Apolipoprotein A-I Tryptophan Substitution Leads to Resistance to Myeloperoxidase-Mediated Loss of Function

Dao-Quan Peng, Gregory Brubaker, Zhiping Wu, Lemin Zheng, Belinda Willard, Michael Kinter, Stanley L. Hazen, Jonathan D. Smith

Objective—Apolipoprotein A-I (apoAI) acts as an ABCA1-dependent acceptor of cellular phospholipids and cholesterol during the biogenesis of HDL, but this activity is susceptible to oxidative inactivation by myeloperoxidase. We tried to determine which residues mediated this inactivation and create an oxidant-resistant apoAI variant.

Methods and Results—Mass spectrometry detected the presence of tryptophan, methionine, tyrosine, and lysine oxidation in apoAI recovered from human atheroma. We investigated the role of these residues in the myeloperoxidase-mediated loss of apoAI activity. Site-directed mutagenesis and chemical modification were used to create variants of apoAI which were tested for ABCA1-dependent cholesterol acceptor activity and oxidative inactivation. We previously reported that tyrosine modification is not required for myeloperoxidase-induced loss of apoAI function. Lysine methylation did not alter the sensitivity of apoAI to myeloperoxidase, whereas site-specific substitution of apoAI methionine to valine increased the sensitivity of apoAI to myeloperoxidase. ApoAI tryptophan residues were identified as essential in apoAI function and oxidant sensitivity as substitution of all four apoAI tryptophan residues to leucine led to loss of function, but the conservative substitution to phenylalanine retained full function and was resistant to oxidative inactivation.

Conclusions—Tryptophan modification of apoAI is primarily responsible for the myeloperoxidase-mediated loss of the cholesterol acceptor activity of apoAI. (Arterioscler Thromb Vasc Biol. 2008;28:000-000)

Key Words: 

High levels of high density lipoprotein (HDL) and apolipoprotein A-I (apoAI), the major HDL protein, are associated with decreased risk for cardiovascular disease. The protection against cardiovascular disease afforded by apoAI and HDL may in part be attributed to their role in reverse cholesterol transport, although they have additional antiinflammatory, antioxidant, and endothelial cell regenerative properties that may also play protective roles. However, all HDL may not be functionally equivalent. Fogelman and colleagues have reported that HDL samples from patients with cardiovascular disease have a diminished capacity to inhibit LDL-induced monocyte migration in a coculture system. Previously, work from our group and subsequently from the group led by Heinecke have found that apoAI serves as a preferred target for myeloperoxidase (MPO)-catalyzed oxidative modification, leading to chlorination and nitrification of specific tyrosine residues and concomitant loss of ABCA1-mediated cholesterol acceptor activity. In addition, elevated content of chlorotryptophan and nitrotyrosine residues are observed in plasma apoAI recovered from human atheroma. We also reported that the degree of tyrosine modification of plasma apoAI, isolated from cardiology patients, correlates with its cholesterol acceptor activity. Thus, tyrosine modification of apoAI by MPO is associated with loss of apoAI function as an ABCA1-dependent acceptor of cellular lipids and can serve as a fingerprint to monitor the extent of apoAI modification. However, our prior studies using a tyrosine-free apoAI derivative (7YF, all seven tyrosines substituted by phenylalanine) show that it is equally susceptible to MPO-mediated loss of function compared to wild-type apoAI. We also demonstrated in vitro that MPO could modify apoAI lysine residues into lysine chloramines and aminoacidic acid, and apoAI tryptophan residues were converted into the mono- and di-oxygenated derivatives. It has also been previously shown that MPO modification of HDL led to a time- and dose-dependent decrease in bulk tryptophan fluorescence. However, whether such modifications occurred in vivo and might be responsible for oxidative inactivation of apoAI cholesterol efflux activity remains unknown. In the current study we report the presence of multiple site-specific tryptophan, methionine, and lysine modifications in apoAI isolated from human atheroma. We thus sought to determine the effects of altering the MPO sensitive lysine, methionine, and tryptophan residues in apoAI and whether such modifications...
might account for the observed oxidative inactivation of apoAI in vivo. We report here that replacement of the four apoAI tryptophan residues with leucines led to loss of its cholesterol acceptor function, whereas the replacement of tryptophan with phenylalanines not only preserved apoAI function but rendered it resistant to MPO-mediated loss of cholesterol acceptor and lipid binding activities. The apoAI with tryptophan to phenylalanine substitutions, though it retained its activity, was still sensitive to MPO-mediated cross-linking and loss of \( \alpha \)-helical content. The present studies thus suggest that apoAI tryptophan residues are responsible for MPO-dependent oxidative loss of apoAI function in vivo.

