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Impact of HDL Oxidation by the Myeloperoxidase System on Sterol Efflux by the ABCA1 Pathway

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Abstract

Protein oxidation by phagocytic white blood cells is implicated in tissue injury during inflammation. One important target might be high-density lipoprotein (HDL), which protects against atherosclerosis by removing excess cholesterol from artery wall macrophages. In the human artery wall, cholesterol-laden macrophages are a rich source of myeloperoxidase (MPO), which uses hydrogen peroxide for oxidative reactions in the extracellular milieu. Levels of two characteristic products of MPO—chlorotyrosine and nitrotyrosine—are markedly elevated in HDL from human atherosclerotic lesions. Here, we describe how MPO-dependent chlorination impairs the ability of apolipoprotein A-I (apoA-I), HDL's major protein, to transport cholesterol by the ATP-binding cassette transporter A1 (ABCA1) pathway. Faulty interactions between apoA-I and ABCA1 are involved. Tandem mass spectrometry and investigations of mutated forms of apoA-I demonstrate that tyrosine residues in apoA-I are chlorinated in a site-specific manner by chloramine intermediates on suitably juxtaposed lysine residues. Plasma HDL isolated from subjects with coronary artery disease (CAD) also contains higher levels of chlorinated and nitrated tyrosine residues than HDL from healthy subjects. Thus, the presence of chlorinated HDL might serve as a marker of CAD risk. Because HDL damaged by MPO *in vitro* becomes dysfunctional, inhibiting MPO *in vivo* might be cardioprotective.

Keywords

3-Chlorotyrosine; 3-nitrotyrosine; biomarker; apolipoprotein A-I; dysfunctional HDL; coronary artery disease

Introduction

Oxidative modification of biomolecules has been implicated in the pathogenesis of inflammatory diseases (1–3). One important pathway involves reactive intermediates produced by phagocytic white blood cells, the cellular hallmark of inflammation. Protein oxidation may be a key event in these processes, because proteins play fundamental roles as biological catalysts, signaling pathways, and gene regulators. One potent oxidative enzyme is myeloperoxidase (MPO), which is expressed by neutrophils, monocytes, and some populations of macrophages. This heme protein, which is found at high levels in human

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atherosclerotic tissue (4), uses hydrogen peroxide to convert reducing substrates to an array of reactive intermediates, including hypochlorous acid, nitrogen dioxide radical, and tyrosyl radical (5). The physiological role of MPO is to kill invading bacterial and fungal pathogens (6–8). During acute or chronic inflammation, however, the reactive intermediates it spawns might also oxidize host proteins (4).

Lipoproteins, which play critical roles in atherosclerosis (9), may be one important target for oxidation. For example, low density lipoprotein (LDL), the major carrier of cholesterol in the blood and an important risk factor for coronary artery disease, is the source of cholesteryl ester in the macrophage foam cells of the artery wall that play key roles in both the initiation and progression of atherosclerosis. Paradoxically, high concentrations of LDL fail to convert cultured macrophages into macrophage foam cells (9). In contrast, macrophages rapidly take up and degrade oxidized LDL (10, 11). Moreover, many lines of evidence implicate oxidized LDL in the pathogenesis of atherosclerosis in animal models (10, 11), though the pathway's role in human disease is less clear (12).

In contrast to LDL, high density lipoprotein (HDL) is cardioprotective, partly because its major protein, apolipoprotein A-I (apoA-I), removes cholesterol from macrophages (13–16). In this article, we review the evidence that MPO promotes the oxidation of HDL in humans, describe how MPO oxidizes apoA-I at specific sites, and show how such damage impairs HDL's ability to transport cholesterol by the ATP-binding cassette transporter A1 (ABCA1) pathway.

Activated phagocytes secrete myeloperoxidase, which produces reactive chlorinating and nitrating species

Activated phagocytic white blood cells (neutrophils, monocytes, macrophages, and eosinophils) use NOX2, a membrane-associated NADPH oxidase, to produce extracellular superoxide ($O_2^{\bullet -}$), which dismutates to form hydrogen peroxide (H_2O_2) (17, 18). Activated neutrophils, monocytes, and certain macrophage populations also secrete MPO (4), while eosinophils secrete eosinophil peroxidase (EPO) (19). Both MPO and EPO are heme enzymes that use H_2O_2 as an oxidizing substrate to generate reactive intermediates (19). For MPO, the major end product at plasma concentrations of chloride ion is generally thought to be hypochlorous acid (HOCl), a potent anti-microbial agent (17, 18). The reaction involves the two-electron oxidation of chloride ion (Cl^-) by compound I, a complex of the heme group of MPO with H_2O_2.

