Decreased Protection by HDL From Poorly Controlled Type 2 Diabetic Subjects Against LDL Oxidation May Be Due to the Abnormal Composition of HDL

Maya S. Gowri, Deneys R. Van der Westhuyzen, Susan R. Bridges, James W. Anderson

Abstract—High plasma triglyceride concentrations in diabetic subjects increase their risk for developing coronary heart disease. Numerous studies have shown that the high density lipoprotein (HDL) composition is abnormal in type 2 diabetic subjects. One study has shown that HDL (lipoprotein A-I) isolated from subjects with non–insulin-dependent diabetes mellitus exhibits a decreased capacity to induce cholesterol efflux. The current study examined the effect of HDL₂ and HDL₁ subfractions from poorly controlled type 2 diabetic and control subjects on THP-1 macrophage-mediated low density lipoprotein (LDL) oxidation. The composition and protective effects of HDL₂, but not of HDL₁, differed significantly between control and diabetic subjects. HDL₂ from diabetics were triglyceride enriched and cholesterol depleted compared with those from controls. Control HDL₂ inhibited LDL oxidation, as assessed by lipid peroxides and electrophoretic mobility, significantly (P<0.05) more than did diabetic HDL₂ in both the fasting and postprandial state. In addition, HDL₁ from diabetics did not protect against apolipoprotein B-100 fragmentation in LDL. Cross-linking in apolipoprotein A-I, oxidized in the presence of LDL, was extensive in HDL₁ from diabetics compared with that from controls. Serum triglyceride concentrations were negatively correlated with protection by HDL₂ (r = −0.673, P<0.05) in diabetic but not in control subjects. HDL₂-associated platelet-activating factor acetylhydrolase activity was positively correlated with protection by HDL₂ in control (r = 0.872, P<0.002) but not in diabetic subjects. In conclusion, compositional alterations in HDL₂ from poorly controlled type 2 diabetic subjects may reduce its antiatherogenic properties. (Arterioscler Thromb Vasc Biol. 1999;19:2226-2233.)

Key Words: diabetes ■ hypertriglyceridemia ■ atherosclerosis ■ oxidized LDL ■ HDL

Diabetic individuals have a significantly higher risk for atherosclerosis than do nondiabetic individuals. Persons with type 2 diabetes have significantly increased serum triglyceride (TG) and decreased HDL cholesterol concentrations. High plasma TG concentrations among diabetic subjects appear to be an independent predictive factor of mortality from coronary artery disease (CAD). Over the last decade, the abnormal HDL composition in type 2 diabetes has been well studied and is characterized by TG enrichment, cholesterol depletion, and decreased apo A-I concentrations. In subjects with type 2 diabetes, total serum TG concentrations were found to be inversely correlated with HDL cholesterol and positively correlated with HDL-TG concentrations. Such abnormalities in HDL might contribute to the well-established high risk for atherosclerosis in diabetes.

Type 2 diabetic subjects also have a decreased lipoprotein lipase activity that might cause decreased clearance of TG-rich lipoproteins, leading to accumulation of these lipoproteins in the plasma. Elevated levels of TG-rich lipoproteins may increase the cholesteryl ester transfer protein (CETP) activity that may in turn cause TG enrichment of HDL at the expense of core cholesteryl esters. Increased CETP activity has been reported in type 2 diabetes.

In type 2 diabetic subjects, enhanced LDL oxidation occurs in vivo, since high titers of autoantibodies to oxidatively modified LDL are present in the plasma. Oxidative modification of LDL appears central to foam cell formation, the earliest lesion of atherosclerosis. HDL may protect from cardiovascular disease by a number of mechanisms, one of which is by inhibiting LDL oxidation in the subendothelial space. HDL prevents the formation of minimally modified LDL in cocultures of artery wall cells by facilitating hydrolysis of active, oxidized phospholipids to lysophospholipids, thereby destroying the biologically active lipids in minimally modified LDL. Platelet-activating factor acetylhydrolase (PAF-AH) and paraoxonase (PON) are 2 enzymes associated with HDL that are known to protect LDL from oxidation.

