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Site-specific Oxidation of Apolipoprotein A-I Impairs Cholesterol Export by ABCA1, a Key Cardioprotective Function of HDL

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Abstract

The mechanisms that deprive HDL of its cardioprotective properties are poorly understood. One potential pathway involves oxidative damage of HDL proteins by myeloperoxidase (MPO) a heme enzyme secreted by human artery wall macrophages. Mass spectrometric analysis demonstrated that levels of 3-chlorotyrosine and 3-nitrotyrosine—two characteristic products of MPO—are elevated in HDL isolated from patients with established cardiovascular disease. When apolipoprotein A-I (apoA-I), the major HDL protein, is oxidized by MPO, its ability to promote cellular cholesterol efflux by the membrane-associated ATP-binding cassette transporter A1 (ABCA1) pathway is diminished. Biochemical studies revealed that oxidation of specific tyrosine and methionine residues in apoA-I contributes to this loss of ABCA1 activity. Another potential mechanism for generating dysfunctional HDL involves covalent modification of apoA-I by reactive carbonyls, which have been implicated in atherogenesis and diabetic vascular disease. Indeed, modification of apoA-I by malondialdehyde (MDA) or acrolein also markedly impaired the lipoprotein's ability to promote cellular cholesterol efflux by the ABCA1 pathway. Tandem mass spectrometric analyses revealed that these reactive carbonyls target specific Lys residues in the C-terminus of apoA-I. Importantly, immunochemical analyses showed that levels of MDA-protein adducts are elevated in HDL isolated from human atherosclerotic lesions. Also, apoA-I co-localized with acrolein adducts in such lesions. Thus, lipid peroxidation products might specifically modify HDL *in vivo*. Our observations support the hypotheses that MPO and reactive carbonyls might generate dysfunctional HDL in humans.

Keywords

Myeloperoxidase; malondialdehyde; acrolein; dysfunctional HDL; 3-chlorotyrosine; coronary artery disease

1. Introduction

Despite enormous progress in preventing and treating cardiovascular disease, atherosclerosis remains the leading cause of death in industrialized societies (1). One important risk factor is an elevated level of low-density lipoprotein (LDL), the major carrier of cholesterol in the blood of humans on a Western diet (2). However, a high LDL level may not by itself be

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sufficient, because *in vitro* studies suggest that LDL must be modified to be atherogenic (3–5). For example, macrophages become foam cells, a hallmark of atherosclerosis, by taking up excess lipid, but they fail to accumulate lipid *in vitro* when incubated with high concentrations of native LDL. In contrast, they rapidly take up oxidized LDL.

Macrophage foam cells are abundant at all stages of the atherosclerotic process. Moreover, oxidative modifications of LDL are known to promote cholesterol uptake by the macrophage scavenger receptors that trigger foam cell formation (3). Significantly, oxidized LDL has also been detected in human atherosclerotic lesions, raising the possibility that oxidative modification of lipoproteins may be clinically important.

Unlike LDL, high-density lipoprotein (HDL) protects the artery wall from atherosclerosis (6). Many potential mechanisms have been proposed for HDL's anti-atherogenic effects, including the ability of HDL to inhibit inflammation and regulate NO production by endothelial cells (7–9). However, the best established cardioprotective effect of HDL is its role in reverse cholesterol transport (10). In this scenario, HDL removes excess cholesterol from artery wall macrophages and transports it back to the liver for excretion in bile. Apolipoprotein A-I (apoA-I), the major protein of HDL, plays a critical role in the first step of reverse cholesterol transport by enhancing the sterol efflux from macrophages. Thus, a severe deficiency of apoA-I increases CAD in both humans and mice (11). In contrast, overexpression of apoA-I in transgenic mice and rabbits increased HDL levels and consistently reduced CAD (12–14). Administering small apolipoprotein-mimetic peptides to mice also reduced atherogenesis (15, 16). These findings support the possibility that the arterial supply or activity of apoA-I and HDL particles can influence the progression and/or regression of lesions.

Active removal of cholesterol from macrophages to HDL is mediated by ABCA1 and ABCG1, two membrane-bound proteins called ATP-binding cassette (ABC) transporters (17–19). Another membrane-bound protein termed scavenger receptor B1 (SR-B1) can also play a role in removing cholesterol from macrophages (20). However, factors that might prevent apoA-I and HDL from interacting with cholesterol transporters have not yet been identified. One important pathway may involve reactive intermediates that modify artery wall proteins.

In this article, we review evidence that several reactive intermediates, including malondialdehyde (MDA), acrolein, and oxidants generated by myeloperoxidase (MPO), modify HDL in humans. We emphasize our recent findings that these reactive intermediates target specific amino acid residues in apoA-I, and discuss how such damage impairs HDL's ability to transport cholesterol by the ABCA1 pathway.

2. One key cardioprotective property of HDL involves reverse cholesterol transport

HDL—originally classified by its density on ultracentrifugation—is a noncovalent assembly of proteins and lipids (21). About ~70% of its protein mass is apoA-I (22), a 28-kDa peptide. Although most circulating apoA-I is found in HDL, ~5% of the apoA-I in plasma is thought to be lipid-free or lipid-poor (22, 23). ApoA-I has 243 amino acid residues, and its 10 repeated sequences of 11 or 22 amino acids (often termed helical repeats) play a key role in its secondary and tertiary structure (24).

Clinical, epidemiological, and animal studies have demonstrated a strong inverse relationship between HDL level and risk for coronary artery disease (25–27), suggesting that HDL is cardioprotective. This property has been attributed to several distinct pathways (6,

25). Many lines of evidence, including studies of myeloid cells deficient in ABC transporters, indicate that one key protective mechanism is cholesterol removal from macrophages by HDL and/or apoA-I (17, 28, 29). HDL components can remove cellular cholesterol by multiple mechanisms *in vitro* (30, 31). For example, plasma membrane cholesterol can be transferred to HDL phospholipids by passive diffusion, a process that is facilitated by interaction of HDL particles with SR-B1 (20).

An energy-dependent pathway for reverse cholesterol transport to apoA-I involves ABCA1 and ABCG1. ABCA1 is highly expressed in the liver and tissue macrophages (32, 33). When mutated, it can cause Tangier disease, which involves abnormally low levels of HDL and premature atherosclerosis (6, 17, 18, 34–37). Moreover, myeloid-specific deletion of ABCA1 markedly increased atherosclerosis in mice without affecting plasma lipoprotein levels (6). The ABCA1 pathway mediates the transport of cholesterol, phospholipids, and other lipophilic molecules across cellular membranes, where they are removed from cells mainly by lipid-free or lipid-poor apoA-I (17, 38).

The other major transporter, ABCG1, is highly expressed in tissue macrophages, and it mediates cholesterol transport from cells to mature, lipid-containing HDL particles (39, 40). The lungs of mice deficient in ABCG1 accumulate massive quantities of sterol- and lipid-laden macrophages (41), indicating that the transporter is also involved in cholesterol efflux from macrophages *in vivo* (39, 40). However, selective ABCG1 deficiency does not always promote atherosclerosis in hypercholesterolemic mouse models (42–44). In contrast, atherosclerosis is markedly enhanced when mouse macrophages lack both ABCA1 and ABCG1 (45, 46). Thus, the two pathways appear to work together in mice to prevent foam cell formation and atherosclerosis.