Methods

**Mass Spectrometry**

Human atheroma-derived apoAI was isolated by immunoaffinity chromatography as previously described. ApoAI was eluted in glycine buffer (pH 2.5) and subjected directly to trypsin digestion or first separated by SDS-PAGE and subjected to gel trypsin digestion. Mass spectrometry was performed and collision-induced dissociation (CID) spectra were obtained, as previously described. Chlorotyrosine and 2-amino adipic acid analyses were performed after acid hydrolysis with high isotope internal standards as previously described using duplicate assays of apoAI from human atheroma or from plasma of healthy volunteers isolated by immunoaffinity chromatography.

**Site-Directed Mutagenesis and Recombinant**

**ApoAI Production**

The pET-20b bacterial expression vector containing the cDNA of 6-His tagged recombinant human apoAI (rh-apoAI) was previously described. Point mutations to tryptophan (8, 50, 72, 108) and methionine (86, 112, 148) residues were made using QuickChange Mutagenesis Kit from Stratagene and confirmed by DNA sequencing. Plasmids were transformed into Escherichia coli strain BL21 (DE-3) pLysS and apoAI expression and purification was performed as described previously.

**ApoAI Modifications in Human Atheroma**

We previously demonstrated the presence of nitro- and chlorotyrosine in apoAI isolated from human atheroma tissue. We extended these studies to determine whether we could also identify modified tryptophan, methionine, and lysine residues in apoAI isolated from human atheroma, all modifications which we or others have previously identified after in vitro MPO treatment of apoAI. Using tandem mass spectrometry, we were able to detect monohydroxytryptophan residues at all four tryptophan positions, 8, 50, 72, and 108, and dihydroxytryptophan at position 108 within apoAI tryptophan residues with leucines led to loss of its cholesterol acceptor function, whereas the replacement of tryptophan with phenylalanines not only preserved apoAI function but rendered it resistant to MPO-mediated loss of cholesterol acceptor and lipid binding activities. The apoAI with tryptophan to phenylalanine substitutions, though it retained its activity, was still sensitive to MPO-mediated cross-linking and loss of \( \alpha \)-helical content. The present studies thus suggest that apoAI tryptophan residues are responsible for MPO-dependent oxidative loss of apoAI function in vivo.

**Results**

**ApoAI Modifications in Human Atheroma**

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Figure 1. Identification of modified apoAI in human atheroma by mass spectrometry. CID spectra were acquired after direct or in gel tryptic digest of immunopurified apoAI derived from human atheroma. Doubly charged ions were detected and fragmented in an LC-tandem mass spectrometry experiment. A, Peptide D1–R10 containing monohydroxytryptophan at residue 8. B, Peptide L46–K59 containing monohydroxytryptophan at residue 50. C, Peptide E62–K77 containing monohydroxytryptophan at residue 72. D, Peptide W108–R116 containing monohydroxytryptophan at residue 108 and methionine sulfoxide at residue 112. E, The same peptide as in D, but the tryptophan at residue 108 is converted to dihydroxytryptophan. F, Peptide L41–R49 containing methionine sulfoxide as at residue 48.
Apoptosis, which is a form of programmed cell death, can be induced by a variety of stimuli, including oxidants. These stimuli can damage cellular proteins, including apoAI, leading to a reduction in its function. 

