Clinical Track

Humans With Atherosclerosis Have Impaired ABCA1 Cholesterol Efflux and Enhanced High-Density Lipoprotein Oxidation by Myeloperoxidase

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Rationale: The efflux capacity of high-density lipoprotein (HDL) with cultured macrophages associates strongly and negatively with coronary artery disease status, indicating that impaired sterol efflux capacity might be a marker—and perhaps mediator—of atherosclerotic burden. However, the mechanisms that contribute to impaired sterol efflux capacity remain poorly understood.

Objective: Our aim was to determine the relationship between myeloperoxidase-mediated oxidative damage to apolipoprotein A-I, the major HDL protein, and the ability of HDL to remove cellular cholesterol by the ATP-binding cassette transporter A1 (ABCA1) pathway.

Methods and Results: We quantified both site-specific oxidation of apolipoprotein A-I and HDL’s ABCA1 cholesterol efflux capacity in control subjects and subjects with stable coronary artery disease or acute coronary syndrome. Subjects with coronary artery disease and acute coronary syndrome had higher levels of chlorinated tyrosine 192 and oxidized methionine 148 compared with control subjects. In contrast, plasma levels of myeloperoxidase did not differ between the groups. HDL from the subjects with coronary artery disease and acute coronary syndrome was less able to accept cholesterol from cells expressing ABCA1 compared with HDL from control subjects. Levels of chlorinated tyrosine and oxidized methionine associated inversely with ABCA1 efflux capacity and positively with atherosclerotic disease status. These differences remained significant after adjusting for HDL-cholesterol levels.

Conclusions: Our observations indicate that myeloperoxidase may contribute to the generation of dysfunctional HDL with impaired ABCA1 efflux capacity in humans with atherosclerosis. Quantification of chlorotyrosine and oxidized methionine in circulating HDL might be useful indicators of the risk of cardiovascular disease that are independent of HDL-cholesterol. (Circ Res. 2014;114:1733-1742.)

Key Words: 3-chlorotyrosine ■ acute coronary syndrome ■ cardiovascular diseases ■ mass spectrometry ■ peroxidase

Clinical and epidemiological studies show a robust, inverse association of high-density lipoprotein cholesterol (HDL-C) levels with cardiovascular disease (CVD) risk. In mouse models of atherosclerosis, HDL’s ability to remove cholesterol from macrophages in the artery wall is a key cardioprotective function. A well-established pathway involves apolipoprotein A-I (apoA-I), HDL’s major protein. Lipid-free apoA-I promotes cholesterol efflux from macrophages via ATP-binding cassette transporter A1 (ABCA1), a membrane-associated transporter. Humans lacking ABCA1 have Tangier disease, which is characterized by low levels of HDL-C and accumulation of cholesterol-loaded macrophage in many different tissues. Ablation of ABCA1 in mouse myeloid cells promotes atherosclerosis in hypercholesterolemic mice without affecting HDL-C levels, indicating that cholesterol efflux by the ABCA1 pathway is of central importance in the cardioprotective effects of HDL. Inflammation impairs macrophage sterol efflux in animal models, rendering HDL dysfunctional. However, the underlying mechanisms and relevance of these findings to human atherosclerosis remain unclear.

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Many lines of evidence indicate that oxidative stress plays a role in the pathogenesis of CVD. One important mechanism may involve damage to HDL by myeloperoxidase, a heme protein expressed at high levels by inflammatory macrophages in...
human atherosclerotic tissue. The enzyme uses H₂O₂ and chloride ion to produce hypochlorous acid (HOCl), which converts tyrosine to 3-chlorotyrosine. It also oxidizes methionine residues. When lipid-free apoA-I is oxidized by myeloperoxidase in vitro, its ability to remove excess cellular cholesterol by the ABCA1 pathway is decreased. Moreover, in vitro studies with a mutated form of apoA-I and biochemical studies with methionine sulfoxide (Met(O)) reductase suggest that chlorination of tyrosine 192 in concert with oxidation of methionine impairs ABCA1 transport activity. Oxidation of methionine in apoA-I also impairs its ability to activate lecithin–cholesterol acyltransferase, which plays a key role in maturation of HDL. Elevated levels of chlorotyrosine have been detected in HDL isolated from subjects with coronary disease, and tyrosine 192 is the major chlorination site in apoA-I of HDL isolated from human atherosclerotic lesions. These observations suggest that oxidation might impair human HDL’s cardioprotective functions, such as cholesterol clearance from macrophages in the artery wall.

Inflammation and metabolic disorders have been proposed to convert HDL to a dysfunctional form lacking antiatherogenic properties. Consistent with this proposal, the ability of serum HDL to promote sterol efflux from cultured macrophages varies markedly, despite similar levels of HDL-C and apoA-I. Therefore, HDL-C is not necessarily the major determinant of HDL’s macrophage sterol efflux capacity. One proposed factor is HDL’s activity with the ABCA1 pathway. Importantly, the efflux capacity of serum HDL with cultured macrophages associated strongly and negatively with coronary artery disease (CAD) status, and that association was independent of HDL-C and apoA-I levels. Taken together, these observations suggest that serum HDL’s capacity to promote sterol efflux from macrophages reflects its functionality, raising the possibility that this function of HDL provides insight into the relationship between HDL biology and CAD risk.

The factors that control the efflux capacity of serum HDL remain poorly understood. In the current study, we isolated HDL from control subjects and subjects with stable CAD or acute coronary syndrome (ACS) and used tandem mass spectrometry (MS) to explore the relationship between site-specific oxidation of apoA-I and the protein’s ability to promote cholesterol efflux by the ABCA1 pathway. We show that levels of chlorinated and oxidized apoA-I are elevated in subjects with CAD and ACS, and that they correlate inversely with HDL’s cholesterol efflux capacity. Moreover, HDL from subjects with CAD and ACS was less able to accept sterol from cells expressing ABCA1 compared with HDL from control subjects. Thus, myeloperoxidase may help generate dysfunctional HDL in humans.

