Antioxidative Activity of HDL Particle Subspecies Is Impaired in Hyperalphalipoproteinemia: Relevance of Enzymatic and Physicochemical Properties

Anatol Kontush, Eliana Cotta de Faria, Sandrine Chantepie, M. John Chapman

Objective—Hyperalphalipoproteinemia (HALP) is characterized by elevated plasma levels of high-density lipoprotein (HDL) particles with altered composition, metabolism, and function. The impact of such modification on antioxidative activities of HDL subfractions is indeterminate.

Methods and Results—Gradient fractionation revealed that buoyant HDL2b and 2a and small dense HDL3b and 3c levels were elevated up to 2.0-fold in HALP subjects (n=9; mean plasma HDL cholesterol, 79 mg/dL) with low hepatic lipase activity. HDL2a, 3a, 3b, and 3c displayed lower specific antioxidative activity (sAA) during low-density lipoprotein (LDL) oxidation (−15% to −86%, on a unit particle mass basis) than their normolipidemic counterparts (n=13). LDL oxidation was delayed by control HDL3a, 3b, and 3c (up to −79%) but specifically by HDL3c (−54%) in HALP. Paraoxonase activity was deficient in all HALP HDL subfractions. Paraoxonase, PAF-AH, and LCAT activities together accounted for ~50% of variation in sAA. Abnormal chemical composition of HDL3b and 3c (cholesterol-deficient, triglyceride-enriched) in HALP was associated with impaired sAA. Systemic oxidative stress (as plasma 8-isoprostanones) tended to be elevated (1.5-fold) in HALP and negatively correlated with sAA (as TBARS).

Conclusions—Intrinsic antioxidative activity of HDL subspecies is impaired in HALP, reflecting altered enzymatic and physicochemical properties. (Arterioscler Thromb Vasc Biol. 2004;24:526-533.)

Key Words: oxidative stress ■ HDL particle heterogeneity ■ LDL oxidation ■ lipid hydroperoxides ■ atherosclerosis

It is well established that plasma concentrations of high-density lipoprotein cholesterol (HDL-C) are inversely correlated with the risk of atherosclerosis and cardiovascular (CV) disease.1 Marked elevation in HDL-C levels is typical of hyperalphalipoproteinemia (HALP), which is characterized by plasma levels of HDL-C above the 90th percentile for an age- and sex-matched general population.2,3 Primary HALP can arise from genetic deficiency of plasma high-density lipoprotein (HDL), as well as from increased production of apolipoprotein A-I (apoA-I), a major HDL apolipoprotein;2 equally, however, HALP may be of unknown etiology. HALP involves modification of the physicochemical properties, metabolism, and function of HDL; for example, large CE-rich HDL2 particles that accumulate in familial CETP deficiency possess diminished capacity for cholesterol efflux from macrophages.2 In a transgenic mouse model of HALP overexpressing human lecithin–cholesterol acyltransferase (LCAT), dysfunctional HDL exhibits diminished capacity for reverse-cholesterol transport (RCT).4

The antiatherogenic actions of HDL reflect functional biological properties of HDL particle subpopulations rather than absolute plasma levels of HDL-C.5 A spectrum of antiatherogenic activities is associated with HDL particles, which include the capacity to transport cholesterol from arterial wall cells to the liver in the RCT pathway and to exert antiinflammatory and antioxidative activities.5,6 Oxidation of low-density lipoprotein (LDL) in the arterial wall is a central proinflammatory event in atherogenesis.6 LDL particles carry enzymes that may inhibit LDL oxidation, including paraoxonase (PON), platelet-activating factor acetylhydrolase (PAF-AH), and LCAT.7,8 These enzymes hydrolyze proinflammatory molecular species of oxidized lipids and prevent their accumulation in LDL. In addition, apoA-I can bind oxidized lipids and remove them from LDL.9 Significantly, antioxidative activity of HDL can be severely compromised under some pathological conditions, such as inflammation,7 which may in turn result in accelerated LDL oxidation and enhanced atherosclerosis.