3. HDL's anti-inflammatory properties may contribute to its cardioprotective effects

Many lines of evidence support this proposal (47). For example, systemic inflammation and marked atherosclerosis are evident in hypercholesterolemic mice that are also deficient in apoA-I (48). Recent studies indicate that the animals' immune system also is abnormal (49). Macrophages harvested from mice that lack ABCA1 alone or both ABCA1 and ABCG1 release markedly higher levels of inflammatory cytokines when stimulated with lipopolysaccharide (LPS), a bacterial ligand for the TLR4 receptor (31, 50, 51).

HDL also suppresses the type 1 interferon response in macrophages (9), which is of central importance in atherogenesis (52). 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 (53). Indeed, inflammation significantly remodels both HDL lipids and proteins during acute inflammation (54, 55). Both mouse and human studies suggest that inflammatory HDL can become dysfunctional and lose its cardioprotective effects (28, 47, 56, 57), though the underlying mechanisms are poorly understood. One important pathway could involve oxidative damage (58–60).

4. Myeloperoxidase (MPO), a heme enzyme that produces reactive chlorinating and nitrating species, modifies artery wall proteins in humans

One potential pathway for generating dysfunctional HDL involves myeloperoxidase (MPO), a heme enzyme in neutrophils, monocytes, and some macrophage populations. Co-localization of macrophages with immunostaining for MPO has been observed in human atherosclerotic lesions, which also yield active enzyme (61). These observations suggest that

lipid-laden macrophages, the cellular hallmark of the atherosclerotic lesion, express MPO in response to inflammatory stimuli associated with atherosclerosis.

Macrophages also produce reactive oxygen species, such as superoxide and hydrogen peroxide (H_2O_2). MPO uses H_2O_2 to generate reactive intermediates (62). The major end product at plasma concentrations of chloride ion is generally thought to be hypochlorous acid (HOCl), a potent anti-microbial agent (63, 64). HOCl is a powerful chlorinating reagent that reacts with a wide variety of biomolecules (65). For example, HOCl produced by MPO converts free and protein-bound tyrosine (Tyr) residues to 3-chlorotyrosine (66, 67), which is generated by acute inflammation in mice. 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 to proteins (67–69).

Another pathway for oxidizing artery wall proteins involves the nitric oxide (NO) to promote vasodilation (70, 71). NO reacts rapidly with superoxide ($O_2^{\bullet -}$) to form peroxynitrite ($ONOO^-$), a reactive nitrofen species (72). Macrophages are a rich source of both $O_2^{\bullet -}$ and NO, suggesting that $ONOO^-$ may be an important reactive nitrogen species *in vivo*. Furthermore, oxidation of NO produces nitrite (NO_2^-), which MPO and H_2O_2 convert to nitrogen dioxide radical ($NO_2^{\bullet}$), another potent nitrating intermediate (73, 74). Both $ONOO^-$ and $NO^{\bullet}$ generate 3-nitrotyrosine when they react with tyrosine residues. Such reactive nitrogen species might promote inflammation by nitrating lipoproteins and other artery wall proteins.

Mass spectrometric analyses have detected high levels of 3-chlorotyrosine and 3-nitrotyrosine in LDL and HDL isolated from human atherosclerotic tissue (69), strongly suggesting that MPO is one pathway for oxidative damage in the human artery wall. Moreover, tyrosine chlorination and nitration are impaired when mice are deficient in MPO (68). Thus, the MPO pathway might both nitrate and chlorinate lipoproteins *in vivo*.

4.1 MPO oxidizes HDL in humans with cardiovascular disease

Despite considerable interest in the atherogenic potential of oxidized lipoproteins, the pathways that promote HDL oxidation *in vivo* remain unclear One potential mechanism for the formation of dysfunctional HDL involves reactive intermediates generated by MPO. Indeed, recent studies from two different groups strongly suggest that MPO oxidizes HDL in humans (58–60).

Our group isolated HDL from plasma of subjects with established CAD and from age- and sex-matched healthy subjects (58, 59). Using isotope dilution mass spectrometry (MS), we detected much higher levels of protein-bound 3-chlorotyrosine and 3-nitrotyrosine in circulating HDL from the CAD patients than in HDL from the controls (58, 59). Moreover, levels of both abnormal amino acids were significantly higher in HDL isolated from atherosclerotic lesions than in plasma HDL (58, 59). Other investigators have reported similar results (60). As 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 (68, 74), these observations strongly support the hypothesis that MPO oxidizes HDL in humans.

Because MPO oxidation products were undetectable in plasma LDL or total plasma proteins in these studies, it is unlikely that HDL was oxidized in the circulation. However, it might have been damaged before entering the circulation, perhaps in a microenvironment rich in MPO and depleted of antioxidants. One likely environment is the inflamed atherosclerotic lesion. It is important to note that HDL is thought to be cardioprotective by entering the artery wall, interacting with macrophages to remove cholesterol, and then leaving the artery

wall and returning the cholesterol to the liver for excretion. In this scenario, chlorinated HDL detected in the plasma is derived from atherosclerotic lesions. 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 (58, 59). Thus, plasma HDL in subjects with established CAD, but not in control subjects, may be enriched with chlorotyrosine, a marker of damage by MPO.

4.2 Macrophage-specific expression of MPO promotes atherosclerosis in mice

Mouse models have provided major insights into atherogenesis. However, *macrophages in mouse atherosclerotic tissue do not express MPO* (75). Thus, it has not been possible to use knockout mice to study the role of MPO in atherosclerosis because this only affects levels of the enzyme in circulating neutrophils and monocytes.

To address this important limitation of mouse models, we generated a transgenic mouse that expresses human MPO selectively in macrophages (76). This MPO transgenic (MPO-Tg) mouse line allowed us to directly examine the impact of MPO expression in macrophages on atherosclerosis. After LDL receptor knock-out ($LDLR^{-/-}$) mice received bone marrow from these MPO-Tg mice and ate a high-fat, high-cholesterol diet, they had significantly more atherosclerosis than mice transplanted with bone marrow from either wild type or $LDLR^{-/-}$ mice. These observations strongly suggest that expression of MPO in macrophages promotes atherosclerosis in this mouse model of hypercholesterolemia.

4.3 MPO-mediated chlorination, but not nitration, of apoA-I impairs cholesterol transport by ABCA1

For oxidized HDL to promote atherosclerosis, oxidation would need to compromise HDL's cardioprotective functions, such as cholesterol removal. In the artery wall, membrane-associated ABCA1 interacts with lipid-free or poorly-lipidated apoA-I to remove excess cholesterol from resident macrophages (17, 18, 77, 78). ApoA-I contains amphipathic α-helices, a structural motif that promotes lipid association (22, 24, 79, 80). Synthetic peptides that have α-helices but differ in primary sequence from apoA-I can also interact with ABCA1 to promote reverse cholesterol transport from cells. Therefore, interaction with ABCA1 appears to require α-helices, and agents that disrupt helical structure might inhibit cholesterol removal.

