiments with duplicate
incubations.

**FIGURE 5.** Effects of amines on the production of 5-chlorouracil by N*-chloro-N*-acetyllysine. The reaction was initiated by adding N*-chloro-N*-acetyllysine (100 nmol)
to 1 ml of buffer A supplemented with 10 μM of the indicated amine and 1 mM uracil. The
reaction mixture was incubated for 5 h at 37 °C. Following the addition of 6 mM L-methi-
onine, levels of 5-chlorouracil were quantified by HPLC analysis. Results represent the
means ± S.D. of two independent experiments with duplicate incubations. *, p < 0.0001;
**, p = 0.0003.
TABLE 1

| Antioxidant       | [5-Chlorouracil] μM (% inhibition) |
|-------------------|-----------------------------------|
| None              | 10.4 ± 0.1 (0)                    |
| Glutathione       | <1.4 (>87)                        |
| Nα-Acetylcysteine | <1.4 (>87)                        |
| Ascorbate         | <1.4 (>87)                        |
| Taurine           | 3.5 ± 1.0 (66)                    |
| Trolox            | 9.4 ± 0.2 (10)                    |
| Mannitol          | 10.1 ± 0.3 (3)                    |
| Phenol            | 10.2 ± 0.2 (2)                    |
| Sodium nitrite    | 11.6 ± 0.2                        |
| Azide             | 14.8 ± 0.1                        |

This observation is consistent with the observed lower reactivity of N-chlorotaurine in our system and the facile ability of active chlorine to exchange between different amino groups (36).

Effects of pH and Chloride Ion on the Formation of 5-Chlorouracil by Chloramines—To better understand how N-chloramines produce 5-chlorouracil, we examined the pH dependence of the reaction. Chlorination in the absence of nicotine was minimal at pH 4–5. Chlorination increased markedly with increasing acidity and modestly toward neutral pH (Fig. 6, A–C). In the presence of nicotine, chlorination was minimal at pH 4 and increased with both increasing and decreasing pH (Fig. 6, D–F). Nicotine only enhanced the chlorination reaction at pH >4.

Our previous work showed that, under acidic conditions, Cl2− markedly enhances chlorination of uracil by HOCl (28), implicating a Cl2−like species in the reaction (Reaction 2).

\[
\text{Cl}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HOCl} + \text{Cl}^- + \text{H}^+ 
\]

To determine if Cl− enhances chlorination by N-chloramines, we examined its effects on the formation of 5-chlorouracil at various pHs. Both in the presence and absence of nicotine, Cl−-promoted uracil chlorination by N-chloramines under acidic conditions (pH < 4) but not at higher pH. This suggests that chlorination by N-chloramines under acidic conditions may also involve a Cl2−-like species. Under more basic conditions, the increased reactivity of N-chloramines with uracil may be attributed to preferential halogenation of the uracil anion (pK_a ~ 9.5) (61).

Human Atherosclerotic Tissue Contains Elevated Levels of 5-Chlorouracil—To explore the possibility that uracil chlorination is relevant to the artery wall in vivo, we measured levels of uracil, 5-chlorouracil, and 5-bromouracil in the intima and media of normal and atherosclerotic human aortic tissue. All atherosclerotic lesions analyzed were intermediate to advanced lesions, and half came from patients suffering from abdominal aortic aneurysm. Both normal (n = 5) and diseased (n = 8) tissue samples contained comparable amounts of uracil (~5–27 nmol of uracil/g of tissue), a substrate for myeloperoxidase chlorination. GC/MS analysis revealed peaks of material that exhibited major ions and retention times identical to those of authentic 5-chlorouracil (Fig. 7A) and 5-bromouracil (data not shown). Selected ion monitoring demonstrated that the ions derived from 5-chlorouracil and 5-bromouracil coeluted with those derived from 5-chloro[13C2,15N2]uracil and 5-bromo[13C2,15N2]uracil, respectively.