### Methods

#### Subjects

Plasma samples were collected prospectively over 10 months from 3 groups of subjects at the University of Washington: control subjects (n=20), subjects with stable CAD (n=20), and subjects with ACS (n=20). The control subjects, who were recruited by advertisement, had no clinical history of CVD, no family history of premature CAD, and were not receiving lipid-lowering therapy. Subjects with stable CAD had clinically established atherosclerotic vascular disease but had been stable for ≥3 months. Subjects with CAD also had at least one ≥50% stenotic lesion on coronary angiography or a history of myocardial infarction, percutaneous coronary intervention, or coronary artery bypass grafting. All subjects with ACS exhibited acute ECG changes consistent with myocardial ischemia or elevated troponin levels. ACS was confirmed with urgent coronary angiography; all subjects had at least one ≥50% stenotic lesion with ruptured plaque or thrombus. All subjects provided signed informed consent, and all protocols were approved by the University of Washington Institutional Review Board.

#### Plasma Levels of Lipids, High-Sensitivity C-Reactive Protein, and Myeloperoxidase

Lipid profiles and high-sensitivity C-reactive protein (hs-CRP) were determined by the Northwest Lipid Metabolism and Diabetes Research Laboratories, University of Washington. All lipid analyses were standardized using the Centers for Disease Control and Prevention Reference Methods. Myeloperoxidase levels in plasma were measured by the Cleveland Heart Laboratory (Cleveland, OH).

#### HDL Isolation From Plasma

Blood was collected from overnight-fasted subjects into ice-cold tubes containing EDTA (6 mmol/L final concentration). To protect methionine residues from oxidation, plasma was prepared immediately by centrifugation (2500g for 15 minutes) and frozen at −80°C until analysis. HDL (density, 1.063–1.210 g/mL) was isolated by sequential ultracentrifugation from freshly thawed plasma, using buffers supplemented with 100 μmol/L diethylenetriaminepentaacetic acid, 100 μmol/L butylated hydroxytoluene, and a protease inhibitor mixture (Sigma, St Louis, MO). All studies were performed on samples that had been stored for <12 months, and freshly prepared methionine (10 mmol/L final) was added to each sample before work-up for MS analysis.

#### Isotope-Labeled ApoA-I

₁⁵N-labeled apoA-I was prepared by growing bacteria that stably express human apoA-I in minimal medium supplemented with [₁⁵N]H₄Cl. Mass Spectrometric Analysis With Selected Reaction Monitoring

The protein concentration of HDL was determined using the Lowry assay (BioRad), with albumin as the standard. After the addition of freshly prepared methionine (10 mmol/L final concentration), proteins were reduced with dithiothreitol and alkylated with iodoacetamide. After digestion of apoA-I with sequencing-grade modified trypsin (Promega) or endoproteinase Glu-C (from Staphylococcus aureus V8; Roche Applied Science), site-specific oxidation of apoA-I was quantified by selected reaction monitoring, a quantitative and sensitive MS/MS technique for detecting peptides and their
post-translational modifications, in peptide digests of HDL with a nano-LC-MS/MS as described. Additional details are provided in the Online Data Supplement.

Ex Vivo Oxidation of HDL
Preliminary studies demonstrated that tyrosine chlorination was negligible in recombinant apoA-I and reconstituted HDL analyzed by tandem MS, indicating that sample work-up and MS analysis did not contribute significantly to ex vivo oxidation. However, when reconstituted HDL disks made from apoA-I and phospholipid-containing linoleic acid (which is highly susceptible to oxidation) were isolated by ultracentrifugation, oxidation of methionine 86 and methionine 112 in apoA-I increased significantly (Online Figure I). In contrast, there was no evidence of increased oxidation of methionine 148 (Online Figure I). Moreover, there was no additional oxidation of methionine 148 in 15N-apoA-I when reconstituted HDL (made from 15N-apoA-I) was incubated in plasma and then isolated by ultracentrifugation (Online Figure II). The addition to plasma of azide (10 mmol/L), a potent inhibitor of myeloperoxidase, did not affect methionine oxidation or tyrosine chlorination of HDL or reconstituted HDL (Online Figures II and III). These data suggest that lipid oxidation products, but not plasma myeloperoxidase or other heme proteins, can contribute to ex vivo oxidation of methionine 86 and methionine 112, but not methionine 148, as previously described.

ABCA1 Efflux Capacity of Serum HDL and In Vitro Oxidized HDL
Serum was derived from plasma by adding calcium. Polyethylene glycol was then used to precipitate lipoproteins containing apolipoprotein B, and the supernatant was centrifuged to generate serum HDL. ABCA1-specific sterol efflux to serum HDL was quantified using baby hamster kidney cells expressing mifepristone-inducible human ABCA1. HDL isolated by ultracentrifugation was oxidized with HOCI and reduced with piB as described. Additional details are provided in the Online Data Supplement.

Statistical Analysis
Continuous variables are presented as mean and SD, and categorical variables as frequencies and percentages. Because levels of chlorotyrosine 192 and Met(O) 148 exhibited a nonnormal distribution in our study population, we used logarithmic transformation in all analyses. Linear regression analysis with continuous variables used Pearson coefficient. Multiple logistic regression was used to estimate the association between 3-chlorotyrosine 192, Met(O) 148, cholesterol efflux capacity, and CVD status after adjustment for HDL-C levels. Odds ratios are reported for 1 SD change for continuous variables. Significant \( P \) values were \( <0.05 \) on 2-tailed analysis. Statistical analyses were performed with SPSS (Windows version 19; Chicago, IL) or OriginPro (version 8.6; Origin Laboratory, Northampton, MA).