Cavallero and colleagues showed that LpA-I (HDL containing only apo A-I) isolated from subjects with non–insulin-dependent diabetes mellitus (NIDDM) exhibited a decreased...
capacity to induce cholesterol efflux in both the fasting and postprandial state and hence, had a decreased ability to accomplish their antiatherogenic role. The ability of compositionally abnormal HDL particles from type 2 diabetic subjects to perform their antiatherogenic function of protecting LDL against oxidation has not been critically examined. In our study, we examined the protection exhibited by HDL2 and HDL3 from subjects with poorly controlled type 2 diabetes against macrophage-mediated oxidation of LDL compared with nondiabetic healthy controls. We found that the HDL2 subfraction from diabetic subjects was abnormal in composition, as previously reported, and also exhibited decreased protection against LDL oxidation when compared with controls.

Methods

Materials

Total cholesterol and free cholesterol kits (COD-PAP) and the total phospholipid kit (phospholipids B) were purchased from Wako Chemicals. 1-Palmitoyl-2-[U-14C]-nitrobenzoxadiazoloylaminocaproyl phosphatidylcholine (C18:1, NBD PC) was purchased from Avanti Polar Lipids. Rabbit anti-human apo A-I and goat anti-human apo A-II were purchased from Calbiochem. Electrophoresis reagents were purchased from Bio-Rad. FCS, phorbol 12-myristate 13-acetate, peroxidase-conjugated anti-rabbit IgG and anti-goat IgG, paraoxon, glucose enzymatic kit (glucose oxidase), and the TG enzymatic kit (INT reagent) were purchased from Sigma Chemical Co.

Study Protocol

Subjects

Ten poorly controlled type 2 diabetic men who had serum cholesterol concentrations ≤240 mg/dL and serum TG concentrations ≤300 mg/dL were recruited from the Veterans Administration outpatient clinic, and 10 matched male control subjects were also recruited from the Veterans Administration Medical Center and University of Kentucky Medical Center staff. Nine subjects were white and 1 was African-American in each group. Medications taken by the diabetic subjects at the time of the study were as follows: sulfonylurea (all 10 subjects), biguanide (3 subjects), nitrates (4 subjects), loop diuretics (3 subjects), β-blockers (2 subjects), α-blockers (1 subject), and angiotensin-converting enzyme inhibitors (5 subjects). Two of the diabetic subjects had no diabetic complications, 1 subject had cerebral artery occlusion, 4 subjects had CAD, and 3 subjects had hypertension. Except for 1 diabetic subject who smoked <½ pack of cigarettes per day, all other subjects were nonsmokers. None of the subjects took any antioxidant supplements or probucol.

Test Meal

We gave our subjects a fat load to see whether there was any difference between fasting and postprandial HDLs in their antiatherogenic role of protecting LDL against oxidation. Each individual arrived at 8 AM after an overnight fast. Fifty milliliters of blood was collected for lipid analysis and lipoprotein separation from the HDL2 subfraction from diabetic subjects was abnormal in composition, as previously reported, and also exhibited decreased protection against LDL oxidation when compared with controls.

Serum Measurements

Serum glucose concentrations were determined by using an enzymatic kit (glucose oxidase). Glycosylated hemoglobin (HbA1c) values were determined with an ion-exchange mini-column chromatographic procedure (Bio-Rad Laboratories); normal values are 4.2% to 6.4%. Total cholesterol was measured with an enzymatic kit from Wako Chemicals (COD-PAP) and total TGs by an enzymatic kit from Sigma (INT reagent). HDL cholesterol was quantitated after precipitation of apo B-containing lipoproteins with magnesium/phosphotungstic acid. HDL2 cholesterol was quantitated after a dual-precipitation method with combinations of magnesium/phosphotungstic acid and dextran sulfate. HDL3 cholesterol was calculated as the difference between total HDL cholesterol and HDL2 cholesterol.20 LDL cholesterol was calculated by the Friedewald formula.21