Plasma HDL particles are structurally, metabolically, and functionally heterogeneous.10 Small, dense, lipid-poor HDL3 display higher capacity to accept cholesterol,11 to inhibit expression of adhesion molecules on endothelial cells in vitro,12 and to protect LDL from oxidative stress13–15 as...
compared with large, light, lipid-rich HDL2. The intravascular metabolism and particle heterogeneity of HDL are altered in HALP. The impact of such modification on the antioxidative activities of HDL remains, however, to be established. Therefore, we evaluated the capacity of distinct HDL subfractions (HDL2b, 2a, 3a, 3b, and 3c) from normocholesterolemic HALP subjects to protect LDL from oxidation. Our data reveal that the intrinsic antioxidative activity of HDL particle subspecies is decreased in HALP subjects with low HL activity, and that this deficiency is partially compensated by elevated particle number.

Methods

Subjects
HALP (n=9) and normolipidemic control (n=13) male subjects were recruited at the Campinas University Hospital (Campinas, Brazil). HALP was defined using inclusion criteria according to Baldassarre et al.1 and notably as a plasma level of HDL-C higher than the 90th percentile (>65 mg/dL) for the local population (n=1700). All subjects recruited for the study were non-smokers and either abstainers or moderate alcohol consumers (Table 1). To minimize the influence of non-HDL-C parameters on lipid metabolism, the HALP and control groups were matched according to plasma levels of triglycerides and LDL cholesterol (LDL-C) (Table 1). In addition, there were no significant differences in the level of inflammation (as assessed by plasma CRP level) and plasma activities of CETP and phospholipid-transfer protein between the groups (data not shown). HL activity was significantly decreased in HALP subjects as compared with controls (2.44±1.04 versus 3.93±1.46 μmol free fatty acids/mL per hour; P=0.02); decreased HL activity may therefore be responsible for HALP in our study population, consistent with the classification of Yamashita et al.2

Antioxidative Activities of HDL Subfractions

We assessed two different antioxidative activities for each isolated HDL subfraction, “specific antioxidative activity” and “total antioxidative activity.” Specific antioxidative activity was measured at the same final concentration of each subfraction (10 mg total mass/dL) to assess the intrinsic capacity of HDL subfractions to protect LDL from oxidation by a water-soluble generator of free radicals, 2,2′-azobis-(2-amidinopropane) hydrochloride (AAPH), at an HDL/LDL ratio within the physiological range (2 to 6 mol/mol). Total antioxidative activity was measured at a 7-fold dilution of each subfraction and took into account interindividual variation in plasma levels of HDL subfractions.

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

Plasma Lipids and Apolipoproteins

Plasma levels of HDL-C were significantly (+55%) higher in HALP subjects (n=9) in comparison with the normolipidemic group (n=13; Table 1). In addition, TC (+25%), apoA-I levels (+24%), and the ratio of HDL-C to apoA-I (0.42±0.05 versus 0.34±0.06; P<0.01) were significantly elevated in HALP individuals, whereas the ratio of TC to HDL-C was greater in controls (3.27±0.59 versus 2.64±0.41; P<0.02). In contrast, plasma levels of LDL-C, apoB100, and TG did not differ significantly between the HALP group and controls. HDL-C negatively correlated with HL activity (r=−0.51, P=0.01), consistent with the key role of HL deficiency in HALP.

Systemic Oxidative Stress

Levels of systemic oxidative stress assessed as plasma 8-isoprostanes by ELISA tended to be elevated in HALP subjects as compared with controls (76±46 versus 51±38 ng/L; P=0.19).

Plasma Levels and Chemical Composition of HDL Subfractions

Plasma levels of large buoyant HDL2 particles (HDL2b and 2a) and also those of small dense HDL3 (HDL3b and 3c) were significantly elevated in the HALP group in comparison with normolipidemic controls (Figure 1). By contrast, concentrations of the HDL3a subfraction at the center of the HDL density distribution were alike. Differences between HALP and control subjects in total HDL mass—and therefore HDL particle number—decreased: HDL2b (2.00-fold) >HDL3c (1.43-fold) >HDL3b (1.38-fold) >HDL2a (1.20-fold) >HDL3a (1.10-fold).

