Reconstituted High-Density Lipoprotein Attenuates Platelet Function in Individuals With Type 2 Diabetes Mellitus by Promoting Cholesterol Efflux

Anna C. Calkin, PhD; Brian G. Drew, PhD; Akiko Ono, PhD; Stephen J. Duffy, MBBS, PhD; Michelle V. Gordon, MBBS; Simone M. Schoenwaelder, PhD; Dmitri Sviridov, PhD; Mark E. Cooper, MBBS, PhD; Bronwyn A. Kingwell, PhD; Shaun P. Jackson, MBBS, PhD

Background—Individuals with diabetes mellitus have an increased risk of cardiovascular disease and exhibit platelet hyperreactivity, increasing their resistance to antithrombotic therapies such as aspirin and clopidogrel. Reconstituted high-density lipoprotein (rHDL) has short-term beneficial effects on atherosclerotic plaques, but whether it can effectively reduce the reactivity of diabetic platelets is not known.

Methods and Results—Individuals with type 2 diabetes mellitus were infused with placebo or rHDL (CSL-111; 20 mg · kg⁻¹ · h⁻¹) for 4 hours, resulting in an ∼1.4-fold increase in plasma HDL cholesterol levels. rHDL infusion was associated with a >50% reduction in the ex vivo platelet aggregation response to multiple agonists, an effect that persisted in washed platelets. In vitro studies in platelets from healthy individuals revealed that the inhibitory effects of rHDL on platelet function were time and dose dependent and resulted in a widespread attenuation of platelet function and a 50% reduction in thrombus formation under flow. These effects could be recapitulated, in part, by the isolated phospholipid component of rHDL, which enhanced efflux of cholesterol from platelets and reduced lipid raft assembly. In contrast, the apolipoprotein AI component of rHDL had minimal effect on platelet function, cholesterol efflux, or lipid raft assembly.

Conclusion—These findings suggest that rHDL therapy is highly effective at inhibiting the heightened reactivity of diabetic platelets, partly through reducing the cholesterol content of platelet membranes. These properties, combined with the known short-term beneficial effects of rHDL on atherosclerotic lesions, suggest that rHDL infusions may be an effective approach to reduce atherothrombotic complications in diabetic individuals.

Clinical Trial Registration Information—URL: http://www.clinicaltrials.gov. Unique identifier: NCT00395148. (Circulation. 2009;120:2095-2104.)

Key Words: cholesterol ■ diabetes mellitus ■ platelets

Type 2 diabetes mellitus is a well-known risk factor for cardiovascular disease and is associated with a 3- to 4-fold increase in the incidence of fatal coronary and cerebral ischemic events.¹² It is increasingly appreciated that the elevated cardiovascular risk observed in diabetes mellitus is due not only to an increase in atherosclerotic burden but also to enhanced platelet activity, leading to a prothrombotic tendency. Diabetic platelet “hyperreactivity” is caused by a multitude of factors, including increased expression of surface receptors and adhesion molecules, enhanced production of thrombin and thromboxane A₂, and dysregulated platelet calcium homeostasis.³⁻⁶ This increase in platelet reactivity reduces the effectiveness of commonly used antiplatelet agents such as aspirin and clopidogrel.⁷ As a consequence, more potent antiplatelet therapeutic strategies are increasingly being used to reduce thrombotic complications in diabetes mellitus; however, these approaches have minimal impact on the atherosclerotic lesions themselves, leading to persistent plaque instability.

Clinical Perspective on p 2104

A potentially important physiological regulator of atherosclerotic plaques is high-density lipoprotein (HDL). Large epidemiological trials have established that plasma HDL cholesterol levels correlate inversely with adverse
The cardioprotective effects of HDL are generally attributed to its ability to mediate reverse cholesterol transport and its anti-inflammatory and antioxidative actions. Increasing HDL levels long term via the use of statins or fibrates is associated with a reduction in cardiovascular events in type 2 diabetes mellitus. HDL cholesterol levels have been inversely correlated with thrombosis, and HDL has been shown to mediate various antithrombotic effects. Although native and reconstituted HDLs (rHDLs) have direct effects on platelet function, albeit through ill-defined mechanisms, it is generally considered that the major antithrombotic effects of HDL occur indirectly, via effects on the vasculature. For example, HDL reduces endothelial cell surface expression of adhesion molecules, modulates blood flow through alterations in nitric oxide production, and decreases tissue factor production.

