Reconstituted High-Density Lipoprotein Increases Plasma High-Density Lipoprotein Anti-Inflammatory Properties and Cholesterol Efflux Capacity in Patients With Type 2 Diabetes

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Objectives
Our aim was to investigate the effects of reconstituted high-density lipoprotein (rHDL) infusions on plasma high-density lipoprotein (HDL) anti-inflammatory properties and ex vivo cholesterol efflux in patients with type 2 diabetes.

Background
The anti-inflammatory effects of HDL contribute to protection from cardiovascular events. Individuals with type 2 diabetes are at elevated risk for cardiovascular disease, and typically have low HDL with reduced anti-inflammatory properties.

Methods
Thirteen fasting male patients (mean age 52 years) with type 2 diabetes mellitus received both rHDL (80 mg/kg of apolipoprotein A-I) and a saline placebo on separate occasions in a randomized cross-over design study. Changes in the ability of isolated HDL to influence the expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in human coronary artery endothelial cells was the main outcome measure. Other outcome measures included expression of the key integrin, CD11b on patient monocytes, adhesiveness of patient neutrophils to fibrinogen, and the ability of plasma to promote cholesterol efflux to THP-1 macrophages.

Results
Four and 72 h post-rHDL infusion, the anti-inflammatory properties of isolated HDL increased in parallel to their concentration in plasma (by up to 25%, p < 0.01). Participants’ peripheral blood monocyte CD11b expression and neutrophil adhesion to a fibrinogen matrix was also reduced 72 h post-rHDL, compared with that seen in placebo (p = 0.02). rHDL increased the capacity of plasma to receive cholesterol from THP-1 macrophages by 1 h up to 72 h post-infusion (by 40% to 60%, p < 0.05).

Conclusions
rHDL infusions have significant, potentially atheroprotective effects in individuals with diabetes, including suppression of inflammation and enhancement of cholesterol efflux. (J Am Coll Cardiol 2009;53:962–71) © 2009 by the American College of Cardiology Foundation

Large epidemiologic studies have established that low serum levels of high-density lipoprotein (HDL) are a significant and independent predictor of adverse cardiovascular events (1–6). These cardioprotective effects of HDL are thought to be due, in part, to their ability to mediate reverse cholesterol transport (7) and to inhibit athero-inflammation (8,9), thereby potentially reducing atherosclerotic plaque burden and vulnerability. Indeed, recent trials have demonstrated that infusions of reconstituted high-density lipoprotein (rHDL) and recombinant HDL in humans with known coronary artery disease significantly (albeit modestly) reduced plaque volume and had favorable effects on plaque morphology as assessed by intravascular ultrasound (10,11). Moreover, infusions of rHDL have beneficial effects on...
endothelial function in patients with type 2 diabetes (12) and hypercholesterolemia (13). The effects of rHDL on vascular inflammation, however, have not previously been studied in humans.

We hypothesized that infusions of rHDL in humans would enhance the anti-inflammatory properties of plasma HDL. To test this hypothesis, we examined the effects of rHDL infusions on key aspects of HDL structure and anti-inflammatory properties. This study was conducted in the context of type 2 diabetes mellitus, where HDL levels are typically low and HDL particles may be subject to modification by advanced glycation end products and thus, may be less effective in their ability to mediate cholesterol efflux and suppress inflammation (14–17). We also assessed the effects of rHDL infusion on other important aspects of atherogenesis ex vivo, including patient leukocyte integrin expression and cholesterol-effluxing capacity of plasma. Our results indicate that rHDL infusions significantly increase the amount of HDL in plasma, leading to a parallel increase in the anti-inflammatory properties of the isolated plasma HDL lasting up to 72 h. We also report important beneficial effects on leukocyte function as well as significant enhancement of plasma cholesterol-effluxing capacity.