Significantly higher absolute amounts of CE in HDL2b and 2a, free cholesterol (FC) in HDL2b, phospholipids (PL) in

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**TABLE 1. Clinical and Biological Characteristics of Normocholesterolemic HALP Subjects and Normolipidemic Controls**

<table>
<thead>
<tr>
<th></th>
<th>Control Subjects (n=13)</th>
<th>HALP Subjects (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>41.7±16.2</td>
<td>55.6±15.3</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>22.6±1.3</td>
<td>24.8±4.5</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>13/0</td>
<td>9/0</td>
</tr>
<tr>
<td>Smoking (Y/N)</td>
<td>0/13</td>
<td>0/9</td>
</tr>
<tr>
<td>Alcohol (Y/N)</td>
<td>0/7</td>
<td>3/6</td>
</tr>
<tr>
<td>Plasma TC (mg/dL)</td>
<td>165±23</td>
<td>207±29†</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>77±19</td>
<td>69±26</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>97±23</td>
<td>111±22</td>
</tr>
<tr>
<td>ApoB100 (mg/dL)</td>
<td>83±19</td>
<td>95±31</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>51±7</td>
<td>79±11*</td>
</tr>
<tr>
<td>ApoA-I (mg/dL)</td>
<td>153±17</td>
<td>190±21*</td>
</tr>
</tbody>
</table>

*P<0.001, †P<0.01 vs control subjects. Y/N indicates yes/no.
their molecular weights (Mr) (see online supplement) and SDS-PAGE (data not shown).

Significant difference in the relative contents of major lipids (TG and total protein) as compared with controls. No subjects were selectively depleted of CE and FC and enriched significantly higher percent contents of CE in HDL2b, TG in HDL3a, and total protein in HDL 2b, 3b, and 3c subfractions as compared with controls (Table 2). When chemical composition was expressed as a percentage of total mass, then significantly higher percent contents of CE in HDL2b, TG in HDL3a, 3b, and 3c, total protein in HDL3b and 3c and 3c and lower percent contents of CE in HDL3b and 3c, FC in HDL3b and 3c, and total protein in HDL2b subfractions were observed in the HALP group as compared with control subjects (Table 2). These between-group differences clearly were observed in the HALP group as compared with control subjects.

When HDL subfractions (HDL3a, 3b, and 3c) from control subjects were added to reference LDL (isolated from one control subject and used throughout the study) directly before addition of AAPH, LDL oxidation was significantly delayed (Figure IIA). In contrast, only the HDL3c subfraction from HALP subjects was able to significantly delay oxidation of reference LDL relative to that of LDL oxidized alone (Figure IB, available online at http://atvb.ahajournals.org).

In the control group, protection of LDL during AAPH-induced oxidation mediated by the same concentration of each HDL subfraction, ie, “specific antioxidative activity,” decreased in the order HDL3c> HDL3b> HDL3a> HDL2a > HDL2b (Figure 2: Figures I and II, available online at http://atvb.ahajournals.org). In control subjects, small dense HDL subfractions (HDL3a, 3b, and 3c) significantly decreased LDL oxidation rate in the propagation phase (<19%, P=0.01; <47%, P<0.001; and <69%, P<0.001, respectively: Figure 2A) and equally prolonged this phase (+16%, P<0.05; +56%, P<0.05; and +79%, P<0.001, respectively: Figure II A). In contrast, only HDL3c significantly (−54%, P<0.01) decreased LDL oxidation rate in the propagation phase among HDL subfractions in HALP subjects (Figure 2A); no effect of any subfraction on the duration of the propagation phase was found (Figure II A). As a result, HDL2a, 3a, 3b, and 3c subfractions isolated from HALP subjects were significantly less active (−15 to −86%) in

### TABLE 2. Concentrations of Lipids (mg/dL) and Apolipoproteins (mol/mol HDL), Percent Chemical Composition and PAF-AH and LCAT activities in HDL subfractions in Normocholesterolemic HALP Subjects and Normolipidemic Controls