LDL and HDL Subfraction Preparation

HDLS from the study subjects and LDL from a healthy donor were isolated by sequential ultracentrifugation.22 LDL was isolated from the same healthy donor for every oxidation experiment. In brief, the density of serum was adjusted to 1.09 g/mL and centrifuged for 11 hours, 15 minutes at 50 000 rpm in a VTi50 rotor at 4°C. To isolate LDL, the density of the VLDL-LDL fraction was adjusted to 1.3 g/mL and gradient ultracentrifugation was carried out at 50 000 rpm for 2.5 hours at 4°C. To isolate HDL, the density of the bottom fraction was adjusted to 1.25 g/mL with solid KBr and centrifuged in a VTi50 rotor at 50 000 rpm for 20 hours at 4°C. Gradient ultracentrifugation was carried out at 4°C for 15 hours at 50 000 rpm for fractionation of HDL.23 LDL, HDL2, and HDL3 were dialyzed extensively overnight against 3 changes of PBS (pH 7.4), sterilized through a 0.45-μm-filter unit (Millipore), and stored at 4°C under N2. Aliquots of HDL subfractions were frozen at −70°C for analysis of HDL phospholipid, cholesterol ester, and free cholesterol concentrations.

HDL Subfraction Composition

Total cholesterol and free cholesterol concentrations were measured by enzymatic methods with the use of commercially available kits (COD-PAP), and cholesteryl esters were calculated as the difference between total and free cholesterol. TG concentrations were determined by using an enzymatic kit (INT reagent). Total phospholipids were measured by an enzymatic method that uses phospholipase D–choline oxidase and peroxidase (phospholipids B).

Total protein contents of the HDL subfractions were measured by the Lowry method.24 Apo A-I and A-II in HDL subfractions were quantitated by SDS–polyacrylamide gradient gel electrophoresis (PAGE) followed by Western blotting.25 In brief, electrophoresis was carried out on a 5% to 20% acrylamide/SDS gel and a 25 mmol/L Tris, 192 mmol/L glycine electrophoresis buffer (pH 8.4) containing 0.1% SDS. The proteins were transferred onto a 0.2-μm nitrocellulose membrane by using a 25 mmol/L Tris, 192 mmol/L glycine transfer buffer (pH 8.4) containing 15% (vol/vol) methanol.23 The blots were blocked for 1 hour with 5% (wt/vol) nonfat dry milk in PBS (pH 7.4). The blots were then exposed to the primary and secondary antibodies for 1 hour each. The primary antibody for apo A-I was rabbit anti-human apo A-I, and for apo A-II, goat anti-human apo A-II. The secondary antibody was a peroxidase-conjugated anti-rabbit IgG and anti-goat IgG, respectively. The antibodies were then detected with the enhanced chemiluminescence (ECL) detection reagents (Amersham), and the blots were exposed to x-ray film. The bands were scanned with a densitometer.

HDL Subfraction Enzyme Activities

PON and PAF-AH activities were measured in fresh HDL samples within 2 days of isolation. HDL PON activity was measured with a 1.0 mmol/L paraoxon substrate in 50 mmol/L Tris-HCl buffer (pH 7.4) containing 1.0 mmol/L CaCl2 in a total volume of 800 μL.25 One hundred nanograms of HDL2 or HDL3 was added to start the reaction, and the increase in absorbance at 412 nm was recorded continuously for 10 minutes. The amount of 4-nitrophenol formed was calculated from the molar extinction coefficient of 12 800 M−1 cm−1. The blank contained substrate without HDL. One unit of PON activity is defined as 1 nmol of 4-nitrophenol formed per minute under the above assay conditions.

PAF-AH activity was measured by using a fluorescent substrate, CNBD PC, by a modification of the method of Steinbrecher and
TABLE 1. Baseline Characteristics and Serum Lipid Profiles of Control and Diabetic Subjects*

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Diabetics</th>
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<tr>
<td></td>
<td>Fasting (CF)</td>
<td>Postprandial (CF)</td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Age, y</td>
<td>48.7±2.6</td>
<td>. . .</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.1±1.2</td>
<td>. . .</td>
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<tr>
<td>HbA₁c, %</td>
<td>5.3±0.16</td>
<td>. . .</td>
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<tr>
<td>Fasting glucose, mg/dL</td>
<td>86±2</td>
<td>. . .</td>
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<tr>
<td>Serum lipids, mg/dL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG</td>
<td>120.6±24.3</td>
<td>197.7±38.6§</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>194.3±9.4</td>
<td>201±14</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>132.6±9.7</td>
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</tr>
<tr>
<td>HDL cholesterol</td>
<td>37.6±2.2</td>
<td>36.4±1.7</td>
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</tbody>
</table>

*Values are mean±SEM.
mg/dL: P vs control: †<0.002; ‡<0.0001; §<0.02; CF vs CF; ||<0.002, DF vs DP; ¶<0.01, CF vs DF; and #<0.005, CP vs DP.