Infusion of rHDL is associated with various cardiovascular benefits, including restored endothelial dysfunction, increased levels of circulating endothelial progenitor cells, enhanced HDL anti-inflammatory properties, reduced monocyte adhesion, and improved metabolic control. Moreover, rHDL infusion directly modulates plaque composition, reducing plaque lipid content, macrophage infiltration, and inflammatory properties. Given the ability of rHDL to modulate various pathways associated with atherosclerosis, we aimed to investigate the efficacy of rHDL therapy at attenuating platelet reactivity in high-risk individuals with type 2 diabetes mellitus and to gain further insight into its antiplatelet effects.

**Methods**

**Human rHDL Infusion Study: Inclusion and Exclusion Criteria**

The present investigation is based on a previously described protocol that examined the anti-inflammatory and metabolic actions of rHDL. This study, approved by the Alfred Hospital Ethics Committee and performed in accordance with the Declaration of Helsinki, involved 13 male individuals with type 2 diabetes mellitus. Written informed consent was obtained from all participants. Criteria for participation included a fasting plasma glucose greater than 7.1 mmol/L or 2-hour blood glucose level of greater than 11.1 mmol/L after a 70-g oral glucose load (oral glucose tolerance test). Individuals with a previous history of major illness, including coronary heart disease, were excluded. Those taking peroxisome proliferator–activated receptor agonists, metformin, lipid-modifying therapies, or antiplatelet agents were also excluded from participation. Four individuals were being treated with glipizide, 1 was taking glyburide, and 1 was being treated with gliclazide, which were withdrawn for 5 half-life periods before participation.

**Human rHDL Infusion Study Design**

rHDL (CSL-111) was supplied by CSL Behring AG (Bern, Switzerland). We have investigated 10 individuals receiving placebo and 7 receiving rHDL. Of these, 4 individuals received both interventions. Because samples for both interventions were not obtained in all individuals, data were treated in a cross-sectional manner for statistical purposes. Venous blood was sampled at baseline, at the conclusion of the 4-hour infusion, and at 72 hours after infusion with a 19-gauge butterfly needle.

**Human rHDL Infusion Study: Lipoproteins**

HDL cholesterol and apolipoprotein AI (apoAI) protein were analyzed as previously described. Blood Collection: In Vitro Assays See the online-only Data Supplement.

**Platelet Preparation**

Washed platelets and platelet-rich plasma were prepared as previously described.

**Preparation of Lipids**

See the online-only Data Supplement.

**Aggregation Assays**

Platelet aggregation studies were initiated by stirring the suspensions at 600 rpm for 10 minutes at 37°C in a 4-channel automated platelet analyzer (AggRAM, Helena Laboratories, Beaumont, Tex). See the online-only Data Supplement for further details.

**Integrin αIIbβ3 Activation**

See the online-only Data Supplement.

**P-Selectin Expression**

See the online-only Data Supplement.

**Integrin**

**Western Blotting**

See the online-only Data Supplement.

**Cholesterol Efflux**

See the online-only Data Supplement.

**Lipid Raft Abundance**

Washed platelets (5 × 10^7/mL) were incubated with FITC-conjugated cholera toxin B (10 μg/mL; Molecular Probes, Eugene, Ore) in the presence or absence of ADP (10 μmol/L; BDH Chemicals, Poole, UK) for 30 minutes. Platelets were subsequently fixed with paraformaldehyde (2% final concentration). All samples were resuspended in PBS and analyzed on a FACSCalibur (BD Biosciences, San Jose, Calif).

**Statistical Analysis**

Data are presented as mean ± SEM. Normally distributed data were analyzed for statistical significance with an unpaired t test, ANOVA, or repeated-measures MANOVA with post hoc testing (Dunnnett or Bonferroni) as appropriate. Nonnormally distributed data were analyzed by the Mann-Whitney rank-sum test or Kruskal-Wallis 1-way ANOVA with the Dunn post hoc test. The null hypothesis was rejected at P < 0.05.