<table>
<thead>
<tr>
<th>Group</th>
<th>HDL2b</th>
<th>HDL2a</th>
<th>HDL3a</th>
<th>HDL3b</th>
<th>HDL3c</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE</td>
<td>16.7±5.2 (25.2)</td>
<td>18.3±3.6 (25.6)</td>
<td>12.9±2.9 (24.0)</td>
<td>4.6±1.0 (18.9)</td>
<td>2.0±0.9 (13.2)</td>
</tr>
<tr>
<td>HALP</td>
<td>38.1±12.0 (25.5*)</td>
<td>23.1±5.6* (27.1)</td>
<td>14.6±4.8 (24.3)</td>
<td>5.2±2.1 (15.2)</td>
<td>1.7±1.1 (7.8*)</td>
</tr>
<tr>
<td>FC</td>
<td>3.3±1.5 (4.9)</td>
<td>2.2±0.7 (3.1)</td>
<td>1.3±0.4 (2.4)</td>
<td>0.5±0.2 (2.1)</td>
<td>0.2±0.1 (1.6)</td>
</tr>
<tr>
<td>HALP</td>
<td>5.3±1.6* (4.0)</td>
<td>2.3±0.5 (2.8)</td>
<td>1.3±0.5 (2.3)</td>
<td>0.4±0.1 (1.2)</td>
<td>0.1±0.1 (0.6)</td>
</tr>
<tr>
<td>PL</td>
<td>19.2±7.4 (28.6)</td>
<td>22.3±3.7 (31.5)</td>
<td>15.0±4.7 (27.7)</td>
<td>5.0±1.2 (20.8)</td>
<td>2.0±0.6 (14.0)</td>
</tr>
<tr>
<td>HALP</td>
<td>42.7±9.7‡ (32.8)</td>
<td>26.9±5.8* (31.6)</td>
<td>16.1±4.2 (27.5)</td>
<td>6.5±2.1* (19.4)</td>
<td>2.5±1.3 (11.5)</td>
</tr>
<tr>
<td>TG</td>
<td>4.0±1.4 (6.2)</td>
<td>3.1±0.9 (4.4)</td>
<td>2.2±0.6 (4.2)</td>
<td>1.1±0.5 (4.7)</td>
<td>0.8±0.4 (5.4)</td>
</tr>
<tr>
<td>HALP</td>
<td>7.5±1.6‡ (5.8)</td>
<td>4.7±1.2‡ (5.7)</td>
<td>3.2±0.9* (5.6*)</td>
<td>2.2±0.7* (6.7*)</td>
<td>1.8±0.7* (8.9*)</td>
</tr>
<tr>
<td>Total protein</td>
<td>22.9±5.6 (35.1)</td>
<td>25.1±3.3 (35.3)</td>
<td>22.2±3.2 (41.7)</td>
<td>12.9±2.2 (53.5)</td>
<td>9.6±2.1 (65.8)</td>
</tr>
<tr>
<td>HALP</td>
<td>38.2±9.8‡ (29.9‡)</td>
<td>28.1±7.0 (32.9)</td>
<td>23.4±6.1 (40.3)</td>
<td>19.0±4.9‡ (57.5*)</td>
<td>14.8±2.8‡ (71.2*)</td>
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<tr>
<td>PAF-AH activity§</td>
<td>2.67±1.01</td>
<td>0.79±0.29</td>
<td>0.85±0.18</td>
<td>2.13±0.51</td>
<td>3.62±0.79</td>
</tr>
<tr>
<td>HALP</td>
<td>1.28±0.57†</td>
<td>0.80±0.35</td>
<td>1.05±0.33</td>
<td>2.04±0.71</td>
<td>3.24±1.03</td>
</tr>
<tr>
<td>LCAT activity¶</td>
<td>6.34±3.45</td>
<td>6.82±3.38</td>
<td>7.15±2.85</td>
<td>8.52±4.14</td>
<td>24.41±12.69</td>
</tr>
<tr>
<td>ApoA-I</td>
<td>11.17±5.66</td>
<td>11.17±5.32</td>
<td>9.61±4.40</td>
<td>11.87±4.16</td>
<td>13.93±3.10*</td>
</tr>
<tr>
<td>HALP</td>
<td>3.81±1.31</td>
<td>4.56±0.82</td>
<td>3.55±1.09</td>
<td>2.88±1.33</td>
<td>2.64±1.33</td>
</tr>
<tr>
<td>ApoA-II</td>
<td>4.85±0.95</td>
<td>4.78±1.11</td>
<td>4.30±1.92</td>
<td>3.72±0.89</td>
<td>2.95±0.41</td>
</tr>
</tbody>
</table>

Data are shown for 13 normolipidemic controls and nine HALP subjects. Apolipoproteins, PAF-AH, and LCAT activities were measured in a subset of 8 controls and 8 HALP subjects. Numbers in parentheses denote % chemical composition (wt/wt).

*P<0.05, †P<0.01, ‡P<0.001 vs corresponding control value.