To confirm the identity of 5-chlorouracil, we exploited the isotopic distribution of [35Cl and [37Cl. Mass spectra demonstrated the isotopic ratio (~3:1) of the ions at m/z 325 and 327 expected for a monochlorinated compound (Fig. 7B). Similarly, the isotopic distribution of [79Br and [81Br demonstrated the peak area ratio (~1:1) of the ions at m/z 369 and 371 expected for a monobrominated compound (data not shown). The m/z of the material, isotopic patterns, and GC retention times were all consistent with the presence of 5-chlorouracil and 5-bromouracil in atherosclerotic tissue.

To ensure that the 5-chlorouracil or 5-bromouracil we detected in the samples were not generated artifically during sample preparation (62), we added [13C2,15N2]uracil to the tissue samples prior to homogenization. Any uracil that was artifically halogenated during the sample preparation would have been detected as 5-chloro-[13C2,15N2]uracil or 5-bromo-[13C2,15N2]uracil. Quantification of endogenous 5-halouracils was corrected for this small ex vivo component of oxidized nucleobases.

Six of the eight atherosclerotic lesions contained 5-chlorouracil as monitored by isotope dilution GC/MS analysis. The two lesions with undetectable 5-chlorouracil were acellular and calcified advanced lesions. 5-Chlorouracil was undetectable at the limit of detection in all five normal aortic tissue samples analyzed. Quantification by isotope dilution GC/MS with artifact monitoring demonstrated that, on average, 5-chlorouracil levels were 10-fold higher in atherosclerotic lesions than in normal aortic tissue (Fig. 7C). 5-Bromouracil was detected in five of six atherosclerotic lesions and in three of the five normal aortas. However, levels of 5-bromouracil in normal and atherosclerotic tissue did not differ significantly (Fig. 7D). Previous studies have shown that 5-bromouracil can be generated by either eosinophil peroxidase or myeloperoxidase, both of which preferentially brominate uracil at plasma concentrations of halide (100 mM Cl−, 100 μM Br−) (51, 63). Because eosinophil peroxidase is not thought to participate in atherogenesis, these observations suggest that Br− levels in the vascular tissues were not high enough to favor bromination by myeloperoxidase.
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FIGURE 7. Detection of 5-chlorouracil in human atherosclerotic lesions, using negative-ion electron capture GC/MS. A, detection of endogenous 5-chlorouracil (m/z 327), internal standard (m/z 330), and 5-chlorouracil generated ex vivo (m/z 333) by selected ion monitoring. Note that 5-chlorouracil coelutes with the internal standard. B, full scan mass spectrum of the material eluting with the retention time of 5-chlorouracil (4.06 min). Note the prominent (M + 2) isotopic pattern characteristic of a chlorinated compound. C and D, quantification of 5-chlorouracil and 5-bromouracil in human atherosclerotic lesions and normal aortic tissue. *, p < 0.05.

DISCUSSION

Oxidative damage to DNA is implicated in atherogenesis (25–27), but the underlying pathways remain undefined. Our detection of elevated levels of 5-chlorouracil in human atherosclerotic lesions implicates myeloperoxidase as one potential mechanism. In fact, myeloperoxidase colocalizes with macrophages in human atherosclerotic lesions (10). In the current study, we showed that GM-CSF-stimulated human monocyte-derived macrophages chlorinate uracil in vitro. The process required activation of the cells with phorbol ester and was inhibited by azide, catalase, and methionine but not taurine. These observations are consistent with the idea that myeloperoxidase and N-chloramines participate in the chlorination reaction pathway. Collectively, our findings identify macrophages as one potential source for the chlorination of uracil during atherogenesis.

