Myeloperoxidase Impairs ABCA1-dependent Cholesterol Efflux through Methionine Oxidation and Site-specific Tyrosine Chlorination of Apolipoprotein A-I*

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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-chlorotyrosine. Myeloperoxidase (MPO) is the only known source of 3-chlorotyrosine in vivo, indicating that MPO oxidizes HDL in humans. We previously reported that Tyr-192 is the major site that is chlorinated in apolipoprotein A-I (apoA-I), the chief protein in HDL, and that chlorinated apoA-I loses its ability to promote cholesterol efflux from cells by the ATP-binding cassette transporter A1 (ABCA1) pathway. However, the pathways that promote the chlorination of specific Tyr residues in apoA-I are controversial, and the mechanism for MPO-mediated loss of ABCA1-dependent cholesterol efflux of apoA-I is unclear. Using site-directed mutagenesis, we now demonstrate that lysine residues direct tyrosine chlorination in apoA-I. Importantly, methionine residues inhibit chlorination, indicating that they can act as local, protein-bound antioxidants. Moreover, we observed normal cholesterol efflux activity when Tyr-192 of apoA-I was mutated to Phe and the oxidized protein was incubated with methionine sulfoxide reductase. Thus, a combination of Tyr-192 chlorination and methionine oxidation is necessary for depriving apoA-I of its ABCA1-dependent cholesterol transport activity. Our observations suggest that biologically significant oxidative damage of apoA-I involves modification of a limited number of specific amino acids, raising the feasibility of producing oxidation-resistant forms of apoA-I that have enhanced anti-atherogenic activity in vivo.

High density lipoprotein (HDL) protects against atherosclerosis by removing cholesterol from cells of the artery wall (1, 2). Apolipoprotein A-I (apoA-I), which accounts for ~70% of the total protein in HDL, promotes cholesterol and phospholipid efflux largely by an active transport process mediated by ATP-binding cassette transporter A1 (ABCA1). However, oxidation of apoA-I severely impairs cholesterol efflux by the ABCA1 pathway (3–5). Moreover, HDL is chlorinated in human atherosclerotic lesions and the blood of subjects with established coronary artery disease, suggesting that apoA-I oxidation might promote atherogenesis.

One potential pathway for apoA-I oxidation involves myeloperoxidase (MPO), a heme protein expressed by macrophages in human atherosclerotic tissue (6–8). MPO secreted by phagocytes uses hydrogen peroxide (H2O2) and chloride (Cl–) to generate the powerful oxidant hypochlorous acid (HOCl). HOCl converts tyrosine to 3-chlorotyrosine (8–10), and MPO is the only known source of this halogenated amino acid during acute inflammation in mice (11), indicating that MPO oxidizes HDL in vivo. We previously showed that MPO or HOCl targets tyrosine residue 192 (Tyr-192) when it chlorinates apoA-I, regardless of whether the protein is free or associated with HDL (12, 13). Moreover, apoA-I loses its ability to remove cholesterol from cells as it becomes oxidized in this manner (3–5, 13), indicating that Tyr chlorination might be important for this impaired activity.

The pathways that promote the MPO-dependent oxidation of specific residues in apoA-I and loss of its ABCA1 activity are controversial. The mechanism we have proposed is based on the observation that Tyr-192 lies in an XXY motif—Y = Tyr, K = Lys, X = unreactive amino acid—and therefore is adjacent to a Lys residue on the same face of an amphipathic α-helix (12, 14). Moreover, HOCl reacts rapidly with the ε amino group of lysine to form long-lived chloramines (8, 12). Using synthetic peptides, we demonstrated that lysine residues can direct the regiospecific chlorination of Tyr residues by a pathway involving chloramine formation. In this model, the site-specific chlorination of Tyr-192 in apoA-I requires the participation of a nearby lysine residue (12).

An alternative proposal is that MPO must bind directly to the region of apoA-I containing Tyr-192 to promote site-specific chlorination of the residue (5). Based on studies of mutated apoA-I, it was also concluded that tyrosine chlorination is not a prerequisite for loss of ABCA1 transport activity when MPO oxidizes apoA-I (15).

Methionine sulfoxide (Met(O)) has also been detected in circulating HDL (16). ApoA-I can reduce lipid hydroperoxides to alcohols in concert with oxidation of Met residues to Met(O) (17, 18). HDL is the major carrier of lipid hydroperoxides in the blood of humans (19), suggesting that this pathway may be physiologically relevant. The alkyl thiol of Met is the most HOCl-reactive moiety in the 20 common amino acids (20, 21), but the role of oxidation of Met residues in the impaired cholesterol efflux activity of MPO-oxidized apoA-I has received surprisingly little attention.

Met(O) residues in proteins are reduced to methionine by a family of intracellular enzymes termed methionine sulfoxide reductases (22). In the current studies, we used mutated apoA-I and methionine sulfoxide reductase to probe the role of specific amino acids residues in Tyr chlorination and the loss of ABCA1-cholesterol efflux activity that occurs when apoA-I is exposed to MPO.