$$\mathrm{MPO}+\mathrm{H_2O_2} \rightarrow \text{compound I}$$
$$\mathrm{Cl^-}+\text{compound I}+\mathrm{H^+} \rightarrow \mathrm{HOCl}+\mathrm{H_2O}+\mathrm{MPO}$$

HOCl is a powerful chlorinating reagent that reacts with a wide variety of biomolecules (20). For example, HOCl produced by MPO converts free and protein-bound tyrosine (Tyr) residues to 3-chlorotyrosine (21, 22), and studies of mice have revealed that acute inflammation generates 3-chlorotyrosine. Importantly, mice deficient in MPO fail to produce 3-chlorotyrosine (7), demonstrating that this abnormal amino acid is a molecular fingerprint that implicates the enzyme in oxidative damage (7, 22, 23).

Phagocytes also use nitric oxide synthase 2 (NOS2) to generate nitric oxide (NO), which plays an important role in killing intracellular pathogens (24). NO can decompose to form nitrite (NO_2^-) or react with $O_2^{\bullet -}$ to produce peroxynitrite ($ONOO^-$), a potent nitrating intermediate (25, 26).

$$O_2^{\bullet -} + NO \rightarrow ONOO^-$$

MPO converts NO_2^- to nitrogen dioxide radical ($NO_2^\bullet$) (27–29). The reaction likely involves the direct one-electron oxidation of nitrite to nitrogen dioxide radical by compound I.

$$NO_2^- + \text{compound I} \rightarrow NO_2^\bullet + \text{compound II} + H_2O$$

Nitrogen dioxide radical ($NO_2^\bullet$), which can be derived from $ONOO^\bullet$ or the MPO^--NO_2^--H_2O_2 system, generates 3-nitrotyrosine when it reacts with tyrosine residues (26–29).

HDL is cardioprotective

In atherosclerosis, the leading cause of death in Western industrialized societies, deposition of inflammatory macrophages in the vessel wall narrows arterial blood vessels. Among the factors that greatly increase the risk for this disease is an elevated level of LDL (30). In contrast, high-density lipoprotein (HDL) protects the artery wall (13, 16, 31, 32). Indeed, clinical, epidemiological and animal studies have demonstrated a strong inverse relationship between HDL level and risk for coronary artery disease (33).

Several distinct pathways have been implicated in HDL's cardioprotective effects (32, 33). One key mechanism involves cholesterol removal from macrophages (13, 34, 35). The first step in this active transport process (termed reverse cholesterol transport) is mediated by a membrane-bound protein called ABCA1 (13, 36–38). Strong evidence for the importance of reverse cholesterol transport was provided by the demonstration that myeloid-specific deletion of ABCA1 markedly increases atherosclerosis in mice without affecting plasma lipoprotein levels. Moreover, recent studies indicate that HDL also regulates the proliferation of hematopoietic stem cells (35), which in turn controls leukocytosis and the number of circulating monocytes—key precursors of macrophages in the artery wall.

Many lines of evidence support the proposal that HDL's anti-inflammatory properties also contribute to its cardioprotective actions (39). For example, HDL suppresses the type 1 interferon response in macrophages (40), which is of central importance in atherogenesis (41). Moreover, proteins carried by HDL play key roles in the acute-phase response, proteolysis, and the complement system, strongly linking the lipoprotein to modulation of inflammation (42). Indeed, inflammation significantly remodels both HDL lipids and proteins during acute inflammation (43, 44). Two important targets are apoA-I and serum amyloid A. The apoA-I content of HDL decreases while the content of SAA1 and SAA2 markedly increase. However, the precise role of changes in the protein and lipid composition of HDL in altering sterol efflux by macrophages *in vivo* remains unclear (34, 45–47). Both mouse and human studies suggest that inflammatory HDL can become dysfunctional and lose its cardioprotective effects (34, 39, 48, 49), though the underlying mechanisms are poorly understood. One important pathway could involve oxidative damage (50–52).

HDL is an attractive model system for studying protein oxidation

HDL is a noncovalent assembly of proteins and lipids that was originally defined by its density on ultracentrifugation (53). ApoA-I, a 28 kDa peptide containing 243 amino acid residues, accounts for ~70% of HDL's protein mass (54). Most circulating apoA-I is found in HDL, but ~5% of the apoA-I in plasma is thought to be lipid-free (15, 54).