**Results**

**Clinical Study**

**Metabolic Parameters**

Study volunteers exhibited elevated fasting plasma glucose levels (10.5 ± 1.0 mmol/L), elevated glycohemoglobin (7.8 ± 0.6%), and an average body mass index of 34.3 kg/m² (Table 1). Consistent with their type 2 diabetes mellitus status, rHDL infusion was associated with an approximate 1.3-fold increase from baseline in plasma HDL cholesterol levels by the conclusion of the 4-hour infusion. This continued to rise to 1.4-fold above baseline levels 72 hours after the conclusion of the infusion.
after infusion (Table 2). \textsuperscript{14,25} Plasma apoAI levels were increased >2-fold above baseline at the conclusion of the infusion and by 72 hours were 1.7-fold above baseline (Table 2). Neither of these parameters was significantly altered with placebo infusion.

**Ex Vivo Platelet Response to rHDL Infusion**

Platelet-rich plasma derived from blood sampled at the conclusion of the 4-hour rHDL infusion demonstrated a 50% to 75% attenuation in the platelet aggregation response induced by ADP and collagen relative to placebo (repeated-measures MANOVA treatment effect, \textit{P}=0.001; Bonferroni posthoc test at 4 hours, \textit{P}=0.03; Figure 1A and repeated-measures MANOVA treatment effect, \textit{P}=0.005; Bonferroni posthoc test at 4 hours, \textit{P}=0.02; Figure 1B). rHDL infusion resulted in reduced maximal aggregation and enhanced reversibility, leading to almost complete disaggregation of ADP-stimulated platelets, whereas those obtained 72 hours after infusion exhibited a normal aggregation response (Figure 1A and 1B), demonstrating that the inhibitory effects of rHDL were reversible. Interestingly, when platelets derived from blood sampled at the conclusion of the rHDL infusion were washed and resuspended in buffer, the inhibitory effects associated with the rHDL infusion persisted (Figure 1C and 1D). Indeed, a marked attenuation in platelet fibrinogen binding, a direct marker of integrin \(\alpha_{\text{IIb}}\beta_3\) activation, was observed in platelets from individuals receiving rHDL infusion compared with those receiving placebo in response to both ADP and collagen-related peptide. These findings demonstrate that a single infusion of rHDL is associated with a marked decrease in agonist-induced integrin \(\alpha_{\text{IIb}}\beta_3\) activation and platelet aggregation.

**In Vitro Studies**

**The Effect of rHDL on Platelet Function Is Dose and Time Dependent and Persists When rHDL Is Removed**

To gain further insight into the mechanisms by which rHDL modulates platelet function, we performed in vitro studies on isolated, washed human platelets. Similar to the ex vivo clinical data, incubation of washed platelets from male diabetic subjects with rHDL directly attenuated fibrinogen binding to ADP-stimulated platelets, to an extent similar to that observed with the rHDL infusion (Figure I of the online-only Data Supplement). These inhibitory effects were not specific to diabetic platelets because incubation of washed platelets from healthy donors with rHDL directly attenuated the maximal and sustained platelet aggregation response induced by ADP (Figure 2A). The inhibitory effects of rHDL on platelet aggregation were time dependent, with minimal inhibition in the first 30 minutes of incubation; however, between 60 and 300 minutes, there was a progressive decrease in platelet aggregation associated with rHDL (repeated-measures MANOVA treatment effect, \textit{P}<0.001; Figure 2C). The inhibitory effects of rHDL were also dose dependent, with maximal inhibition observed at rHDL doses \(\approx 25 \mu g/mL\) (Figure 2B). Unless otherwise stated, in all subsequent studies, platelets were incubated with a subphysiological concentration of rHDL (50 \(\mu g/mL\); 5 mg/dL)\textsuperscript{25} for at least 60 minutes before the functional studies were performed. To investigate whether the inhibitory effects of rHDL on platelet function required the presence of rHDL or persisted once it was removed, preincubation assays were performed. Consistent with the ex vivo studies, preincubation of platelets with rHDL for 1 or 2 hours (before washing in the absence of rHDL) resulted in a time-dependent inhibition of ADP-induced aggregation (Figure 2D).

