Tyrosine 192 in Apolipoprotein A-I Is the Major Site of Nitration and Chlorination by Myeloperoxidase, but Only Chlorination Markedly Impairs ABCA1-dependent Cholesterol Transport*

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High density lipoprotein (HDL) isolated from human atherosclerotic lesions and the blood of patients with established coronary artery disease contains elevated levels of 3-nitrotyrosine and 3-chlorotyrosine. Myeloperoxidase (MPO) is the only known source of 3-chlorotyrosine in humans, indicating that MPO oxidizes HDL in vivo. In the current studies, we used tandem mass spectrometry to identify the major sites of tyrosine oxidation when lipid-free apolipoprotein A-I (apoA-I), the major protein of HDL, was exposed to MPO or peroxyxinitrite (ONOO\(^-\)). Tyrosine 192 was the predominant site of both nitration and chlorination by MPO and was also the major site of nitration by ONOO\(^-\). Electron paramagnetic spin resonance studies of spin-labeled apoA-I revealed that residue 192 was located in an unusually hydrophilic environment. Moreover, the environment of residue 192 became much more hydrophobic when apoA-I was incorporated into discoidal HDL, and Tyr\(^{192}\) residue 192 became much more hydrophobic when hydrophilic environment. Moreover, the environment of residue 192 was located in an unusually hydrophilic environment. Furthermore, the environment of residue 192 became much more hydrophobic when apoA-I was incorporated into discoidal HDL, and Tyr\(^{192}\) of HDL-associated apoA-I was a poor substrate for nitration by both myeloperoxidase and ONOO\(^-\), suggesting that solvent accessibility accounted in part for the reactivity of Tyr\(^{192}\). The ability of lipid-free apoA-I to facilitate ATP-binding cassette transporter A1 cholesterol transport was greatly reduced after chlorination by MPO. Loss of activity occurred in concert with chlorination of Tyr\(^{192}\). Both ONOO\(^-\) and MPO nitrotyrinated Tyr\(^{192}\) in high yield, but unlike chlorination, nitration minimally affected the ability of apoA-I to promote cholesterol efflux from cells. Our results indicate that Tyr\(^{192}\) is the predominant site of nitration and chlorination when MPO or ONOO\(^-\) oxidizes lipid-free apoA-I but that only chlorination markedly reduces the cholesterol efflux activity of apoA-I. This impaired biological activity of chlorinated apoA-I suggests that MPO-mediated oxidation of HDL might contribute to the link between inflammation and cardiovascular disease.

Many lines of evidence indicate that high density lipoprotein (HDL) protects the artery wall from atherosclerosis. One important pathway involves HDL apolipoproteins that remove cellular cholesterol and phospholipids by an active transport process mediated by ATP-binding cassette transporter A1 (ABCA1) (1–5). Lipid-laden macrophages represent the cellular hallmark of the atherosclerotic lesion, and ABCA1 plays a critical role in removing cholesterol from macrophages in vivo (1–5). The most abundant apolipoprotein in HDL is apolipoprotein A-I (apoA-I), which accounts for 70% of the total protein content. Lipid-poor apoA-I promotes cellular efflux of cholesterol and phospholipids exclusively by the ABCA1 pathway (1–5).

Several other mechanisms, including the ability of HDL to inhibit low density lipoprotein oxidation, reduce lipid hydroperoxides, and transport oxidized lipids to the liver for excretion, may also be cardioprotective (6–14). Methionine and phenylalanine residues in apoA-I are oxidized by reactive intermediates (8–10, 15–17), but it is unclear if oxidation of these residues affects the ability of the apolipoprotein to remove cholesterol from cells. Whether specific tyrosine residues in apoA-I are also vulnerable to oxidation in vivo is unclear, although its tyrosine residues are readily converted to \(o\),\(^\prime\)-dityrosine by tyrosyl radical in vitro (7).

Macrophages might be an important source of oxidants that damage HDL. One pathway involves myeloperoxidase, a phagocyte heme protein that is expressed at high levels in human atherosclerotic tissue (19, 20). The enzyme uses hydrogen peroxide (\(\text{H}_2\text{O}_2\)) for oxidative reactions in the extracellular milieu (21–24). The major end product at plasma concentrations of chloride ion (\(\text{Cl}^-\)) is generally thought to be hypochlorous acid (HOCl).

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

**REACTION 1**

LDL isolated from human atherosclerotic lesions contains elevated levels of 3-chlorotyrosine, a product characteristic of HOCl (25–27), indicating that myeloperoxidase is one pathway for oxidizing lipoproteins in the human artery wall.

Myeloperoxidase can also produce nitrogen dioxide radical (\(\text{NO}_2\)), a reactive species that converts tyrosine to 3-nitrotyrosine (28–30). We have recently shown that myeloperoxidase nitrates HDL in vitro (31). The reaction probably involves HDL apolipoproteins that remove cellular cholesterol and phospholipids by an active transport process mediated by ATP-binding cassette transporter A1; apoA-I, apolipoprotein A-I; CIY, chlorotyrosine; DTPA, diethylenetriaminepentaacetic acid; ESI, electrospray ionization; MS, mass spectrometry; NO\(_2\), nitrotyrosine; SDSL-EPR, site-directed spin label electron paramagnetic resonance spectroscopy; HPLC, high pressure liquid chromatography; LC, liquid chromatography; BHK, baby hamster kidney.
volves direct one-electron oxidation of nitrite (NO$_2^-$) by compound I, a complex of myeloperoxidase and H$_2$O$_2$.

$$\text{NO}_2^- + \text{compound I} \rightarrow \text{NO}_3^- + \text{compound II}$$

**REACTION 2**

LDL isolated from atherosclerotic lesions is enriched in 3-nitrotyrosine (32), indicating that myeloperoxidase or other pathways that generate reactive nitrogen species also operate in the atherosclerotic artery wall.