Methods

Inclusion and exclusion criteria. Studies were approved by the Alfred Hospital Ethics Committee, performed in accordance with the Declaration of Helsinki, and informed written consent was obtained from all participants. Potential participants were screened for type 2 diabetes mellitus using standard criteria (fasting plasma glucose >7.1 mmol/l or a 2-h blood glucose level of >11.1 mmol/l after a 70-g oral glucose load [oral glucose tolerance test]). Those with a previous history of major illness including coronary heart disease were excluded. Patients on peroxisome proliferator-activated receptor agonists (fibrates or thiazolidinediones), metformin, lipid-modifying therapy, or antiocoagulants were also excluded. Patient characteristics, medications, and safety profiles are presented in Table 1.

rHDL constituents and preparation. rHDL was supplied by CSL Behring AG (Bern, Switzerland) (18). The rHDL was prepared fresh for each study by the Alfred Hospital Pharmacy. rHDL consisted of apolipoprotein (apo) A-I isolated from human plasma and phosphatidyl choline from soy bean. ApoA-I and phosphatidyl choline were combined in the presence of sodium cholate in a molar ratio of 1:150 and form disc-shaped, noncovalently associated particles resembling nascent HDL. The final product was lyophilized. Before infusion, the lyophilized product was reconstituted with sterile water for injection. The final concentration was 20 mg of apoA-I per milliliter of solution. This preparation has been demonstrated to produce biological responses analogous to native HDL in previous studies (13,19,20).

Table 1 Participant Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± SEM</th>
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<tbody>
<tr>
<td>Age (yrs)</td>
<td>51.8 ± 2.0</td>
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<tr>
<td>Height (cm)</td>
<td>176.9 ± 2.0</td>
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<tr>
<td>Weight (kg)</td>
<td>107.8 ± 7.8</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>34.3 ± 2.3</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>10.5 ± 1.0</td>
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<tr>
<td>HbA₁c (%)</td>
<td>7.8 ± 0.6</td>
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<tr>
<td>2-h OGTT (mmol/l)</td>
<td>13.9 ± 0.05</td>
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<tr>
<td>HDL-C (mmol/l)</td>
<td>1.02 ± 0.04</td>
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<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.98 ± 0.26</td>
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<tr>
<td>Triglycerides (mmol/l)</td>
<td>3.06 ± 0.27</td>
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<tr>
<td>Alanine transaminase (U/l)</td>
<td>39.6 ± 3.2</td>
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<tr>
<td>Gamma-glutamyl transpeptidase (U/l)</td>
<td>47.0 ± 3.4</td>
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<tr>
<td>Alkaline phosphatase (U/l)</td>
<td>82.6 ± 5.8</td>
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All continuous variables are expressed as mean ± standard error of the mean (SEM) (n = 13). BMI = body mass index; HbA₁c = glycylated hemoglobin; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; OGTT = oral glucose tolerance test.

rHDL infusion protocol. Thirteen patients with type 2 diabetes mellitus were included in a placebo (saline)-controlled, double-blind, cross-over study of the acute effects of rHDL. The 2 infusions were administered in random order and separated by at least 4 weeks. For each visit, participants fasted overnight and arrived at the laboratory at 8 AM. Two catheters were inserted into antecubital veins, 1 in each arm. The left arm catheter was utilized to administer the drug infusion (rHDL 80 mg/kg; or placebo [saline] over 4 h) by calibrated infusion pump while the right arm catheter was dedicated for blood sampling. Each participant was required to return 72 h post-infusion for further blood sampling. There was no interaction between order of intervention (rHDL and placebo) and time or treatment for any of the variables tested.

Plasma lipid parameters and inflammatory markers. Blood from participants was collected into the appropriate anticoagulant, and plasma was immediately separated by centrifugation and snap frozen. HDL cholesterol was measured by...
colorimetric assay per manufacturer instructions (WAKO Pure Chemical Industries, Osaka, Japan). ApoA-I protein was analyzed in plasma by enzyme-linked immunosorbent assay per manufacturer instructions (Alerchek, Portland, Oregon). Plasma soluble intercellular adhesion molecule (sICAM)-1 and soluble vascular cell adhesion molecule (sVCAM)-1 were measured by enzyme-linked immunosorbent assay per the manufacturer instructions (Bender Med-Systems, Vienna, Austria). Plasma C-reactive protein was measured by high-sensitivity turbidimetric assay (Roche, Basel, Switzerland).