Results
The clinical characteristics and lipid values of subjects are shown in the Table. Analysis with 1-way ANOVA indicated a significant difference in HDL-C levels among the groups (\( P=0.0003 \)). Further analysis with Fisher least significance difference (LSD) test demonstrated that the groups with stable CAD (42±11 mg/dL; \( n=20 \)) and ACS (47±17 mg/dL; \( n=20 \)) had significantly lower HDL-C levels (\( P=0.0001 \) and 0.003, respectively) compared with the healthy control group (61±16 mg/dL; \( n=20 \)). Low-density lipoprotein cholesterol (LDL-C) levels were similar in the control and ACS groups but lower in the CAD group (\( P=0.009 \); Fisher LSD test; CAD versus control), likely because all subjects with CAD were on statin therapy. Plasma levels of myeloperoxidase were similar in the control and CAD groups, but higher in the ACS group. However, the differences in myeloperoxidase levels between the groups were not significant by 1-way ANOVA. In contrast, the ACS and CAD groups had significantly higher levels of hs-CRP compared with the control group (\( P=0.02 \) and <0.0001, respectively; Fisher LSD test).

Subjects With Stable CAD and ACS Have Elevated Levels of Chlorinated and Oxidized ApoA-I
To quantify oxidation sites in HDL isolated by ultracentrifugation from human plasma of controls and subjects with CAD and ACS, we supplemented the HDL with 15N-apoA-I chlorinated with reagent HOCI. Levels of 3-chlorotyrosine and Met(O) in peptide digestes were quantified by tandem MS with selected reaction monitoring and isotope dilution. This sensitive and quantitative approach detected all 7 peptides that

| Table. Clinical Characteristics of Study Subjects |
|-----------------|--------|--------|
| Control (n=20)  | Stable CAD (n=20) | ACS (n=20) |
| Age, y          | 56±8   | 64±11*  | 63±12 |
| Male sex, %     | 15 (75%) | 14 (70%) | 12 (60%) |
| Patients with hypertension, % | 2 (10%) | 15 (75%)† | 10 (50%)‡ |
| Patients with diabetes mellitus, % | 0 (0%) | 3 (15%) | 9 (45%)† |
| Smokers, %      | 4 (20%) | 13 (65%)† | 12 (60%)‡ |
| Total cholesterol, mg/dL | 203±35 | 168±61* | 186±41 |
| HDL-cholesterol, mg/dL | 61±16 | 42±11† | 47±17 † |
| LDL-cholesterol, mg/dL | 117±25 | 94±52 | 114±42 |
| Triglycerides, mg/dL | 123±118 | 158±93 | 126±72 |
| MPO, pmol/L     | 809±1431 | 920±2033 | 1610±2267 |
| hs-CRP, μg/dL   | 88±92 | 290±406 * | 1,369±3442‡ |

Values are mean±SD. Myeloperoxidase (MPO) and high-sensitivity C-reactive protein (hs-CRP) values were log-transformed before 1-way ANOVA. ACS indicates acute coronary syndrome; CAD, coronary artery disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; and N/A, not available. *†, ‡P<0.05, 0.001, or 0.01, respectively, vs control subjects by Fisher least significant difference test (after 1-way ANOVA with \( P<0.05 \)).

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contain tyrosine, all 3 peptides that contain methionine, and the corresponding chlorinated and oxidized products.

Chlorinated peptides were readily detectable in the proteolytic digests of apoA-I prepared from HDL isolated from human plasma (Figure 1A). Moreover, we identified tyrosine 192 as the major chlorination site in apoA-I of HDL isolated from both the diseased and control subjects ($P<0.0001$ versus tyrosine 18; Student $t$ test), as previously demonstrated for HDL isolated from human atherosclerotic lesions.27 The average level (mean±SD) of 3-chlorotyrosine 192 in all the subjects was 62±26 μmol/mol tyrosine. Tyrosine 18 was the second major site of chlorination, with an average level of 29±11 μmol/mol tyrosine. Lower levels of 3-chlorotyrosine (<10 μmol/mol tyrosine) were detected at the other 5 tyrosine residues.

To determine whether the healthy and diseased groups had different levels of 3-chlorotyrosine in the apoA-I from their plasma HDL, we calculated the overall levels of tyrosine chlorination for all 7 tyrosine residues. The total level of protein-bound 3-chlorotyrosine in the control group was 14±2.5 μmol/mol tyrosine (n=20). In the CAD (n=20) and ACS (n=20) groups, it was 17±3.9 and 20±8.2 μmol/mol tyrosine, respectively. Analysis by 1-way ANOVA indicated that total chlorotyrosine levels differed among the groups ($P=0.009)$. Total levels of 3-chlorotyrosine in apoA-I of HDL from the subjects with CAD or ACS were significantly higher than in healthy control subjects ($P=0.04$ and 0.002; Fisher LSD test). Levels of 3-chlorotyrosine ranged from 10 to 19 μmol/mol tyrosine in the control group, 11 to 24 μmol/mol tyrosine in the CAD group, and 10 to 41 μmol/mol tyrosine in the ACS group.

Subjects With CAD and ACS Have Elevated Levels of Chlorinated Tyrosine 192 and Oxidized Methionine 148

Because tyrosine 192 was the major chlorination site in apoA-I of HDL isolated from human plasma, we determined whether subjects with CAD or ACS had higher levels of that chlorinated residue (Figure 1B). The average level of 3-chlorotyrosine 192 in the control group was 50±8.5 μmol/mol tyrosine. After log transformation of 3-chlorotyrosine 192 values, levels differed significantly among the groups ($P=0.01$; 1-way ANOVA). Levels were significantly higher (Fisher LSD test) in subjects with CAD (64±20 μmol/mol tyrosine; $P=0.02$) and ACS (71±36 μmol/mol tyrosine; $P=0.004$) than in the control subjects. However, the difference between the CAD and ACS levels was not significant.

We quantified the levels of oxidation of all 3 methionine residues in apoA-I (Online Figure IV). However, we focused on Met(O) 148 because oxidation of that residue strongly associates with impaired ABCA1-mediated cholesterol efflux activity and reduced ability to activate lecithin–cholesterol acyltransferase.25,26 Importantly, preliminary studies indicated that methionine 148 was resistant to ex vivo oxidation (Methods in the Online Data Supplement; Online Figures I and II). In the control group, the average level of Met(O) 148 was 75±13 mmol/mol methionine (Figure 1C). After log transformation of Met(O) 148 values, levels were significantly higher in the subjects with CAD (120±69 mmol/mol methionine; $P=0.002$) and ACS (110±62 mmol/mol methionine; $P=0.02$) than in the control subjects.