**rHDL Causes Widespread Inhibition of Platelet Function**

Agonist dose-response studies revealed that rHDL-treated platelets had a reduced platelet aggregation response over

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**Table 1. Participant Characteristics**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean±SEM</th>
</tr>
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<tbody>
<tr>
<td>Age, y</td>
<td>51.8±2.0</td>
</tr>
<tr>
<td>Body mass index, kg/m(^2)</td>
<td>34.3±2.3</td>
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<tr>
<td>Fasting glucose, mmol/L</td>
<td>10.5±1.0</td>
</tr>
<tr>
<td>HbA(_{1c}), %</td>
<td>7.8±0.6</td>
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<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.0±0.0</td>
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<tr>
<td>LDL cholesterol, mmol/L</td>
<td>3.1±0.3</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.0±0.3</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>2.1±0.4</td>
</tr>
</tbody>
</table>

\(\text{HbA}_{1c}\) indicates hemoglobin \(\text{A}_{1c}\); HDL, high-density lipoprotein; LDL, low-density lipoprotein. \(n=13\).

**Table 2. Plasma Levels of HDL and ApoAI in Study Volunteers**

<table>
<thead>
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<th>Parameter</th>
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<th>4</th>
<th>72</th>
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<tbody>
<tr>
<td>Placebo</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>0.94±0.05</td>
<td>0.90±0.06</td>
<td>0.84±0.04</td>
<td>0.95±0.06</td>
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<tr>
<td>ApoAI, mg/dL</td>
<td>73.5±3.6</td>
<td>74.0±2.6</td>
<td>83.5±6.4</td>
<td>81.5±4.5</td>
</tr>
<tr>
<td>rHDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>0.99±0.05</td>
<td>1.16±0.06</td>
<td>1.29±0.05</td>
<td>1.44±0.1</td>
</tr>
<tr>
<td>ApoAI, mg/dL</td>
<td>73.4±4.1</td>
<td>121.3±10.7</td>
<td>168.5±14.7</td>
<td>125.7±11.5</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM (\(n=10\) per group for placebo, \(n=7\) per group for rHDL).

*\(P<0.01\), †\(P<0.001\) between treatments.
a broad range of ADP and collagen-related peptide concentrations, with less inhibition noted at higher concentrations (Figure 3A and 3B). Furthermore, the inhibition was not selective to aggregation because thrombin (repeated-measures MANOVA treatment effect, \( P<0.001 \)) and collagen-related peptide–induced platelet α-granule release (Figure 3C and 3D) and platelet adhesion and subsequent spreading on fibrinogen (Figure 3E) and collagen (data not shown) were also decreased, suggesting a widespread attenuation of platelet function associated with rHDL.

**Thrombus Formation Is Markedly Attenuated by rHDL**

Platelet adhesion and aggregation at sites of vascular injury in vivo are regulated by blood flow, with increasing shear leading to enhanced thrombus growth. To investigate the impact of rHDL on platelet thrombus formation under physiological blood flow conditions, in vitro flow-based assays were performed on a type I fibrillar collagen matrix. At a high wall shear rate of 1800 seconds\(^{-1}\), large, discrete platelet thrombi formed on immobilized collagen fibrils. In contrast, whole blood treated with rHDL formed significantly smaller thrombi, with an \( \approx 50\% \) reduction in thrombus height and volume (\( P<0.01 \); Figure 4A through 4D). Real-time analysis of thrombus formation revealed a reduction in primary platelet adhesion to the collagen substrate and reduced platelet incorporation into developing thrombi, suggesting that the reduction in thrombus size was due primarily to a defect in initial thrombus growth rather than increased thrombus instability (data not shown).
Inhibitory Effects of rHDL Occur Independently of ATP Binding Cassette Transporter A1 or Scavenger Receptor-B1

Many of the known vascular protective effects of HDL are mediated by binding to specific cell surface receptors, including ATP binding cassette transporter A1 (ABCA1) and scavenger receptor-B1 (SR-B1). Consistent with previous reports, we demonstrated the presence of SR-B1 on platelets (~57 kDa; nonglycosylated SR-B1; Figure 5B), but we did not detect significant levels of ABCA1 (Figure 5A). To determine whether SR-B1 mediated the inhibitory effects of rHDL on platelet function, we performed blocking studies. A blocking antibody against SR-B1, known to cross-react with SR-B1, was used. Figure 3 shows the inhibitory effect of rHDL on platelet function.

**Figure 3.** The inhibitory effect of rHDL on platelet function is widespread. Platelets incubated with vehicle or rHDL (50 μg/mL for 2 hours) were examined for (A) aggregation in response to ADP or (B) collagen-related peptide (CRP), P-selectin expression in response to (C) thrombin (1U/mL) over time (n=9 per group) and (D) CRP at 30 minutes (n=6 per group). E. Attachment and spreading on fibrinogen (10 μg/mL for 15 minutes). Data are expressed as mean±SEM. *P<0.05, **P<0.01, ***P<0.001 vs control (C).