Because HDL isolated from humans with CAD contains elevated levels of 3-chlorotyrosine and 3-nitrotyrosine (58–60), we determined how oxidation affected apoA-I's ability to promote cholesterol efflux from cells (58, 81). Oxidizing the protein with H_2O_2 had no effect. In striking contrast, exposing it to increasing concentrations of H_2O_2 in the presence of MPO and NaCl (the MPO-H_2O_2-NaCl system, which generates HOCl) progressively and severely impaired its ability to remove cellular cholesterol from BHK cells transfected with ABCA1 (Fig. 1B; ref. (81)). Similar results were observed with increasing concentrations of reagent HOCl (Fig. 1A, ref. (81)). This impairment correlated closely with the extent to which one particular tyrosine residue—Tyr192—was chlorinated (Fig. 2A; ref. (81, 82)). Although Zheng *et al.* reported similar results (60), they also found that nitrating apoA-I with MPO impaired its activity with ABCA1. In contrast, we found that nitrating apoA-I with $ONOO^-$ or H_2O_2 in the presence of MPO and $NaNO_2$ (the MPO-H_2O_2-$NaNO_2$ system) barely affected cholesterol efflux activity (Fig. 1; ref. (81)), despite extensive nitration of Tyr192 (Fig. 2B; ref. (81, 83)).

Our observations suggest that oxidation of apoA-I by HOCl or the MPO chlorinating system, but not by the MPO nitrating system, selectively impairs the protein's ability to remove cholesterol from cells by a pathway requiring ABCA1. Thus, HOCl generated by

MPO might be a mechanism for generating dysfunctional HDL, for impairing ABCA1-dependent cholesterol efflux from macrophage foam cells in the human artery wall, and therefore for promoting the development of atherosclerotic plaque.

4.4 The YXXK motif directs site-specific tyrosine chlorination in apoA-I

Most studies of protein oxidation have focused on the vulnerability of individual amino acid side chains. Remarkably little is known about the influence of nearby residues or specific motifs. To explain the selective oxidation of Tyr192 (58, 81, 82), we hypothesized that Lys residues located in a YXXK motif (Y = tyrosine, K = lysine, X = unreactive amino acid) promote Tyr chlorination in proteins. Thus, Tyr would be oxidized permanently but Lys only transiently.

When we tested our model by using synthetic peptides, we found that lysine residues located in a YXXK motif can direct the regiospecific chlorination of nearby tyrosine residues by a pathway involving chloramine formation (82). Long-lived chloramines (63), which have been proposed to chlorinate Tyr in synthetic peptides (66, 82), form when HOCl (or other chlorinating agents) reacts rapidly with the ε-amino group of Lys. As Tyr192 is two residues away from Lys195 in apoA-I's primary sequence, we proposed that site-specific chlorination of Tyr192 in apoA-I by MPO requires the participation of Lys195, which is located in a YXXK motif.

Using hydrogen-deuterium exchange, Zheng *et al*. showed that MPO interacts with the region of apoA-I that contains Tyr192 (60). 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, perhaps via interactions with positively charged amino acids such as Lys. To distinguish between their model and ours, we engineered a series of mutations in the cDNA of human apoA-I, using site-directed mutagenesis (84). Studies with those mutations provided strong evidence that YXXK can direct the regiospecific chlorination of tyrosine residues in apoA-I. For example, chlorination of Tyr192 was blocked when we mutated Lys195 to arginine. Also, tyrosine residues that normally resist chlorination were chlorinated in high yield when we introduced the KXXY motif into that region of the protein.

Importantly, we observed virtually identical results when we used reagent HOCl or the complete MPO chlorinating system (84). The reaction with reagent HOCl clearly cannot involve direct interaction or binding of MPO with apoA-I. These observations strongly support our proposal that the YXXK motif orchestrates the regiospecific chlorination of tyrosine residues in proteins. They argue against the hypothesis that MPO must interact directly with apoA-I to selectively chlorinate Tyr192.

4.5 MPO impairs ABCA1-dependent cholesterol export from cells by oxidizing methionine residues and promoting site-specific chlorination of tyrosine 192 in apoA-I

Amino acid analysis identified tyrosine, methionine, and phenylalanine residues as the major targets when HOCl oxidizes apoA-I *in vitro* (85–87). However, those studies did not determine whether residues at specific locations are especially vulnerable to chlorination. Using tandem MS, we identified a single tyrosine residue, Tyr192, as the major chlorination site when HOCl oxidizes apoA-I (Fig. 2A) (81–83). Moreover, we noted a strong linear association between the extent of Tyr192 chlorination and loss of ABCA1 transport activity (81).

To determine whether tyrosine chlorination in apoA-I reduces ABCA1 activity, we converted the Tyr192 in apoA-I to phenylalanine (Tyr192Phe). The relationship between protein concentration and ABCA1-dependent cholesterol efflux was virtually identical for the mutant protein and the wild type protein (Fig. 3A; ref. (84)), strongly suggesting that the

Tyr192Phe mutation had no major impact on the structure of lipid-free apoA-I. When the mutant protein was exposed to either HOCl or the MPO chlorinating system, the substitution offered a small but significant protection against inactivation (Fig. 3B) (84). Other investigators replaced all seven tyrosine residues in apoA-I with phenylalanine, which is resistant to chlorination. As with wild type apoA-I, this mutant was unable to promote ABCA1-dependent cholesterol efflux after being oxidized by MPO (88). However, our Tyr192Phe mutant appeared somewhat resistant to inactivation when the concentration of oxidant was high (Fig. 3B) (84). The differences between the two mutant proteins may reflect our use of transfected cells that express high levels of ABCA1, which may be more sensitive to subtle changes in apoA-I's activity.

HOCl reacts more strongly with the alkylated thiol of methionine than with Tyr residues (89). Moreover, methionine sulfoxide [Met(O)] has been detected in circulating HDL (90). However, the role of methionine oxidation in apoA-I's cholesterol efflux activity has been unclear. Using tandem MS, we confirmed that HOCl or the complete MPO system quantitatively oxidized all three methionine residues in apoA-I to Met(O) (84). To determine whether Met(O) contributed to the loss of ABCA1 activity, we used a bacterial methionine sulfoxide reductase that converts both the R- and S-forms of Met(O) residues back to methionine (91). Methionine sulfoxide reductase completely reversed methionine oxidation in apoA-I that had been exposed to MPO (84). However, the protein's cholesterol efflux activity was only partly restored (Fig. 3B; ref (84)), implying that oxidation of Met residues alone is insufficient to eliminate that apoA-I function. Thus, neither inhibition of Tyr192 chlorination nor reduction of Met(O) alone markedly protected apoA-I from oxidative inactivation.

We next determined if a combination of Tyr chlorination and Met oxidation contributes to the loss of ABCA1 activity when apoA-I is exposed to MPO. When Tyr192Phe apoA-I was exposed to HOCl or the MPO system, subsequent treatment with methionine sulfoxide reductase almost completely restored its ability to promote cholesterol efflux by ABCA1 (Fig. 3B, ref. (84)). It is noteworthy that Tyr192 is one of eight amino acid residues that are completely conserved in every species of apoA-I sequenced (24). Moreover, the conserved pair of amino acids Glu191-Tyr192 has been proposed to play a key role in a solvent-exposed loop-helix-loop that regulates the structure and function of helical repeats 9 and 10 (24). These observations indicate that a combination of Tyr192 chlorination (84) and methionine oxidation (90)—perhaps together with other structural changes—impairs the ability of apoA-I to activate sterol efflux by the ABCA1 pathway (84).

HOCl can also oxidize tryptophan residues in apoA-I (87, 92). However, oxidation failed to prevent a mutant apoA-I in which Phe had replaced all four Trp residues from activating ABCA1 (93). Analysis by circular dichroism suggested that the α-helical content of lipid-free control protein was 56% while that of the mutant protein was 71% (93), which matches that (~70%) of lipid-associated native apoA-I (85). These observations indicate that the tryptophan substitutions significantly alter the tertiary structure of lipid-free apoA-I. Thus, the mutant protein's oxidation resistance might result from structural alterations.