Studies in myeloperoxidase-deficient mice strongly suggest that myeloperoxidase is the only generator of 3-chlorotyrosine during acute inflammation.38 In contrast, other oxidative...
pathways can produce Met(O).\textsuperscript{21} To determine whether myeloperoxidase might be an important pathway for promoting methionine oxidation in humans, we assessed the relationship between 3-chlorotyrosine 192 levels and Met(O) 148 levels (Figure 1D) in all the subjects (n=60). Linear regression analysis demonstrated a strong correlation (Pearson $r=0.57$; $P<0.0001$). The levels of 3-chlorotyrosine 192 also correlated with the levels of Met(O) 86 ($r=0.52$; $P<0.0001$; Online Figure IVD) and Met(O) 112 ($r=0.58$; $P<0.0001$; Online Figure IVE). Our observations suggest that myeloperoxidase contributes to methionine oxidation in vivo in human apoA-I.

We also assessed the relationship between plasma myeloperoxidase levels and levels of 3-chlorotyrosine 192 and oxidized methionine residues in HDL (Online Figure V). Linear regression analysis revealed that myeloperoxidase levels were modestly correlated with levels of 3-chlorotyrosine 192 ($r=0.34$; $P=0.008$) but failed to correlate with levels of Met(O) 148 ($r=0.24$; $P=0.06$). Supplementation of plasma with 10 mmol/L azide, a potent inhibitor of myeloperoxidase, had no effect on methionine oxidation or tyrosine chlorination (Online Figures II and III). These observations suggest that myeloperoxidase in plasma has little influence on HDL oxidation.

**Correlations of 3-Chlorotyrosine 192 and Met(O) 148 With Traditional CAD Risk Factors**

We examined the relationship between 3-chlorotyrosine 192 levels in HDL and traditional CAD risk factors in this cohort of control and high-risk patients (Figure 2). Levels of 3-chlorotyrosine 192 did not associate with sex or hypertension; they also failed to correlate with total cholesterol, non–HDL-C, age, or triglycerides (data not shown). However, they were significantly related to smoking status (53.4 versus 70.6 mol/mol; nonsmoker versus smoker; $P=0.007$; 2-tailed Student $t$ test) and diabetes mellitus (56.4 versus 83.2 mol/mol; nondiabetic versus diabetic subjects; $P=0.001$). Levels of 3-chlorotyrosine 192 correlated inversely with HDL-C levels ($r=-0.27$; $P=0.03$; Figure 2A) but not with LDL-C levels ($r=-0.13$; $P=0.31$; Figure 2B). Levels of 3-chlorotyrosine 192 also correlated modestly with levels of hs-CRP ($r=0.38$; $P=0.003$; Figure 2C).

We next examined the relationship between Met(O) 148 levels and traditional CAD risk factors. Sex, age, hypertension, smoking status, total cholesterol, non–HLD-C, and triglyceride did not associate with levels of Met(O) 148. However, Met(O) 148 associated significantly with diabetes mellitus (92 versus 138 mmol/mol methionine; nondiabetic versus diabetic subjects; $P=0.01$). Unlike 3-chlorotyrosine 192, Met(O) 148 was not correlated with HDL-C ($r=-0.23$; $P=0.08$; Figure 2D) but inversely correlated with LDL-C ($r=-0.27$; $P=0.04$; Figure 2E). The correlation between Met(O) 148 and hs-CRP was not significant ($r=0.08$; $P=0.49$; Figure 2F).

**Cholesterol Efflux Capacity of Serum HDL With the ABCA1 Pathway Is Impaired in Subjects With CAD and ACS**

We assessed the capacity of serum HDL to promote cholesterol efflux by the ABCA1 pathway in human isolated HDL using cells that expressed ABCA1. Analysis by 1-way ANOVA indicated that serum HDL efflux capacity differed among the groups ($P=0.0004$).

Serum HDL of subjects with CAD and ACS had significantly less cholesterol efflux capacity (10.8% and 10.0% sterol efflux; $P=0.02$ and <0.0001, respectively; Fisher LSD test) than that of control subjects (11.8% sterol efflux) when cells expressing ABCA1 were cholesterol donors (Figure 3A). There was a trend toward greater impairment of sterol efflux from ABCA1-expressing cells to serum HDL of subjects with ACS than subjects with CAD ($P=0.06$).

**Levels of 3-Chlorotyrosine 192 and Met(O) 148 Inversely Correlate With Serum HDL’s ABCA1 Cholesterol Efflux Capacity**

We investigated the relationship between levels of oxidized residues in apoA-I and the cholesterol efflux capacity of serum HDL using cells that expressed ABCA1. Linear regression analysis of all the subjects (n=60) revealed an inverse correlation with levels of both 3-chlorotyrosine 192 ($r=-0.33$; $P=0.01$; Figure 3B) and Met(O) 148 ($r=-0.32$; $P=0.01$; Figure 3C). The cholesterol efflux capacity of serum HDL
also inversely correlated with levels of Met(O) 86 and Met(O) 112 (Online Figure VI).

To investigate the potential contribution of methionine oxidation in vivo to impaired sterol efflux, we exposed isolated HDL to low levels of HOCl. When 29% of methionine 148 of apoA-I was converted to Met(O) 148, we observed ≈30% loss of cholesterol efflux by the ABCA1 pathway (Figure 4). Met(O) 148 was almost completely reduced back to native methionine when oxidized HDL was incubated with pilB, a Met(O) reductase that reduces both epimers of Met(O). Reduction of Met(O) to methionine completely restored the efflux capacity of HOCl-oxidized HDL (Figure 4). The efflux capacity of control HDL, which contained ≈9% Met(O) 148, was also increased by incubation with pilB. Collectively, these observations provide strong evidence that the levels of Met(O) observed in HDL isolated from subjects with CAD are likely high enough to contribute to impaired sterol efflux by the ABCA1 pathway.