In the amphipathic structure of apoAI, the 21 lysine residues are located between the hydrophilic and hydrophobic faces, playing a role in apoAI’s cholesterol acceptor activity.  

MPO modification of apoAI by MPO is an attractive candidate to be responsible for MPO-induced loss of apoAI function as previously demonstrated by Heinecke and colleagues. To substitute valine for all 3 methionines, we used recombinant human apoAI (rh-apoAI), which adds an additional methionine initiation codon and a 6-His tag to the N terminus. We and others have previously demonstrated that rh-apoAI behaves similarly to plasma-derived apoAI in its cholesterol acceptor activity, lipid binding activity, and its susceptibility to MPO-mediated loss of function. Using site-directed mutagenesis, we created an apoAI expression construct encoding a protein with the 3 internal methionines converted to valine (rh-apoAI 3MV). One cannot substitute for the initiating methionine; however, this methionine and the His tag can be chemically cleaved by formic acid. We determined that the rh-apoAI 3MV, regardless of whether the initiating methionine and His tag were intact or removed, had similar ABCA1-dependent cholesterol acceptor activity compared to wild-type rh-apoAI. In addition, the rh-apoAI 3MV, with or without the N-terminal methionine, and wild-type rh-apoAI were equally competent to interact with lipids and others have previously demonstrated that rh-apoAI behaves similarly to plasma-derived apoAI in its cholesterol acceptor activity, lipid binding activity, and its susceptibility to MPO-mediated loss of function. Using site-directed mutagenesis, we created an apoAI expression construct encoding a protein with the 3 internal methionines converted to valine (rh-apoAI 3MV). One cannot substitute for the initiating methionine; however, this methionine and the His tag can be chemically cleaved by formic acid. We determined that the rh-apoAI 3MV, regardless of whether the initiating methionine and His tag were intact or removed, had similar ABCA1-dependent cholesterol acceptor activity compared to wild-type rh-apoAI. In addition, the rh-apoAI 3MV, with or without the N-terminal methionine, and wild-type rh-apoAI were equally competent compared to wild-type rh-apoAI in the clearance of a DMPC:cholesterol (90:10 mole %) emulsion (supplemental Figure II), demonstrating that the 4WF variant was able to interact with lipids in a cell-free context. We also prepared rHDL by cholate dialysis using POCN and the wild-type or 4WF apoAI. Both yielded a similar pattern of rHDL discs estimated by non-denaturating gels at ~9.8, 12, and 17 nm, without any lipid free apoAI remaining (supplemental Figure IIIA). We tested the wild-type and 4WF rHDL, and both were equally competent...
to mediate ABCA1-independent cholesterol efflux from RAW264.7 cells (supplemental Figure IIIIB), without ABCA1-dependent acceptor activity, as expected for fully lipidated apoAI. We examined the predicted alpha helix content of these proteins by CD and found that the rh-apoAI had 56% alpha helix, whereas the 4WL and 4WF variants both had increased estimated alpha helix contents of 68% and 71%, respectively. Thus, the loss of efflux and lipid-binding activity of the 4WL variant cannot be attributed to loss of helical content.

Both rh-apoAI and the 4WF variant were then subjected to the MPO/Cl⁻/H₂O₂ oxidation system at increasing doses of H₂O₂. Figure 4B shows the result of a study representative of 4 different experiments using 2 independent preparations of each protein. As previously observed, the ABCA1-dependent cholesterol acceptor activity of wild-type apoAI was inhibited by increasing MPO induced oxidation; however, the 4WF variant maintained this activity even as an H₂O₂:apoAI mole ratio of 15 (Figure 4B). The cell-free lipid-binding activity of rh-apoAI 4WF was also resistant to MPO mediated inhibition, compared to rh-apoAI (Figure 4C).