**Figure 4.** Thrombus formation is markedly attenuated by rHDL. Whole blood incubated with vehicle or rHDL (1.6 mg/mL for 1 hour) was perfused over bovine type 1 fibrillar collagen (250 μg/mL)-coated glass capillaries (1800 seconds for 2.5 minutes). Thrombi were fixed, and thrombus volume (A) and height (B) were analyzed (expressed as a percentage of control; n=4 per group). Representative 3-dimensional reconstruction of thrombi in the absence (C) and presence (D) of rHDL. Color bar indicates increase in volume height (top to bottom). Data are expressed as mean±SEM. **P<0.01, ***P<0.001 vs control.
with its human homolog CLA-1, had no significant effect on the attenuation of ADP-induced aggregation observed with rHDL (Figure 5C). Furthermore, the SR-B1 ligands, apoAI (the only protein component of rHDL), and native HDL did not have a significant effect on ADP-induced aggregation (Figure 5D and 5E), consistent with a receptor-independent mechanism.

We investigated the potential contribution of the phospholipid component of rHDL to the inhibitory effect observed on platelet function. In contrast to apoAI, incubation of platelets with soybean phosphocholine (PCsoy) attenuated ADP-induced aggregation to an extent similar to that seen with rHDL (PCsoy, 70.3±3.8%; Figure 5D and 5E). These findings suggest that the phospholipid component of rHDL is primarily responsible for its ability to inhibit platelet function.

**Mechanism of Action: rHDL Enhances Cholesterol Efflux and Reduces Lipid Raft Assembly**

Native HDL differs from rHDL in its cholesterol content, raising the possibility that the differing effects of these particles on platelet function may be related to their ability to act as cholesterol acceptors. Consistent with previous findings, incubation of platelets with methyl-β-cyclodextrin, a membrane cholesterol-depleting agent, significantly attenuated ADP-induced platelet aggregation (85.9±3.3%; Figure 5D and 5E). Conversely, enriching membrane cholesterol content by incubating platelets with cholesterol-loaded methyl-β-cyclodextrin increased ADP-induced platelet aggregation (122.9±5.7%). To directly investigate the impact of rHDL on cholesterol depletion from platelet membranes, we examined the efflux of radiolabeled cholesterol, [1α,2α(n)-3H]-cholesterol, from platelet membranes. rHDL treatment of platelets was associated with a 5-fold increase in cholesterol efflux (P<0.001; Figure 6A). PCsoy was also able to enhance efflux of cholesterol from platelets to a similar level (325±57%; P<0.01). Notably, native HDL also promoted cholesterol efflux, albeit to a lesser extent than rHDL (301±25%; P<0.01). ApoAI had no effect on this parameter, consistent with a lack of effect on platelet function (Figure 6A). Time course studies revealed that efflux of cholesterol by rHDL occurred most rapidly within the first 30 minutes and continued to increase over time, albeit at a slower rate, consistent with rHDL molecules acquiring cholesterol over time, thus reducing the cholesterol gradient (P<0.001; Figure 6B). These changes preceded changes in platelet function (Figure 2C). To further exclude that an active, receptor-mediated process was required for rHDL-induced platelet inhibition via cholesterol efflux, we examined the ability of rHDL to enhance cholesterol efflux in paraformaldehyde-fixed platelets. As demonstrated in Figure 6C, fixing platelets had no inhibitory effect on rHDL-induced cholesterol efflux, confirming that “free diffusion” is likely to be the dominant mechanism underlying rHDL-mediated cholesterol efflux from platelets (Figure 6C).

Alterations in membrane cholesterol content cause disruption to cholesterol-rich signaling microdomains known as
lipid rafts. Lipid rafts have been shown to be integral to platelet signaling pathways and disruption of rafts is associated with reduced platelet activation. We examined the effect of rHDL incubation on the binding of cholera toxin subunit B (CT-B) to the lipid raft marker ganglioside GM1 on the surface of platelets. In resting platelets, rHDL was associated with a significant reduction in CT-B binding, suggesting reduced lipid raft assembly (77.3 ± 1.3%; P < 0.01). No other cholesterol acceptor had any effect on resting CT-B expression levels (Figure 6D). ADP-induced activation was associated with an increase in CT-B expression levels in all groups. Interestingly, both rHDL and PCsoy significantly decreased levels of CT-B in ADP-activated platelets compared with control (control, 169.3 ± 9.7; rHDL, 121.6 ± 7.7; PCsoy, 125.8 ± 4.3; P < 0.01). HDL and apoAI had no effect on this parameter.