EXPERIMENTAL PROCEDURES

Isolation of MPO, PilB, and ApoA-I—MPO (EC 1.11.17.7) was isolated from human neutrophils (23). A truncated gene of PilB of Neisseria gonorrhoeae expressed in Escherichia coli was purified as described (24). Individual substitution mutations within human apoA-I cDNA were introduced by primer directed PCR mutagenesis or by the Mega-Primer PCR method and expressed in Escherichia coli containing the pilBβ688 bacterial expression vector (25, 26). All mutations were verified by dyeode automated fluorescent sequencing.

Oxidation and Methionine Sulfoxide Reduction Reactions—ApoA-I was dialyzed against 10 mm sodium phosphate buffer (pH 7.4). Oxidation reactions were carried out at 37 °C for 1 h in 10 mm sodium phosphate buffer (pH 7.4) containing 100 μm diethylenetriaminepentaacetic acid (27). For the MPO-H2O2-Cl– system, the reaction mixture was supplemented with 50 nm MPO (28). Oxidized apoA-I (6 μmol) was incubated with PilB (4:5:1, apoA-I/enzyme, w/w) for 2 h at 37 °C in Tris–HCl buffer (25 mm (pH 7.4)) containing 15 mM dithiothreitol (DTT).

Liquid Chromatography Electrospray Ionization Mass Spectrometry (LC-ESI-MS)—ApoA-I was incubated overnight at 37 °C with sequencing grade modified trypsin (20:1, protein/enzyme, w/w) or with endoproteinas Glu-C (Staphylococcus aureus V8; 10:1, protein/enzyme, w/w) in 100 mm NH4HCO3 (pH 7.8) (13, 29). Digestion was halted by acidification (pH 2–3). LC-ESI-MS analyses were performed in the positive ion mode with a Finnigan Mat LCQ ion trap instrument (San Jose, CA) coupled to a Waters 2690 HPLC...
Tyrosine chlorination in native and mutated human apoA-I. Isolated recombinant apoA-I or mutated apoA-I was exposed to HOCl (open bars) or to the MPO–H₂O₂ system (filled bars: 10:1, mol/mol, oxidant/apoA-I). The MPO system was supplemented with 100 mM NaCl. A tryptic digest of apoA-I was analyzed by LC-ESI-MS and MS/MS. Results are means (±S.D.) from three independent experiments.

RESULTS

The YXXX Motif Directs Tyr Chlorination in ApoA-I—To determine whether lysine residues in the YXXX motif direct the regiospecific chlorination of Tyr residues in proteins, we used site-directed mutagenesis to engineer a series of mutations in the cDNA of human apoA-I. After isolating the wild-type or mutant apoA-I proteins, we exposed them to reagent HOCl or the MPO–H₂O₂–Cl⁻ system. Reactions were initiated by adding oxidant and terminated by adding Met, a scavenger of HOCl. After digesting the oxidized apoA-I with trypsin, we used LC-ESI-MS and reconstructed ion chromatograms of precursor and product peptides to quantify the yields of chlorinated tyrosine peptides. This approach detected all seven peptides predicted to contain Tyr.

As with apoA-I isolated from human HDL (12, 13), Tyr-192 was the major site of chlorination in recombinant wild-type apoA-I (Fig. 1A). In the K195R mutant, Tyr-192 chlorination was markedly decreased, suggesting that Lys-195 plays a critical role in directing the chlorination of Tyr-192. Tyr-166, which does not reside in a YXXX motif in apoA-I, was chlorinated in low yield when the protein was exposed to HOCl or the complete MPO system (Fig. 1B). However, when Glu-169 was mutated to lysine, chlorination of Tyr-166 increased 20-fold (Fig. 1B). These results demonstrate that lysine residues located in the YXXX motif can direct tyrosine chlorination in apoA-I.

Methionine Residues in the MXXX Motif Inhibit Tyrosine Chlorination—Protein-bound Met residues have been proposed to act as local scavengers of H₂O₂ (32). To determine whether Met residues might similarly scavenge oxidants derived from HOCl, we mutated glutamic acid 198 (which lies 2 residues away from the Lys-195 that directs chlorination of Tyr-192) to Met. In this mutant, Tyr-192 chlorination was dramatically decreased (Fig. 1C), suggesting that Met residues can inhibit Tyr chlorination by scavenging lysine chloramines.