Peroxynitrite (ONOO$^-$) is another nitrating species that might be important in oxidizing lipoproteins during inflammation (18, 33, 34). It is generated when nitric oxide (NO) reacts with superoxide (O$_2^-$).

$$\text{O}_2^- + \text{NO} \rightarrow \text{ONOO}^-$$

**REACTION 3**

Like nitrogen dioxide radical, ONOO$^-$ generates 3-nitrotyrosine when it reacts with tyrosine residues.

We have shown that Tyr$^{192}$ is the single major site of chlorination in apoA-I of HDL exposed to HOCl in vitro (35). Tyr$^{192}$ resides in an YXXX motif, and molecular modeling demonstrates that these tyrosine and lysine residues are adjacent on the same face of an amphipathic $\alpha$-helix in apoA-I. HOCl reacts rapidly with the $\epsilon$ amino group of lysine to form long lived chloramines (23, 24, 35). Studies with synthetic peptides demonstrate that lysine residues can direct the regiospecific chlorination of tyrosine residues by a reaction pathway involving chloramine formation, suggesting that the site-specific chlorination of apoA-I requires the participation of a nearby lysine residue (35).

Recent studies demonstrate that HDL is chlorinated and nitrated in human atherosclerotic lesions (31, 36, 37), indicating that myeloperoxidase is one pathway for oxidizing HDL in vivo. Tandem mass spectrometric (MS) analysis identified myeloperoxidase as a component of lesion HDL (36), suggesting that the enzyme and lipoprotein interact in the artery wall.

Moreover, 3-chlorotyrosine and 3-nitrotyrosine were also detected in circulating HDL (31, 36, 37). Levels of these oxidized amino acids were elevated in HDL isolated from the blood of humans with established coronary artery disease, raising the possibility that circulating levels of chlorinated and nitrated HDL represent a novel marker for clinically significant atherosclerosis (31, 36, 37). Tyrosine chlorination and nitration may be physiologically significant because HDL or apoA-I exposed to HOCl or the myeloperoxidase system is less able to remove cholesterol from cultured cells by the ABCA1 pathway (36, 37).

Remarkably little is known about the factors that control the site-specific nitration and chlorination of tyrosine residues in proteins. In the current study, we use apoA-I, synthetic peptides, and tandem mass spectrometric analysis to investigate the role of reactive nitrogen and chlorine species in modifying apoA-I and altering its ability to remove cholesterol from cells. Our observations indicate that Tyr$^{192}$ is the predominant site of nitration as well as chlorination in apoA-I. However, only chlorination of apoA-I markedly impairs ABCA1-dependent cholesterol transport by the oxidized apolipoprotein.

**EXPERIMENTAL PROCEDURES**

**Materials**

Myeloperoxidase (donor:hydrogen peroxide, oxidoeductase; EC 1.11.1.7) was isolated by lectin affinity and size exclusion chromatographies from human neutrophils (33, 38) and stored at 20 °C. Purified enzyme had an A$_{450}$/A$_{280}$ ratio of 0.8 and was apparently homogeneous on SDS-PAGE analysis; its concentration was determined spectrophotometrically ($\epsilon_{450} = 0.17 \text{ m}^{-1} \text{ cm}^{-1}$) (40). Sodium hypochlorite (NaOCl), trifluoroacetic acid, acetonitrile (CH$_3$CN), and methanol were obtained from Fisher. All organic solvents were HPLC grade. Peptides Ac-GYKRAFE (YKXX), Ac-GEYARKY (YXXKY), and Ac-GEYAREY (YXY-XXX) were prepared by the Protein and Nucleic Acid Chemistry Laboratory, Washington University (St. Louis, MO). Purity of the peptides was confirmed by HPLC and mass spectrometric analysis.

**Methods**

**HDL and ApoA-I Isolation**—Blood collected from healthy adults who had fasted overnight was anticoagulated with EDTA to produce plasma. HDL (density 1.125–1.210 g/ml) was prepared from plasma by sequential ultracentrifugation and was depleted of apolipoprotein E and apolipoprotein B100 by heparin-agarose chromatography (41). ApoA-I was purified to apparent homogeneity from HDL (41). Protein was determined using the Lowry assay (Bio-Rad) with albumin as the standard.

**Oxidation Reactions**—Reactions were carried out at 37 °C in phosphate buffer (20 mM sodium phosphate, 100 mM diethylentriaminepentaacetic acid (DTPA), pH 7.4) containing 5 μM apoA-I. For the myeloperoxidase-H$_2$O$_2$-nitrite system, the reaction mixture was supplemented with 50 nM myeloperoxidase, 100 μM nitrite, and the indicated concentration of H$_2$O$_2$. For the myeloperoxidase-H$_2$O$_2$-chloride system, the reaction mixture was supplemented with 50 nM myeloperoxidase and 100 mM NaCl. Reactions were initiated by adding oxidant and terminated by adding 25 mM methionine. ONOO$^-$ was synthesized from nitrite and H$_2$O$_2$ under acidic conditions, and peroxynitrous acid was stabilized by rapidly quenching the reaction with an excess of sodium hydroxide (42). Concentrations of ONOO$^-$, HOCl, and H$_2$O$_2$ were determined spectrophotometrically ($\epsilon_{390} = 1670 \text{ m}^{-1} \text{ cm}^{-1}$, $\epsilon_{290} = 350 \text{ m}^{-1} \text{ cm}^{-1}$, and $\epsilon_{500} = 39.4 \text{ m}^{-1} \text{ cm}^{-1}$, respectively) (42–44).