ApoA-I's relatively small size facilitates MS/MS sequencing, making it an attractive system for studying the factors that influence protein modification at specific amino acid residues. Other advantages include the availability of both the lipid-free and lipid-associated forms in solution and the ready accessibility of most of the protein's amino acids to reactive intermediates. Moreover, recombinant and mutant forms of apoA-I can be easily isolated (55), which greatly facilitates studies of oxidative reaction mechanisms (56). Importantly, apoA-I is strongly linked to HDL's cardioprotective effects, making it possible to associate specific oxidation sites with changes in the protein's functional properties (32, 48, 54).

MPO targets HDL for oxidation in humans

To assess whether HDL oxidation might contribute to the formation of macrophage foam cells, we first determined whether the human artery wall contains oxidized HDL (50, 51). First, HDL was isolated from human atherosclerotic lesions or from plasma of subjects with established coronary artery disease (CAD) and from age- and sex-matched healthy subjects. Its content of protein-bound 3-chlorotyrosine and 3-nitrotyrosine was then determined, using isotope dilution gas chromatography-mass spectrometry (57). Levels of both abnormal amino acids were higher in HDL isolated from atherosclerotic lesions than in plasma HDL (Fig. 1A and C), strongly suggesting MPO oxidizes HDL in the human artery wall.

Importantly, we also demonstrated that levels of 3-chlorotyrosine and 3-nitrotyrosine were markedly higher in circulating HDL isolated from CAD patients than in HDL isolated from healthy controls (Fig. 1B and D), as Zheng *et al.* also reported (52). Thus, two different groups have shown that levels of chlorinated HDL are higher in plasma of subjects with established CAD than in control subjects. In contrast, we were unable to detect chlorotyrosine or nitrotyrosine in plasma LDL or in total plasma proteins of either control or CAD subjects (50, 51), making it unlikely that HDL is oxidized in the circulation.

One possibility is that HDL is damaged in a microenvironment rich in oxidants and depleted of antioxidants before entering the bloodstream. One such environment is the inflamed atherosclerotic lesion. Indeed, using antibodies to 3-nitrotyrosine and to proteins modified by HOCl, we demonstrated that chlorinated and nitrated adducts co-localize with macrophages in human atherosclerotic lesions (50, 51). Because MPO is the only known source of chlorinating intermediates in humans and is a potent source of reactive nitrogen species in a mouse model of acute inflammation (7, 28), these observations strongly support the hypothesis that MPO is one pathway for oxidizing HDL in humans.

MPO impairs the ability of apoA-I to promote cholesterol transport by ABCA1

The lipid-free form of apoA-I interacts with membrane-associated ABCA1 to remove excess cholesterol from artery wall macrophages (13, 38, 58, 59). Because MPO oxidizes HDL *in vivo*, we determined whether reactive intermediates generated by the enzyme affected that process (50). Oxidation by H_2O_2 alone did not. In striking contrast, chlorination of apoA-I by the complete MPO-H_2O_2-Cl^- system or by HOCl alone markedly impaired the protein's ability to promote cholesterol efflux (50, 60). These observations, together with those of other investigators (52), indicate that chlorination of apoA-I selectively impairs the protein's ability to remove cholesterol from cells by a pathway requiring ABCA1. Thus, products generated by MPO might be a mechanism for creating dysfunctional HDL, for impairing ABCA1-dependent cholesterol efflux from macrophage foam cells, and for promoting the development of atherosclerotic plaque.

Tyr192 in apoA-I is the major target for chlorination by HOCl or the MPO chlorinating system

To determine whether HOCl or the MPO-H_2O_2-Cl^- system chlorinates specific tyrosine residues in apoA-I, we exposed lipid-free apoA-I or apoA-I in HDL (lipid-associated apoA-I) to HOCl or the MPO-chloride system (60, 61). After digesting the protein with trypsin or Glu-C, we fractionated the peptides by liquid chromatography (LC), and subjected them to tandem mass spectrometric (MS/MS) analysis. Oxidation reactions were performed at neutral pH in phosphate buffer supplemented with DTPA (to chelate redox-active metal ions) (62).

ApoA-I contains 7 tyrosine residues, and LC-ESI-MS analysis of the tryptic digest of the native protein detected 7 tyrosine-containing peptides. In contrast, MPO-oxidized apoA-I yielded 7 peptides containing tyrosine with masses corresponding to those of the precursor peptides plus 34 amu. This observation suggests the formation of chlorinated amino acids, because chlorination of tyrosine increases the amino acid's mass by 34 amu. Using MS/MS analysis, we confirmed each peptide's sequence and showed that its tyrosine had been chlorinated (63, 64). Figure 2B shows the MS/MS analysis of peptide LAEYHAK (which contains Tyr192) in apoA-I that had been exposed to MPO-H_2O_2-chloride system. Compared with the same peptide from control apoA-I (Fig. 2A), every y ion from y_4 to y_6 had gained 34 amu, as had every b ion from b_4 to b_6. Thus, Tyr192 in the peptide had been converted to 3-chlorotyrosine.