Isolation and characterization of HDL. The total HDL fraction (1.063 < density < 1.21 g/ml) was isolated from plasma at baseline and at 4 and 72 h post-infusion by sequential ultracentrifugation and dialyzed against endotoxin-free phosphate-buffered saline (21). All compositional analyses were performed on a Roche Diagnostics/Hitachi 902 autoanalyzer (Roche Diagnostics, Mannheim, Germany). Protein, triglyceride, and phospholipid concentrations were determined using established techniques (22–24). Total cholesterol concentrations were quantitated enzymatically using a Roche Diagnostics kit (Basel, Switzerland). Unesterified (free) cholesterol concentrations were assayed enzymatically (25). Cholesteryl ester concentrations were determined by subtracting the concentration of unesterified cholesterol from the total cholesterol concentration (in mmol/l). The mass of unesterified cholesterol was calculated from the molar concentration using the molecular weight of cholesterol. The cholesteryl ester mass (including both the cholesterol and fatty acid components) was calculated from the molar concentration using a typical HDL cholesteryl ester (approximately 650). Phospholipid and triglyceride mass was similarly calculated using known molecular weights of phospholipid and triglyceride. ApoA-I and apoa-II composition was determined immunoturbidometrically using sheep anti-human apoA-I (26) and apoA-II (27) polyclonal antibodies, respectively. HDL size was determined by nondenaturing polyacrylamide gel electrophoresis (28) and quantified using ImageQuant TL (GE Healthcare, Piscataway, New Jersey) software. HDL surface charge was determined by agarose gel electrophoresis (29).

Ex vivo endothelial cell adhesion molecule expression. HDL was isolated from equivalent plasma volumes at 0, 4, and 72 h post-infusion. Because the plasma apoA-II concentration did not change with infusion of either saline or rHDL (Fig. 1, bottom), HDL was incubated with cultured human coronary artery endothelial cells (Lonza Bioscience, Basel, Switzerland) at increasing HDL apoA-II concentrations (1 to 8 μmol/l), in growth media, for 16 h at 37°C. Cells were then stimulated with tumor necrosis factor-alpha (0.1 ng/ml), and 5 h later the cellular expression of the adhesion molecules VCAM- and ICAM-1 were determined by flow cytometry (30) (Beckman Coulter FC 500, Fullerton, California). Cell viability was determined by a lactate dehydrogenase release assay and found to be >90% in all experiments.

Plasma monocyte activation. CD11b expression on peripheral blood monocytes from patients was measured in whole blood as a marker of monocyte activation before and 72 h after both infusions. Peripheral blood anticoagulated with citrate was incubated with an anti-human CD11b antibody (Serotec, Clone ICRF44, Oxford, United Kingdom) for 15 min at 37°C. Cells were fixed and lysed with OptiLyse B (Immunotech, Prague, Czech Republic) for 10
min. The lysis process was completed with the addition of distilled water. Analyses were controlled using the isotype-matched negative control antibody (FITC-antimouse IgG, Serotec, Clone W3/25). CD11b expression on monocytes was measured by flow cytometry (FACS Calibur, Becton Dickinson, Franklin Lakes, New Jersey); the monocyte population was identified by size and granularity profiles. Analysis was conducted using Cell Quest Pro software (version 3.3, BD BioSciences, Franklin Lakes, New Jersey).

**Plasma neutrophil adhesion to immobilized fibrinogen.** Neutrophils are more abundant in coronary disease patients (31,32), and their adhesion at the site of atheroma contributes to disease progression (33) and acute events (34). Adhesion of neutrophils from patients before and 72 h after both infusions to immobilized fibrinogen was assessed. Following the method of Woollard et al. (35), glass cover slips (Ø 12 mm) in 24-well plates were coated with fibrinogen (100 µg/ml) at 4°C overnight. Neutrophils (1 × 10⁶/ml) were added for 15 min at 37°C in 300 µl of plasma. Cover slips were then removed and adhered cells fixed in 4% formaldehyde for 30 min. Cover slips were washed by careful submersion in water, dried, and mounted on glass slides using mounting medium (Dako, Glostrup, Denmark). Neutrophil adhesion was quantified by phase microscopy (×40 objective), counting 5 random fields from each slide. Treatments were carried out in triplicate.