Carbon dioxide reacts rapidly with ONOO$^-$ to form ONOOHCO$_2$, whose reactivity differs from that of ONOO$^-$ (45). In preliminary experiments, we determined that adding 25 mM NaHCO$_3$ to the reaction mixture failed to alter yields of oxidized amino acids in apoA-I when ONOO$^-$ was the reactant. This probably reflects the presence of bicarbonate in the phosphate buffer used for oxidation reactions.

**HPLC Analysis of Peptides**—Peptides were separated at a flow rate of 0.5 ml/min on a reverse-phase column (Vydac C18 MS, 4.6 × 250 mm) using a Beckman HPLC system (Fullerton, CA) with UV detection at 290 nm. The peptides were eluted using a gradient of solvent A (0.06% trifluoroacetic acid in H$_2$O) and solvent B (0.05% trifluoroacetic acid in 90% CH$_3$CN, 10% H$_2$O). Solvent B was increased from 10 to 45% over 50 min.

**Protolytic Digestion of Proteins**—Native or oxidized apoA-I was incubated overnight at 37 °C with sequencing grade modified trypsin (Promega, Madison, WI) at a ratio of 25:1 (w/w protein/peptide) and analyzed by HPLC-ESI-MS (49). Synthetic or tryptic digest peptides were separated at a flow rate of 0.2 ml/min on a reverse-phase column (Vydac C18 MS; 2.1 × 250 mm) using a gradient of solvent A (0.2% trifluoroacetic acid in H$_2$O) and solvent B (0.05% trifluoroacetic acid in 90% CH$_3$CN, 10% H$_2$O). Solvent B was increased to 2% for 5 min, increased to 10% in 1 min, and then increased to 35% over 36 min for synthetic peptides or to 45% over 66 min for tryptic digest peptides from apoA-I. The electrospray needle was held at 4500 V. Nitrogen, the sheath gas, was set at 80 units. The collision gas was helium. The temperature of the heated capillary was 220 °C.

**Production of Recombinant Human ApoA-I**—Individual Cys substitution mutations within apoA-I cDNA were created by primer-directed PCR mutagenesis or the Mega-Primer PCR method (48). Mutations were verified by dideoxy automated fluorescent sequencing. ApoA-I was expressed using the pET-29b (Novagen, Inc., Madison, WI)-based vector pNFXex in Escherichia coli strain BL21(DE-3) pLyS3 and isolated with a His-Trap chelating column (Amersham Biosciences) (49). During the isolation procedure, expressed proteins were maintained in 3 mM guanidine hydrochloride, 20 mM phosphate, 0.5 M NaCl, and pH 7.4. Eluted protein was dialyzed extensively against Tris-buffered saline (150 mM NaCl, 20 mM Tris, pH 8) supplemented with 1 mM benzamidine and 1 mM EDTA and then filter-sterilized. ApoA-I preparations contained no detectable phospholipid.

**Site-directed Spin Labeling of ApoA-I with Methionine Thiosulfonate**—ApoA-I labeling was performed with 5 mg of cysteine-substituted apoA-I, as described (50). ApoA-I was loaded onto a 1-mL His-Trap chelating column preloaded with 0.1 M NiSO$_4$, extensively washed, derivatized on column with methionine thiosulfonate, and eluted. The
We previously showed that Tyr192 in apoA-I is a nonpolar relaxer (20 mM chromium oxalate or oxygen in equilibrium with atmospheric levels, respectively) was measured at room temperature (54). Oxidation reactions were carried out in the presence of chloride ion (to prevent chlorination reactions) and at neutral pH in phosphate buffer containing the metal chelator DTPA. We used various ratios of oxidant (H₂O₂ or ONOO⁻, mol/mol, oxidant/apoA-I) for 60 min at 37 °C and then terminated the reaction with a molar excess (relative to oxidant) of methionine. Because apoA-I contains 7 tyrosine residues and 243 amino acids, a 10:1 ratio of oxidant to apoA-I gave a 1:1 ratio of oxidant to tyrosine residues and a 1:24 ratio of oxidant to total amino acids.

We first showed that LC-ESI-MS analysis of the tryptic digest of native apoA-I detected peptides that collectively covered ~80% of the protein’s sequence and included all seven peptides predicted to contain tyrosine. To determine which tyrosine residues had been nitrated, we used reconstructed ion chromatograms to detect (i) each of the peptides that contained tyrosine and (ii) any tyrosine-containing peptides that had gained 45 atomic mass units (addition of one NO₂ group and loss of one hydrogen). We quantified product yields using the ion current of each precursor and product peptide and reconstructed ion chromatograms.

LC-ESI-MS and MS/MS analysis of the tryptic digest of oxidized apoA-I detected seven peptides whose mass corresponded to the mass of the oxidized peptide plus 45 atomic mass units, suggesting the formation of nitrated amino acids. We also detected one peptide that had gained 61 atomic mass units, suggesting the addition of both a nitro group and an oxygen atom (45 + 16 atomic mass units). Using LC-ESI-MS/MS analysis, we confirmed each peptide’s sequence (Table I) and showed that its tyrosine had been targeted for nitration (Fig. 1; peptide LAEYHAK containing Tyr192). A methionine-containing peptide (WQEEMELYR) had an oxygen on its methionine residue (Met + 16) and had been nitrated on its tyrosine residue (Tyr + 45). No peptides containing 3-nitrotyrosine together with an unoxidized methionine residue were identified with the MPO system. However, we did detect WQEEMELI(NO₂)YR when apoA-I was exposed to reagent ONOO⁻.