Pritchard. 26 Four micromoles of the substrate C6NBD PC was incubated with 10 µg of HDL₂ or HDL₃ protein in 1 mL of PBS (pH 7.4) at 37°C for 90 minutes. The reaction was terminated by vortexing with 1 mL of methanol and 1 mL of chloroform for ~1 minute, and the mixture was then centrifuged at 2500g for 10 minutes. The fluorescence of the aqueous phase was measured at 470 nm excitation and 533 nm emission in a fluorospectrometer. The mass of fluorescent substrate hydrolyzed was calculated by using a standard curve generated by using C6 NBD fatty acid diluted in methanol.

**Cell Culture**

Dr Mark Kindy (Department of Biochemistry) provided the THP-1 monocyctic cells. THP-1 cells were grown in suspension in RPMI-1640 medium containing 10% FCS, 100 U/mL streptomycin, and 100 U/mL penicillin. Cells were maintained at a density of 10⁶/mL by pelleting the cells twice weekly. Forty-eight hours before cell-mediated oxidation studies, 2×10⁵ cells were seeded per well in a 12-well tissue-culture plate and induced to differentiate into macrophages by the addition of 10⁻¹⁰ mol/L phorbol 12-myristate 13-acetate. 27

**Macrophage-Mediated Oxidation of Lipoproteins**

The differentiated macrophages were washed 3 times with PBS before incubation with lipoproteins. Macrophage cells were incubated with 100 µg of LDL protein, with or without 100 µg of HDL₂ or HDL₃ protein, in 1 mL of serum-free Ham’s F-10 medium containing gentamicin (50 µg/mL) at 37°C in a 5% CO₂ incubator for 24 hours. 28 Additionally, 100 µg of HDL₂ or HDL₃ protein was incubated separately with the cells. Oxidation was arrested after 24 hours by the addition of 200 µmol/L EDTA and 20 µmol/L BHT, and the medium was spun at 1200 rpm for 5 minutes at 4°C. Aliquots of the medium were used for determining lipid peroxides, electrophoretic mobility, apo B-100 fragmentation in LDL, and apo A-I cross-linking in HDL subfractions.

**Assessment of Oxidative Damage**

Lipid peroxides in the oxidized lipoproteins were determined by mixing 0.1 mL of the medium containing the modified lipoproteins with 1.0 mL of iodine-color reagent, incubating the mixture for 30 minutes at room temperature in the dark, and reading the absorbance at 365 nm. 29 The concentration of lipid peroxides was calculated using a molar absorption coefficient of 2.46×10⁻⁶ M⁻¹ cm⁻¹. Lipid peroxides present in HDL samples oxidized separately were used as a correction factor for the lipid peroxides formed in the LDL samples containing HDL during oxidation.

Electrophoretic mobility was determined using 1.0% agarose gels. Oxidized LDL was subjected to electrophoresis in 0.05 mol/L barbital buffer (pH 8.6) at 90 V for 45 minutes. 30 The gel was dried and stained in Sudan black B. Electrophoretic mobility was measured as the distance from the origin to the median point of the lipoprotein peak distribution. The increase in the electrophoretic mobility of each oxidized LDL sample was calculated as a relative ratio to that of native LDL and expressed as relative electrophoretic mobility.

**Apo B-100 Fragmentation**

Fragmentation and aggregation of apo B-100 in the medium containing 1 µg of LDL, oxidized in the absence or presence of HDL₂ or HDL₃, was determined using 5% to 20% SDS-polyacrylamide gradient gels under reducing conditions as detailed above. The gels were fixed for 1 hour and stained with AgNO₃ (Bio-Rad).