**Ex vivo cholesterol efflux.** The capacity of plasma samples to support cholesterol efflux was analyzed ex vivo using the human monocyte cell line THP-1. Cells were activated with an LXR agonist (TO901317, 1 µmol/l) to enhance the expression of ABC transporters, but were not loaded with excess cholesterol. THP-1 human monocyte cells were seeded into 12 well plates at density of 8 × 10⁶ cells per well. Cells were differentiated to macrophages with phorbol 12-myristate-13-acetate (final concentration 100 ng/ml), and cellular cholesterol was labeled by incubation in serum-containing medium with [1α,2α(3H)]-cholesterol (GE Healthcare, Buckinghamshire, United Kingdom) (final radioactivity 0.5 MBq/ml) for 48 h in a CO₂ incubator. Cells were then washed and incubated for 18 h at 37°C in serum-free medium containing the LXR agonist, TO901317 (final concentration 1 µmol/l). Cells were washed again and incubated for another 2 h at 37°C in serum-free medium containing patient plasma at the final concentration of 2%. Medium was collected and centrifuged, then aliquots of supernatant were counted in liquid scintillation on a beta-counter. Cells were also harvested, and radioactivity counted as for supernatant. Cholesterol efflux was expressed as the proportion of [3H]-cholesterol transferred from cells to medium. Background values (i.e., the efflux in the absence of plasma) were subtracted.

**Statistics.** Time course data were compared using repeated measures analysis of variance to both baseline and with placebo as indicated in the text and figure legends. Comparisons between treatments included treatment order as a between-subject variable. Individual mean comparisons were made using a least significant difference post hoc test. Pearson correlation coefficients were calculated to determine the relationship between concentration of HDL constituents and cell adhesion molecule expression. All data were analyzed using SPSS for Windows (version 15, SPSS Inc., Chicago, Illinois) and Sigmastat for Windows (version 3.5, Systat, San Jose, California) and are expressed as mean ± standard error of the mean. A value of p < 0.05 was deemed significant.

**Results**

**Plasma HDL and apoA-I levels.** HDL was significantly elevated from 3 h (p = 0.009) into the rHDL infusion (relative to placebo), continued to rise throughout the 4-h infusion to 51 ± 5% above placebo (p < 0.001), and remained at 55 ± 8% (p < 0.001) above placebo at 72 h post-infusion (ptreatment < 0.001) (Fig. 1, top). ApoA-I was elevated from 1 h (p = 0.005) into the rHDL infusion (relative to placebo) and continued to rise to 126 ± 12% above placebo at 4 h (p < 0.001). By 72 h, apoA-I levels had declined but remained 61 ± 7% (p < 0.001) above placebo (p<sub>0.001</sub>) (Fig. 1, middle). Plasma apoA-II concentrations did not significantly change with infusion of either saline or rHDL (Fig. 1, bottom).

**HDL size.** The size of the plasma HDL particles, isolated from participants infused with rHDL or saline, was assessed by subjecting aliquots of the isolated HDL (10 µl) to nondenaturing gradient gel electrophoresis. At baseline, 1 population of particles was observed (8.2 nm in diameter). At 4 h post-infusion, 2 new populations of HDL particles were formed (8.0 and 8.9 nm in diameter), with disappearance of the original population. By 72 h, only a single HDL population of particles 8.3 nm in diameter was apparent. The sizes of the new HDL particles formed post-rHDL infusion were clearly distinct from the infused rHDL (11.8 to 14.8 nm) (Fig. 2). Saline infusion did not affect the size of the isolated HDL (not shown). Infusion with either saline or rHDL had no effect on HDL surface charge (not shown), with isolated HDL found in the alpha-migrating position only, with no pre-beta particles visualized.