When apoA-I was exposed to either the myeloperoxidase-H₂O₂-nitrite system or reagent ONOO⁻ (Fig. 2), the predominant tyrosine oxidation product was LAE(NO₂)YHAK. For the myeloperoxidase-H₂O₂-nitrite system, nitration of Tyr192 was optimal at a molar ratio of 10:1 (50 μM H₂O₂) of oxidant to apoA-I (Fig. 2A). Approximately 50% of Tyr192 was nitrated under those conditions; the yield of 3-nitrotyrosine decreased at lower or higher concentrations of H₂O₂. For ONOO⁻, nitration increased in a hyperbolic manner with increasing concentra-
tions of oxidant (Fig. 2B). At a molar ratio of 5:1 or 50:1 (25 or 250 μM ONOO⁻) of oxidant to apoA-I, ~40% or >90% of Tyr₁⁹² residues were nitrated, respectively. When ~50% of the Tyr₁⁹² residues were nitrated by ONOO⁻ (10:1, mol/mol, oxidant/protein) for 60 min at 37 °C in phosphate buffer (20 mM sodium phosphate, 100 μM DTPA, pH 7.4) supplemented with 50 mM myeloperoxidase and 100 μM nitrite, after the reaction was terminated with l-methionine, apoA-I was digested with trypsin, and the peptides were analyzed with LC-ESI-MS/MS.

Myeloperoxidase Preferentially Chlorinates Tyrosine 192 in Lipid-free ApoA-I—To determine whether Tyr₁⁹² is the major site of chlorination when the myeloperoxidase system oxidizes apoA-I, we exposed the lipid-free apolipoprotein to myeloperoxidase, chloride ion, and various ratios of oxidant (mol/mol, H₂O₂/apoA-I) for 60 min at 37 °C and then terminated the reaction by adding 2.5 mM methionine. To prevent nitration, all reactions were carried out in buffer that lacked nitrite.

LC-ESI-MS and MS/MS analyses of a tryptic digest of the oxidized protein confirmed that Tyr₁⁹² was the predominant site of chlorination by the myeloperoxidase-H₂O₂-chloride system (Fig. 3A). A low level of chlorination was also observed at Tyr₂⁹, Tyr₁₁₅, Tyr₁₆₆, and Tyr₂₃₆. The patterns for tyrosine chlorination by the enzymatic system and HOCl were similar (Fig. 3, compare A and B). Chlorination of ~50% of Tyr₁⁹² required a 50:1 ratio (mol/mol) of oxidant to protein for the enzymatic system or reagent HOCl. These findings indicate that Tyr₁⁹² in peptide LAEYHAK is the major chlorination site in apoA-I by the myeloperoxidase-H₂O₂-chloride system. Moreover, chlorination of apoA-I tyrosine residues by myeloperoxidase is much more selective than nitration.

Oxidation by Reactive Nitrogen or Chlorine Changes the Apparent Mr of ApoA-I—Analytical SDS-PAGE under reducing conditions detected proteins of increased apparent Mr in ApoA-I that had been modified by either the myeloperoxidase-H₂O₂-nitrite system or the myeloperoxidase-H₂O₂-chloride system (Fig. 4A). A broad band of material (45–60 kDa) was apparent in apoA-I that had been exposed to the myeloperoxidase-H₂O₂-chloride system. Species with modestly increased or decreased apparent mass of ~28 kDa were also observed when apoA-I was exposed to nitrating or chlorinating species generated by myeloperoxidase (Fig. 4A), perhaps due to conforma-
Nitration and Chlorination of Tyrosine Residues in ApoA-I

Reagent ONOO\(^{-}\)/H\(_{2}\)O\(_{2}\) yielded apoA-I species with apparent molecular mass values of 60 kDa, which were similar to those observed in apoA-I exposed to reactive nitrating species generated by the enzymatic systems (Fig. 4A). Reagent HOCl, but not ONOO\(^{-}\)/H\(_{2}\)O\(_{2}\), produced a broad band of material (45–60 kDa) and apoA-I species with a modest increase or decrease in apparent molecular mass (Fig. 4B). These observations suggest that reactive nitrogen and chlorine species can induce conformational changes and aggregation or cross-linking of apoA-I.

Tyrosine 192 of Lipid-free ApoA-I Resides in a Hydrophilic Environment—The aromatic ring of tyrosine’s side chain makes the amino acid residue strongly hydrophobic, but the polar hydroxyl group can form strong hydrogen bonds with water and other polar molecules. Tyrosine can thus inhabit both hydrophobic and hydrophilic environments. To investigate the hydrophobicity of the local environments of tyrosine residues in apoA-I, we used site-directed spin label EPR spectroscopy (SDSL-EPR) (50). To incorporate a spin label at a particular site of interest, we took advantage of the absence of endogenous cysteines in apoA-I and the sulfhydryl-specific reactivity of methionine thiosulfonate. The apoA-I cysteine substitutions Y18C, Y29C, Y115C, Y166C, Y192C, and Y236C were bacterially expressed, purified, and labeled as described (50). By examining the accessibility of the spin-labeled sites to polar or nonpolar relaxers (chromium and oxygen, respectively), we quantified the hydrophobicity of the environment surrounding the examined residues, as represented by the contrast function \(\Phi\).

The contrast function \(\Phi = \ln(\text{collision frequency}_{\text{nonpolar}}/\text{collision frequency}_{\text{polar}})\) provides a measure of the hydrophobicity of the local environment of the labeled side chain (50). We determined \(\Phi\) for each derivatized apoA-I species under lipid-free conditions. Of the tyrosines examined, position 192 was the most accessible to the polar relaxer, with a \(\Phi\) value of –3.08 (Fig. 5). This low value indicates that position 192 is fully exposed to the aqueous environment. In addition, we examined the solvent accessibility of the three lysine residues (Lys\(^{119}\),

Fig. 3. Identification of chlorinated tyrosine residues in lipid-free apoA-I exposed to the myeloperoxidase-H\(_{2}\)O\(_{2}\)-chloride system or reagent HOCl. ApoA-I (5 \(\mu\)M) was exposed to the myeloperoxidase-H\(_{2}\)O\(_{2}\)-chloride system or HOCl at the indicated molar ratio of oxidant/protein for 60 min at 37 °C in phosphate buffer. The myeloperoxidase system was supplemented with 100 mM NaCl. A tryptic digest of apoA-I was analyzed by LC-ESI-MS and MS/MS. Chlorinated peptides were detected and quantified using reconstructed ion chromatograms of precursor and product peptides. Peptide sequences were confirmed using MS/MS. Results are representative of those from two independent experiments.