To determine which tyrosines are the major chlorination sites when HOCl or the MPO-H_2O_2-chloride system oxidizes lipid-free or lipid-associated apoA-I, we quantified product yields of 3-chlorotyrosine, using the ion current of each precursor and product peptide (within the same sample) and reconstructing ion chromatograms (63). When apoA-I was exposed to either oxidizing system, Tyr192 was the major site of chlorination, with lower levels of chlorination at Tyr115 and Tyr236. We observed much less chlorination at the other four tyrosine residues (Fig. 3A; (64)). These observations indicate that Tyr192 is the predominant chlorination site when lipid-free or lipid-associated apoA-I is exposed to HOCl or the complete MPO enzymatic system (60, 63, 64).

Chloramines promote protein tyrosine chlorination by MPO

Currently, two models attempt to explain MPO's ability to selectively chlorinate Tyr192 in apoA-I. In our model, residues suitably juxtaposed to Tyr192 promote chlorination. Our proposal was based on the observation that HOCl reacts rapidly with the ε-amino group of lysine (Lys) to form long-lived chloramines (17), which chlorinate tyrosine in synthetic peptides (21, 61). As Tyr192 is two residues away from Lys195 in apoA-I's primary sequence and as that region is directly adjacent to an alpha-helical domain, the two residues likely are in close proximity. We used synthetic peptides to test our model. These investigations showed that lysine residues can direct the chlorination of tyrosine residues by a pathway involving chloramine formation (61). Therefore, we proposed that the YXXK motif (Y = tyrosine, K = lysine, X = unreactive amino acid) can direct regiospecific tyrosine chlorination in proteins. In this model, site-specific chlorination of Tyr192 in apoA-I by MPO requires the participation of a nearby lysine residue, Lys195, which is located in a YXXK motif.

In contrast, Zheng *et al.* proposed that MPO must bind directly to apoA-I (52). Using hydrogen-deuterium exchange, Zheng *et al*. showed that MPO interacts with the region of apoA-I that contains Tyr192 (52). Based on that observation, the group proposed an

alternative model in which MPO must bind directly to that region to promote site-specific chlorination of Tyr192.

To distinguish between these two models, we used site-directed mutagenesis to engineer a series of mutations in the cDNA of human apoA-I (56). Studies with those mutations provided strong evidence that the YXXK motif can direct the regiospecific chlorination of tyrosine residues. For example, chlorination of Tyr192 was blocked when Lys195 was mutated to arginine. Also, tyrosine residues that normally resist chlorination were chlorinated in high yield when we introduced the YXXK motif into the protein. Importantly, we observed virtually identical results when we used reagent HOCl to chlorinate apoA-I (56), and the reaction with HOCl clearly cannot involve direct interaction of MPO with apoA-I. These observations strongly support the proposal that the YXXK motif promotes tyrosine chlorination in apoA-I. They argue against the hypothesis that MPO must interact directly with the apolipoprotein to selectively chlorinate Tyr192.

Tyrosine chlorination in concert with methionine oxidation impairs reverse cholesterol transport by apoA-I

There is a strong linear association between the extent of Tyr192 chlorination and loss of ABCA1 transport activity (60). Tyr192 is one of the eight residues in apoA-I that is conserved in all 31 species of animals in which the gene has been sequenced (65). Molecular dynamics simulations indicate that Y192 is in a solvent-exposed loop-helix-loop in the lipid-associated form of apoA-I, which is 100% conserved in all species (65, 66), strongly suggesting that this site in apoA-I has an important biological function. Importantly, this region of the protein resides next to helix 10, which plays a key role in promoting sterol efflux by ABCA1 (67–69).

To test the idea that chlorination of Tyr192 might strongly impair cholesterol transport, we replaced Tyr192 in apoA-I with Phe (Tyr192Phe) and monitored the mutant protein's ABCA1 activity (56). The ABCA1-dependent cholesterol efflux of the mutant and wild-type proteins were virtually identical, suggesting that mutating Tyr192 to Phe has little impact on the structure of lipid-free apoA-I. When this mutant protein was exposed to either HOCl or the MPO chlorinating system, however, the substitution provided a small but significant protection against inactivation (56), suggesting that Tyr192 chlorination normally contributes to the loss of apoA-I function but that other factors are also important.