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

Many lines of evidence support the hypothesis that lipid removal by apolipoproteins involves three steps. First, apolipoproteins bind directly to ABCA1, stabilizing the protein and trigger sterol enrichment of the exofacial leaf of the plasma membrane. Next, apoA-I binds to exovesiculated lipid domains formed by ABCA1. Finally, apoA-I promotes the excretion of exovesiculated lipids (17, 94–96). Any of these steps could be impaired if apoA-I were oxidized by the MPO pathway.

4.6.1 Oxidation impairs apoA-I binding to ABCA1—To investigate potential mechanisms, we used a competitive assay to test whether chlorination reduces the direct binding of apoA-I to ABCA1 (83). BHK cells transfected with ABCA1 were incubated with ^{125}I-apoA-I in the absence or presence of unlabeled and unmodified apoA-I or with oxidized apoA-I. The cells were then treated with the cross-linking agent DSP. Following immunoprecipitation of ABCA1 and then SDS-PAGE, we detected any ^{125}I-apoA-I that had cross-linked to ABCA1, using autoradiography and phosphorimaging. 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 (83). These observations indicate that chlorinated apoA-I is less able to bind to ABCA1 than is native or nitrated apoA-I. Thus, MPO-mediated chlorination of apoA-I impairs the first step in cholesterol transport.

Most of the apoA-I binding sites on the surfaces of cells that express ABCA1 are lipid domains formed by ABCA1 (95–98). To determine how oxidation by the MPO pathway affected apoA-I's ability to bind to such cells, we exposed apoA-I to HOCl or H_2O_2 plus MPO and NaCl. Chlorination of apoA-I by either HOCl or the MPO-H_2O_2-NaCl system impaired its ability to compete with ^{125}I-apoA-I for binding to BHK cells expressing ABCA1 (83). In contrast, apoA-I nitrated by $ONOO^-$ or the MPO-nitrite system was able to bind normally. These results indicate that chlorination—but not nitration—severely impairs the high-affinity binding of apoA-I to cells that express ABCA1.

When apoA-I stabilizes ABCA1, it does not need to bind directly to the transporter (99). We therefore compared the abilities of control and oxidized apoA-I to stabilize ABCA1 protein. For these studies, we used J774 macrophages that express ABCA1, because those cells rapidly degrade ABCA1 protein in the absence of apolipoproteins and ABCA1 inducers (99–101). Indeed, when macrophages were treated with 8-Br-cAMP (to induce ABCA1) and subsequently incubated without 8-Br-cAMP, most of the induced ABCA1 protein disappeared within 4 h (83). When we included control, chlorinated, or nitrated apoA-I in the media, this loss was minimized (83). Thus, although chlorinated apoA-I was markedly less able to interact with ABCA1 and to remove lipid, it stabilized ABCA1 protein just as effectively as untreated or nitrated apoA-I.

4.6.2 Oxidation of apoA-I has variable effects on phospholipid binding—Different investigators have reached different conclusions regarding the impact of apoA-I oxidation on lipid binding, a key step in sterol export by ABCA1 (83, 102, 103). For example, Stocker and colleagues demonstrated that oxygenation of methionine residues in apoA-I resulted in a greater affinity for lipid, as determined by the rate of clearance of multilamellar phospholipid vesicles (102). However, Kinter, Smith and colleagues found that chlorination or nitration of apoA-I impaired its ability to bind lipid, using an assay that monitored the ability of the apoA-I to inhibit LDL aggregation (103). Using two different assays, we found that chlorination or nitration of apoA-I could either improve or impair lipid binding (83).

The conflicting results of the different studies suggest that oxidation of apoA-I has complex effects on phospholipid binding, perhaps because different method were used to assess protein-lipid interaction. In future studies it will be important to clarify the impact of apoA-I oxidation on its ability to interact with phospholipids.

5. Reactive carbonyls: potential intermediates for generating dysfunctional HDL in humans

Reactive carbonyls, which can also modify proteins (104, 105), have been implicated in atherogenesis and diabetic vascular disease (3, 106, 107). They can result from lipid peroxidation or oxidation of carbohydrates or amino acids (104, 105, 108). Major carbonyl products of carbohydrate oxidation *in vivo* are thought to be glyoxal, methylglyoxal, and glycolaldehdye, which are precursors of advanced glycation end products (AGEs). Lipid peroxidation yields a different spectrum of reactive carbonyl compounds, including malondialdehyde (MDA), 4-hydroxynonenal (HNE), and acrolein (106, 108), which have been termed advanced lipoxidation end products (ALEs; ref. (104)). Figure 4A shows the structure of major physiologically relevant reactive carbonyls. Importantly, ALE and AGE levels are elevated in diabetes, a disease that greatly increases the risk for CAD (104, 109).

We therefore determined whether reactive carbonyls can interrupt cholesterol efflux by the ABCA1 pathway. Treating cultured macrophages with glycolaldehyde or glyoxal—two AGEs—strongly inhibited ABCA1-dependent transport of cholesterol from cells to apoA-I (110). Glycolaldehyde and glyoxal destabilized ABCA1 and nearly abolished its binding to apoA-I, indicating that these carbonyls inhibit cholesterol export by directly modifying ABCA1. Reactive carbonyls may also impair cholesterol transport by modifying HDL lipoproteins, because HDL is the major carrier of lipid hydroperoxides in plasma and may be constantly exposed to reactive carbonyls (111). Moreover, when plasma is oxidized *ex vivo*, apoA-I is a major target for lipid oxidation products (112). Reactive carbonyls are also known to impair HDL's function *in vitro.* For example, high concentrations of carbonyls modified apoA-I and inhibited LCAT activation by HDL, but the underlying mechanisms were not identified (113). When apoA-I incorporated into discoidal reconstituted HDL was exposed to methylglyoxal, it lost its ability to activate lecithin:cholesterol acyltransferase (LCAT) (114). This loss associated with modification of arginine, lysine, and tryptophan residues. These observations suggest that reactive carbonyls can potentially generate dysfunctional HDL.

5.1 Acrolein impairs ABCA1-dependent cholesterol export from cells by modifying C-terminal lysine residues of apoA-I

Acrolein is the most reactive α,β-unsaturated aldehyde, and it rapidly modifies biological nucleophiles (115). This potent electrophile may be produced in the body through lipid peroxidation or peroxidation of threonine by MPO, and high levels are found in cigarette smoke (116–118). Biomarkers of lipid peroxidation products, renal disease, and cigarette smoking all strongly associate with an increased risk of vascular disease (5, 104, 119). Moreover, epitopes recognized by a monoclonal antibody specific for lysine adducts of acrolein have been detected in macrophages of atherosclerotic lesions (118). Thus, acrolein might be another reactive species that damages artery wall proteins.

We investigated the possibility that acrolein might promote the formation of macrophage foam cells in the artery wall by impairing apoA-I's ability to remove cholesterol from cells by the ABCA1 pathway. When BHK cells transfected with ABCA1were incubated with apoA-I and exposed to increasing concentrations of acrolein, the apolipoprotein progressively lost its ability to promote cholesterol and phospholipid efflux (Fig. 5A) (120). When increasing amounts of apoA-I were incubated with those cells, acrolein-modified apoA-I was significantly less able than untreated apoA-I to remove cholesterol (Fig. 5B) (120). Thus, modification by acrolein, like chlorination by MPO, reduces apoA-I's ability to promote cholesterol and phospholipid efflux by the ABCA1 pathway.