Together, these findings demonstrate that rHDL enhances cholesterol removal from platelets in a receptor-independent manner, resulting in the disruption of lipid rafts and a reduction in platelet activation.

**Discussion**

The studies presented here demonstrate the effectiveness of a single infusion of rHDL in attenuating the heightened reactivity of platelets from individuals with diabetes mellitus. Moreover, they demonstrate the far greater platelet inhibitory properties of rHDL relative to native HDL, an unexpected finding given that both forms of HDL typically elicit similar cellular effects via apoAI stimulation of cell surface receptors. Our studies suggest that cholesterol depletion is likely to be the predominant mechanism by which rHDL attenuates platelet function, disrupting lipid rafts and inhibiting platelet activation. This, combined with the well-defined antiinflammatory and antioxidant properties of rHDL on the vasculature, suggests that infusions of rHDL may represent an effective approach to reduce atherothrombotic complications in diabetic individuals.

A major clinical problem with type 2 diabetes mellitus is heightened platelet reactivity, leading to a prothrombotic phenotype. As a consequence, diabetic platelets are less sensitive to the antithrombotic benefits of aspirin and clopidogrel. There is growing evidence that more potent antithrombotic strategies, including combination antiplatelet therapies, high-dose clopidogrel, prasugrel, or the use of glycoprotein IIb/IIIa inhibitors, are necessary to effectively limit platelet aggregation in diabetic individuals. However, these antithrombotic approaches have little impact on the underlying stability of vulnerable plaques and do not target pathways that are specifically linked to the phenomenon of platelet hyperreactivity. The findings presented here demonstrate that rHDL is highly effective at reversing a key process linked to platelet hyperreactivity, namely the excessive accumulation of cholesterol in platelet membranes. Notably, platelets from individuals with diabetes mellitus exhibit reduced membrane fluidity, presumably because of increased cholesterol content, and this is considered one of the mechanisms responsible for the heightened platelet sensitivity associated with this condition. Recently, Shaw et al have demonstrated that rHDL infusion was associated with a reduction in cholesterol content in femoral plaques of individuals with peripheral vascular disease. Indeed, it is becoming increasingly apparent that rHDL mediates many of its inhibitory actions through the removal of cholesterol, whether via SR-B1, ABCA1, or ABCG1/4 in different cell types or via free diffusion, as we have demonstrated in platelets.

The flux of free cholesterol is known to occur passively in a bidirectional manner and is dependent on the cholesterol gradient. Because of the absence of cholesterol in either rHDL or phospholipid vesicles, exposure of these molecules...
to cells results largely in unidirectional transfer of cholesterol to these particles. In the present study, rHDL infusion was associated with an increase in size and a change in composition of HDL particles upon incorporation of rHDL. The phospholipid content of HDL particles almost doubled by the conclusion of the rHDL infusion. Thus, it is anticipated that these phospholipid-rich particles would have an increased ability to accept cholesterol from cells, consistent with the marked inhibitory effect of rHDL observed 4 hours after infusion. Seventy-two hours after infusion, particle composition was similar to that seen at baseline, consistent with a lack of effect of rHDL on platelet function observed at this time point. It is becoming increasingly apparent that phospholipids themselves can mediate various effects, and it is interesting to speculate that infusion of phospholipids alone may result in an attenuation of platelet signaling. Phospholipids are an attractive alternative to rHDL; however, the way in which phospholipid micelles would be metabolized in vivo remains to be determined. Interestingly, phospholipid infusion is associated with a reduction in atherosclerosis in vivo.