We therefore investigated the effect of methionine (Met) oxidation, as this residue contains an alkylated thiol that reacts much more strongly with HOCl than does the phenolic group of Tyr (70). Although the product, methionine sulfoxide [Met(O)], has been detected in circulating HDL (71), it is unclear whether oxidation of methionine residues by MPO impairs the cholesterol efflux activity of apoA-I. Using tandem mass spectrometry, we confirmed that MPO quantitatively converts all three methionine residues in apoA-I to Met(O) (56). To investigate the loss of efflux activity, we used PilB, a bacterial methionine sulfoxide reductase that converts both the R- and S-forms of Met(O) residues back to methionine (72). MS/MS analysis demonstrated that PilB completely reversed methionine sulfoxide back to methionine in apoA-I that had been exposed to MPO or HOCl (56). However, the protein's cholesterol efflux activity was only partially restored (56). Thus, Met(O) alone is insufficient to markedly impair apoA-I's function.

We next determined whether the combination of Tyr chlorination and Met oxidation contributes to the loss of ABCA1 activity when apoA-I is exposed to MPO. Remarkably, subsequent treatment with PilB of the Tyr192Phe mutant that had been exposed to HOCl or

the MPO system almost completely restored the protein's ability to promote cholesterol efflux by the ABCA1 pathway (56).

These observations indicate that neither Tyr192 chlorination (56) nor methionine oxidation (71) alone deprives apoA-I of its cholesterol efflux activity. Instead, a combination of the two—perhaps together with other structural changes—is required to completely destroy that activity (56). Based on these observations, and the demonstration that the highly conserved Tyr192 residue resides in a helix-loop-helix domain next to helix 10 (65, 66), we have proposed that chlorination of Tyr192 in concert with oxygenation of Met residues impairs the remodeling of lipid-free apoA-I (48, 73), which in turn alters the ability of the protein to interact with ABCA1 (Fig. 4).

HOCl can also oxidize tryptophan residues in apoA-I (63, 74). It is noteworthy that a mutant form of apoA-I in which all four Trp residues were replaced with Phe was resistant to oxidative inactivation by MPO (75). Those investigators proposed that oxidation of tryptophans in apoA-I is responsible for MPO-mediated loss of ABCA1 function. Circular dichroism demonstrated that 71% of the lipid-free form of the mutant protein had an alpha-helical structure (75), which is markedly higher than in lipid-free wild-type apoA-I (50–57%) (54). Moreover, it is well established that the alpha-helical content of apoA-I also increases markedly when the protein associates with lipid (54), a key step in reverse cholesterol transport by the ABCA1 pathway. These observations indicate that the tryptophan substitutions significantly alter the secondary and tertiary structure of lipid-free apoA-I. Thus, the mutant protein's apparent resistance to oxidation might result from other structural changes.

Tyrosine 192 is the major site of nitration

To determine whether $ONOO^-$ or the MPO-H_2O_2-nitrite system nitrates tyrosine residues in apoA-I, we exposed the lipid-free apolipoprotein to those nitrating agents (63). LC-ESI-MS and MS/MS analysis of the tryptic digest of oxidized apoA-I detected 7 peptides with masses corresponding to those of their precursor peptides plus 45 amu, suggesting nitration of amino acids. Using LC-ESI-MS/MS, we confirmed each peptide's sequence and showed that its tyrosine had been targeted for nitration (63). Figure 2C shows the MS/MS analysis of peptide LAE<u>Y</u>HAK (containing Tyr192) from apoA-I exposed to the MPO-H_2O_2-nitrite system. Compared with the same peptide from control apoA-I (Fig. 2A), every y ion from y_4 to y_6 of the exposed peptide had gained 45 amu, as had every b ion from b_4 to b_6. Thus, the peptide's Tyr192 had been converted to 3-nitrotyrosine (Fig. 2C).

To determine whether reactive nitrogen species selectively target Tyr192 in apoA-I, we quantified product yields of 3-nitrotyrosine, using the ion current of each precursor and product peptide and reconstructed ion chromatograms (63). When apoA-I was exposed to reagent $ONOO^-$, Tyr192 was the predominant nitration site (Fig. 3B; (64)). That was also the case when apoA-I was exposed to the MPO-H_2O_2-nitrite system, but all of the protein's other tyrosine residues were also significantly nitrated (Fig. 3B; (64)). These findings indicate that reagent $ONOO^-$ and the MPO-H_2O_2-nitrite system nitrate all 7 tyrosine residues in apoA-I, that the major site nitrated by both sources of reactive nitrogen species is Tyr192, and that reagent $ONOO^-$ is a more selective nitrating agent than the MPO-H_2O_2-nitrite system.