**HDL composition.** Saline infusion did not significantly alter HDL composition (not shown). After rHDL infusion, the phospholipid content of isolated HDL was significantly increased at 4 and 72 h relative to other HDL constituents (Table 2). The apoA-I, apoA-II, triglyceride, and free and esterified cholesterol content of isolated HDL were all significantly decreased at 4 h post-rHDL infusion. At 72 h, the proportion of apoA-I and apoA-II remained decreased relative to what was observed at baseline.

**Endothelial cell adhesion molecule expression.** To determine if infusions of rHDL affected the anti-inflammatory properties of the plasma HDL fraction, the HDL was isolated from equivalent volumes of plasma by sequential ultracentrifugation at 0, 4, and 72 h post-infusion. Saline infusion had no effect on the anti-inflammatory properties.
of isolated HDL. At a final apoA-II concentration of 4 μmol/l, VCAM-1 expression was inhibited by 9 ± 2%, 11 ± 3%, and 7 ± 2% at 0, 4, and 72 h post-saline infusion, respectively. Similarly, ICAM-1 expression was inhibited by 5 ± 2%, 7 ± 4%, and 9 ± 5%, at 0, 4, and 72 h post-saline infusion, respectively.

Post-rHDL infusion, HDL isolated at 4 h and 72 h inhibited VCAM- and ICAM-1 expression to a significantly greater extent, compared with HDL isolated at baseline. These differences were apparent at an HDL apoA-II concentration of 4 μmol/l (Figs. 3A and 3D). A similar pattern was observed when these experiments were conducted at HDL apoA-II concentrations of 1, 2, and 8 μmol/l. VCAM- and ICAM-1 expression correlated significantly with HDL apoA-I concentration (R = 0.68 and R = 0.51, respectively, p < 0.01 for both) (Figs. 3B and 3E), HDL phospholipid concentration (R = 0.58 and R = 0.46 respectively, p < 0.01 for both), and HDL free cholesterol concentration (R = 0.66 and R = 0.51, respectively, p < 0.01 for both), but not with HDL apoA-II concentration (R = 0.13 and R = 0.16, respectively). The magnitude of VCAM- and ICAM-1 inhibition is, therefore, proportional to the amount of HDL that was included in the incubations, as evidenced by significant correlations with all HDL constituents (apart from apoA-II) and CAM expression.

Plasma sICAM- and sVCAM-1. Plasma sVCAM-1 levels were significantly reduced after the 4-h rHDL infusion, but had returned to baseline 72 h after the infusion (analysis of variance time effect and treatment-time interaction both p = 0.04) (Fig. 4, top). There was no significant effect of rHDL on circulating sICAM-1 levels (Fig. 4, bottom). There was also no significant effect of rHDL on plasma CRP at either 4 or 72 h (placebo 2.74 ± 0.54 mg/l vs. rHDL 2.98 ± 0.87 mg/l post-infusion).

Leukocyte CD11b expression and neutrophil adhesion. Monocyte CD11b expression was expressed as a percentage change from baseline levels 72 h after the placebo and rHDL infusions. While there was a trend for increased expression with placebo, CD11b expression was significantly reduced relative to placebo after rHDL (p = 0.021) (Fig. 5, top). This corresponded with a significant reduction in neutrophil adhesion to fibrinogen with rHDL (p = 0.026) (Fig. 5, bottom).

Cholesterol efflux. Relative to baseline, rHDL enhanced the capacity of plasma to support cholesterol efflux by 35 ± 7% after only 1 h (p = 0.002) into the infusion and remained elevated at this level during the remaining infusion time and 72 h (p = 0.018) after the infusion) (Fig. 6, top). This effect was also observed when assessed relative to placebo 3 h into the infusion and 72 h post-infusion (Fig. 6, bottom). Cholesterol efflux was significantly higher after the rHDL infusion by approximately 40% (p = 0.021) and 60% (p = 0.002) at 3 and 72 h, respectively (p < 0.007). Interestingly, the capacity of plasma to support cholesterol efflux did not increase further during the continued infusion despite a further rise in plasma apoA-I and HDL-C levels (Fig. 1). Consequently there was no statistically significant correlation between cholesterol efflux and plasma apoA-I or HDL cholesterol levels.