§nmol/min per mg protein.

†Percent hydrolysed substrate.

### Antioxidative Action of HDL Subfractions During LDL Oxidation

When HDL subfractions (HDL3a, 3b, or 3c) from control subjects were added to reference LDL (isolated from one control subject and used throughout the study) directly before addition of AAPH, LDL oxidation was significantly delayed (Figure I, available online at http://atvb.ahajournals.org). In contrast, only the HDL3c subfraction from HALP subjects was able to significantly delay oxidation of reference LDL relative to that of LDL oxidized alone (Figure II, available online at http://atvb.ahajournals.org).
delaying LDL oxidation in the propagation phase than their counterparts from normolipidemic subjects; the "specific antioxidative activity" of HDL2a, 3a, 3b, and 3c was therefore diminished in HALP. Differences in the "specific antioxidative activity" between HDL subfractions from normolipidemic and HALP subjects were equally pronounced when calculated on a per particle basis rather than on the basis of equal total mass. For example, reduction in the oxidation rate in the propagation phase calculated per 1 μmol/L of HDL3b and 3c, respectively, equalled 87% and 113% in control subjects versus 39% and 89% in HALP subjects; antioxidative actions of light HDL2 subfractions were less pronounced.

Oxidative protection of LDL by HDL subfractions was most pronounced at late stages of oxidation (Figure 2); no significant inhibition of LDL oxidation by any HDL subfraction was observed in the lag phase (data not shown). The apparent increase in maximal diene formation observed in the presence of HDL subfractions was caused by the oxidation of HDL subfractions themselves, because subtraction of oxidation time courses recorded for HDL subfractions in the absence of LDL (see later) from those for LDL+HDL mixtures revealed no increase in maximal diene formation (data not shown).

To confirm that the distinct antioxidative properties of HDL subfractions were unrelated to the origin of the LDL preparation used, we assessed the capacity of HDL subfractions to protect autologous LDL, rather than reference LDL, from oxidative stress. HDL subfractions isolated from three selected HALP subjects were significantly less efficient, regarding their specific antioxidative activity, than their counterparts from control subjects (n=3) in delaying oxidation of autologous LDL isolated from the same individuals (data not shown). In addition, significant correlations between oxidation parameters measured for the same HDL subfractions in the presence of autologous and of reference LDL were found (eg, r=0.72, P<0.01, r=0.47, P<0.01, and r=0.5, P<0.05 versus control subjects, REM and TBARS were measured in a subset of 7 controls and 7 HALP subjects).

Differences in HDL-mediated protection of LDL from oxidation detected by measurement of conjugated dienes between HALP and control groups were confirmed by measurement of relative electrophoretic mobility (REM) of LDL. Again, dense HDL3a, 3b, and 3c subfractions from HALP subjects were less efficient in delaying LDL oxidation than those from controls (Figure 3A). Qualitatively similar results were obtained when accumulation of thiobarbituric acid-reactive substances (TBARS) was measured (Figure 3B).
HDL subfractions isolated from HALP individuals and from control subjects were also distinct in their total antioxidative activity (thereby reflecting plasma levels) to oxidation of reference LDL. All 5 HDL subfractions from controls and from HALP subjects significantly decreased LDL oxidation rate in the propagation phase (Figure 2B). Five HDL subfractions from controls and 2 (HDL2a and 3a) from the HALP group significantly prolonged the propagation phase (Figure IIB). As a result, HDL2a, 3a, and 3b subfractions isolated from HALP subjects revealed significantly lower “total antioxidative activity” as compared with their counterparts in controls. Note that the “total antioxidative activity” of HDL subfractions tended to be higher than their “specific activity” in the HALP group, because of higher levels of HDL—and greater particle numbers—present in the assay used to measure “total activity.” Therefore, differences between HALP subjects and controls in “total antioxidative activity” were considerably less pronounced than those detected in “specific antioxidative activity” (Figure 2A and 2B), ie, low HDL “specific activity” in the HALP group was partly, but not significantly correlated (eg, r = 0.42, P < 0.001, for the oxidation rate in the propagation phase and r = 0.36, P < 0.001, for the duration of the propagation phase).