Like other α,β-unsaturated aldehydes, acrolein reacts with the sulfhydryl group of cysteine, the imidazole group of histidine, and the ε-amino group of lysine (106, 121, 122). Indirect evidence suggests that it might also modify arginine (123). To investigate the reactions of amino acid side chains with acrolein, we synthesized model peptides containing Lys, His, and Arg (apoA-I contains no cysteine). We found that acrolein reacted with Lys and His but not with Arg residues in the peptides (120).

In vitro studies have identified a number of acrolein adducts with His and Lys in proteins (Fig. 4B). ApoA-I contains five His, 21 Lys, and 19 Arg. We determined that all 21 Lys residues are modified by acrolein, suggesting that Lys residues are acrolein's major targets in apoA-I protein (120). Importantly, our approach demonstrated that Lys226 was the major residue modified by acrolein (120), strongly implying that the local amino acid environment and perhaps the α-helical structure of apoA-I significantly influence acrolein's reactions with specific residues in this protein.

To determine whether modification of Lys226 might account for the loss of apoA-I function, we examined the relationship between the disappearance of the peptide containing this residue and loss of ABCA1 activity. Loss of the peptide associated strongly with a decrease in the ability of acrolein-modified apoA-I to promote cholesterol efflux (120). Moreover, there was a strong linear correlation between the appearance of MP-Lys226 and decreased lipid efflux from cells (120).

We cannot exclude the possibility that other modifications to apoA-I helped impair cholesterol efflux, but our observations suggest that modification of Lys226, which lies in helix 10, is particularly important. Previous studies demonstrated that deleting helix 10 and/or helices 9 plus 10 of apoA-I greatly diminishes ABCA1-dependent cholesterol efflux (124, 125). Moreover, a synthetic peptide that mimics the 9/10 helix of apoA-I mediated high-affinity cholesterol efflux via ABCA1 (126). Those investigators suggested that a specific structural element containing a linear array of acidic residues spanning two apoA-I amphipathic α-helices mediates cholesterol efflux and stabilizes ABCA1. It is noteworthy that modification of Lys226 could disrupt this linear array, which might account for acrolein's ability to impair cholesterol transport.

Importantly, immunohistochemical studies with a monoclonal antibody that reacts specifically with MP-Lys (118, 121) found that acrolein adducts co-localized with apoA-I in human atherosclerotic lesions (120), suggesting that apoA-I is targeted and modified by acrolein in atherosclerotic intima. Thus, our observations suggest the following model. Lipid peroxidation and amino acid oxidation by MPO in the artery wall generate acrolein (116, 127). The aldehyde then reacts with apoA-I. Modification of Lys residues in helix 10 impairs apoA-I's ability to remove cholesterol from lipid-laden macrophages, contributing to the formation of atherosclerotic lesions. Our findings suggest that acrolein production by phagocytes is a physiological mechanism for modifying apoA-I to produce dysfunctional HDL and promote atherogenesis.

5.2 Malondialdehyde impairs ABCA1-dependent cholesterol efflux by pathways associated with cross-linking C-terminal lysine residues in apoA-I

We next investigated whether other physiologically relevant reactive carbonyls resulting from lipid peroxidation (MDA and HNE) or carbohydrate oxidation (glycolaldehyde, glyoxal, and methylglyoxal) also impair apoA-I's ability to promote cholesterol efflux. When we transfected BHK cells with ABCA1 and incubated them with apoA-I that had been exposed to carbonyls, the only carbonyl that affected cholesterol efflux was MDA (Fig. 6A) (128).

Exposure to increasing concentrations of MDA progressively and dramatically deprived apoA-I of its ability to promote cholesterol efflux (Fig. 6B) (128). When increasing amounts of apoA-I were incubated with BHK cells transfected with ABCA1, cholesterol removal was dramatically lower with MDA-modified apoA-I than with native apoA-I (Fig. 6D) (128). Thus, MDA significantly reduces apoA-I's ability to promote cholesterol efflux by the ABCA1 pathway. In contrast, modification by HNE, glyoxal, methylglyoxal, or glycolaldehdye has little effect.

We used tandem MS to elucidate the molecular basis for apoA-I's selective vulnerability to MDA. Previous studies identified Lys residues as major targets, and the adducts included *N*-propenal-lysine (K+54, Fig. 4C) and dihydropyridine (DHP)-lysine (K+134) (106, 129). To determine which adducts form in apoA-I, we exposed the protein to MDA, digested it, and analyzed the resulting peptides. Tandem MS detected K+54 (128). In contrast, we failed to detect DHP-Lys when we exposed apoA-I to a wide range of MDA concentrations.

The *N*-propenal-Lys adduct contains a carbonyl group that could cross-link with a second Lys residue to form Lys-MDA-Lys (Fig. 4C). Indeed, when apoA-I was exposed to MDA, tandem MS identified K+36+K (128), the Lys-1-amino-3-iminopropene-Lys (Lys-MDA-Lys) cross-link in which two Lys residues have gained 36 amu (130). To determine whether MDA targets specific Lys residues in apoA-I, we used isotope dilution MS to quantify the product yields of modified Lys residues (128). Certain Lys residues were converted in high yield to MDA-Lys (K+54) or Lys-MDA-Lys (K+36+K). Moreover, six of the 10 most reactive Lys residues resided in helical repeats 7 to 10 of apoA-I's C-terminus (128), suggesting that ABCA1 activity might be seriously impaired when this region is modified.

Consistent with this proposal, we identified the C-terminus of apoA-I as the major site for cross-linking by MDA (128). A molecular dynamic model based on energy minimization suggested that cross-linking drastically condenses the C-terminus of apoA-I, affecting its conformation and conformational adaptability. Importantly, studies of deletion mutants of apoA-I suggest that repeat 10 of the C-terminus helps activate ABCA1 (131, 132). Taken together, these observations support the proposal that the protein loses its ability to promote cholesterol efflux by the ABCA1 pathway when MDA modifies its C-terminus.

MDA is one of the most abundant products of lipid peroxidation (106). Moreover, MDA levels appear to be elevated in diseases such as diabetes mellitus that increase the risk of cardiovascular disease (133). Importantly, immunochemical analyses of HDL with MDA2, a monoclonal antibody that binds with high affinity to a variety of MDA-modified proteins (134), demonstrated that HDL isolated from atherosclerotic tissue contained higher levels of MDA-modified proteins than HDL from plasma of apparently healthy humans (128). This suggests that MDA modifies HDL in human atherosclerotic tissue. It also suggests that cross-linking of Lys residues in apoA-I's C-terminus by MDA in the artery wall could promote the formation of macrophage foam cells, because cholesterol efflux by the ABCA1 pathway would be impaired.

5.3 HNE modifes His residues in apoA-I without affecting cholesterol efflux by ABCA1

HNE (a reactive carbonyl generated by lipid peroxidation) (106) can react with imidazoles (His), thiols (Cys), or free amino (Lys) groups of proteins to form stable Michael adducts with hemiacetal structures (Michael adducts form when a nucleophile is added to an α,β-unsaturated carbonyl compound, Fig. 4D) (130, 135). After we exposed apoA-I to a 50:1 molar ratio of HNE, all 5 peptides containing His were lost in near-quantitative yield (~90%) (128). Tandem MS revealed that the His residue in each peptide had gained 158 amu or 156 amu without $NaBH_4$ reduction (128), suggesting that His had been converted to the hemiacetal adduct (136). Remarkably, this concentration of HNE had no effect on apoA-I's

ability to promote cholesterol efflux by the ABCA1 pathway, likely because all five His residues lie in the protein's central region, which does not appear to play a major role in activating the transporter.