**Apo A-I Cross-Linking**

Apolipoprotein A-I cross-linking in macrophage-modified HDL₂ or HDL₃ was determined by subjecting the medium containing 1 µg of HDL protein to SDS-PAGE under reducing conditions, 23 followed by Western blotting with an antibody against apo A-I.

**Data Analysis**

Statistical analysis for comparison between groups was performed using a 2-way ANOVA followed by post hoc testing with Tukey’s multiple comparisons test by using SYSTAT statistics software. 31 Results are expressed as mean±SEM. Correlation coefficients were determined by simple linear regression analysis. Values of P<0.05 were considered to be statistically significant.

**Results**

**Subject Characteristics**

Table 1 provides the clinical characteristics and also the serum lipid profiles of the diabetic and control subjects in both the fasting and postprandial states. The diabetic subjects were poorly controlled and had high HbA₁c and fasting serum glucose concentrations. Although body mass index did not differ between the diabetic and control subjects, the diabetic subjects were significantly older (P<0.002). Diabetic and control subjects had similar serum total and LDL cholesterol concentrations. Diabetic subjects had slightly but not significantly higher serum TG concentrations, compared with controls, in both the fasting and postprandial states. However, HDL cholesterol and HDL₃ cholesterol concentrations were significantly decreased in the diabetics in both the fasting (P<0.01) and postprandial (P<0.005) states. Serum TG
TABLE 2. Fasting and Postprandial HDL2 and HDL3 Compositions in Control and Diabetic Subjects

<table>
<thead>
<tr>
<th></th>
<th>HDL2</th>
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<th>HDL3</th>
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<tr>
<td></td>
<td>Fasting (CF)</td>
<td>Postprandial (CP)</td>
<td>Fasting (DF)</td>
</tr>
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<td>n</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Protein, %</td>
<td>42.6±2.2</td>
<td>43.7±2.5</td>
<td>44.6±2.2</td>
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<tr>
<td>Phospholipid, %</td>
<td>29.3±3.2</td>
<td>29.5±3.1</td>
<td>28.3±3.3</td>
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<tr>
<td>Free cholesterol, %</td>
<td>3.77±0.22</td>
<td>4.41±0.21</td>
<td>2.96±0.15</td>
</tr>
<tr>
<td>Cholesterol ester, %</td>
<td>19.0±0.8</td>
<td>16.0±0.6</td>
<td>14.9±1.1</td>
</tr>
<tr>
<td>Triglyceride, %</td>
<td>5.30±0.66</td>
<td>6.40±0.69</td>
<td>9.33±0.70</td>
</tr>
<tr>
<td>Apo A-I#</td>
<td>0.489±0.02</td>
<td>0.497±0.02</td>
<td>0.422±0.01</td>
</tr>
<tr>
<td>PON activity**</td>
<td>1.35±0.39</td>
<td>1.34±0.32</td>
<td>1.1±0.30</td>
</tr>
<tr>
<td>PAF-AH activity**</td>
<td>437±85</td>
<td>350±68</td>
<td>569±86</td>
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</table>

*Values are mean±SEM.  †P<0.01 CF vs CP; ‡ P<0.05 DF vs DP; § P<0.05 CF vs DF; |P<0.05 CF vs DF; and ¶ P<0.05 CP vs DP.
#Values are in mg/mg HDL protein.
**Values are in nmol · min⁻¹ · mg⁻¹ apo Al.

Composition of HDL2 and HDL3

Table 2 illustrates composition of HDL2 and HDL3 from control and diabetic subjects. Two-way ANOVA indicated that diabetic subjects exhibited significant compositional abnormalities in their HDL2 fraction in both fasting and postprandial states compared with controls. Diabetics had (1) a decrease in HDL2 free cholesterol concentrations and (2) an increase in HDL2-TG concentrations in both fasting (P<0.05) and postprandial (P<0.05) states compared with controls. Apo A-I concentrations were significantly decreased in HDL2 from diabetic subjects compared with controls in both fasting (P<0.05) and postprandial (P<0.05) states. There was a slight but nonsignificant increase in the HDL3 apo A-II concentrations in diabetics compared with control subjects. PAF-AH and PON activities in HDL2 were not significantly different between control and diabetic subjects.