Fig. 4. SDS-PAGE of lipid-free apoA-I modified by reactive nitrogen and chlorine species. ApoA-I (5 \(\mu\)M for MPO and 25 \(\mu\)M for reagent HOCl or ONOO\(^{-}\)) was exposed to the myeloperoxidase-H\(_{2}\)O\(_{2}\)-chloride system (MPO-Cl\(_{\text{aq}}\)), the myeloperoxidase-nitrite system (MPO-NO\(_{2}\)), ONOO\(^{-}\), or HOCl at the indicated molar ratio of oxidant/protein for 60 min at 37 °C in phosphate buffer. The myeloperoxidase-nitrite system and myeloperoxidase-chloride system were supplemented with 100 \(\mu\)M nitrite and 100 mM NaCl, respectively. After the reaction was terminated with methionine, apoA-I modified by the indicated conditions was subjected to electrophoresis under denaturing and reducing conditions.
Lys195, and Lys238) that are situated within the YXXK motif of apoA-I (35). Position 195 was in the most hydrophilic environment, having a $\Phi$ value of 3.93, consistent with a residue fully exposed to solvent. The positions of the other lysine residues had significantly higher $\Phi$ values ($\Phi$ 1.67 for residue 119 and 1.92 for residue 238). Thus, both Tyr192 and Lys195 are highly exposed to the aqueous environment in lipid-free apoA-I.

To evaluate the effect of lipidation on the local environment of the tyrosine residues in apoA-I, we prepared protein-lipid (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) complexes by the cholate dialysis method (51, 52), which were isolated by ultracentrifugation and analyzed by SDSL-EPR spectroscopy (Fig. 5). In lipid-bound apoA-I, the $\Phi$ value for position 192 was significantly higher ($\Phi$ 1.13) than in the lipid-free counterpart, indicating that this residue resides in a markedly more nonpolar environment upon lipid association. Lipidation also greatly increased the hydrophobicity of the local environment around Tyr192 ($\Phi$ of ~0.80). These observations strongly suggest that the lipid association of apoA-I renders Tyr192 and Lys195 less accessible to aqueous solvent.

Tyrosine 192 in ApoA-I of HDL Is Resistant to Nitration but not Chlorination—Accessibility to solvent may be one important factor controlling the susceptibility of tyrosine residues to nitration (57). Our SDSL-EPR studies suggested that lipid association greatly increases the hydrophobicity of the local environment around Tyr192. Thus, the residue is likely to become much less accessible to aqueous phase reactive intermediates. To determine whether this process might affect the susceptibility of Tyr192 to nitration or chlorination, we exposed plasma isolated HDL to myeloperoxidase or ONOO$^-$, maintaining the ratio of oxidant to apoA-I (mol/mol) at 25:1 for chlorination and 10:1 for nitration. The myeloperoxidase system was supplemented with 100 mM NaCl or 100 μM nitrite. A tryptic digest of apoA-I was analyzed by LC-ESI-MS and MS/MS. Chlorinated and nitrated peptides were detected and quantified using reconstructed ion chromatograms of precursor and product peptides. Peptide sequences were confirmed using MS/MS. Results are representative of those from two independent experiments.
3-chlorotyrosine at residue 192 was ~80% of that observed in lipid-free apoA-I under our experimental conditions. Because the molar ratio of oxidant to apoA-I was ~40% lower in HDL than in lipid-free apoA-I, these observations suggest that Tyr192 reacts strongly with HOCl generated by myeloperoxidase when apoA-I is either lipid-free or associated with lipids in HDL.

Tyr192 was also the major site of nitration when lipid-free apoA-I was incubated with either the myeloperoxidase-H2O2-nitrite system or reagent ONOO− (Fig. 2). However, Tyr192 in apoA-I was not nitrated when HDL was exposed to the myeloperoxidase-H2O2-nitrite system (Fig. 6B), although we did detect a low level of nitration of Tyr18, Tyr29, Tyr30, and Tyr236. When HDL was exposed to ONOO−, tyrosine residues 18, 29, 115, 166, 192, and 236 of apoA-I were nitrated (Fig. 6C), and the overall product yield of 3-nitrotyrosine was markedly lower. These observations, in concert with the EPR studies of lipid-free and lipid-associated apoA-I, suggest that solvent accessibility is a major factor controlling tyrosine nitration by reactive nitrogen species.

**Lysine Residues Fail to Direct the Regiospecific Nitrination of Tyrosine Residues in Peptides**—Five of the 7 tyrosine residues in apoA-I lie in amphipathic helices that are thought to play critical roles in lipid binding, lipoprotein stability, and reverse cholesterol transport (1, 58). The helical wheel representation of amphipathic helices predicts that tyrosine and lysine residues in a KY motif will lie next to each other on the same face of the α-helix (58). The predominant site of nitration in apoA-I, Tyr192, resides in a KYXX motif.