We also demonstrated that long-lived N-chloramines halogenate uracil in vitro and that the N-chloramine reactions required myeloperoxidase- or HOCl-derived N-chloramines. These results were unanticipated, because earlier studies have suggested that N-haloamines are highly selective oxidants with specificity for thiols and thioethers under neutral and slightly alkaline conditions (36, 64, 65), although recent studies suggest that N-chloramines derived from lysine residues can direct the regiospecific chlorination of tyrosines in proteins (15). It is important to note that HOCl reacts rapidly with primary amines to yield N-chloramines and that primary amines on taurine, free amino acids, the N terminus of proteins, and the side chains of lysine residues are abundant in the extracellular environment. Indeed, the total concentration of primary amines in plasma (~50 mM (36, 58)) is roughly 100-fold greater than the combined concentration of ascorbate (~50 µM (66)) and free thiols (~500 µM, primarily albumin-associated (66)), the major scavengers of HOCl (59). These observations suggest that primary amines are likely to be important targets for HOCl generated by myeloperoxidase, generating long-lived N-chloramines that can mediate subsequent chlorinating reactions.

In agreement with previous studies, we found N°-chloro-N°-acetyllysine and N°-chloroglycine to be more potent chlorinating agents than N°-chlorotaurine (36, 59). This is likely due, in part, to the relative stabilities of the N-chloramines themselves. The stabilities of the three N-chloramines at neutral pH as monitored spectrophotometrically (data not shown) were consistent with this proposal. Two factors likely contribute to this stability. First, free amino acids, such as glycine, that possess amino and carboxylic acid groups on the α-carbon readily eliminate HCl from the N-chloramine, forming an imine (67). This unstable intermediate subsequently hydrolyzes to the aldehyde with release of ammonia. Thus, HOCl rapidly converts tyrosine and serine to p-hydroxyphenylacetalddehyde and glycoaldehyde (68), respectively, removing the oxidizing equivalent from the N-Cl bond while forming a reactive carbonyl. Second, the electron-withdrawing effects of the sulfonic acid group should stabilize the N-chloro derivative of taurine, a β-amino acid that does not readily form the imine (67). These factors suggest that N°-chloro-N°-acetyllysine should be a more effective chlorinating agent than N°-chloroglycine or N°-chlorotaurine, which is what we observed when we exposed uracil to the various N-chloramines.

Our in vitro studies focused on halogenation of uracil by myeloperoxidase. However, it is important to note that the 5-chlorouracil we detected in vivo may have been derived from other sources. For example, 5-chlorouracil is readily deaminated both enzymatically and under acidic conditions to form 5-chlorouracil (33, 69, 70). Indeed, previous studies have shown that brominated, chlorinated, or fluorinated deoxyuridine is deaminated to the corresponding deoxyuridine following injection into humans or animals (71, 72). Therefore, the 5-chlorouracil we detected in arterial tissue might be derived in part from 5-chlorouracil (73, 74). Another potential source is phagocytosed cellular debris (28, 33). Neutrophils, monocytes, and macrophages avidly phagocytose invading pathogens and apoptotic cells, and this material is exposed to high local concentrations of H2O2 and myeloperoxidase-derived oxidants in the phagolysosome. In this microenvironment, which is depleted of antioxidants and contains high concentrations of HOCl, 5-chlorouracil might be derived from DNA, RNA, and pyrimidine nucleosides, nucleotides, or bases (33, 74).

Unlike oxidized bases such as 8-hydroxydeoxyguanosine, halogenated pyrimidines can be taken up by cells and then erroneously used for DNA synthesis (75, 76). The best-known base analog mutagen is 5-bromodeoxyuridine (BrdUrd), a thymidine analog. 5-Chlorodeoxyuridine (ClUdU) is also a thymidine analog, because the halide group mimics the 5-methyl group of the thymine ring. Thymine normally forms a base pair with adenine in double-stranded DNA. However, the electron-withdrawing effect of 5-halo-pyrimidines increases the likelihood that a tautomer and/or anion will form (61). As a result, thymidine
analogs can pair with guanine, creating transition mutations in the new DNA.

After 5-chlorouracil and 5-bromouracil are taken up by mammalian cells and tissues, they are converted to their corresponding deoxynucleosides by thymidine phosphorylase (77, 78). DNA polymerase then incorporates the resulting CldU and BrdUrd into DNA (75, 76). MS, flow cytometric analysis, and immunohistochemical studies revealed that 5-bromouracil is incorporated into the DNA of dividing mammalian cells exposed to 5-BrdUrd produced by eosinophil peroxidase (51). Thus, our detection of halogenated uracil in atherosclerotic lesions may be significant, because 5-CldU and 5-BrdUrd can induce sister chromatid exchanges (79 – 81) and mispair with guanine, causing GC to AT and GC to CT transition mutations (61).

