Small, Dense HDL Particles Exert Potent Protection of Atherogenic LDL Against Oxidative Stress

Anatol Kontush, Sandrine Chantepie, M. John Chapman

Objectives—The relationship of the structural and functional heterogeneity of HDL particles to protection of LDL against oxidative stress is indeterminate.

Methods and Results—HDL subfractions of defined physicochemical properties were isolated by density gradient ultracentrifugation from normolipidemic human serum (n = 8), and their capacity to protect LDL from oxidation was evaluated. Under mild oxidative stress induced by AAPH or Cu(II), HDL subfractions (at equal cholesterol or protein concentration or equal particle number) significantly decreased LDL oxidation rate (−20% to −85%) in the propagation phase (234 nm), which was prolonged by up to 82% with decreased maximal diene formation. Antioxidative activity of HDL subfractions increased with increment in density, as follows: HDL2b < HDL2a < HDL3a < HDL3b < HDL3c (confirmed by thiobarbituric acid–reactive substance content and LDL electrophoretic mobility). Concordantly, antioxidative activity of small HDL prepared by FPLC was significantly higher (+56%) than that of large HDL. Antioxidative action of HDL subfractions was primarily associated with inactivation of LDL lipid hydroperoxides. The potent protective activity of small HDL could not be accounted for exclusively by enzymatic activities (PON1, platelet-activating factor acetylhydrolase, and lecithin-cholesterol acyltransferase).

Conclusions—Small, dense HDL exhibit potent antioxidant activity, which may arise from synergy in inactivation of oxidized LDL lipids by enzymatic and nonenzymatic mechanisms, in part reflecting distinct intrinsic physicochemical properties.

Key Words: HDL subfractions ■ LDL oxidation ■ small, dense HDL ■ enzymatic activities ■ physicochemical properties

It is now established that oxidation of LDL constitutes a key event in inflammation and atherogenesis.15 Mechanisms of LDL oxidation in vivo involve concerted modification by oxidants produced by arterial wall cells, such as reactive nitrogen species, reactive chlorine species, hydroxyl radicals, and lipid-soluble free radicals.26 Such a spectrum of chemically diverse oxidants implies that any single low-molecular-weight antioxidant, such as vitamins E and C, even at physiologically relevant doses, may not provide complete oxidative protection of LDL in vivo1,2 and that biological systems that can neutralize diverse forms of oxidative stress could be more efficient.

Plasma HDLs possess a spectrum of antiatherogenic actions, including potent antioxidant and antiinflammatory activities.3 Although HDLs can themselves undergo oxidative modification,4 several enzymes that may cleave oxidized lipids and thereby inhibit LDL oxidation are associated with HDL particles; these include paraoxonase (PON) in its major isoform, PON1,5 platelet-activating factor acetylhydrolase (PAF-AH),6 lecithin-cholesterol acyltransferase (LCAT),7 and glutathione selenoperoxidase.8 In addition, apolipoprotein A-I (apoA-I), a major HDL apolipoprotein, can remove oxidized lipids from LDL, suggesting that HDL can function as an acceptor of oxidized lipids.9 Other HDL apolipoproteins, such as apoA-II,10 apoA-IV,11 apoE,12 and apoJ,13 also function as antioxidants in vitro. The diversity of antioxidative actions of HDL particles suggests that HDL provide efficient protection of LDL from oxidation in vivo.

Plasma LDL are heterogeneous in their physicochemical properties and consist of 3 major particle subclasses, large, buoyant LDL, intermediate LDL, and small, dense LDL; such LDL subfractions are distinct in their atherogenic and oxidative properties.14,15 Equally, circulating HDL particles are heterogeneous in physicochemical properties, intravascular metabolism, and biological activity.16,17 Indeed, HDL particle phenotype is qualitatively and quantitatively altered in dyslipidemias associated with premature atherosclerosis, including hyperlipidemias of Types IIA, IIB, and IV, and Type II diabetes.17 However, the relationship of HDL particle phenotype to antiatherogenic activity is indeterminate.