Serum HDL’s cholesterol efflux capability with macrophages associates with both HDL-C levels and the functionality of HDL particles. We, therefore, determined the relationship between HDL-C levels and the ABCA1 activity of serum HDL for all of the subjects (n=60) using linear regression analysis (Figure 5A). Efflux capacity correlated with HDL-C levels (P=0.02) and accounted for approximately the same variance in the data (r=0.31) as was observed for apoA-I chlorination (r=−0.33). Thus, the impaired cholesterol efflux in the CAD and ACS groups likely reflects in part lower HDL-C levels. After we controlled for HDL-C level, logistic regression analysis revealed that the inverse correlations between 3-chlorotyrosine 192 or Met(O) 148 with efflux capacity remained significant (r=−0.27; P=0.04 and r=−0.27; P=0.04, respectively). Therefore, modification of apoA-I by myeloperoxidase correlated with impaired HDL-C efflux capacity via the ABCA1 pathway, even after adjusting for HDL-C levels. In contrast, we observed no correlation of the efflux capacity of serum HDL with plasma levels of myeloperoxidase (r=−0.07; P=0.60; Figure 3D).

We also examined the relationship between ABCA1 cholesterol efflux capacity and traditional risk factors for atherosclerosis in our subjects (Figure 5). Cholesterol efflux correlated weakly with HDL-C (r=0.31; P=0.02; Figure 5A), consistent with the observation that lipid-free or poorly lipidated apolipoproteins are the major ligands for ABCA1. Efflux capacity also correlated with LDL-C (r=0.26; P=0.04; Figure 5B), but adjusting for HDL-C levels attenuated that relationship (r=0.24; P=0.07). Cholesterol efflux did not correlate with triglyceride levels (r=0.17; P=0.19; Figure 5C). However, after we controlled for HDL-C levels, that relationship became significant (r=0.49; P=0.001). Efflux did not associate with sex or hypertension. Efflux did not correlate with age or total cholesterol. However, it related significantly to diabetes mellitus (11.2% versus 10.0%; nondiabetic versus diabetic subjects; P=0.01; 2-tailed Student t test). Efflux associated with smoking status (13.3% versus 10.6%; nonsmoker versus smoker; P=0.047); it also correlated with hs-CRP levels (r=−0.35; P=0.006; Figure 5D).

3-Chlorotyrosine 192, Met(O) 148, and Efflux Capacity Associate With CVD Status

We investigated the relationships among apoA-I oxidation, ABCA1 efflux capacity, traditional cardiovascular risk factors, and CVD status (Figure 6). To increase power, and because the levels of oxidized amino acids and sterol efflux capacity of HDL were similar in subjects with CAD and ACS,
we included both subjects with CAD and ACS in the CVD group.

Compared with control subjects, subjects with CVD had significantly lower levels of HDL-C (mean values for case and control subjects, 45 and 61 mg/dL, respectively; \( P = 0.0003 \)). Cholesterol efflux capacity of serum HDL was significantly lower in CVD subjects compared with control subjects (mean values for case and control subjects, 10.4% and 11.8%, respectively; \( P = 0.001 \)). Increased efflux capacity by the ABCA1 pathway associated with a decreased risk of CVD (odds ratio per 1 SD change, 0.30; 95% confidence interval, 0.14–0.66; \( P = 0.003 \)). This association remained robust after adjusting for HDL-C levels (adjusted odds ratio, 0.33; 95% confidence interval, 0.14–0.66; \( P = 0.003 \)). Elevated levels of 3-chlorotyrosine 192 and Met(O) 148 positively correlated with ABCA1 efflux capacity with CVD status, and this association persisted after adjustment for HDL-C. Levels of 3-chlorotyrosine 192 and Met(O) 148 positively associated with CVD status. Moreover, serum HDL’s ability to promote sterol efflux by the ABCA1 pathway correlated inversely with levels of 3-chlorotyrosine 192 and Met(O) 148. Because chlorination of tyrosine 192 in concert with oxidation of methionine residues in vitro impairs the ABCA1 activity of apoA-I, and 3-chlorotyrosine is a characteristic chemical fingerprint of myeloperoxidase, our observations suggest that myeloperoxidase contributes to the generation of dysfunctional HDL with impaired ABCA1 efflux capacity in humans with atherosclerotic vascular disease.

A key issue is whether the levels of oxidized apoA-I we detected in apoA-I of HDL of subjects with CAD and ACS were high enough to potentially reduce HDL’s sterol efflux capacity. When HDL oxidized in vitro with HOCl contained levels of Met(O) similar to those observed in vivo, its ability to promote sterol efflux by the ABCA1 pathway was significantly impaired. Importantly, the ability of oxidized HDL to promote sterol efflux by the ABCA1 pathway was completely restored when Met(O) was converted to methionine by Met(O) reductase. In contrast, the levels of 3-chlorotyrosine we detected in HDL were low and unlikely to contribute directly to impaired sterol efflux capacity by the ABCA1 pathway. Collectively, these observations provide strong evidence that the levels of Met(O) observed in HDL isolated from subjects with CAD likely impair sterol efflux. In future studies, it will be important to determine whether other modifications of apoA-I, such as lipid adduction and tryptophan oxidation, contribute to impaired sterol efflux by the ABCA1 pathway.