MPO modification of apoAI leads to extensive cross linking resulting in dimers, multimers, and presumably intramolecular cross links as well, and we previously have
shown that the MPO-mediated apoAI cross linking pattern was not altered in the variant with all 7 tyrosine residues converted to phenylalanine.9 On subjecting rh-apoAI and the 4WF variant to MPO/Cl\textsubscript{2}/H\textsubscript{2}O\textsubscript{2} oxidation at increasing doses of H\textsubscript{2}O\textsubscript{2}, (using the identical protein products that were used for efflux in Figure 4B), we observed altered migration of these proteins in denaturing gels consistent with intermolecular cross linking (Figure 5). However, the migration patterns were different, with the 4WF variant giving a sharp predominant zone at \( \approx 70 \) kDa, whereas the wild-type protein yielded a less distinct predominant zone between 55 and 65 kDa (Figure 5). The migration of the monomer was altered for both proteins, which could be indicative of intramolecular cross links or other amino acid modifications. Although the 4WF variant is resistant to MPO-mediated loss of cholesterol acceptor activity, this variant was more susceptible to MPO-induced cross linking, particularly at low doses of H\textsubscript{2}O\textsubscript{2} (Figure 5). We also subjected these MPO-modified proteins to structural analysis by CD (supplemental Table II), and found that both were susceptible to loss of alpha helical content, although the 4WF variant started with a higher value.

The MPO/Cl\textsubscript{2}/H\textsubscript{2}O\textsubscript{2} oxidation system generates HOCI,\textsuperscript{20} the active reagent of bleach, and we and others have previously demonstrated that HOCI treatment of apoAI results in loss of cholesterol acceptor and lipid-binding activity.\textsuperscript{5,6} Thus, we subjected wild-type apoAI and the 4WF variant to increasing doses of HOCI. Similar to the findings with the MPO modification system, the cholesterol acceptor activity of the 4WF variant was resistant to this treatment, whereas the efflux activity of wild-type rh-apoAI was impaired by increasing doses of HOCI (Figure 6).

We used a quantitative mass spectrometry method, with heavy isotope internal standards, to detect total chlorotyrosine from MPO-modified rh-apoAI 4WF (H\textsubscript{2}O\textsubscript{2}:apoAI = 15:1), and we detected 1.2 mole\% conversion of tyrosine into chlorotyrosine, comparable to the highest levels of chlorotyrosine detected in apoAI recovered from human atheroma.\textsuperscript{4} Thus, the apoAI 4WF variant had fully functional efflux capacity (see Figure 4B) at physiological levels of tyrosine chlorination found within the highly oxidative environment of human atheroma tissues.

**Discussion**

ApoAI is a selective target for MPO-mediated modification as demonstrated by its high chlorotyrosine content in human plasma and atheroma.\textsuperscript{4,5} Moreover, it is clear that in vitro modification of apoAI by MPO leads to reduced activity of apoAI as both a lipid-binding protein and an acceptor of cellular lipids through the ABCA1 pathway.\textsuperscript{4,5,7,6} This has led to the concept that apoAI in some subjects may be dysfunctional, and in fact the cholesterol acceptor activity of apoAI varies in different subjects and is correlated with tyrosine chlorination, which is a unique indicator of MPO-mediated modification.\textsuperscript{4,16} These basic findings have been independently observed by our group and Heinecke’s group. However, the precise mechanism by which MPO modification renders apoAI dysfunctional is controversial. We previously reported that a tyrosine-free apoAI variant was equally susceptible as wild-type apoAI to MPO-mediated loss of apoAI function, and we identified MPO-induced in vitro modifications of lysine and tryptophan residues of unknown physiological and functional consequences.\textsuperscript{5} In the current work, we identified site-specific in vivo modifications of tryptophan, methionine, and lysine in atheroma-derived apoAI and then used chemical modification and site-directed mutagenesis to determine which of these residues is associated with the MPO-mediated loss of apoAI function. We found that replacing apoAI tryptophan residues with phenylalanine led to formation of a fully functional apoAI variant with marked resistance against oxidative inactivation by pathophysiological exposure levels of MPO-generated oxidants.