Two-way ANOVA indicated that the only significant differences in HDL3 composition were associated with the high-fat meal and not due to diabetes. We found no difference in the HDL3 composition between the control and diabetic subjects. In both control and diabetic subjects, HDL3 had a significant increase in proteins (P<0.01) and phospholipids (P<0.05) but a significant decrease in cholesteryl esters (P<0.01) postprandially compared with the fasting state. Free cholesterol concentrations in HDL3 were significantly increased in the postprandial state (P<0.05) in control but not in diabetic subjects. There was no significant difference in either apo A-I and A-II concentrations or PON and PAF-AH activities in HDL3 from control and diabetic subjects.

Protection Against Macrophage-Mediated LDL Oxidation

Figure 1 illustrates lipid peroxide accumulation in LDL oxidized in the absence and presence of HDL2 or HDL3 from control and diabetic subjects in both fasting and postprandial states. Lipid peroxides in LDL oxidized alone with the cells was taken as 100%. Lipid peroxides in LDL oxidized in the presence of HDL2 from diabetic subjects were significantly higher, both in the fasting (53.3±3.8% LDL response versus 29.2±8.1%; P=0.049) and postprandial (63.3±8.8% versus 35.3±7.7%; P=0.01) states compared with controls. Lipid peroxide accumulation in LDL oxidized in the presence of HDL3 was not significantly different between control and diabetic subjects. HDL2 was significantly more protective than HDL3 both in fasting (29.2±8.1% versus 63.0±8.4%; P=0.002) and in postprandial (35.3±7.7% versus 57.9±7.7%; P<0.02) states in controls only but not in diabetic subjects. We found a significant increase in the electrophoretic mobility of LDL oxidized in the presence of HDL2 from diabetic compared with control subjects, in both
fasting (1.39 ± 0.05 versus 1.22 ± 0.06; *P* < 0.05) and postprandial (1.52 ± 0.06 versus 1.32 ± 0.07; **P** < 0.05) states (Figure 2). Again, HDL2 was more efficient in reducing the electrophoretic mobility of LDL compared with HDL3, both in fasting (1.22 ± 0.06 versus 1.49 ± 0.07; **P** = 0.001) and postprandial (1.32 ± 0.07 versus 1.53 ± 0.07; *P* = 0.05) states in controls only but not in the diabetic subjects. During oxidation, there was no difference in either the lipid peroxides or the electrophoretic mobility of HDL2 or HDL3 from control and diabetic subjects, in both the fasting and postprandial state (data not shown).

Figure 3 illustrates the fragmentation of apo B-100 in LDL when oxidized in the absence or presence of HDL2 from control and diabetic subjects. HDL2 from control subjects, in both fasting and postprandial states, protected the apo B-100 band in LDL from fragmentation during oxidation to a significant extent. However, the apo B-100 band in LDL oxidized in the presence of HDL2 from diabetic subjects was almost completely fragmented.

Figure 4 illustrates that HDL2 from controls, when oxidized in the presence of LDL, had only minimal apo A-I cross-linking in the fasting state, which was amplified in the postprandial state. However, both fasting and postprandial HDL2 from diabetic subjects, oxidized in the presence of LDL, showed extensive apo A-I cross-linking when compared with control HDL2.