The functional heterogeneity of HDL particles may be assessed by several methodological approaches, including

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Percent Weight of Chemical Composition, Aporotein Content, PAF-AH Activity, and Cu(II)-Binding Capacity of HDL Subfractions From Normolipidemic Subjects

<table>
<thead>
<tr>
<th></th>
<th>HDL2b</th>
<th>HDL2a</th>
<th>HDL3a</th>
<th>HDL3b</th>
<th>HDL3c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesteryl esters</td>
<td>28.8±1.3</td>
<td>26.6±3.1</td>
<td>24.4±2.9</td>
<td>22.6±2.9</td>
<td>17.1±5.2</td>
</tr>
<tr>
<td>Free cholesterol</td>
<td>4.2±1.2</td>
<td>3.2±1.0</td>
<td>2.3±0.7</td>
<td>1.0±0.5</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>30.1±2.4</td>
<td>32.7±3.6</td>
<td>29.0±4.9</td>
<td>23.6±4.2</td>
<td>16.0±3.4</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>4.1±1.1</td>
<td>3.9±1.3</td>
<td>3.1±1.4</td>
<td>2.2±1.1</td>
<td>0.8±1.1</td>
</tr>
<tr>
<td>Total protein</td>
<td>32.8±3.5</td>
<td>33.7±4.1</td>
<td>41.2±5.2</td>
<td>50.5±4.2</td>
<td>66.0±8.1</td>
</tr>
<tr>
<td>ApoA-I, mol/mol HDL</td>
<td>3.65±1.36</td>
<td>4.14±1.43</td>
<td>4.39±0.77</td>
<td>3.75±1.00</td>
<td>3.03±0.09</td>
</tr>
<tr>
<td>ApoA-II, mol/mol HDL</td>
<td>0.79±0.24</td>
<td>1.06±0.80</td>
<td>1.41±0.28</td>
<td>0.99±0.46</td>
<td>0.36±0.13</td>
</tr>
<tr>
<td>LCAT, % hydrolyzed substrate</td>
<td>24.5±5.8</td>
<td>24.6±5.7</td>
<td>35.9±39.1</td>
<td>31.1±10.0</td>
<td>28.0±7.0</td>
</tr>
<tr>
<td>PAF-AH, nmol/min per mg protein</td>
<td>1.17±0.20</td>
<td>2.02±1.43</td>
<td>0.93±0.18</td>
<td>2.72±1.82</td>
<td>4.16±0.30</td>
</tr>
<tr>
<td>Cu(II)-binding capacity, mol/mol HDL</td>
<td>12.7±7.2</td>
<td>10.3±3.7</td>
<td>12.1±1.4</td>
<td>12.6±7.8</td>
<td>9.7±8.9</td>
</tr>
</tbody>
</table>

Data are shown for EDTA plasma of 4 to 8 healthy male donors.

ultracentrifugation, size exclusion or affinity chromatography, electrophoresis, and selective precipitation. Each of these methods is dependent on distinct physical, chemical, and immunological parameters; thus, HDL particle subspecies are isolated that are not strictly comparable between fractionation methods in terms of their structure, lipid and protein composition, and biological activity.

The question as to whether distinct HDL particle subfractions may differ in their capacity to protect LDL against oxidation is of immediate relevance to the inflammatory dimension of atherogenesis. Ultracentrifugally isolated HDL3 exerts greater inhibition of adhesion protein expression in endothelial cells than HDL2. By contrast, the potential heterogeneity of antioxidative activity of HDL particle subfractions is controversial. Isopycnic density gradient centrifugation allows reproducible isolation of 5 physicochemically defined, highly purified, major HDL subfractions, HDL2b, 2a, 3a, 3b, and 3c. By this approach, we presently demonstrate that small, dense HDL particles exert potent protection of atherogenic LDL against oxidative stress in normolipidemic subjects.

Methods

Details of blood samples, isolation of lipoproteins, characterization of native and oxidized lipoproteins, and statistical analysis are available online at http://atvb.ahajournals.org.