**Oxidative Resistance of HDL Subfractions**

When HDL subfractions from either HALP or normolipidemic subjects were subjected to AAPH-induced oxidation in the absence of LDL, their oxidative resistance decreased in the order HDL3c > HDL3b > HDL3a > HDL2a = HDL2b, thereby mirroring their protective activity during LDL oxidation (data not shown). HDL subfractions from HALP subjects were similarly susceptible to AAPH-induced oxidation as compared with their corresponding counterparts from controls (data not shown).

**Protein Components of HDL Possessing Antioxidative Activity**

In both HALP and control subjects, PON1 activity with phenyl acetate as substrate decreased in the order HDL3c > HDL3b > HDL3a > HDL2a = HDL2b (Figure 4), consistent with data reported previously.16 PON1 activity in each HDL subfraction was significantly lower (up to –84%) in the HALP group than that in control subjects. Consistent with these data, PON1 activity was significantly lower in total serum LDL (1.85 ± 0.60 versus 3.34 ± 1.17 μmol/min/mg apoA-I, P = 0.03) and tended to be lower in serum of HDL subfractions (4.56 ± 3.95 versus 9.19 ± 4.61 μmol/min per mg apoA-I, P = 0.09) as compared with controls (n = 6 in each group).

Ca(II) is a key factor in PON1 activity;8 equally, EDTA is known to partially deactivate PON1. The PON1 activity of HDL subfractions isolated from serum was therefore compared with that of HDL subfractions isolated from EDTA plasma and found to be significantly higher (up to 56-fold) in serum (Table I, available online at http://atvb.ahajournals.org). Accordingly, HDL subfractions isolated from serum were slightly more potent in protecting LDL from oxidation than their counterparts isolated from EDTA plasma. Addition of CaCl2 to the LDL + HDL mixture to the same concentration as that used in the assay for PON1 activity (1 mM/L) did not influence the oxidation time course observed in the presence of HDL subfractions isolated from EDTA plasma (data not shown). Protection of LDL mediated by HDL subfractions from serum of both HALP and control subjects decreased in the same order as that observed for HDL subfractions isolated from EDTA plasma, ie, HDL3c > HDL3b > HDL3a > HDL2a = HDL2b. Consistent with data obtained for EDTA plasma, PON1 activity of HDL subfractions isolated from serum was lower in the HALP group than in control subjects (Table I). PON1 activities of HDL subfractions isolated from serum and from EDTA plasma were strongly correlated (r = 0.88, n = 30, P < 0.0001, for 3 normolipidemic controls and 3 HALP subjects).

Activities of 2 other HDL-associated enzymes with antioxidative properties were decreased in HALP. PAF-AH activity was significantly lower in the light HDL2b subfraction and LCAT activity was significantly lower in the dense HDL3c subfraction from HALP subjects as compared with controls (Table 2).

Absolute concentrations of apoA-I and apoA-II in HDL subfractions were consistently higher in HALP subjects as compared with controls (data not shown). To express contents of apoA-I and apoA-II on a per particle basis, the molarity data for apoA-I and apoA-II were corrected for minor differences in the M, values of corresponding HDL subfractions between the 2 groups (see online supplement). Contents of apoA-I and apoA-II expressed on a per particle basis did not reveal significant differences between groups (Table 2), thereby indicating that the unit HDL particle content of apoA-I and apoA-II was similar in corresponding HDL subfractions from controls and HALP subjects.