Discussion

In this study, we show for the first time that infusions of rHDL in patients with type 2 diabetes mellitus increase the anti-inflammatory properties of the resulting plasma HDL

<p>| Table 2 Composition of High-Density Lipoproteins After rHDL Infusion |
|----------------------|------------------|------------------|</p>
<table>
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<tr>
<th></th>
<th>Baseline</th>
<th>4 h</th>
<th>72 h</th>
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<tr>
<td>ApoA-I</td>
<td>32.7 ± 1.9</td>
<td>24.9 ± 1.7</td>
<td>27.6 ± 3.3</td>
</tr>
<tr>
<td>ApoA-II</td>
<td>15.4 ± 3.6</td>
<td>4.0 ± 0.5</td>
<td>9.2 ± 1.3</td>
</tr>
<tr>
<td>PL</td>
<td>27.0 ± 2.4</td>
<td>57.3 ± 3.5</td>
<td>36.5 ± 2.3</td>
</tr>
<tr>
<td>CE</td>
<td>19.1 ± 2.5</td>
<td>10.1 ± 1.3</td>
<td>21.2 ± 2.4</td>
</tr>
<tr>
<td>FC</td>
<td>1.7 ± 0.4</td>
<td>1.3 ± 0.1</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>TG</td>
<td>4.0 ± 0.5</td>
<td>2.6 ± 0.8</td>
<td>3.9 ± 0.6</td>
</tr>
</tbody>
</table>

Composition (mass %) of high-density lipoproteins isolated from participants (n = 10) at baseline and 4 and 72 h after infusion of reconstituted high-density lipoprotein (HDL) expressed as mean ± standard error of the mean (measures in triplicate). *p < 0.05 by comparison with baseline values. apo = apolipoprotein; CE = cholesterol ester; FC = free cholesterol; PL = phospholipid; TG = triglyceride.
fraction, as demonstrated by its ability to inhibit the cellular expression of VCAM-1 and ICAM-1 on stimulated human coronary endothelial cells. Furthermore, the magnitude of inhibition was in proportion to the increase in the plasma concentration of HDL. Infusion of rHDL also resulted in reduced CD11b expression on circulating monocytes and reduced adhesion of patient neutrophils to fibrinogen and enhanced ex vivo cholesterol efflux (20) capacity of plasma.

There are now robust data demonstrating the anti-inflammatory properties of HDLs (36). In vitro studies have shown that discoidal rHDL consisting of apoA-I complexed with phosphatidylcholine inhibits the cellular expression of VCAM-1 and ICAM-1 on stimulated endothelial cells (30,37,38). Similarly, HDL isolated from human plasma inhibits cell adhesion molecule expression ex vivo, though this inhibition varies between individuals (36).

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**Figure 3** Endothelial Cell Adhesion Molecule Expression

Participants (n = 13) were infused with reconstituted HDL (80 mg/kg), and plasma was collected at 0, 4, and 72 h post-infusion. The HDL was ultracentrifugally isolated as described in the Methods section. Cultured human coronary artery endothelial cells were pre-incubated at 37°C for 16 h with the ultracentrifugally isolated HDL at a final apolipoprotein A-I concentration of 4 μmol/l. The cells were then stimulated with tumor necrosis factor-alpha (final concentration 0.1 ng/ml) for 5 h. Expression of vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 on the endothelial surface was determined by flow cytometry. Panels A and B show VCAM-1 and ICAM-1 expression (mean ± standard error of the mean) in the absence of HDL and in the presence of isolated HDL at 0, 4, and 72 h post-reconstituted HDL infusion. The solid squares in C and D show VCAM-1 and ICAM-1 expression as a function of the apoA-I concentration (mean ± standard error of the mean) of the HDL preparations (R = 0.68 and R = 0.51, respectively, p < 0.01 for both). The solid squares in E and F show VCAM-1 and ICAM-1 expression as a function of the phospholipid concentration (mean ± standard error of the mean) of the HDL preparations (R = 0.58 and R = 0.46, respectively, p < 0.01 for both). *p < 0.01 compared with baseline (0 h). PL = phospholipid; other abbreviations as in Figure 1.
Furthermore, in rabbits, infusions of rHDL significantly inhibit vascular inflammation induced by insertion of a nonocclusive silastic collar around the carotid artery (8). The apoA-I mimetic peptide D-4F has also been shown to have anti-inflammatory properties in animal models (39–42), and rHDL has anti-inflammatory properties in the context of human endotoxemia (43). Although previous studies infusing rHDL in humans have focused on atheroma regression and plaque morphology, as assessed by intravascular ultrasound (10), no study has specifically investigated the potential vascular anti-inflammatory effects of rHDL infusion in humans.