Because chlorination impairs the ABCA1 activity of apoA-I, we determined whether reactive nitrogen species affected apoA-I's ability to promote cholesterol efflux from cells (50). In contrast to chlorination, neither the complete MPO-H_2O_2-nitrate system nor reagent $ONOO^-$ alone significantly affected that activity (60, 64). Interestingly, we also failed to

observe generation of Met(O) residues under those conditions, raising the possibility that lack of Met oxidation protects the nitrated protein from loss of ABCA1 activity.

Chlorination of apoA-I reduces cholesterol efflux by impairing the ability of the protein to interact directly with ABCA1

Our studies demonstrated that MPO-mediated chlorination (but not nitration) of apoA-I markedly reduces the protein's ability to remove cholesterol from cells by the ABCA1 pathway. This process has been proposed to involve a series of steps. Initially, apoA-I binds to ABCA1 (76, 77); then apoA-I interacts with and solubilizes membrane lipids to generate nascent HDL particles (78–81).

A competitive assay was used to test whether chlorination reduces the direct binding of apoA-I to ABCA1 (64). ABCA1-transfected BHK cells were incubated with ^{125}I-apoA-I in the absence or presence of unlabeled unmodified apoA-I or oxidized apoA-I. The cells were then treated with the cross-linking agent DSP. Following immunoprecipitation of ABCA1 and SDS PAGE, ^{125}I-apoA-I cross-linked to ABCA1 was detected by autoradiography. Unlabeled control apoA-I or nitrated apoA-I reduced the amount of complex detected, indicating that these forms of apoA-I competed equally well with ^{125}I-apoA-I for binding to ABCA1. In contrast, apoA-I exposed to either HOCl or the MPO-H_2O_2-Cl^- system was much less effective, suggesting impaired binding of chlorinated apoA-I to ABCA1.

Other investigators have shown that the loss (caused by MPO) of the ABCA1-dependent cholesterol efflux activity of apoA-I associated with a decrease in the protein's lipid binding activity (82). To address this issue, we used both a kinetic turbidimetric method and size-exclusion chromatography. In the turbidimetric study, both chlorinated and nitrated apoA-I converted multilamellar liposomes to single unilamellar vesicles (SUV) more rapidly than did control apoA-I (64). Similar results have been reported for apoA-I containing oxidized methionine residues (83). These observations suggest that oxidizing apoA-I does not reduce its ability to solubilize phospholipids as assessed by this assay.

In the second study, we incubated apoA-I or oxidized apoA-I with SUV prepared from egg phosphatidylcholine and trace amounts of radiolabeled free cholesterol (64). Then we used size-exclusion chromatography to separate lipid-free or poorly lipidated apoA-I from apoA-I that was associated with SUV (84). These experiments suggested that oxidation of apoA-I by the MPO-H_2O_2-chloride system only modestly affects the protein's ability to interact with phospholipids (64). Therefore, our observations support the proposal that chlorination of apoA-I by MPO impairs cholesterol transport by impairing the direct interaction between apoA-I and ABCA1 but not by impairing apoA-I's ability to solubilize phospholipids.

Quantitative analysis of site-specific chlorination and nitration of proteins

Tandem mass spectrometry (MS/MS) is a powerful tool for identifying posttranslational modifications of proteins (85). There has also been growing interest in developing mass spectrometric methods for quantification of posttranslational modifications of proteins (86). Using apoA-I, the major protein in HDL as a model system, we developed an LC-ESI-MS/MS method to characterize and quantify tyrosine chlorination and nitration in apoA-I exposed to chlorinating and nitrating systems (60, 63). This approach demonstrated that all seven tyrosine residues in apoA-I can be chlorinated or nitrated (Fig. 2) (64). To explore whether specific tyrosine residues are the major sites of chlorination and nitration, we quantified the yield of chlorination or nitration of tyrosine residues in apoA-I, using the ion current of each precursor and product peptide and reconstructed ion chromatograms (63). In this approach, the product yield is defined as the percentage of the peak area of the product

ion(s) relative to the sum of the peak areas of the precursor and product ions. Our observation demonstrated that Tyr192 is the major site for chlorination and nitration when apoA-I was exposed to chlorinating and nitrating systems (Fig. 2) (60, 64). This approach is also readily applied to other proteins. The analytical strategy is also well-suited for quantifying site-specific modifications when multiple sites in a protein are involved, as is often the case when proteins are oxidized by reactive intermediates.