6. Mechanisms for generating dysfunctional HDL in humans

Our *in vitro* studies have shown that chlorination of apoA-I by MPO hampers the lipoprotein's ability to transport cholesterol by the ABCA1 pathway. Modification of one or more Met residues and chlorination of Tyr192 are responsible for this impairment. Moreover, chlorination of apoA-I by MPO hinders the direct interaction between apoA-I and ABCA1, while apoA-I's ability to solubilize phospholipids is minimally affected. Our studies further demonstrated that plasma HDL isolated from humans with CAD contains higher levels of 3-chlorotyrosine and 3- nitrotyrosine than HDL from healthy subjects, suggesting that chlorinated and/or nitrated HDL might serve as a marker of active cardiovascular disease in humans. Thus, MPO could potentially generate dysfunctional HDL in humans. In future studies, it will be important to identify the specific sites of chlorination and nitration of apoA-I *in vivo*. It will also be critical to establish whether chlorinated and nitrated Tyr192 and oxidized Met residues in plasma apoA-I can indeed serve as CAD markers. Studies using MPO inhibitors will be necessary to determine whether HDL oxidation is causally linked to atherosclerosis in humans.

We also demonstrated that MDA and acrolein impaired apoA-I's ability to transport cholesterol by the ABCA1 pathway. The mechanisms likely involve modification and cross-linking of Lys residues in repeats 9 and 10 in the protein's C-terminus. Our observations raise the possibility that modification of apoA-I by MDA and acrolein generates dysfunctional HDL that can no longer remove cholesterol from macrophages. In future studies, it will be of interest to determine whether these carbonyls impair cholesterol transport by impairing the direct interaction between apoA-I and ABCA1, as chlorination does. We would also like to determine whether reactive carbonyls inhibit other key steps in reverse cholesterol transport—such as LCAT activation—that depend on apoA-I. It will be crucial to obtain direct evidence that HDL is modified by MDA and acrolein *in vivo* by detecting MDA and acrolein modification and/or cross-linking products in HDL isolated from humans. It will also be important to determine whether HDL modified by MDA and acrolein can serve as markers for CAD.

7. Conclusions

Our studies suggest that MPO and reactive carbonyls are potential mechanisms for generating dysfunctional HDL in humans. Oxidation of lipid-free apoA-I by MPO or reactive carbonyls impairs cholesterol efflux by the ABCA1 pathway. This deficit in cholesterol removal might promote foam cell formation and therefore atherogenesis.

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The abbreviations used are

ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1

AGE	advanced glycation end products
ALE	advanced lipoxidation end products
apoA-I	apolipoprotein A-I
BHK	baby hamster kidney
CAD	coronary artery disease
DHP-lysine	dihydropyridine-lysine
DTPA	diethylenetriaminepentaacetic acid
HDL	high-density lipoproteins
HNE	4-hydroxynonenal
HOCl	hypochlorous acid
H_2O_2	hydrogen peroxide
LCAT	lecithin:cholesterol acyltransferase
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LDL	low-density lipoprotein
Lys-MDA-Lys	Lys-1-amino-3-iminopropene-Lys
Met(O)	methionine sulfoxide
MDA	malondialdehyde
MPO	myeloperoxidase
MP-lysine	N^{ε}-(3-methylpyridinium)lysine
MS	mass spectrometry
NO	nitric oxide
$NO_2^{\bullet}$	nitrogen dioxide radical
$ONOO^-$	peroxynitrite
PilB	methionine sulfoxide reductase
SR-B1	scavenger receptor B1
SUV	single unilamellar vesicles
WT	wild type
Y192F	Tyr192Phe apoA-I mutant

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Research highlights

- MPO impairs ABCA1 cholesterol efflux by modifying Met residues and Tyr192 in apoA-I.
- Chlorination of apoA-I by MPO impairs the initial interactions with ABCA1.
- Acrolein impairs cholesterol efflux by modifying Lys226 in apoA-I.
- MDA impairs cholesterol export by cross-linking of C-terminal Lys residues in apoA-I.
- MPO and reactive carbonyls might generate dysfunctional HDL in humans.

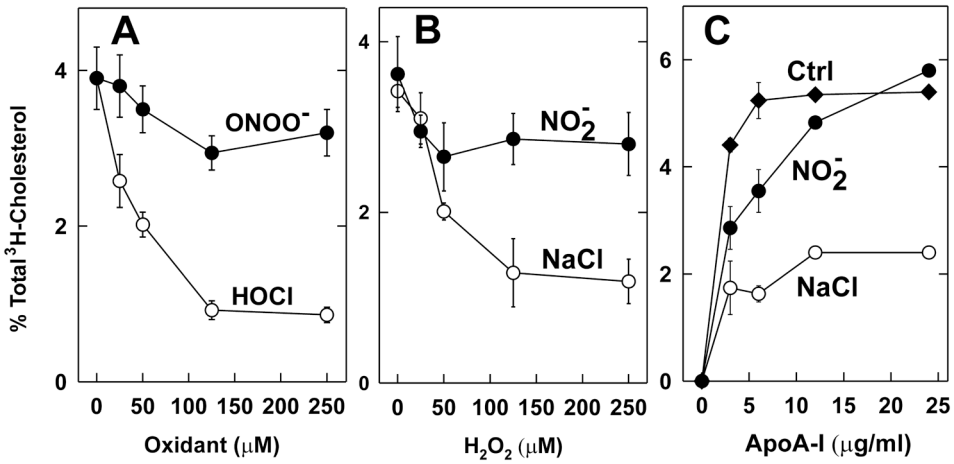

Figure 1. Cholesterol efflux activities of lipid-free apoA-I oxidized with HOCl, $ONOO^-$, MPO-H_2O_2-chloride, or MPO-H_2O_2-nitrite

ApoA-I (5 μM) was incubated with the indicated concentrations of HOCl, $ONOO^-$, or H_2O_2 for 60 min at 37 °C in phosphate buffer. The reaction was terminated by adding methionine. Where indicated, the system was supplemented with 50 nM myeloperoxidase (MPO) and 100 μM nitrite (NO_2^-) or 100 mM NaCl (NaCl). (A, B) [^{3}H]Cholesterol-labeled ABCA1-transfected BHK cells were incubated for 2 h with native (0 μM oxidant), HOCl-oxidized, $ONOO^-$-oxidized, or MPO-H_2O_2-oxidized apoA-I (5 μg/mL). (C) [^{3}H]Cholesterol-labeled ABCA1-transfected BHK cells were incubated with the indicated concentration of apoA-I for 2 h. ApoA-I was incubated with MPO and 0 μM oxidant (Ctrl), 125 μM H_2O_2 plus 100 μM nitrite (NO_2), or 100 mM NaCl (NaCl). At the end of the incubation, [^{3}H]cholesterol efflux to the acceptor apolipoprotein was measured (81).