**Correlations**

When the “specific antioxidative activity” of HDL subfractions (expressed as the oxidation rate of LDL as well as phase duration in the presence of each HDL subfraction) was correlated with HDL levels and activities of antioxidative proteins, PON1, PAF-AH, and LCAT activities were signifi-
icantly negatively correlated with the oxidation rate in the propagation phase and maximal amount of dienes but positively correlated with the duration of both the propagation phase and the lag phase (Table II and Figure III, available online at http://atvb.ahajournals.org). Protein content (as % of total mass) in HDL subfractions was significantly negatively correlated with the oxidation rates and positively correlated with the phase durations, whereas percent CE, percent TG, and percent PL contents showed opposite correlations (data not shown). Plasma levels of 8-isoprostanes were negatively correlated with the duration of LDL propagation phase in the presence of HDL3a ($r = -0.47, P = 0.03$) and 3c ($r = -0.48, P = 0.02$) subfractions and with percent PL content in HDL3a ($r = -0.45, P = 0.04$), 3b ($r = -0.59, P = 0.005$), and 3c ($r = -0.49, P = 0.02$) subfractions. Plasma levels of 8-isoprostanes were positively correlated with the oxidation rate in the propagation phase in the presence of the HDL3a subfraction ($r = 0.46, P = 0.04$), with TBARS accumulation in the presence of HDL2b ($r = 0.58, P = 0.04$), 3a ($r = 0.62, P = 0.02$), 3b ($r = 0.77, P = 0.002$), and 3c ($r = 0.75, P = 0.003$) subfractions, with percent TG content in HDL3a ($r = 0.50, P = 0.02$) and 3b ($r = 0.53, P = 0.01$) subfractions, with percent TP content in HDL3b ($r = 0.56, P = 0.008$) and 3c ($r = 0.51, P = 0.02$) subfractions as well as with LDL-C ($r = 0.46, P = 0.03$). By contrast, no correlation was found between plasma 8-isoprostanes and any LDL oxidation parameter measured in the absence of HDL subfractions (data not shown). Similarly, no significant correlation was found between 8-isoprostanes, PON1, PAF-AH, and LCAT activities and “total antioxidative activity” of HDL subfractions, or between the antioxidative and enzymatic activities of HDL subfractions and apoA-I level, apoA-II level, or age (data not shown).

**Discussion**

Our present studies reveal that small dense HDL3a, 3b, and 3c subfractions, together with the CE-rich HDL2a subfraction, exhibit impaired antioxidative activity (up to $-86\%$) in HALP associated with HL deficiency, as compared with their normolipidemic counterparts; moreover, this deficit primarily involves the capacities of these particle subfractions to protect LDL from oxidative stress on a unit mass basis (“specific antioxidative activity”). Indeed, only HDL3c from HALP subjects significantly decreased LDL oxidation rate, in contrast to normolipidemic subjects in which all 3 small, dense HDL3 subspecies (HDL3a, 3b, 3c) possess potent capacity to protect LDL from oxidation. Importantly, plasma levels of 8-isoprostanes, markers of oxidative stress, tended to be elevated (1.5-fold) in HALP and were positively correlated with the specific antioxidative activity of HDL subfractions. These data suggest the existence of a functional relationship between systemic oxidative stress, the oxidative susceptibility of LDL in the presence of HDL subfractions, and the intrinsic antioxidative capacity of HDL subfractions themselves.

Of the enzymes transported by HDL that can delay or prevent formation of oxidized lipids, PON1 activity was deficient in all HDL subfractions, PAF-AH in HDL2b, and LCAT in the HDL3c subfraction in HALP. Importantly, activities of PON1, PAF-AH, and LCAT significantly correlated with specific antioxidative activity of HDL subfractions. The correlation coefficients (Table II) allowed us to estimate that PON1 accounted for $\approx 25\%$ of the variation in specific antioxidative activity, whereas PAF-AH and LCAT accounted for $\approx 12\%$ each. Therefore, PON1, PAF-AH, and LCAT are potentially key factors in the deficient intrinsic antioxidative activity of HALP HDL subfractions, accounting together for $\approx 50\%$ of its variation. The possibility that these enzymes may act synergistically to protect LDL cannot, however, be excluded. The key role of enzymes other than PON1 is consistent with the observation 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 as compared with that in PON1 activity (Table I). Indeed, PON1-independent inhibition of LDL oxidation by HDL has been previously demonstrated.\textsuperscript{17}

The physiological significance of HDL-associated enzymes with antioxidative activity is emphasized by the intimate association between low plasma PON activity, which is exclusively localized to HDL,\textsuperscript{18} and CV disease.\textsuperscript{19} In addition, overexpression of PON1,\textsuperscript{20} PAF-AH,\textsuperscript{21} and LCAT\textsuperscript{22} decreases oxidative stress and reduces atherosclerosis in mice. Subnormal PON1 activity in HALP HDL particles may theoretically reflect decreased PON1 mass and prevalence of a genetic background associated with reduced PON1 activity, such as the 192 Arg/Gln polymorphism.\textsuperscript{8} Equally, PON1 might be inhibited or even degraded by products of lipid oxidation\textsuperscript{23} during the prolonged plasma residence time of LDL when HL activity is low.\textsuperscript{24} By contrast, decreased LCAT and PAF-AH activities in distinct HDL subfractions in HALP appear to be related to their redistribution between plasma lipoproteins, consistent with earlier data.\textsuperscript{25,26}