Future directions: Quantifying site-specific modification of apoA-I *in vivo* by isotope dilution

Detection and quantification of oxidative modifications of proteins *in vivo* require specific and sensitive approaches. To detect site-specific modification of apoa-I in HDL isolated from humans, we developed an LC-ESI-MS/MS analysis that uses selected reaction monitoring (SRM). This tandem MS technique is extraordinarily sensitive because only the selected precursor and product (fragmentation) ions are quantified. Therefore, chemical noise is significantly reduced and the signal to noise ratio can be markedly improved (87, 88). Moreover, virtually the entire duty cycle of the instrument is used to detect ions of interest. To further increase power, we use isotope-labeled [^{15}N]apoA-I protein (89). It is important to note that the SRM approach is a powerful one for detecting site specific modification of other proteins *in vivo*.

In our initial experiments, we analyzed apoA-I that we had exposed to trace amounts of HOCl, $ONOO^-$, or H_2O_2 in the MPO system. To confirm the identity of the oxidized peptides and to quantitatively assess the site-specific modifications, chlorinated and nitrated ^{15}N-labeled apoA-I was added to apoA-I before digestion. After digesting the proteins, we analyzed the resulting peptides with SRM. These studies confirmed that Tyr192 is the major site of chlorination and nitration when apoA-I is exposed to MPO *in vitro*. They also validated our approach to quantifying chlorinated and nitrated tyrosine residues or other modified amino acids in apoA-I of HDL isolated from humans.

It will be of great interest to determine whether the sites in apoA-I that are oxidatively modified *in vitro* are similar to those that are modified *in vivo*.

A model for generating dysfunctional HDL via oxidative pathways

Our *in vitro* studies have shown that chlorination of apoA-I by MPO impairs the lipoprotein's ability to transport cholesterol by the ABCA1 pathway. Furthermore, they suggest that chlorination impairs the direct interaction between apoA-I and ABCA1 while minimally affecting apoA-I's ability to solubilize phospholipids. Thus, MPO could potentially generate dysfunctional HDL in humans.

We therefore propose the following model for generating dysfunctional HDL in the human artery wall. Activated phagocytes and macrophages use NADPH oxidase to produce high concentrations of H_2O_2 near the plasma membrane. MPO secreted by the cells then converts H_2O_2 to HOCl, which modifies specific Tyr (Tyr192) and Met residues in apoA-I. Oxidation of lipid-free apoA-I by MPO impairs cholesterol efflux by the ABCA1 pathway. This reduction in cholesterol removal promotes foam cell formation and atherogenesis.

Our studies further demonstrate that plasma HDL isolated from humans with coronary artery disease (CAD) contains higher levels of 3-chlorotyrosine and 3-nitrotyrosine than HDL from healthy subjects. Thus, chlorinated and/or nitrated HDL might serve as a marker—and perhaps a mediator—of active cardiovascular disease in humans. In future studies, it will be important to determine the sites of chlorination and nitration of apoA-I *in vivo*. It will also

be critical to establish whether chlorinated and nitrated Tyr192 of apoA-I in plasma can indeed serve as markers for CAD.

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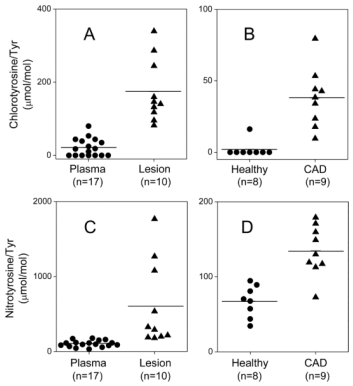

Figure 1. MS quantification of 3-chlorotyrosine and 3-nitrotyrosine in HDL isolated from human atherosclerotic lesions and plasma

Plasma was obtained from healthy humans and humans with established CVD. Human atherosclerotic tissue was obtained at surgery from subjects undergoing carotid endarterectomy. HDL was isolated from plasma and tissue by sequential ultracentrifugation. Oxidized amino acids isolated from hydrolyzed HDL proteins were quantified by isotope dilution GC/MS with selected ion monitoring (50, 51). (A) The levels of protein-bound 3-chlorotyrosine in lesion HDL was 8-fold higher than in circulating HDL ($P < 0.0001$). (B) The level of protein-bound 3-chlorotyrosine was 13-times higher in circulating HDL from the patients with established CVD than in circulating HDL from the healthy subjects ($P < 0.0001$). (C) The levels of protein-bound 3-nitrotyrosine in HDL isolated from the atherosclerotic lesions was six times higher than that in circulating HDL ($p < 0.01$). (D) The levels of protein-bound 3-nitrotyrosine in circulating HDL isolated from patients with established CVD was 2-fold higher than in circulating HDL isolated from the healthy subjects ($p < 0.01$).