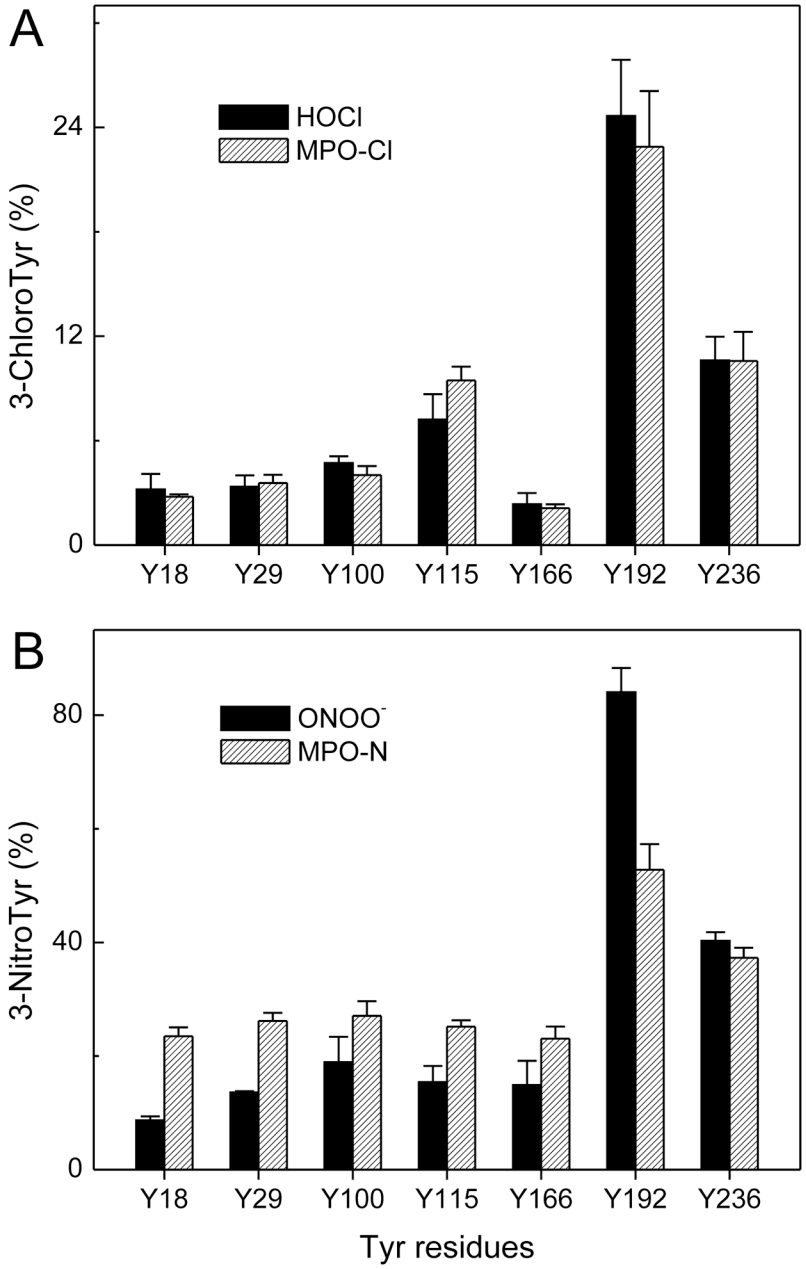

Figure 2. Site-specific chlorination of tyrosine residues in apoA-I exposed to HOCl or the MPO-H_2O_2-NaCl system (A) and nitration of tyrosine residues in apoA-I exposed to $ONOO^-$ or the MPO-H_2O_2-$NaNO_2$ system (B)

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 MS and tandem 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 tandem MS. Results are from 3 independent experiments (mean ± SD) (83).

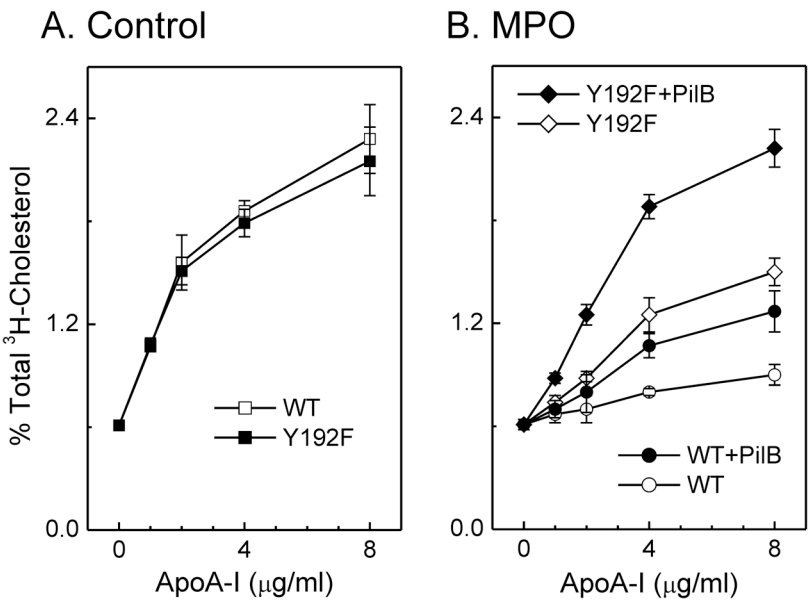

Figure 3. Cholesterol efflux activities of apoA-I (WT) and Tyr192Phe (Y192F) apoA-I exposed to the MPO system and reduced by PilB

(A) [^{3}H]Cholesterol-labeled ABCA1-transfected BHK cells were incubated with the indicated concentration of apoA-I (WT) or Tyr192Phe apoA-I (Y192F) for 2 h. At the end of the incubation, [^{3}H]cholesterol efflux to the acceptor apolipoprotein was measured. (B) ApoA-I or Tyr192Phe apoA-I (5 μM) was oxidized by the MPO-H_2O_2-NaCl system (25:1, mol/mol, H_2O_2/apoA-I) for 60 min at 37 °C in phosphate buffer (100 μM DTPA, 20 mM sodium phosphate, pH 7.4). The reaction was terminated by adding methionine. Where indicated, apoA-I was incubated with the methionine sulfoxide reductase PilB. [^{3}H]Cholesterol-labeled ABCA1-transfected BHK cells were incubated with the indicated concentration of lipoproteins, and cholesterol efflux was measured at the end of the incubation. Results are means ± SD of 3 determinations and are representative of 3 independent experiments (84).