### Linear Regression Analysis
Simple linear regression analysis was performed to assess which components in the control and diabetic HDL2 might be associated with their protective effects against LDL oxidation. Protection by control HDL2 against lipid peroxide accumulation in LDL was strongly and positively correlated with its associated PAF-AH activity both in the fasting (r = 0.872, *P* = 0.002) (Figure 5a) and postprandial (r = 0.818, *P* = 0.007) state (data not shown), whereas diabetic HDL2-associated PAF-AH activity was not correlated with its protection in either the fasting (Figure 5b) or postprandial state (data not shown). We found that protection by control HDL2 against lipid peroxide accumulation in LDL was positively correlated with PAF-AH activity when expressed either per milligram of apo A-I or per milligram of HDL protein. No such correlation existed in the diabetic subjects, even when the PAF-AH activity was expressed either per milligram of apo A-I or per milligram of HDL protein. We did not see any correlation between HDL2-associated PON activity and protection by HDL2 from control or diabetic subjects against lipid peroxide accumulation in LDL. In controls, PAF-AH activity was significantly higher in HDL2 than in HDL1 (437 versus 247 nmol min⁻¹ mg⁻¹ apo A-I, *P* < 0.05), whereas PON activity was significantly higher in HDL1 than in HDL2 (7.66 versus 1.35 nmol min⁻¹ mg⁻¹ apo A-I, *P* < 0.05).
Correlation analysis indicated that protection by HDL₂ from control subjects against lipid peroxide accumulation in LDL was not correlated with serum TG, HDL₂-TG, cholesterol, phospholipid, or apoprotein concentrations. In contrast, protection by HDL₂ from diabetic subjects was inversely correlated with serum TG concentrations in both the fasting \((r = -0.673, P<0.05)\) and postprandial \((r = -0.798, P<0.02)\) states (Figure 6a and 6b). Similarly, protection by HDL₂ from lipid peroxide accumulation in the diabetic subjects in the fasting state was inversely correlated with HDL₂-TG concentration \((r = -0.636, P<0.05)\) (Figure 6c) and positively correlated with HDL₂ free cholesterol concentration \((r = 0.820, P<0.005)\) (Figure 6d). In the diabetic subjects, we did not see any correlation between the level of glycemic control and HDL₂ and HDL₃ composition, PON and PAF-AH activities, or protection against LDL oxidation (data not shown).

**Discussion**

Type 2 diabetic subjects present an increased risk for CAD that is related to their degree of hypertriglyceridemia. Compositional abnormalities in HDL are well characterized in NIDDM. To determine whether abnormal HDL particles in NIDDM are able to maintain antiatherogenic functions, we examined the protective effects of HDL₂ and HDL₃ from poorly controlled type 2 diabetic and nondiabetic control subjects in both fasting and postprandial states against macrophage-mediated LDL oxidation.

Control subjects had a slight increase in phospholipid, free cholesterol, and protein concentrations in both HDL₂ and HDL₃ postprandially compared with the fasting state. Alimentary lipemia resulted in an increase in concentrations of phospholipids and proteins in HDL, particularly in the HDL₂ subfraction. The magnitude of postprandial lipemia determines the proportion of TGs in postprandial HDL₂. In our study, HDL₂ from diabetic subjects were enriched in TGs and depleted of free cholesterol and apo A-I compared with that in controls. Other studies reported similar compositional abnormalities in type 2 diabetes. Similar to our results, Cavallero et al reported a significant postprandial decrease in free cholesterol and cholesteryl ester concentrations and an increase in TG concentrations in HDL₂ from type 2 diabetics. Increased cholesteryl ester transfer rates mediated by CETP.
in type 2 diabetes\textsuperscript{10,11,36} appear important for the TG enrichment and cholesterol depletion seen in HDL\textsubscript{2}. In the diabetic subjects, we found a positive correlation between serum TG concentrations and HDL\textsubscript{2}-TG concentrations (data not shown), as previously reported by Biesbroeck and colleagues.\textsuperscript{9} Postprandially, we found a significant TG enrichment of HDL\textsubscript{2} in diabetic subjects only, although both control and diabetic subjects had significant increases in their serum TG concentrations postprandially compared with the fasting state. Abbott et al\textsuperscript{37} have shown that serum PON activity is lower in type 2 diabetic subjects than in controls. However, we did not see a significant difference in HDL PON or PAF-AH activity between diabetic and control subjects.

Effects of the individual HDL\textsubscript{2} and HDL\textsubscript{3} subfractions against LDL oxidation in relation to their PAF-AH and PON activities are poorly understood. When we examined the protective effects of HDL subfractions against LDL oxidation from control subjects, HDL\textsubscript{2} was significantly more efficient than HDL\textsubscript{3} in inhibiting LDL oxidation in all parameters assayed. Navab and colleagues\textsuperscript{38} have shown that virtually all HDL-mediated inhibition of monocyte transmigration induced by minimally modified LDL can be accounted for by HDL\textsubscript{2}, whereas HDL\textsubscript{3} had no effect. The greater protective effect of HDL\textsubscript{2} seen in our study may be due to a higher PAF-AH activity associated with HDL\textsubscript{2} compared with HDL\textsubscript{3}. In our study, HDL\textsubscript{4} and HDL\textsubscript{3} were not good substrates for macrophage oxidation compared with LDL. This may be due to a decreased lipid content in HDL. Other studies have also shown that unlike LDL, HDL is not susceptible to modification by endothelial cells\textsuperscript{14} or in cocultures of artery wall cells.\textsuperscript{38}