Tyr-115, which also resides in a YXXX motif in apoA-I, was chlorinated poorly (Fig. 1D) by HOCl or the complete MPO system. When we mutated Met-112 to Ala, however, HOCl generated a high yield of chlorinated Try-115 (data not shown). Thus, chlorination of Tyr-115 appears to be inhibited by the adjacent Met residue in wild type apoA-I. When Met-112 was mutated to Lys, chlorination of Tyr-115 also increased markedly (Fig. 1D). Collectively, these observations suggest that protein-bound Met residues that reside in MXXX or KXXX motifs can inhibit Tyr chlorination by scavenging lysine chloramines. Methionine Sulfoxide Reductase Converts Met(O) to Methionine in Oxidized ApoA-I—ApoA-I contains 3 Met residues, but it is unknown how oxidizing them with HOCl affects apoA-I-mediated cholesterol efflux by the ABCA1 pathway. When we exposed apoA-I to HOCl or the MPO system and then digested it with trypsin or Glu-C, LC-ESI-MS/MS analysis showed that each Met had been targeted for oxidation to Met(O) (Fig. 2, A and B). In contrast, methionine sulfone was not detected in oxidized apoA-I. When apoA-I was first exposed to the complete MPO system and then incubated with the methionine sulfoxide reductase PilB, Met(O) was converted back to methionine (Fig. 2C).
Tyr-115 in apoA-I exposed to MPO increased. When we introduced a Met residue 2 residues away from the Lys residue in apoA-I, Tyr-192 chlorination was inhibited. Our results strongly support the hypothesis that protein-bound Met residues act as local antioxidants (32) by scavenging chlorinating intermediates.

Met oxidation might affect Tyr chlorination by additional mechanisms. The /H9251/helical structure of apoA-I depends critically upon hydrophobic amino acids, which form the lipid-associating face of the amphipathic helix (14). Oxygenation markedly decreases the hydrophobicity of Met. By disrupting the secondary structure of the apolipoprotein, Met oxidation could alter the ability of nearby Lys residues located in the YXXK motif to promote the site specific chlorination of apoA-I.

A key question is whether or not Tyr chlorination impairs the ability of apoA-I to promote ABCA1-dependent cholesterol efflux. We had noted the strong linear association between the extent of Tyr-192 chlorination and loss of biological activity, which suggests that Tyr oxidation might be an important contributor (3, 13). However, recent studies of a mutant form of apoA-I in which Phe replaced all 7 Tyr residues led to the proposal that Tyr chlorination is irrelevant to the loss of ABCA1-dependent cholesterol efflux that occurs when MPO oxidizes apoA-I (15).

To address this issue, we measured the cholesterol efflux activity of apoA-I containing a Phe substituted for Tyr-192, which makes this residue completely resistant to chlorination. In contrast to the previous report with murine macrophages (15), we found using ABCA1-transfected BHK cells that the mutation had a small but reproducible protective effect against apoA-I inactivation by either HOCl or the MPO system. The difference between our study and the previous one may reflect the fact that our transfected BHK cells express much higher ABCA1 levels than murine macrophages, allowing us to detect modest changes in apoA-I activity.

Our observation that protein-bound Met is a potent local scavenger of chlorinating intermediates has implications for the role of Met in protection against oxidative inactivation of apoA-I. Further studies are needed to elucidate the precise mechanisms by which Met residues act as local antioxidants and to understand the role of Met oxidation in the regulation of apoA-I function.
HOCl led us to investigate the role of Met oxidation in the biological activity of apoA-I. When we exposed apoA-I to HOCl or to H₂O₂ with the complete MPO system, we observed near-quantitative oxidation of the 3 Met residues in apoA-I. Met oxidation was completely reversed by treatment with PilB, a methionine sulfoxide reductase that reduces both the (R) and (S) epimers of Met(O) (22). Remarkably, when the Tyr-192 → Phe mutation was exposed to HOCl or the complete MPO system and then incubated with the methionine reductase PilB, its ability to promote cholesterol efflux by the ABCA1 pathway was almost completely restored. These observations indicate that Met oxidation and Tyr chlorination together, in contrast to Met oxidation alone (33), are necessary for depriving apoA-I of its cholesterol efflux activity.

This synergism between the Tyr-192 → Phe mutation and Met(O) reduction implies that the functional defect induced by oxidation of apoA-I arises from a cooperative interaction between oxidation of Tyr-192 and one or more Met residues. Alternatively, it is possible that other structural modifications in oxidized apoA-I are involved in the loss of ABCA1 activity. It is noteworthy that Tyr-192 resides in the middle of the random coil loop that changes its conformation when it encounters lipid, driving the remodeling of apoA-I to its lipid-associated form (34). Chlorination of this Tyr residue, in concert with Met oxidation, may stabilize a conformational variant that fails to either associate strongly with lipid or interact productively with ABCA1.

In conclusion, oxidation by MPO impairs the ability of apoA-I to promote cholesterol efflux by the ABCA1 pathway, suggesting that this oxidative process might contribute to foam cell formation and atherogenesis. Studies with mutant forms of apoA-I strongly support the proposal that specific amino acid sequences direct the regioselective chlorination of Tyr. Moreover, oxidation of Met and Tyr residues plays a critical role in the oxidative inactivation of the protein. Our observations raise the possibility that modified forms of apoA-I that are resistant to oxidation might be especially anti-atherogenic in vivo.

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