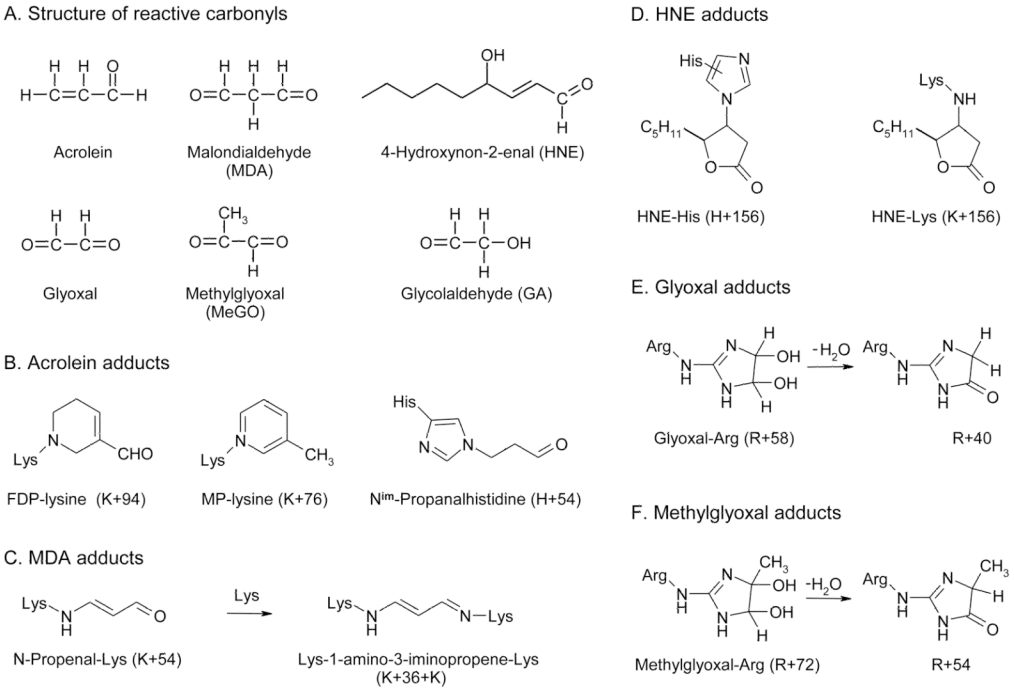

Figure 4.
Structures of physiologically relevant reactive carbonyls and carbonyl adducts detected by mass spectrometry.

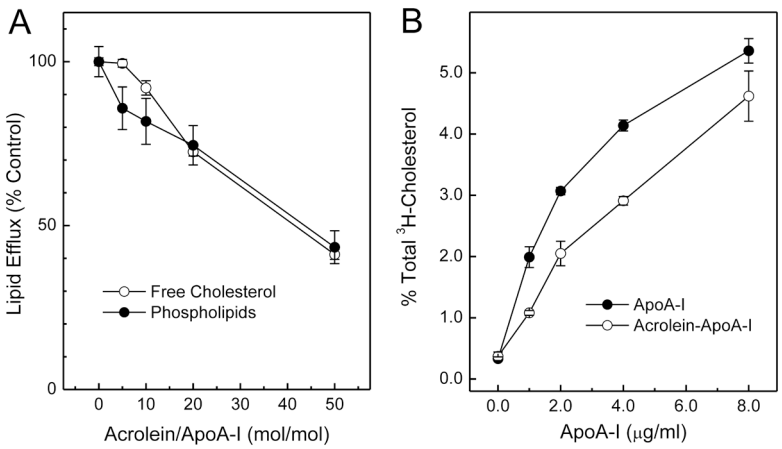

Figure 5. Cholesterol and phospholipid efflux activities of acrolein-modified apoA-I

ApoA- I (25 μM) was incubated with the indicated concentrations of acrolein (A) or with 500 μM acrolein (B) for 24 h at 37°C in 50 mM sodium phosphate buffer (pH 7.4) containing 100 μM DTPA. Reactions were initiated by adding acrolein, and terminated by adding a 20-fold molar excess (relative to acrolein) of aminoguanidine. (A) [^{3}H]Cholesterol- or phospholipid-labeled ABCA1-transfected BHK cells were incubated for 2 h with 5 μg/ml of native (0 μM acrolein) or acrolein-modified apoA-I. (B) [^{3}H]Cholesterol-labeled ABCA1-transfected BHK cells were incubated with the indicated concentrations of untreated or acrolein-treated apoA-I (20:1, mol/mol, acrolein/protein) for 2 h. At the end of the incubation, [^{3}H]cholesterol efflux to the acceptor apolipoprotein was measured. Results represent those from 2 independent experiments (120).

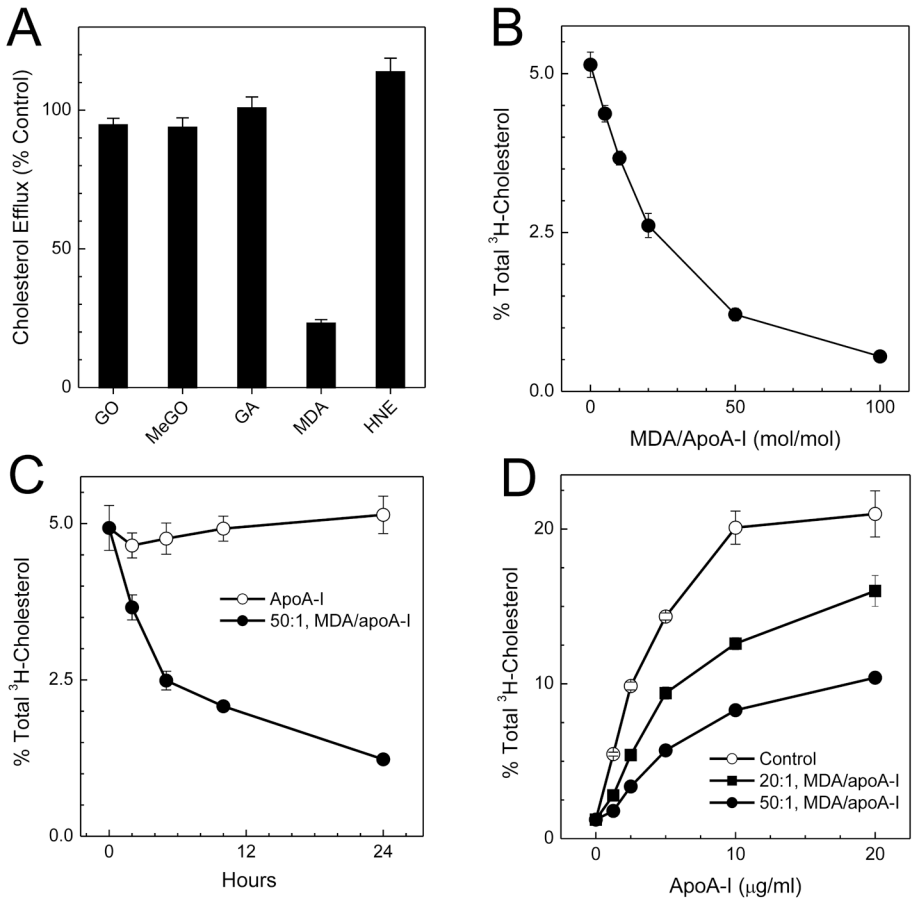

Figure 6. Impact of carbonyl modification on apoA-I's ability to promote cholesterol efflux by ABCA1

ApoA-I (5 μM) was incubated with 250 μM glyoxal, methylglyoxal, glycolaldehdye, MDA, or HNE (A), or with the indicated concentrations of MDA for 24 h (B and D), or with 250 μM MDA for the indicated times (C) at 37°C in 50 mM sodium phosphate buffer (pH 7.4) containing 100 μM DTPA. Reactions were initiated by adding carbonyl, and terminated by adding a 20-fold molar excess (relative to carbonyl) of aminoguanidine. (A, B, and C) [^{3}H]Cholesterol-labeled ABCA1-transfected BHK cells were incubated for 2 h with 3 μg/mL of control (0 μM carbonyl) or carbonyl-modified apoA-I. (D) [^{3}H]Cholesterol-labeled ABCA1- transfected BHK cells were incubated with the indicated concentrations of untreated or MDA- treated apoA-I (20:1 or 50:1, mol/mol, MDA/protein) for 2 h. At the end of the incubation, [^{3}H]cholesterol efflux to the acceptor apolipoprotein was measured. Results represent 2 independent experiments (128).