In light of these findings, it is possible that halogenated uracils produced by macrophages are incorporated into the DNA of endothelial cells, smooth muscle cells, and T lymphocytes, and perhaps macrophages themselves, in atherosclerotic intima, and possibly even in the smooth muscle cells in the media, where they contribute to atherogenesis via mutational changes. This is of interest, because myeloperoxidase and apoptotic macrophages colocalize with ruptured plaques in humans (10, 11). The death of macrophages has also been hypothesized to promote the formation of the necrotic core (82), and myeloperoxidase is present at high concentrations in this location (10). Smooth muscle cell apoptosis has been suggested to promote plaque rupture (83), thrombosis (84), and monocyte recruitment (85). Thus, nucleo-base halogenation by macrophages might promote atherogenesis in several ways.

The deoxynucleosides BrdUrd and CldU have been implicated in regulating gene expression in various mammalian cell types. For example, BrdUrd prevents differentiation of a myoblast cell line by blocking the expression of the myogenic determination gene, MyoD1 (86). BrdUrd and CldU have also been found to induce cellular senescence in mammalian cells, where CldU changes expression of nuclear matrix proteins (87, 88). It is therefore conceivable that 5-chlorouracil, once converted to CldU, could alter the gene expression patterns of various cellular components of the atheroma. One potential mechanism for phenotypic modulation of smooth muscle cells could involve alterations of gene expression patterns by halogenated uracils that may or may not require incorporation of the base analogs into DNA.

Previous work has shown that tertiary amines such as nicotine, trimethylamine, and quinine enhance chlorination of various biological substrates by HOCl (29, 30, 55, 56). Cigarette smoke, a major risk factor for accelerated atherosclerosis and sudden death, contains high concentrations of nicotine, and in the current study this tertiary amine markedly enhanced uracil chlorination by macrophages, the myeloperoxidase-H2O2-Cl− system, and N-chloramines. It has been proposed that tertiary amines react with HOCl to form quaternary chlorammonium ions (R3N+Cl−) that subsequently chlorinate nucleobases (30, 89, 90). Our results suggest that nicotine reacts in a similar manner with N-chloramines to enhance uracil chlorination.

Our observations of enhanced 5-chlorouracil formation by N-chloramines in the presence of 10 μM nicotine could be pathophysiologically relevant, because nicotine concentrations in the plasma of active and passive smokers range from 10−6 to 10−8 μM (91). It is likely that nicotine concentrations in the respiratory tract reach even higher concentrations (92).

Cigarette smoking is a well known risk factor for heart disease (1), but its exact role in atherogenesis remains unknown. Given our findings, it is possible that nicotine from cigarettes enhances DNA damage in the inflammatory milieu of the developing atheroma. It is noteworthy that DNA damage is also implicated in oncogenesis and that polymorphisms in the myeloperoxidase gene associate strongly with smokers’ risk of lung cancer (34, 35, 93). Thus, our findings may have important implications for other chronic inflammatory diseases such as lung cancer, chronic obstructive pulmonary disease, and Crohn’s disease, for which cigarette smoking is a major risk factor.

Our detection of elevated levels of 5-chlorouracil in human atherosclerotic lesions implicates myeloperoxidase as one mechanism for oxidizing nucleobases during atherogenesis. We also demonstrated that myeloperoxidase-expressing macrophages chlorinate uracil. These findings suggest that myeloperoxidase-derived macrophages is a potential source of the elevated level of 5-chlorouracil we detected in human atherosclerotic lesions and that a pathway involving N-chloramines may be involved. 5-Chlorouracil, in turn, may promote atherogenesis by a number of mechanisms, including mutagenesis, cytotoxicity, and phenotypic modulation.

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