Although methionine can be oxidized by lipid hydroperoxides in HDL as well as other pathways, the correlation between levels of Met(O) 148 and levels of 3-chlorotyrosine 192 (a unique product of myeloperoxidase) indicates that myeloperoxidase likely contributes to the oxidation of methionine residues in vivo. In contrast to our observations with the ABCA1 pathway and lecithin–cholesterol acyltransferase activation, the oxidation of methionine residues in apoA-I does not impair sterol efflux from human monocyte-derived macrophages. However, the contribution of the ABCA1 pathway to efflux in these cells is uncertain, and the clinical relevance of these observations is unclear, because there is no evidence that impaired efflux with human macrophages associates with CVD status. In a secondary analysis, we also found that levels of 3-chlorotyrosine 192 and Met(O) 148 associated with smoking and diabetes mellitus status. Indeed, increased levels of oxidized methionine residues in apoA-I of isolated HDL were observed in a study of subjects with type 1 diabetes mellitus. In future studies, it will be of interest to determine whether smoking and diabetes mellitus—2 major risk factors

**Figure 4. Methionine oxidation and cholesterol efflux capacity of high-density lipoprotein (HDL) exposed to hypochlorous acid (HOCl) in vitro. A, Cholesterol efflux capacity of HDL exposed to HOCl and reduced by pilB. B, Levels of methionine sulfoxide [Met(O)] 148 in HDL exposed to HOCl and reduced by pilB. HDL (1 mg/mL) was oxidized by HOCl (180 μmol/L) at 37°C for 1 h. Where indicated, HDL (0.5 mg/mL) was incubated subsequently with the Met(O) reductase pilB (4:1; HDL/pilB; wt/wt) at 37°C for 2 h. Cholesterol efflux from ATP-binding cassette transporter A1–expressing baby hamster kidney cells to control HDL, HOCl-oxidized HDL, and pilB-reduced HDL was measured as described in Methods section. Levels of Met(O) 148 were quantified by tandem mass spectrometry with selected reaction monitoring. Results are mean±SD of 3 determinations and are representative of 2 independent experiments.**
for CAD—associate with impaired sterol efflux capacity and HDL modification by myeloperoxidase.

There was a strong trend (P=0.06) toward greater impairment of serum HDL's efflux capacity with the ABCA1 pathway in subjects with ACS than in subjects with CAD. However, the 2 groups contained similar levels of 3-chlorotyrosine and Met(O) in their HDLs. These observations suggest that additional factors impair sterol efflux in subjects with ACS. One important contributor may be acute inflammation, a prominent component of unstable angina and acute myocardial infarction. Consistent with this suggestion, we observed a significant, inverse correlation between hs-CRP levels and the sterol efflux capacity of serum HDL in our cohort. Acute inflammation alters the concentration of HDL-associated enzymes and remodels the human HDL proteome, and animal models and human studies provide strong support for the hypothesis that inflammation converts HDL into a dysfunctional form. Cholesterol efflux to HDL may be one important target because lipopolysaccharide, a potent inducer of acute inflammation, impairs cholesterol efflux from macrophages in mice.

Our detection of higher levels of 3-chlorotyrosine and Met(O) in plasma HDL from subjects with CAD and ACS might seem surprising, given that blood is richly endowed with antioxidants that scavenge HOCl. Moreover, levels of the oxidized residues were similar in the 2 groups, suggesting that acute inflammation is unlikely to promote oxidation of circulating HDL. Thus, the oxidized HDL in plasma might be generated elsewhere, perhaps in a microenvironment that is depleted of antioxidants but enriched in macrophages, myeloperoxidase, and HOCl. Many lines of evidence indicate that atherosclerotic lesions provide such a milieu. One intriguing possibility is that HDL is oxidized in atherosclerotic lesions (or other inflamed tissues rich in leukocytes), resulting in a feedback loop in which reduced sterol efflux prompts macrophages to become foam cells, accelerating atherosclerosis. In turn, these events promote additional HDL oxidation and the appearance of myeloperoxidase-oxidized HDL in blood. Regardless of whether it originates in blood or lesions, however, 3-chlorotyrosine and Met(O) in circulating HDL might...
be a useful indicator of the risk of CVD and the efficacy of antioxidant interventions.

The levels of chlorotyrosine 192 and Met(O) 148 were similar in patients with ACS and stable CAD, raising the possibility that the subjects with ACS had elevated levels of the 2 oxidized amino acids before they developed clinical symptoms. In future studies, it will be of great interest to determine whether levels of myeloperoxidase-oxidized HDL can identify apparently healthy subjects who are at increased risk of CAD, and whether HDL oxidation is enhanced in conditions that predispose humans to clinically significant atherosclerosis.

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Disclosures

Dr Heinecke is named as a coinventor on patents from the US Patent Office on the use of oxidation markers to predict the risk of cardiovascular disease. Dr Heinecke has served as a consultant for Merck, Amgen, Bristol Meyer Squibb, and Insilicos. Dr Zhao is the recipient of research support from Bristol Meyer Squibb and Kowa. The other authors report no conflicts.

References


**Novelty and Significance**

**What Is Known?**

- The ability of high-density lipoprotein (HDL) to accept cholesterol from macrophages is negatively associated with cardiovascular disease status.
- HDL receives cholesterol from ATP-binding cassette transporter A1 (ABCA1), a membrane protein that exports the sterol from macrophages.
- HDL can be damaged by myeloperoxidase, which oxidizes methionine residues and chlorinates tyrosine 192 of apolipoprotein A-I in HDL, impairing the ability of HDL to accept cholesterol from the ABCA1 pathway.

**What New Information Does This Article Contribute?**

- Subjects with stable coronary artery disease or acute coronary syndrome have elevated levels of oxidized HDL.
- Levels of chlorinated tyrosine 192 and oxidized methionine 148 in apolipoprotein A-I strongly associate with cardiovascular disease status and the degree to which HDL is unable to accept cholesterol from cells via the ABCA1 pathway.
- Because chlorinated tyrosine 192 and oxidized methionine 148 are produced by myeloperoxidase, the enzyme may help generate dysfunctional HDL in humans.