The rHDL dose (80 mg/kg) was based on previous studies demonstrating beneficial effects on endothelial function (12,13,19) and plaque morphology (10). Tardif et al. (10) compared 40 mg/kg versus 80 mg/kg rHDL, and although there was no difference in the overall frequency of major adverse events between the treatment and placebo groups, alanine aminotransferase levels exceeded 100-fold of the upper limit of normal in some patients in the 80 mg/kg group. The patients in the current trial were early stage, well-controlled diabetic patients, and rHDL treatment at 80 mg/kg was not associated with any significant liver function abnormalities (Table 1, bottom). Since we show that anti-inflammatory effects parallel HDL concentration, we would expect a proportionally lesser, but clinically significant, anti-inflammatory response at a dose of 40 mg/kg.

In the current study, experiments on cell adhesion molecule expression were conducted with isolated HDL, as opposed to whole plasma. We thus avoided the potentially confounding effects of other plasma components, which enabled us to observe direct HDL-mediated changes on these cells. The anti-inflammatory properties of the plasma HDL were significantly increased at 4 h and even at 72 h post-rHDL infusion. These inhibitory effects were associated with increases in all HDL constituents apart from apoA-II, suggesting that the anti-inflammatory properties of these particles in plasma post-infusion is related to the total amount of HDL in plasma, and not to an increased concentration in one particular constituent. Our results do not suggest that rHDL infusions lead to an increased anti-inflammatory capacity of plasma HDL particles, independent of their plasma concentration.

Changes in isolated HDL size were also observed post-rHDL infusion, from a single population of particles pre-infusion, to a new larger population with an additional slightly smaller population at 4 h, before returning to a single population similar to baseline at 72 h. The presence of HDL significantly increased at 4 h and even at 72 h post-rHDL infusion. These inhibitory effects were associated with increases in all HDL constituents apart from apoA-II, suggesting that the anti-inflammatory properties of these particles in plasma post-infusion is related to the total amount of HDL in plasma, and not to an increased concentration in one particular constituent. Our results do not suggest that rHDL infusions lead to an increased anti-inflammatory capacity of plasma HDL particles, independent of their plasma concentration.

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of the larger population of particles at 4 h may reflect incorporation of infused apoA-I and phospholipids into the endogenous HDL as suggested by Nanjee et al. (20). Indeed, our HDL compositional data, demonstrating a significant increase in phospholipid content relative to other constituents, suggests that it may play a particular role in transiently increasing plasma HDL size post-rHDL infusion. The remodeling of the larger particles by cholesterol ester transfer protein, hepatic lipase (44,45), and/or phospholipid transfer protein (46) may account for the presence of the smaller HDL particles at 4 h. The improvement in HDL anti-inflammatory capacity only occurred in proportion to the amount of HDL in plasma, irrespective of whether it was altered in size (4 h) or similar in size to baseline (72 h). However, it has been postulated that the anti-inflammatory capacity of HDL may be dependent on its subclasses, where HDL3 has been shown to be more potent than HDL2 in inhibiting adhesion molecule expression on endothelial cells. There is some evidence that phospholipid content may account for these observations (47).