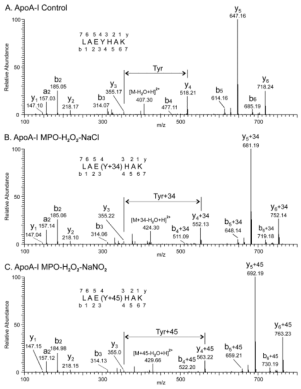

Figure 2. MS/MS identification of the major site of tyrosine chlorination and nitration in apoA-I exposed to MPO-H_2O_2-NaCl and MPO-H_2O_2-$NaNO_2$

MS/MS analysis of (A) [LAE<u>Y</u>HAK + 2H] $^{+2}$ (*m/z* 416.4), (B) [LAE(<u>Y</u>-Cl)HAK + 2H] $^{+2}$ (*m/z* 433.4), and (C) [LAE(<u>Y</u>-NO_2)HAK + 2H] $^{+2}$ (*m/z* 438.9) in apoA-I that had been oxidized with MPO-H_2O_2-NaCl or MPO-H_2O_2-$NaNO_2$. Lipid-free or lipid-associated apoA-I (5 μM) was exposed to H_2O_2 (125 μM) in the MPO-chloride or the MPO-nitrite system for 60 min at 37°C in phosphate buffer (20 mM sodium phosphate, 100 μM DTPA, pH 7.4). After the reaction was terminated with L-methionine, apoA-I was digested with trypsin, and the peptides were analyzed by LC-ESI-MS/MS.

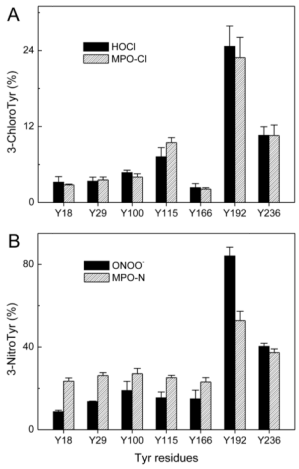

Figure 3. Site-specific chlorination of tyrosine residues in apoA-I exposed to HOCl or the MPO-H_2O_2-NaCl system (A) and nitration of tyrosine residues in apoA-I exposed to $ONOO^-$ or the MPO-H_2O_2-$NaNO_2$ system (B)

ApoA-I (10 μM) was exposed to HOCl (A, solid bars), $ONOO^-$ (B, solid bars), or H_2O_2 in the MPO-chloride system (A, single-line shaded bars) or MPO-nitrite system (B, single-line shaded bars) at molar ratio of 25:1 (oxidant/apoA-I) for 60 min at 37 °C in phosphate buffer (100 μM DTPA, 20 mM sodium phosphate, pH 7.4). After the reaction was terminated with L-methionine, a tryptic digest of oxidized apoA-I was analyzed by LC-ESI-MS and MS/MS and the oxidized peptides were detected and quantified using reconstructed ion chromatograms of precursor and product peptides. Product yield (%) = peak area of product ion / sum (peak area of precursor ion + peak areas of product ions) × 100. Peptide sequences were confirmed using MS/MS. Results are from 3 independent experiments (mean ± SD) (64).

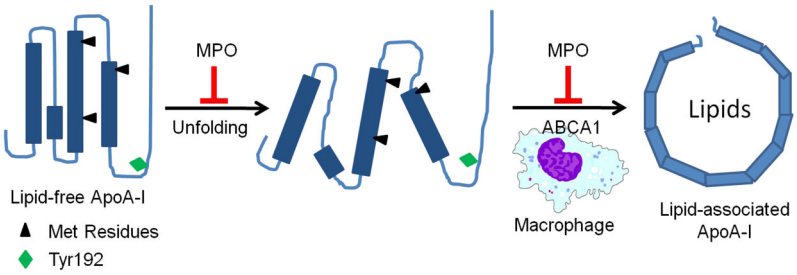

Figure 4. Oxidation may alter the remodeling pathway of apoA-I and impair its interaction with ABCA1

The conversion of lipid-free apoA-I to a lipid-associated form has been proposed to involve remodeling around the protein's hairpin loops (54, 90). Based on this model, we have proposed that MPO could inactivate the ABCA1 activity of apoA-I by oxidizing residues in or near its loop regions (73), which serve as hinges when the protein unfolds and refolds. **Black triangle**, Met residues (M86, M112, and M148); **Green diamond**, Tyr192.