The ability of HDL to remove cholesterol from artery wall macrophages is one of its key cardioprotective functions. The sterol efflux capacity of HDL shows a robust negative association with coronary artery disease status, suggesting that loss of efflux capacity contributes to HDL dysfunction. However, the underlying mechanisms remain poorly understood. We found that subjects with coronary artery disease or acute coronary syndrome have higher levels of oxidized and chlorinated HDL compared with control subjects, and that the oxidation pattern—chlorinated tyrosine 192 and oxidized methionine 148—that produced by myeloperoxidase. HDL from subjects with coronary artery disease and acute coronary syndrome was also less efficient in promoting sterol efflux by the ABCA1 pathway. Converting oxidized methionine residues of HDL back to methionine improved sterol efflux capacity in vitro. Importantly, the levels of oxidized and chlorinated HDL were inversely correlated with ABCA1 efflux capacity and positively associated with atherosclerotic status. These observations indicate that dysfunctional HDL with impaired sterol efflux capacity could be generated by myeloperoxidase. Thus, inhibiting HDL oxidation by myeloperoxidase in vivo might be a new approach for treating atherosclerosis.
Humans With Atherosclerosis Have Impaired ABCA1 Cholesterol Efflux and Enhanced High-Density Lipoprotein Oxidation by Myeloperoxidase

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SUPPLEMENTAL MATERIAL

DETAILED METHODS

Preparation of reconstituted high-density lipoprotein (rHDL).
Discoidal rHDL (9.6 nm diameter) was prepared by the sodium cholate dialysis method as previously described 1. 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine (PLPC, from Avanti Polar Lipids, Inc. Pelham, Alabama), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, from Avanti Polar Lipids, Inc. Pelham, Alabama) and free cholesterol were dissolved in chloroform-methanol (3:1, v/v), dried under a N 2 stream, and the residual solvent was removed by overnight lyophilization. Solubilization buffer (Tris-buffered saline (TBS, pH 8.0) containing 19 mM sodium deoxycholate (DC), diethylenetriaminepentaacetic acid (DTPA, 100 µM) and methionine (10 mM) was added to the dried lipids and incubated at 37 °C until clear. Unlabeled or 15N-labeled apoA-I was then added to final molar ratios of PLPC:POPC:FC:apoA-I of 40:40:4:1. To generate discoidal rHDL the mixture was incubated for 1 h at 37 °C under argon; deoxycholate was then removed by dialysis against TBS (pH 8.0; 2000:1, vol/vol, TBS:rHDL) at 4 °C with two changes per day for 3 days.

Isolation of rHDL by ultracentrifugation.
rHDL containing apoA-I was isolated by sequential ultracentrifugation 2, using buffers supplemented with 100 µM DTPA, 100 µM butylated hydroxytoluene (BHT), and a protease inhibitor mixture (Sigma, St. Louis, MO).

Incubation of 15N-apoA-I rHDL with plasma and isolation by ultracentrifugation.
rHDL containing 15N-labeled apoA-I was incubated with plasma (supplemented with DTPA [100 µM] and methionine [10 mM]) under argon at 37 °C for 1 h. Sodium azide (10 mM) was also included when indicated. HDL (density 1.063–1.210 g/mL) was isolated by sequential ultracentrifugation 2, using buffers supplemented with 100 µM DTPA, 100 µM butylated hydroxytoluene (BHT), and a protease inhibitor mixture (Sigma, St. Louis, MO). Protein concentration of rHDL and HDL were determined using the Lowry assay (BioRad), with albumin as the standard.

In vitro oxidation of HDL and incubation with methionine sulfoxide reductase.
Concentration of HOCl was determined spectrophotometrically (ε292 = 350 M–1 cm–1). Oxidation of ultracentrifuged isolated HDL (1 mg/mL) was carried out at 37°C for 1 h in 20 mM sodium phosphate buffer (pH 7.4) containing 100 µM DTPA. Reactions were initiated by adding HOCl (180 µM) and terminated by adding 5 mM methionine. Truncated pilB of Neisseria gonorrhoeae, a bacterial methionine sulfoxide reductase with activity on both epimers of Met(O), was isolated from Escherichia coli 4. To reduce oxidized Met in apoA-I to methionine, oxidized HDL (0.5 mg/mL) was incubated with pilB (4:1, w/w) for 2 h at 37 °C in Tris-HCl buffer (25 mM [pH 7.4]) containing 15 mM dithiothreitol 5.

Mass spectrometric (MS) analysis with selected reaction monitoring (SRM).
Following the addition of freshly prepared Met (10 mM final concentration), proteins were reduced with dithiothreitol and alkylated with iodoacetamide 6. Then HDL was incubated overnight at 37 °C with 20:1 (w/w) of sequencing grade modified trypsin (Promega) or endoproteinase Glu-C (from Staphylococcus aureus V8, Roche Applied Science) in 50 mM NH4HCO3, pH 7.8 6. Digestion was halted by acidifying the reaction mixture (pH 2–3) with trifluoroacetic acid. Proteolytic digests were desalted with solid-phase extraction, using an Oasis HLB Cartridge (1 mL, 30 µm; Waters) prior to MS analysis.

Site-specific oxidation of apoA-I was quantified by SRM, a quantitative and sensitive MS/MS technique for detecting peptides and their post-translational modifications 7, in peptide digests of HDL with a nano-LC-MS/MS on a Thermo TSQ Vantage coupled to a Waters nanoACQUITY UltraPerformance liquid
chromatography system. The analytical column (15 cm × 75 μm i.d.) was packed in house with C-18 Magic C18 reverse-phase resin (5 μm; 100 Å; Michrom Bioresources). To detect the hydrophilic peptide containing Tyr192 (LAEYHAK) that was derived from tryptic digests of apoA-I, samples (corresponding to 0.5 μg HDL protein) were directly loaded onto the analytical column. Four SRM transitions of each peptide in apoA-I that contained native or oxidized Tyr or Met residues were chosen for quantitative analysis, based on the tandem MS spectrum obtained with the TSQ instrument 2. Precursor and oxidized peptides were detected and quantified as previously described 2. SRM data was analyzed with Skyline, an open source program 8, to obtain the peak area of each transition. The total peak area of 4 selected transitions was used to quantify the concentration of control or oxidized peptides as described 2.

ABCA1 efflux capacity of serum HDL and in vitro oxidized HDL.