Specific antioxidative activity, PON, PAF-AH, and LCAT activities, and oxidative resistance of HDL subfractions were highest in the densest HDL3c subfraction and tended to decrease with decrement in HDL density. HDL-associated enzymes therefore may not only protect LDL but also protect PON/PAF-AH/LCAT-transporting HDL particles themselves against oxidation. It is thus likely that HDL, and especially small dense HDL3 subspecies, are the primary site of the hydrolysis of oxidized lipids by HDL-associated enzymes. HDL can remove lipid hydroperoxides from LDL;\textsuperscript{27} moreover, HDL is a major carrier of lipid hydroperoxides in human plasma.\textsuperscript{28} The antioxidative activities of HDL subfractions that are associated with inactivation of lipid hydroperoxides may account for their observed protective effects on LDL oxidation. This conclusion is supported by the observation that antioxidative effects of HDL subfractions were most pronounced at later stages of LDL oxidation when high levels of lipid hydroperoxides had accumulated; in addition, HDL subfractions were inactive in protecting LDL at early stages of oxidation when levels of lipid hydroperoxides are low.\textsuperscript{29,30} Thus, preferential transfer of oxidized lipids, primarily lipid hydroperoxides, from LDL to small dense HDL during their physical contact on collision with subsequent hydroperoxide cleavage by PON and/or PAF-AH and/or LCAT may be
responsible for the differences observed in the antioxidative properties of HDL subfractions.

This hypothesis is consistent with the higher capacity of dense HDL3 to accept polar lipids than light HDL2, an effect that can be related to protein-independent partition of oxidized lipids from LDL into the surface phospholipids/free cholesterol monolayer of HDL. Indeed, phospholipids exhibit a low degree of order and are loosely packed in small HDL particles. HDL from HALP patients are impaired in their capacity to act as acceptors of cellular free cholesterol; impaired HDL function in HALP may derive from abnormalities in HDL metabolism, and notably from attenuated HDL TG lipolysis by HL. Consistent with this observation, dense HDL subfractions from HALP subjects were enriched in TG and total protein and depleted of CE and FC. The diminished capacity of TG-rich HDL particles to acquire cellular cholesterol has been recently demonstrated, suggesting that they may possess diminished capacity to acquire oxidized CE and FC from LDL. The pro-oxidant role of TG enrichment in HDL subfractions is entirely consistent with positive correlations between plasma levels of 8-isoprostanes and percent TG content of small dense HDL3a and 3b subfractions. These data indicate that HDL-associated enzymes are not the sole factor responsible for the deficient antioxidative activity of HDL subfractions in HALP: structural and physicochemical properties of HDL particles that are relevant for transfer of lipids to HDL may be equally significant, accounting for the remaining 50% of variation in antioxidative activity.

Finally, it is important to emphasize that despite the deficit in the specific antioxidative activity of HDL3 subfractions, the elevated concentrations, and thus increment in particle numbers, in all HDL subfractions (except HDL3a) in HALP resulted in decrements in total antioxidative activity relative to controls that were less pronounced than those in specific activity. Impaired intrinsic antioxidative activities of HDL subfractions in HALP were therefore partially compensated by elevated particle number. In conclusion, our present data add a new dimension to the attenuated biological function of HDL particles in HALP, implying that HDL subfractions possess diminished intrinsic antioxidative capacity in HALP subjects. Such impaired capacity to protect LDL from oxidation in HALP can be partially compensated by elevated numbers of HDL3 particles. Small dense HDL3 possess greater antioxidative activity than large light HDL2; plasma levels of dense HDL3 were a strong predictor of cardiovascular risk in the VA-HIT study. Elevated numbers of dense HDL particles, as occurs in HALP, can therefore contribute to normalization of LDL protection from oxidation in vivo, even if HALP HDL particles have lower intrinsic antioxidative activity as compared with those from normolipidemic subjects. These data are consistent with the observation of Baldassarre et al that HALP is not associated with accelerated development of atherosclerosis. Nonetheless, given the multiple metabolic origins of HALP, the possibility that some forms of HALP may be associated with HDL particle phenotypes characterized by marked biological dysfunction, and concomitantly with accelerated atherosclerosis, cannot be excluded.

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References

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