Results

Chemical Composition of HDL Subfractions

Consistent with published data, the weight composition of major lipid classes showed a distinct trend to decrease with increment in total protein content and particle density across the HDL distribution from HDL2b to HDL3c (Table). AAPH-induced oxidation increased in the order HDL2b < HDL2a < HDL3a < HDL3b < HDL3c (compared on the basis of equal amounts of TC; overall range in TC, 10% to 21% by weight; Table), indicating that inhibition of LDL oxidation was more pronounced in the presence of dense HDL3 than in that of lighter HDL2 subfractions.

HDL subfractions isolated from EDTA plasma were equally able to delay LDL oxidation (Figure 1C). HDL subfractions decreased the oxidation rate in the propagation phase of LDL oxidation. When all calculated parameters were considered, the HDL-mediated protection of LDL to AAPH-induced oxidation increased in the order HDL2b < HDL2a < HDL3a < HDL3b < HDL3c (compared on the basis of equal amounts of TC; overall range in TC, 10% to 21% by weight; Table), indicating that inhibition of LDL oxidation was more pronounced in the presence of dense HDL3 than in that of lighter HDL2 subfractions.
phase (−25%, −42%, and −91% in the presence of HDL2b, 3b, and 3c, respectively, at 1.5 mg TC/dL of each; n=3; P<0.05; Figure 1D) and equally prolonged this phase (+44%, +15%, +35%, and +99% in the presence of HDL2a, 3a, 3b, and 3c, respectively, at 1.5 mg TC/dL of each; n=3; P<0.05). In addition, dense HDL subfractions decreased the maximal amount of conjugated dienes formed on oxidation (−12% and −69% in the presence of HDL3b and 3c, respectively, at 1.5 mg TC/dL of each; n=3; P<0.05), whereas light HDL subfractions tended to increase total diene formation (+11% and +4% in the presence of HDL2b and 2a, respectively; n=6; P<0.05). In contrast, the lag phase was not significantly prolonged by any plasma-derived HDL subfraction (data not shown). The inhibitory effects of HDL subfractions were concentration-dependent (Figure 1D). We next evaluated the possibility that HDL subfractions might afford protection to distinct LDL subfractions; indeed, HDL subfractions from serum or plasma provided efficient protection of both intermediate LDL3 and dense LDL5 from oxidation by AAPH (data not shown). As for HDL subfractions isolated from serum, the plasma HDL-mediated protection of LDL to oxidation induced by AAPH increased in the order HDL2b<HDL2a<HDL3a<HDL3b<HDL3c; however, HDL subfractions from serum tended to provide more efficient protection of LDL compared with EDTA plasma-derived HDL subfractions (Figure 1B versus Figure 1D; online Table I). Antioxidative activities of HDL subfractions isolated from serum and from EDTA plasma showed significant correlation (r=0.64, n=15, P=0.01 for data in online Table I).

Antioxidative Action of HDL Subfractions During Cu(II)-Induced LDL Oxidation

Data obtained using AAPH as a prooxidant were confirmed using Cu(II). Again, oxidation of LDL was markedly delayed by individual serum-derived HDL subfractions at a physiological LDL-TC to HDL-TC ratio between 2:1 and 6:1 (Figure 2A). HDL subfractions (2a, 3a, 3b, and 3c) were
effective in the propagation phase, decreasing oxidation rate \((-26\%, -58\%, -69\%,\) and \(-85\%,\) respectively, at 1.5 mg TC/dL of each subfraction, \(n \geq 3, P < 0.05;\) Figure 2B). In addition, the densest HDL3c subfraction decreased maximal diene formation \((-35\% at 1.5 mg TC/dL; n = 4, P < 0.05.\) All HDL subfractions prolonged the lag phase \((+28\%, +16\% , +49\% , +80\% , +76\% in the presence of HDL2b, 2a, 3a, 3b, and 3c, respectively, at 1.5 mg TC/dL of each, \(n = 4, P < 0.05)\) but also tended to increase initial oxidation rate (data not shown). The latter effect was attributable to the oxidation of HDL subfractions themselves (see above), consistent with the data of Raveh et al.\(^{25}\) Inhibition of LDL oxidation by HDL increased in parallel with elevation in HDL subfraction density at the same LDL-TC to HDL-TC ratio. The inhibitory effects of HDL subfractions were concentration-dependent (Figure 2B).