To explore the influence of lysine residues on the nitration of nearby tyrosine residues, we investigated the reactions of the myeloperoxidase-H2O2-nitrite system or ONOO− with three model peptides: AcGKYRAEY (KYXX), AcGKYARKY, and AcGEYAREY (KYXX). We previously used these peptides to demonstrate that lysine residues direct the regiospecific chlorination of tyrosine by HOCl (35). The three peptides contained the same amino acids (acetyl-G, -Y, -K, -R, -A, and -E), including glutamic acid (E), but the latter replaced lysine (K) in KYXX. Glutamic acid was included because its negative charge ensures aqueous solubility. Arginine was included because its positive charge promotes peptide ionization during mass spectrometric analysis. The N-terminal acetyl group prevented the primary amine from participating in the reaction pathway.

We exposed each peptide to the myeloperoxidase-H2O2-nitrite system or reagent ONOO− in phosphate buffer supplemented with DTPA for 60 min at 37 °C, terminated the reaction with methionine, and analyzed the reaction products by HPLC with UV detection, LC-ESI-MS, and LC-ESI-MS/MS. Oxidation of YYXX with myeloperoxidase (4:1, mol/mol oxidant/peptide) generated three products that individually accounted for 3−9% of the product yield (relative to total oxidant; Table II).

We used HPLC to quantify the product yield and MS/MS to identify the residues that are nitrated when YYXX, YYKY, and YKXXX peptides are exposed to the myeloperoxidase-H2O2-nitrite system or ONOO−. HPLC analysis revealed that the relative product yields were similar when YYXX, YYKY, and YKXXX were exposed to reactive nitrogen species derived from either system (Table II). MS/MS analysis (Fig. 7; YYXX exposed to ONOO−) indicated that the three products of YYXX oxidized with myeloperoxidase and ONOO− were, respectively, AcGEYARE(NO2Y) (YYXXNO2Y), AcGE(NO2Y)AREY (NO2YYXX), and AcGE(NO2Y)AREY (NO2YYXXNO2Y). Similar results were observed with YYXX and YYKY modified by myeloperoxidase or ONOO−; introduction of a lysine residue into the peptide had little effect on either the relative product yields or the sites of tyrosine nitration (Table II). These observations indicate that lysine residues fail to direct the regiospecific nitrination by myeloperoxidase or ONOO− of tyrosine residues in synthetic peptides.

Oxidation of YYXX, YYKY, and YYXXK with HOCl produced a different pattern of products (Table II). Oxidation of YYXX, which contains 2 tyrosine residues and lacks a lysine residue, yielded small and approximately equal amounts of two oxidation products, suggesting the formation of chlorotyrosine (CIY). MS/MS analysis revealed that the two products were monochlorinated on a single tyrosine residue (AcGE/CIYAREY and AcGE/CIYAREY; CIYXXY and YYXXCIY). When the substrate was YYXX or YYKY, HPLC with UV detection revealed a high yield (~40%, mol of product/mol of oxidant) of one major product and a smaller yield (9 and 17%, respectively, mol of product/mol of oxidant) of a minor product (Table II). HPLC and LC-ESI-MS/MS analysis of YYXXK exposed to HOCl demonstrated a major oxidation product that contained a chlorine atom on its first tyrosine residue (AcGE/CYKARYK, CIYXXYY). When the substrate was YYXXK, the major oxidation product contained a chlorine atom on its second tyrosine residue (AcCYKRA/CYK, YYXXCYK). Our results are consistent with our previous observations (35) and confirm that HOCl chlorinates tyrosine residues in peptides containing the motif KYXX or YYXX with high yield. Moreover, chlorination is regiospecific, because it targets the tyrosine residue that lies 2 residues away from a lysine residue.

**Chlorination but Not Nitration of ApoA-I Strongly Inhibits the Ability to Remove Cellular Cholesterol by the ABCA1 Pathway**—We compared the effects of chlorination and nitration of apoA-I on the protein’s ability to promote cholesterol efflux from cells, which occurs exclusively by an ABCA1-dependent process. For these studies, we used [3H]cholesterol-labeled BHK cells that were stably transfected with a mifepristone-inducible ABCA1 cDNA. ApoA-I was exposed to the same mol ratios of oxidants used for our experiments that quantified tyrosine chlorination and nitration (Figs. 2 and 3). Treating apoA-I with increasing concentrations of HOCl progressively and dramatically impaired apoA-I-mediated cholesterol efflux.
from these ABCA1-expressing cells at a concentration of apoA-I (3 μg/ml) that was below the concentration required for maximal efflux (Fig. 8A). Treating apoA-I with increasing concentrations of ONOO⁻ as described in the legend to Table II and analyzed by LC-ESI-MS/MS, inhibited cholesterol efflux only modestly (Fig. 8A). When loss of apoA-I activity was nearly maximal (125 μM oxidant; 25:1, mol/mol, oxidant/protein), an estimated 40% of Tyr192 residues were chlorinated by HOCl, and an estimated 80% were nitrated by ONOO⁻ (compare Fig. 8A with Figs. 2 and 3).

We obtained similar results when we used the myeloperoxidase-H₂O₂ system to oxidize apoA-I with chlorinating or nitrating intermediates (Fig. 8B). In this case, apoA-I exposed to increasing concentrations of H₂O₂ in the presence of myeloperoxidase and plasma concentrations of chloride ion (100 mM NaCl) markedly reduced the cholesterol efflux activity of apoA-I (3 μg/ml) that was below the concentration required for maximal efflux (Fig. 8A). Treating apoA-I with increasing concentrations of ONOO⁻ as described in the legend to Table II and analyzed by LC-ESI-MS/MS, inhibited cholesterol efflux only modestly (Fig. 8A). When loss of apoA-I activity was nearly maximal (125 μM oxidant; 25:1, mol/mol, oxidant/protein), an estimated 35% of Tyr192 residues were chlorinated or nitrated by myeloperoxidase (compare Fig. 8B with Figs. 2 and 3). Thus, although chlorination and nitration modified Tyr192 to similar extents, chlorination was much more effective than nitration at preventing apoA-I from removing cellular cholesterol.