Serum was derived from plasma by adding calcium 9. Polyethylene glycol (PEG) was then used to precipitate lipoproteins containing apolipoprotein B, and the supernatant was centrifuged to generate serum HDL 9, 10. ABCA1-specific sterol efflux to serum HDL or isolated HDL was quantified using baby hamster kidney (BHK) cells expressing mifepristone-inducible human ABCA1 11. BHK cells were radiolabeled with [3H]cholesterol for 24 h. Expression of ABCA1 was induced by incubating the cells for 20 h with DMEM containing 1 mg/mL fatty acids free bovine serum albumin (DMEM/BSA) and 10 nM mifepristone. Efflux of [3H]cholesterol was measured after a 2 h incubation with DMEM/BSA without or with 2% (v:v) serum HDL or 7.5 μg of control HDL, in vitro oxidized HDL or reduced HDL. Preliminary experiments demonstrated that the efflux capacity of HDL is a linear function of the duration of incubation under the conditions used in our assay and 2 h incubation gives us robust signal for sterol efflux. ABCA1-dependent cholesterol efflux was calculated as the percentage of total [3H]cholesterol (medium plus cell) released into the medium by mifepristone-treated BHK after subtraction of the value obtained with BHK cells not expressing ABCA1 (no mifepristone treatment). BHK cells incubated with serum HDL or apoA-I for up to 4 h showed no changes in morphology or in cell protein.

SUPPLEMENTAL REFERENCES


9. de la Llera-Moya M, Drazul-Schrader D, Asztalos BF, Cuchel M, Rader DJ, Rothblat GH. The ability to promote efflux via ABCA1 determines the capacity of serum specimens with similar high-density lipoprotein cholesterol to remove cholesterol from macrophages. *Arterioscler Thromb Vasc Biol*. 2010;30:796-801


Supplemental Figure I. Met148 of apoA-I in rHDL containing linoleic acid is resistant to artifactual oxidation. Reconstituted, discoidal HDL (rHDL, 9.6 nm diameter) generated from native lipid-free apoA-I and phospholipid containing linoleic acid (PLPC) was incubated at 37 °C for 1 h and isolated by ultracentrifugation. Lipid-free apoA-I, rHDL, and ultracentrifuge isolated rHDL were subjected to proteolytic digestion. Peptide digests were analyzed by tandem MS with SRM. Met(O)86 (A), Met(O)112 (B), and Met(O)148 (C) were quantified using reconstructed ion chromatograms of precursor and product peptides. Compared with lipid-free apoA-I, there was no increase in the oxidation of Met(148) in reisolated rHDL under these conditions (n=3). In contrast, oxidation of Met(86) and Met(112) increased. UC, ultracentrifuge isolated.
Supplemental Figure II. Oxidation of Met148 in (A) $^{15}$NapoA-I of rHDL and (B) apoA-I of HDL isolated from plasma, with or without azide. rHDL made with $^{15}$NapoA-I and phospholipid containing PLPC was added into freshly thawed plasma from healthy or CAD subjects (n=3 each group) and incubated under argon for 1 h at 37 °C. Sodium azide (10 mM) was included where indicated. HDL was isolated by ultracentrifugation and then subjected to trypsin digestion. Peptide digests were analyzed by tandem MS with SRM. Oxidation of Met148 in apoA-I of rHDL and HDL was quantified in peptides containing (A) $^{15}$N-Met(O)148 or (B) Met(O)148, respectively, using reconstructed ion chromatograms of precursor and product peptides. Compared with lipid-free apoA-I, there was no significant increase in oxidation of Met148 in rHDL incubated in plasma; azide has no significant effect on the level of oxidation of Met148 in $^{15}$NapoA-I of rHDL. Azide also had no effect on oxidation of Met148 in apoA-I of HDL isolated from either control or CAD plasma.
Supplemental Figure III. Azide has no effect on chlorination of Tyr192 in apoA-I of HDL isolated from plasma. Plasma of control subjects (n=3) or CAD subjects (n=3) stored at -80 °C was thawed, immediately supplemented with azide (10 mM) where indicated, and incubated for 1 h at 37 °C. HDL was isolated by ultracentrifugation and the levels of chlorinated Tyr192 were determined by tandem MS with SRM. Addition of azide to plasma had no significant effect on the level of Tyr192 chlorination.
Supplemental Figure IV. Methionine oxidation in apoA-I of HDL isolated from control, CAD, and ACS subjects. (A) Oxidation of Met residue in apoA-I for all subjects. (B) Levels of Met(O)86 in control subjects, CAD subjects, and ACS subjects. (C) Levels of Met(O)112 in control subjects, CAD subjects, and ACS subjects. (D) Correlation of 3-chloroTyr192 and Met(O)86 in all subjects. (E) Correlation of 3-chloroTyr192 and Met(O)112 in all subjects. (F) Correlation of Met(O)112 and Met(O)148 in all subjects. HDL was isolated by ultracentrifugation from freshly prepared plasma of CAD, ACS, or healthy subjects. Following the addition of oxidized $[^{15}$N]apoA-I as internal standard, HDL was subjected to proteolytic digestion. Peptide digests were analyzed by tandem MS with SRM. Values for 3-chloroTyr192 and Met(O) were logarithmically transformed to achieve a normal distribution.
Supplemental Figure V. Correlation of plasma MPO levels with oxidation products in HDL. MPO levels in plasma were measured by the Cleveland Heart Lab. Levels of 3-chloroTyr192 and Met(O) were quantified as described in the legends of Supplemental Figure II and Figure III. Relationships between plasma MPO levels and (A) 3-chloroTyr192, (B) Met(O)86, (C) Met(O)112 or (D) Met(O)148 were linear regression analyses using Pearson’s correlation coefficient. Values were logarithmically transformed.
Supplemental Figure VI. Correlation of cholesterol efflux capacity of serum HDL with (A) Met(O)86 and (B) Met(O)112. Serum HDL was obtained by polyethylene glycol (PEG) precipitation of serum derived from plasma. Cholesterol efflux from serum HDL to ABCA1-expressing BHK cells was measured as described in Methods. Levels of Met(O)86 and Met(O)112 were quantified as described in the legends of Supplemental Figure IV. Correlation of Met(O)86 (A) or Met(O)112 (B) and ABCA1 efflux capacity were determined by linear regression analysis using Pearson’s correlation coefficient. Values for Met(O) were logarithmically transformed to achieve a normal distribution.