HDL subfractions isolated from EDTA plasma also protected LDL from oxidation. Dense HDL3a, 3b, and 3c were effective in the propagation phase (Figure 2C), decreasing oxidation rate (Figure 2B), and increasing phase duration \((+11\% , +32\% ,\) and \(+148\% ,\) respectively, at 1.5 mg TC/dL of each subfraction; \(n \geq 3, P < 0.05).\) In addition, HDL3c decreased maximal diene formation \((-59\% at 1.5 mg TC/dL; n = 7, P < 0.01)\). No significant inhibition of LDL oxidation was observed in the presence of light HDL2b and 2a. Inhibition of LDL oxidation by EDTA plasma-derived HDL increased in parallel with elevation in HDL subfraction density. In contrast, HDL3 did not exert antioxidant effects in the lag phase (data not shown), whereas HDL2 increased the oxidation rate at this early stage \((+97\% and +87\% in the presence of HDL2b and 2a, respectively; n \geq 3; P < 0.05)\). The inhibitory effects of HDL3 subfractions were concentration-dependent (Figure 2B). Again, HDL subfractions from serum were slightly more efficient in protecting LDL compared with their counterparts from EDTA plasma (Figure 2B versus Figure 2D).

**Influence of HDL Subfractions on Thiobarbituric Acid–Reactive Substance (TBARS) Accumulation and REM of LDL**

Consistent with the data on diene formation, TBARS accumulation was delayed in the presence of dense HDL3b and 3c isolated from either serum or EDTA plasma (Figure 3A), whereas light HDL subfractions \(2b\) and \(2a\) were without significant effect (data not shown). Similarly, increase in REM of LDL after 24-hour oxidation was significantly diminished in the presence of HDL3b and 3c (Figure 3B) but not in that of other HDL subfractions (data not shown). No systematic difference between antioxidative activities of HDL subfractions obtained from serum and plasma was observed.

**Antioxidative Action of HDL Subfractions at Similar Total Protein Content and Similar Particle Number and at Their Physiological Concentration Ratio to LDL**

To confirm our finding that differences in antioxidative properties between HDL subfractions were not dependent on differences in their lipid-to-protein ratio (Table), we incubated LDL in the presence of either the same concentration of HDL protein or the same number of HDL particles isolated from serum. When the concentration of serum-derived HDL subfractions was calculated on a protein basis, HDL antioxidative activity increased in the order HDL2b < HDL2a < HDL3a < HDL3b < HDL3c (online Figure IIA). Again, HDL3c was most effective at late stages of oxidation, increasing the duration of the propagation phase by 30% and decreasing oxidation rate in this phase by 36% at 1.5 mg/dL HDL protein \((n = 3, P < 0.05)\). When serum-derived HDL subfractions were compared on the basis of the same number of particles, HDL3c was again the most potent antioxidant species (online Figure IIB), decreasing the oxidation rate in the propagation phase by 44% and maximal diene formation by 23% at 0.22 \(\mu\)mol/L HDL, ie, at an LDL to HDL molar ratio of 1 to 2 \((n = 2)\). Finally, we compared the antioxidative activity of HDL subfractions on the basis of their circulating levels, which differs considerably. Assuming that HDL2b, 2a, 3a, 3b, and 3c carry on average 15\%, 15\%, 50\%, 15\%, and 5\% of HDL TC,\(^{16}\) we found that serum-derived HDL3a was the most effective inhibitor of LDL oxidation followed by HDL3b and HDL3c (online Figure IIC). This finding is in agreement with the concentration dependence of HDL antioxidative activity as described above (Figure 1B). Data obtained using serum-derived HDL subfrac-
Antioxidative Action of HDL Subfractions Under Strong Oxidative Conditions

When LDL was incubated in the presence of 5.0 μmol/L Cu(II) or 10 mmol/L AAPH, none of the serum-derived HDL subfractions protected LDL from oxidation; moreover, their addition tended to accelerate oxidation (online Figure III), consistent with previously reported data.25 Antioxidative Action of HDL Subfractions Prepared by FPLC

Consistent with data for ultracentrifugally isolated HDL subfractions, antioxidative protection of LDL by small HDL (expressed as decrease in LDL oxidation rate in the propagation phase) was significantly higher than that by large HDL on a protein basis (+56%, n=4, P<0.01; Figure 4).