We constructed apoA-I concentration curves to evaluate the effects of chlorination and nitration on the ability of apoA-I to remove cholesterol by the ABCA1 pathway. Oxidizing apoA-I by the myeloperoxidase-H₂O₂-chloride system greatly reduced the cholesterol efflux activity of apoA-I at all concentrations of apoA-I examined (Fig. 8C). Oxidation by the myeloperoxidase-H₂O₂-nitrite system modestly decreased the apparent affinity of apoA-I for ABCA1 but did not inhibit the ability of apoA-I to remove cellular cholesterol above saturating concentrations. Thus, only chlorination reduced the maximum capacity of apoA-I to remove cellular cholesterol.

We tested the possibility that specific antioxidants could prevent the inhibitory effects of reactive intermediates generated by the myeloperoxidase system. In the absence of myeloperoxidase, treating apoA-I with H₂O₂ plus either chloride (Fig. 9) or nitrite (data not shown) had no effect on its cholesterol efflux activity. The severely impaired apoA-I activity caused by the myeloperoxidase-H₂O₂-chloride system was substantially or completely prevented when the peroxide scavenger catalase, the heme poison azide, or the HOCl scavenger vitamin C or methionine were included in the reaction mixture.

**Fig. 7.** MS/MS analysis of YYYYY peptide (AcGEYAREY) and its reaction products. The peptide was oxidized with ONOO⁻ as described in the legend to Table II and analyzed by LC-ESI-MS/MS. A, MS/MS spectrum of precursor YYYYY. B, MS/MS spectrum of product NO₂YYYY. C, MS/MS spectrum of product YYYYYNO₂. D, MS/MS spectrum of product NO₂YYYYNO₂.
Although the inhibition was markedly lower, a similar pattern was observed when nitrite was substituted for chloride (data not shown). These observations indicate that scavengers of HOCl and H$_2$O$_2$ or inhibitors of heme proteins block the ability of myeloperoxidase to impair the ability of apoA-I to promote cholesterol efflux from cells.

**DISCUSSION**

Our observations indicate that myeloperoxidase and ONOO$^-$ selectively oxidize Tyr$^{192}$ in lipid-free apoA-I, the major protein in HDL. Tyr$^{192}$ was the single major target for both chlorination by the myeloperoxidase-H$_2$O$_2$-chloride system or HOCl and nitration by ONOO$^-$. It also was the predominant site of nitration by myeloperoxidase. EPR studies of spin-labeled apoA-I demonstrated that residue 192 is located in an extremely hydrophilic environment. This observation indicates that Tyr$^{192}$ is highly accessible to polar solvents and raises the possibility that this accessibility is one important factor controlling its reactivity. Moreover, upon incorporation into discoidal HDL, residue 192 was positioned in a much more hydrophobic environment, likely at the lipid-water interface of the particle. Notably, Tyr$^{192}$ of HDL-associated apoA-I was a poor substrate for nitration by both myeloperoxidase and ONOO$^-$. Collectively, these observations indicate that lipid association markedly changes the accessibility of Tyr$^{192}$ to the aqueous environment and that this process in turn reduces the susceptibility of Tyr$^{192}$ to nitration by myeloperoxidase and ONOO$^-$. Remarkably, the ability of apoA-I to promote ABCA1-dependent cholesterol efflux was dramatically impaired when Tyr$^{192}$ was chlorinated. In contrast, nitration of Tyr$^{192}$ had little impact on this biological function. A recent study also showed that nitration was much less effective than chlorination in impairing the cholesterol efflux activity of apoA-I (37). These results indicate that Tyr$^{192}$ is the major aromatic amino acid targeted for oxidation when apoA-I is exposed to reactive chlorine and nitrogen species and that chlorinating and nitrating intermediates exert different effects on the modified apolipoprotein’s ability to promote ABCA1-dependent cholesterol efflux. It is important to note that our observations do not prove that chlorination of Tyr$^{192}$ is responsible for impairing the ability of apoA-I to promote ABCA1-dependent cholesterol efflux, because other amino acids are also modified when apoA-I is exposed to oxidizing intermediates (7–10, 15–17).
Nitration and Chlorination of Tyrosine Residues in ApoA-I

Several factors might account for the differential abilities of chlorinated and nitrated apoA-I to remove cholesterol from cells. One likely possibility is that apoA-I undergoes oxidative modifications distinct from tyrosine chlorination that are uniquely induced by HOCl. It is also possible that nitration and chlorination of Tyr192 exert divergent effects on the ability of apoA-I to promote cholesterol efflux from cells. Alternatively, chlorination but not nitration might produce a conformational alteration in apoA-I that reduces its interactions with ABCA1 or its ability to acquire lipids. In future studies, it will be of interest to determine the specific molecular mechanism underlying the apoA-I chlorination-mediated impairment of ABCA1 activity.

A key question is why Tyr192 is so much more amenable to chlorination and nitration than the six other tyrosine residues in apoA-I. SDS-L-EPR analysis indicates that Tyr192 in lipid-free apoA-I lies in a random coil secondary structure, fully exposed to aqueous solution. However, the secondary structure of this region of apoA-I undergoes a transition to an amphipathic α-helix during discoidal HDL particle formation (50). Because solvent accessibility and loop structures are thought to affect the ability of ONOO− to nitrate tyrosine (18, 57), we employed SDS-L-EPR to determine whether residues 18, 29, 115, 166, 192, and 236 of lipid-free and lipid-associated apoA-I differ in their solvent accessibility (50). In spin-labeled lipid-free apoA-I, residue 192 resided in an extremely hydrophilic environment. In contrast, this residue was positioned in a much more hydrophobic environment on discoidal HDL particles.