Using model peptides, Heinecke and colleagues reported that a lysine downstream of a tyrosine in a YXXK peptide motif can increase tyrosine chlorination by MPO or HOCI.\textsuperscript{22} Shao et al then used site directed mutagenesis of apoAI lysine
bound methionine acting as a scavenger for MPO-generated chlorination of tyrosine 192, consistent with protein MXXY to a KXXY motif), which increased tyrosine 115 substitution of methionine 112 to lysine (going from a able to an altered tertiary structure of this variant leading to the oxidation resistance of the 4WF variant may be attribut-
tional apoAI. Although we cannot exclude the possibility that causative alteration that results in the production of dysfunc-
tional apoAI. Alvarez et al did not directly test whether methionine substitution altered the sensitivity of apoAI to the MPO-mediated loss of function, but they did find that treatment of MPO-modified apoAI with the enzyme methionine sulfide reductase could partially restore the cholesterol acceptor activity of apoAI. In our studies, we directly observed that apoAI methionine residues play a protective scavenging role, as we found a markedly increased susceptibility of the methionine substituted 3MV apoAI variant to low doses of H2O2 in the complete MPO chlorination system. Thus, our site-directed substitution and cholesterol efflux data clearly show that neither methionine nor tyrosine serve as the oxidant sensitive residue involved in MPO-dependent apoAI inactivation. We also performed chemical modification of apoAI lysine residues, which failed to alter the sensitivity of apoAI to MPO-mediated loss of function.

We substituted all 4 apoAI tryptophan residues with either leucine or phenylalanine. The apoAI 4WL variant lost its lipid binding and cholesterol-accepting activities, whereas the 4WF variant retained these activities. Because tryptophan and phenylalanine are the most hydrophobic residues on the Wimley and white scale, our results imply that highly hydrophobic and bulky residues are required at the tryptophan positions on the nonpolar face of apoAI for its lipid binding and accepting functions. The global replacement of tryptophan with phenylalanine, which is far less susceptible to oxidative modification by MPO, created an apoAI that was clearly resistant to the MPO-mediated loss of function, but still susceptible to other modifications that lead to cross linking. In regard to the MPO-mediated cross linking of apoAI, in the current work we observed an altered cross linking pattern comparing the 4WF variant with wild-type apoAI, whereas in our prior study we did not observe an alteration of the cross linking pattern comparing the tyrosine-free 7YF variant. These combined data suggest that: (1) tryptophan residues either contribute directly to the cross links observed in wild-type apoAI, or that tryptophan substitution alters the tertiary structure and this alters the preferred sites of cross linking; and (2) tyrosine residues do not directly participate in the cross links observed in wild-type apoAI.

We conclude that tryptophan oxidation, which we observed in apoAI isolated from human atheroma, is likely to be the causative alteration that results in the production of dysfunctional apoAI. Although we cannot exclude the possibility that the oxidation resistance of the 4WF variant may be attributable to an altered tertiary structure of this variant leading to protection of some other sensitive residue. All of our in vitro MPO treatments were performed with lipid-free apoAI; similar to pre-β particles formed in vivo during lipoprotein remodeling. It is these lipid-free and lipid-poor apoAI particles that are capable to participate in ABCA1-mediated lipid efflux and thus play an important and physiological role in reverse cholesterol transport. We speculate that the 4WF apoAI variant would be a better therapeutic reagent, compared to wild-type apoAI or apoAI Milano, to promote the regression of plaques, a location where the levels of MPO-generated oxidants as well as modified apoAI are high.