Role of Albumin in the Antioxidative Activity of HDL Subfractions

In accordance with previously published data,26 BSA was a weak inhibitor of LDL oxidation by AAPH. Thus, 5 mg/dL BSA induced prolongation of the propagation phase (+43%) and decreased maximal diene formation (~26%; n=2).

Protein Components of Serum-Derived HDL Possessing Antioxidative Activity

PON1 activity with phenyl acetate as substrate increased in the order HDL2b/HDL2a/HDL3a/HDL3b/HDL3c (Figure 5A). PON1 activities of HDL subfractions to phenyl acetate and paraoxon were highly correlated (r=0.85, P<0.001; Figure 5A, inset); PON1 activity to paraoxon was similarly distributed among HDL subfractions (data not shown), consistent with recently published data.19,22,27 However, when HDL was prepared from serum using FPLC, PON1 activity to phenyl acetate was higher in large compared
with small HDL particles (1.21±0.20 versus 0.26±0.07 μmol/min per mg total protein; n=4; P=0.001).

Ca(II) is a key factor in PON1 activity;6 equally, EDTA is known to partially deactivate PON1. The PON1 activity of HDL subfractions from serum was significantly higher (8- to 56-fold) compared with that of HDL subfractions isolated from EDTA plasma (online Table I). However, addition of CaCl2 to the LDL+HDL mixture to the same concentration as that used in the assay for PON1 activity (1 mmol/L) did not afford additional protection of LDL in the presence of plasma-derived HDL subfractions (data not shown). PON1 activities of both serum- and plasma-derived HDL subfractions from 3 donors were strongly correlated (r=0.89, n=15, P<0.001).

On a particle basis, contents of apoA-I and apoA-II were elevated in HDL3a and lowest in HDL3c (Table). PAF-AH activity was significantly increased in small, dense HDL3c (Table). LCAT activity tended to be higher in HDL3 relative to HDL2 subfractions (34.3±18.9% versus 24.9±5.4%, n=4; Table), consistent with previously reported data.28 In contrast and as for PON1, both PAF-AH and LCAT activities were lower in small versus large HDL (~76% and ~58%, respectively) prepared by FPLC. Compared with large LDL, small HDL were selectively enriched in apoA-II (apoA-II/apoAI weight ratio of 0.12±0.06 and 0.28±0.09, respectively; n=5; P<0.05).

**Cu(II)-Binding Capacity and Cu(II)-Reducing Activity of HDL Subfractions**

Differences in Cu(II) binding or Cu(II) reduction can theoretically lead to variation in potency of inhibitory activity to Cu(II)-induced oxidation between HDL subfractions. However, HDL subfractions isolated from EDTA plasma bound similar amounts of Cu(II) (Table). HDL3 exhibited stronger Cu(II)-reducing properties than HDL2 [3.68 and 0.43 μmol/L Cu(I) produced after 10 minutes of incubation in the presence of HDL3a and 2a, respectively, at 5 mg TC/dL, n=2]. In addition, HDL subfractions did not decrease Cu(II) reduction by LDL [8.18, 3.63, and 3.83 μmol/L Cu(I) produced after 10 minutes of incubation in the presence of LDL+HDL3a, LDL+HDL2a, and LDL alone, respectively, at 10 mg LDL-TC and 5 mg HDL-TC/dL, n=2].