In comparing the protective effects of HDL from control and diabetic subjects against LDL oxidation, no differences between HDL\textsubscript{1} from control and diabetic subjects were observed. On the other hand, HDL\textsubscript{2} fractions from diabetic subjects exhibited decreased protection against LDL oxidation compared with controls in both fasting and postprandial states in most of the parameters assayed. There was increased lipid peroxide accumulation and electrophoretic mobility; also, their apo B-100 was almost completely fragmented when LDL was oxidized in the presence of HDL\textsubscript{2} from diabetic compared with control subjects. In controls, in both fasting and postprandial states, HDL\textsubscript{2} exhibited significantly greater protection compared with HDL\textsubscript{3}, but this difference between HDL\textsubscript{2} and HDL\textsubscript{3} was not seen in diabetic subjects. This suggests that HDL\textsubscript{2} from diabetic subjects may have lost its efficiency to protect LDL from oxidation. Correlation analysis indicated that the increase in serum TG concentrations might mediate these effects by altering the composition of HDL\textsubscript{3}. Decreased free cholesterol and increased TG concentrations in HDL\textsubscript{2} from diabetic subjects may contribute importantly to the decreased protection exhibited by diabetic HDL\textsubscript{2}.

Of importance, TG-enriched HDL\textsubscript{2} from diabetic subjects, though not susceptible to oxidation, exhibited decreased protection against LDL oxidation compared with HDL\textsubscript{2} from controls. To determine whether diabetic HDL\textsubscript{2} was being more structurally altered in the presence of LDL compared with control HDL\textsubscript{2}, we studied the apo A-I cross-linking in HDL\textsubscript{2}. When HDL\textsubscript{2} was oxidized in the presence of LDL, there was more extensive apo A-I cross-linking in HDL\textsubscript{2} from diabetic subjects compared with controls. The greater apo A-I cross-linking seen in HDL\textsubscript{2} from diabetic subjects compared with controls, when oxidized along with LDL, suggests that compositionally altered diabetic HDL\textsubscript{2} may not be as efficient as control HDL\textsubscript{2} in inhibiting lipid peroxide generation in LDL. Therefore, the large amounts of aldehydes formed during LDL oxidation may have modified the apo A-I in diabetic HDL\textsubscript{2}. Aldehydes such as 4-hydroxynonenal and malondialdehyde generated during LDL oxidation can cause apo A-I cross-linking in HDL.\textsuperscript{39}

Two possible mechanisms for the decreased protection exhibited by HDL\textsubscript{2} from diabetic subjects include the following: (1) The decreased free cholesterol concentration seen in diabetic HDL\textsubscript{2} may alter its surface fluidity, perhaps thereby facilitating the lipid peroxides generated during LDL oxidation to “seed” the HDL\textsubscript{2}. Once seeded with lipid peroxides, the TG-enriched HDL\textsubscript{2} from diabetic subjects may be more susceptible to oxidation and become less protective. (2) The lipid peroxidation products transferred from oxidized LDL may directly inactivate the PAF-AH activity in HDL\textsubscript{2}. Dentan and colleagues\textsuperscript{40} have demonstrated that 4-hydroxynonenal generated during LDL oxidation may inhibit PAF-AH activity, either by a direct modification of amino acid side chains in PAF-AH or by a modification of the phospholipid environment of the enzyme at the surface of the lipoprotein particle. In conclusion, our study demonstrates that HDL\textsubscript{2} from subjects with poorly controlled NIDDM, in both fasting and postprandial states, exhibits decreased protection against macrophage-mediated LDL oxidation, and this may contribute to accelerated atherosclerosis in type 2 diabetes.

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References
9. Tall A, Sannett D, Granot E. Mechanisms of enhanced cholesteryl ester transfer from high density lipoproteins to apolipoprotein B-containing
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