Integrins and selectins expressed on both the leukocytes and endothelium play an important role in mediating atherosclerosis in response to inflammatory stimuli, through specific roles in the cell adhesion cascade. CD11b present on leukocytes is required for firm adhesion to ICAM-1 on the inflamed endothelium (48,49). Recent evidence suggests that CD11b may also interact with VCAM-1 on the endothelium in the presence of elevated glucose levels (50). Previous studies have demonstrated that CD11b expression on circulating leukocytes is up-regulated in diabetes (51,52) and along with the increased production of pro-inflammatory cytokines (53) suggests an underlying chronic inflammatory state that may contribute to vascular disease progression. We further demonstrate anti-inflammatory actions of rHDL infusion in vivo, with a significant reduction in peripheral blood monocyte CD11b expression and a reduction in CD11b-dependent leukocyte adhesion to fibrinogen matrix after rHDL infusion. This may be relevant in the context of monocyte rolling and adhesion to the endothelial surface, where CD11b expression is associated with atherosclerotic initiation and progression (54). Similar reductions in monocyte CD11b to that observed in the current study occur in hypercholesterolemia after statin administration (55,56). Given the concomitant decreases in adhesion molecule expression on endothelial cells, and CD11b expression and activity on leukocytes, rHDL infusion may contribute to a reduction in leukocyte rolling and adhesion.

Despite clear demonstration of the anti-inflammatory capacity of rHDL infusion on in vitro endothelial cells and circulating leukocytes, we observed only a minor change in plasma sVCAM-1 and no significant change in plasma sICAM-1 or C-reactive protein. These data are not surprising, however, given the diluting effects of total blood volume, thus emphasizing that peripheral blood levels of these markers are relatively insensitive in reflecting endothelial inflammation. This likely also results from the measurement of soluble forms of these proteins, which only reflect the amounts cleaved from endothelial cells.

In contrast to the anti-inflammatory data, the capacity of post-rHDL infusion plasma to efflux cholesterol to THP-1 macrophages was not closely correlated to apoA-I plasma concentration. The pattern of cholesterol efflux in relation to apoA-I and HDL plasma concentration suggests that maximal cholesterol efflux capacity was achieved at plasma concentrations of HDL and apoA-I achieved as early as 1 h into the rHDL infusion. Intracellular cholesterol is determined by both cholesterol efflux and cholesterol influx, plus compensatory changes in cholesterol biosynthesis; only the former was assessed in this study. It is, however, likely that an increased capacity of plasma to promote cholesterol efflux combined with no changes in parameters of forward cholesterol transport (such as plasma LDL cholesterol levels) will shift the balance of cholesterol toward slower accumulation or reduction of intracellular cholesterol content. This was recently confirmed in a separate study where infusion of rHDL caused a significant reduction in the cholesterol
content of superficial femoral artery plaques in patients with peripheral vascular disease (57). **Study limitations.** One limitation in our study is that the ultracentrifugation process results in the isolation of larger alpha-migrating HDL particles, but smaller pre-beta migrating HDL particles are lost. Previous studies have demonstrated that infusions of rHDL in humans do indeed result in the early generation of pre-beta migrating particles (20), which may display anti-inflammatory effects. The capacity for pre-beta migrating HDL particles to inhibit inflammation is not known, possibly because of the complexity of isolating such short-lived particles. However, we cannot eliminate the possibility that these particles may be modulating some of the observed effects.

The conclusions from this study are restricted to the acute effects of rHDL infusion in patients with type 2 diabetes. Whether the observed anti-inflammatory and cholesterol efflux effects are sustained with chronic treatment in other populations and translate to significant athero-protection in vivo remains to be determined.

**Conclusions**

In summary, this study highlights the important and significant anti-inflammatory effects of rHDL infusions on plasma HDL in diabetic individuals, which are related to an increase in its plasma concentration and persist even up to 72 h post-infusion. In addition, rHDL also reduced monocyte activation, neutrophil adhesion, and enhanced ex vivo cholesterol efflux. Thus infusions of rHDL may not only have therapeutic utility in reducing atheroma burden, but may also be used to suppress the widespread vascular inflammation that characterizes the acute coronary and cerebrovascular syndromes.

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