**Correlations**

When the antioxidative activity of serum-derived HDL subfractions was correlated with HDL levels and activities of antioxidative proteins, PON1 activity against phenyl acetate was significantly negatively correlated with the oxidation rate in the propagation phase (r=−0.99, P=0.001; Figure 5B) and maximal diene formation (r=−0.88, P=0.04) in the presence of AAPH; correlations of comparable significance were calculated for PON1 activity against paraoxon as well as for Cu(II)-induced oxidation (data not shown). Similarly, PAF-AH activity negatively correlated with the oxidation rate in the propagation phase (r=−0.98, P=0.004) in the presence of AAPH. Strong correlations were found between total protein content of serum-derived HDL subfractions and their antioxidative activity (eg, r=−0.91, P=0.03 for the oxidation rate in the propagation phase and r=−0.95, P=0.01 for maximal diene formation). No significant correlation was found between the antioxidative activity of HDL subfractions and apoA-I and apoA-II content, LCAT activity, Cu(II)-reducing activity, and Cu(II)-binding capacity (data not shown).

**Discussion**

The present studies have established that both serum-derived and EDTA plasma–derived small, dense HDL particles possess the most potent capacity among HDL subspecies to protect LDL from both metal-dependent and metal-independent oxidation in normolipidemic subjects. The oxidative protection of LDL by ultracentrifugally isolated HDL subfractions (at equal cholesterol or protein concentration or equal particle number) of defined physicochemical properties increased in the order LDL2b<HDLL2a<HDLL3a<HDLL3b<HDLL3c. The inhibitory effects of all HDL subfractions were concentration-dependent; at supraphysiological ratios of LDL to individual HDL subfractions (as TC; 2:1), both small, dense, lipid-poor HDL3 and large, light, cholesteryl ester–rich HDL2 subfractions potently inhibited LDL oxidation (Figures 1B, 1D, 2B, and 2D). HDL subfractions efficiently protected not only total LDL but also intermediate LDL3 (typically the most abundant LDL subfraction in normolipidemic subjects) and small, dense LDL5 (a highly atherogenic LDL subfraction), thereby suggesting that HDL can attenuate oxidation of atherogenic LDL subclasses.

Plasma HDL particles contain antioxidant enzymes with wide substrate specificity, such as PON1,5 PAF-AH,6 and LCAT,7 and apolipoproteins, such as apoA-I,9 apoA-II,10 apoA-IV,11 apoE,12 and apoJ,13 that can delay or prevent formation of oxidized lipids in LDL or inactivate such lipids on formation.9 The antioxidative activities of HDL subfractions that may be enzymatic or nonenzymatic in nature and that are associated with inactivation of phospholipid and cholesteryl ester hydroperoxides may contribute significantly to their observed protective effects on LDL oxidation. This conclusion is supported by the following findings: (1) antioxidative effects of HDL subfractions were most pronounced at later stages of LDL oxidation when high levels of lipid hydroperoxides had accumulated; (2) HDL subfractions exhibited limited capacity to protect LDL at early stages of oxidation when levels of lipid hydroperoxides are low; (3) under mild oxidative conditions [1 mmol/L AAPH or 0.5 μmol/L Cu(II)], all HDL subfractions delayed LDL oxidation; in contrast, none of the HDL subfractions protected LDL under strong oxidative conditions [10 mmol/L AAPH or 5 μmol/L Cu(II)], an observation that may result from saturation of the protective enzymes (or other proteins) by high levels of lipid hydroperoxides or inactivation of the enzymes themselves; (4) total protein content (as percent weight) of HDL subfractions correlated with their antioxidative activity; and (5) dense HDL3 subfractions inhibited TBARS formation and increase in the electrophoretic mobility of LDL. Effects related to the accumulation of highly reactive secondary oxidation products, such as aldehydes, formed on breakdown of lipid hydroperoxides.

When the content of HDL protein components with antioxidative activity, including PON1, PAF-AH, LCAT, apoA-I, and apoA-II, was evaluated, we observed that the antioxidative-
tive activity of density gradient HDL subfractions strongly correlated with their PON1 and PAF-AH activity. The elevated activity of PON1 and PAF-AH in small, dense, protein-rich HDL3 is consistent with earlier data reported for HDL3 isolated by both selective precipitation and ultracentrifugation. These data suggest that both PON1 and PAF-AH may contribute to HDL-mediated oxidative protection of LDL. In contrast, no correlation was found between the antioxidative activity of HDL subfractions and their LCAT activity, apoA-I or apoA-II level, Cu(II)-binding capacity, and Cu(II)-reducing activity. HDL particles may function as preventive antioxidants via the binding of redox-active transition metal ions. However, HDL subfractions were equally effective against both metal-dependent and metal-independent oxidation, indicating that interaction with transition metal ions is not implicated in the oxidative protection of LDL.