These observations indicate that lipid association markedly affects the environment of residue 192 and strongly suggest that Tyr192 in lipid-free apoA-I is highly accessible to aqueous solvent. Consistent with this possibility, Tyr192 in lipid-free apoA-I was the major nitration site for both myeloperoxidase and ONOO−. When apoA-I was incorporated into reconstituted HDL particles, however, nitration of Tyr192 was markedly reduced.

These observations suggest the following model (Scheme 1). In lipid-free apoA-I, Tyr192 lies in a random coil structure in an environment that is highly accessible to aqueous solvent. In this environment, the residue reacts rapidly with the short lived, highly reactive intermediates derived from ONOO− or the myeloperoxidase-H2O2-nitrite system. When apoA-I is associated with lipid, however, Tyr192 partitions into a more hydrophobic environment and is therefore unable to react with nitrating intermediates generated in the aqueous phase by myeloperoxidase and ONOO−. Thus, accessibility to solvent is likely to be an important feature controlling the nitration of Tyr192 in apoA-I.

In contrast to its behavior with nitrating species, Tyr192 was chlorinated in high yield in both lipid-free and lipid-associated apoA-I. This tyrosine resides in a YXXX motif, and we have previously shown that HOCl reacts with lysine residues in peptides to form long lived chloramines that promote the regiospecific chlorination of tyrosine (35). The helical wheel representation of amphipathic helices predicts that tyrosine and lysine residues in the YXXX (and KXXX) motif will lie next to each other on the same face of the α-helix (58), suggesting that lysine residues in the YXXX motif can be converted to chloramines that then direct tyrosine chlorination. In contrast to the free N+ amino group of lysine (pK a −10.5), which exists predominantly as the protonated NH3+ species at neutral pH, the chloramine derived from the lysine N+ amino group is uncharged. Thus, this long lived species could potentially attack the phenolic group of tyrosine in either a hydrophilic or hydrophobic environment. Moreover, Tyr192 lies in an α-helical structure when lipid-associated, which favors positions it for interaction with Lys195. These observations suggest that the chloramine of Lys195 can direct the chlorination of Tyr192 of apoA-I in high yield in both lipid-free and lipid-associated protein.

It has also been proposed that charged amino acid residues help direct tyrosine nitration by ONOO− (18, 57). We used synthetic peptides containing tyrosine and lysine to explore the potential role of primary amines in promoting the regiospecific oxidation of tyrosine by reactive nitrogen species. These studies suggest that Lys195 is unlikely to direct the regiospecific oxidation of Tyr192 by reactive nitrogen species. Instead, the Tyr192 in apoA-I might be so much more amenable to nitration than the other 6 tyrosine residues because of differences in solvent accessibility, spatial orientation, and the local amino acid environment.

Several lines of evidence indicate that myeloperoxidase-mediated apoA-I oxidation may be sufficient to impair ABCA1-dependent cholesterol efflux from macrophage foam cells in the human artery wall. We showed that HDL isolated from human atherosclerotic lesions and plasma from patients with established coronary artery disease contained elevated levels of both 3-chlorotyrosine and 3-nitrotyrosine, which strongly implicates myeloperoxidase as a key contributor to HDL oxidation in vivo (31, 36). The level of tyrosine chlorination in lesion HDL ranged from 100 to 300 μmol of 3-chlorotyrosine/mol of Tyr, suggesting that −1 in every 800 apoA-I molecules was chlorinated. It is likely, however, that there is a gradient in the degree of apoliprotein oxidation between extracellular compartments and the immediate pericellular environment. We previously reported that epitopes for 3-nitrotyrosine, a product of highly reactive nitrogen dioxide radical, co-localize with myeloperoxidase and macrophages in human atherosclerotic tissue (31). Because the nitrogen dioxide radical is a short lived species, this finding implies that proteins in close proximity to macrophages are selectively targeted for oxidative damage. Moreover, apoA-I is poorly nitrated by both myeloperoxidase and ONOO− when it is associated with HDL, raising the possibility that lipid-poor apoA-I, the biologically active ligand for ABCA1, is the major target for nitration in the artery wall. Thus, oxidation of apoA-I appears to be physiologically important, lipid-poor apoA-I may be the primary target for oxidation, and microenvironments depleted of antioxidants might enable oxidation to occur. One such environment surrounds tissue macrophages, which generate high local concentrations of oxidants.
Our results suggest the following model for HDL oxidation and the functional impairment of ABCA1-dependent cholesterol efflux in the human artery wall. Activated macrophages use a membrane-associated NADPH oxidase to produce high pericellular concentrations of superoxide and \( \text{H}_2\text{O}_2 \). Superoxide reacts with NO to generate \( \text{ONOO}^- \) in a diffusion-controlled reaction that may be most favorable near the plasma membrane of the cells. Moreover, myeloperoxidase secreted by activated phagocytes uses \( \text{H}_2\text{O}_2 \) to convert chloride ion into chloride and nitrite into chlorinating and nitrating species. Thus, macrophage-dependent oxidation reactions are likely to be highly restricted in space by local changes in oxidant and enzyme concentrations. Modification of specific amino acid residues in apoA-I may play a critical role in inhibiting the formation of lipid-laden foam cells. In contrast, nitration promotes cholesterol efflux from macrophages, contributing to the formation of lipid-laden foam cells. In contrast, nitration and chlorination of tyrosine residues in apoA-I may play a critical role in inhibiting the ability of the apolipoprotein to promote cholesterol efflux from macrophages, suggesting that it may play a role in inhibiting HDL function and reverse cholesterol transport during atherogenesis.

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Tyrosine 192 in Apolipoprotein A-I Is the Major Site of Nitration and Chlorination by Myeloperoxidase, but Only Chlorination Markedly Impairs ABCA1-dependent Cholesterol Transport

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