Sources of Funding
This work was supported by National Institutes of Health Grants HL66082 (J.D.S.), PS0 HL077107 (S.L.H. and J.D.S.), and PO1 HL076491 (S.L.H.). D-Q.P. was the recipient of an American Heart Association Fellowship Award (0525386B).

Disclosures
None.

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Arterioscler Thromb Vasc Biol. published online August 7, 2008;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplemental Table I

Effect of MPO modification on control and methylated apoAI α-helix content measured by CD.

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<th>Mole ratio</th>
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Method

Far UV Spectral Analysis by Circular Dichroism (CD). Spectra for all apoAI samples were collected by CD using a Jasco J810 Spectropolarimeter (Jasco Incorporated, Easton, MD). The samples were read in a quartz cell with a 0.2 cm path length under a constant nitrogen flush at ambient temperature. Five spectra were collected for each sample from 190 – 250 nm in continuous scanning mode at a bandwidth of 1 nm with a 0.2 nm data pitch. The spectra were normalized to mean residue ellipticity using 115.5 as the mean residue weight for control apoAI. The percent α-helix was predicted using the mean residue ellipticity MRE at 222 nm.
Supplemental Table II
Effect of MPO modification on rh-apoAI and 4WF variant structure as measured by CD.

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<th>H2O2;apoAI Mole ratio</th>
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Supplemental Figure I

Methylated apoAl is susceptible to MPO mediated loss of function. ApoAl (filled circles, solid line) and reductively methylated apoAl (open circles, dashed line) were subjected to modification by MPO at varying H\textsubscript{2}O\textsubscript{2}: apoAl mole ratios. These proteins were then assayed for ABCA1 dependent cellular cholesterol acceptor activity during a 4 hr incubation at 5 µg/ml with cholesterol labeled RAW264.7 cells, which had been treated with 0.3 mM 8Br-cAMP to induce ABCA1. Data are means ± S.D. of triplicate determinations, when no bars appear, the S.D. is within the symbol.
Supplemental Figure II
Clearance of DMPC emulsions by wild type and 4WF rh-apoAI. A. 125 µg of a DMPC:cholesterol emulsion (90:10 by weight) in 0.25 ml of Tris buffered saline (TBS, 10 mM Tris pH 7.4, 100 mM sodium chloride, 0.5 mM EDTA) was pipetted into wells of a 96-well uv plate. 40 µl of TBS (thick line) or TBS containing 25 µg of wild type rh-apoAI (thin line) or rh-apoAI 4WF (dotted line) was added and quickly mixed and read for absorbance at 325 nm over time at 24°C (n=3 per condition, mean ± S.D). The 4WF variant yielded similar DMPC clearance compared to the wild type rh-apoAI.
**Supplemental Figure III**

**Wild type and 4WF rh-apoAI formation of rHDL and efflux acceptor activity.** A. rHDL was prepared by cholate dialysis using POPC and wild type or 4WF rh-apoAI (100:1 molar ratio POPC:apoAI) and run on 4-20% non-denaturing gradient polyacrylamide gels. Lane 1, size standards with diameters listed to left; lane 2, 10 µg wild type rh-apoAI rHDL; lane 3, 10 µg 4WF rh-apoAI rHDL. Both apoAI variants yielded ~9.8, 12, and 17 nm discs on non-denaturing gradient gels. The migration of lipid free rh-apoAI is shown by the arrow on the right side. B. Cholesterol efflux activity of rHDL preparations (4 hr. incubation) from [³H]cholesterol labeled RAW264.7 cells in the presence (filled bars) or absence (open bars) of ABCA1 induction by pretreatment with 0.3 mM 8Br-cAMP. rh-ApoAI (10 µg/ml) yielded ABCA1 dependent cholesterol acceptor activity; however, both the wild type (WT) and 4WF apoAI rHDL preparations (10 µg/ml apoAI) yielded only ABCA1 dependent cholesterol acceptor activity. Bars show mean ± S.D. (n=3).