Consistent with data for ultracentrifugally isolated HDL subfractions, small HDL particles prepared using FPLC possessed higher antioxidative activity (+56%) compared with their large counterparts (Figure 4). However, PON1 and PAF-AH activities were lower in small, compared with large, HDL particles isolated from serum using FPLC, in confirmation of previous data. By contrast, apoA-II was enriched in small versus large HDL, consistent with earlier data. These findings indicate that potent antioxidative activity is an intrinsic property of small HDL particles and that components in addition to PON1 and PAF-AH play key roles in its expression. This conclusion is consistent with the finding that although serum-derived HDL subfractions were more potent in protecting LDL from oxidation than their counterparts from EDTA plasma, this difference was considerably less pronounced compared with that in PON1 activity. Indeed, PON-independent inhibition of LDL oxidation by HDL has been previously demonstrated. Using SDS-PAGE, we found no difference in the levels of apoE and apoA-IV between small and large HDL isolated by FPLC (data not shown); in addition, our attempts to measure activity of glutathione selenoperoxidase in HDL subfractions were unsuccessful because of low assay sensitivity (data not shown), thereby suggesting that none of these proteins with antioxidative properties can account for the distinct antioxidative activities between HDL subfractions.

The distinction between the distribution of PON1 and PAF-AH activities among HDL particles isolated by ultracentrifugation and precipitation versus FPLC can potentially be explained by displacement of weakly bound, dissociable enzymes from large to small HDL by the former methods. Large HDL may be preferentially enriched in weakly bound PON1 and PAF-AH, which dissociate during ultracentrifugation or polyanion precipitation, whereas these enzymes may exist primarily in nondissociable forms in small HDL, as observed by McCall et al for PAF-AH in large versus small LDL. Clearly, then, high ionic strength or g-forces may disrupt PON1- and PAF-AH interaction with large HDL.

The oxidability of HDL subfractions themselves increased in the order HDL2b < HDL2a < HDL3a < HDL3b < HDL3c, mirroring their potency in protecting LDL against oxidation. HDL can remove lipid hydroperoxides from LDL, moreover, HDL is a major carrier of lipid hydroperoxides in human plasma. Thus, preferential transfer of oxidized lipids from LDL to small HDL with subsequent cleavage may be primarily responsible for the distinct antioxidative properties of HDL subfractions. This hypothesis is consistent with the higher capacity of dense HDL3 to accept polar lipids than lighter HDL2, an effect that can be related to protein-independent partition of oxidized lipids from LDL into the surface phospholipid monolayer of HDL. Indeed, phospholipids exhibit a low degree of order and are loosely packed in small HDL particles. The elevated intrinsic antioxidant potency of small, dense HDL may thus arise from mechanistic synergy in inactivation of oxidized lipids because of the presence of (1) multiple enzymatic activities, such as PON1, PAF-AH, and LCAT; (2) specific proteins, such as apoA-I and apoA-II, which interact with oxidized lipids and undergo chemical modification; and (3) lipids, such as phospholipids and plasmalogens, that act as acceptors or targets of reactive LDL oxidation products.

The present evidence for the differential antioxidative properties of HDL subfractions may have important consequences for our understanding of the protective antiatherogenic action of HDL in vivo. Thus, although small, dense HDL3c typically accounts for <15% of total HDL, HDL3c may nonetheless play a pivotal role in the protection of LDL against oxidation, significantly exceeding the protection afforded by low-molecular-weight antioxidants. Indeed, it has been recently shown that HDL3, rather than HDL2, is strongly correlated with the antiatherogenic action of gemfibrozil in the VA-HIT study. Considered together, our present data identify small, dense HDL as a potential pharmacological target for the therapeutic attenuation of atherosclerosis in subjects at high cardiovascular risk associated with increased oxidative stress, as, for example, in the case of type II diabetes and metabolic syndrome.

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