In the present study, the changes in efflux associated with rHDL preceded the attenuation in platelet function. The downstream response to changes in membrane cholesterol content is very often cooperative, requiring cholesterol levels to change beyond threshold. It is possible that removal of a threshold amount of cholesterol is required for subsequent alterations in signaling, which would account for the observed delay. Interestingly, we observed a discrepancy between the effects of native HDL on platelet function and cholesterol efflux. Treatment with native HDL cholesterol was associated with enhanced cholesterol efflux but minimal effect on platelet function. Native HDL contains free cholesterol, allowing this particle to participate in bidirectional flux of cholesterol with platelets (ie, efflux may be accompanied by influx), resulting in minimal net change to the cholesterol content of the platelet membrane. rHDL preparations, on the other hand, do not contain cholesterol; therefore, movement of cholesterol to these particles is unidirectional, resulting in changes in platelet cholesterol content. These findings raise the possibility of measuring subfractions of HDL, in this case, cholesterol-poor HDL particles such as pre-β-HDL, rather than total plasma HDL per se as a marker of platelet reactivity. In keeping with this, it would be anticipated that agents that increase lipid-poor HDL particles such as pre-β-HDL would provide the most benefit with respect to platelet reactivity.

The marked inhibitory effects of rHDL were observed in platelets from healthy donors with varying lipid profiles in which all in vitro experiments were performed. Therefore, it is conceivable that rHDL may have atherothrombotic protective effects in a broad population group, regardless of plasma lipid levels or diabetic status. Acute coronary syndromes are largely a platelet-mediated event, and although current antiplatelet therapies are associated with reduced ischemic events, there is scope for new therapies that reduce plaque instability. rHDL may achieve this via its pleiotropic effects, including improving endothelial function, reducing inflammation and oxidative stress, modulating plaque size, and as demonstrated in this study, reducing platelet hyperreactivity.

Limitations
The clinical study was performed in a small number of diabetic subjects, but the effects were marked and consistent among individuals. Furthermore, because subjects taking antiplatelet therapies were excluded from the study, it is unclear whether administration of rHDL would confer additional protection. Finally, bleeding time was not measured; however, it is conceivable that doses of rHDL can be used that effectively reduce thrombus formation without severely affecting hemostasis.

Conclusions
These findings demonstrate that rHDL therapy is highly effective at inhibiting the reactivity of platelets in individuals with diabetes mellitus, partly via the reduction of cholesterol content of platelet membranes and lipid raft disruption. These properties, combined with the known short-term beneficial effects of rHDL on atherosclerotic lesions and the recently demonstrated benefits on glucose metabolism, suggest that rHDL infusions may be an effective approach to reduce atherothrombotic complications in subjects with type 2 diabetes mellitus. Future studies are required to determine whether rHDL and long-term HDL-raising agents confer additional benefit to current antithrombotic agents without adversely affecting hemostasis.

Acknowledgments
We would like to thank CSL Behring AG, Bern, Switzerland, for supplying the rHDL (CSL-111) and Melissa Formosa and Donna Vizi for assistance with the clinical trial.

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Disclosures
None.

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**CLINICAL PERSPECTIVE**

Individuals with diabetes mellitus have an increased risk of cardiovascular disease and exhibit platelet hyperreactivity, increasing their resistance to antithrombotic therapies such as aspirin and clopidogrel. As a consequence, more potent antiplatelet therapeutic strategies are increasingly being used to reduce thrombotic complications in diabetes mellitus; however, these approaches have minimal impact on atherosclerotic lesions themselves, leading to persistent plaque instability. The studies presented here demonstrate the effectiveness of a single infusion of reconstituted high-density lipoprotein (HDL) in attenuating the reactivity of platelets from individuals with diabetes mellitus. Furthermore, these studies suggest that platelet hyperreactivity associated with cholesterol accumulation in platelet membranes can be reversed by reconstituted HDL through cholesterol depletion and disruption of lipid rafts. The greater platelet inhibitory properties of reconstituted HDL compared with native HDL suggest that agents that increase pre–β-HDL particles would have the greatest efficacy with respect to reducing platelet reactivity. Investigations to determine whether reconstituted HDL infusions or long-term HDL-raising agents confer additional benefits to current antithrombotic agents without adversely affecting hemostasis are required. The present findings add a new dimension to the known antiatherosclerotic actions of reconstituted HDL to provide a rationale for HDL-raising therapies as novel antithrombotic agents.
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ONLINE SUPPLEMENTARY METHODS

Blood collection: *In vitro* assays

Collection of blood from healthy donors was approved by the Monash University Standing Committee on Ethics in Research involving Humans (SCERH; project # CF07/0125 – 2007/0005) as previously described.¹

Preparation of lipids

rHDL (CSL-111) was supplied by CSL Behring AG (Bern, Switzerland). Human native HDL (density=1.085-1.21g/ml) was isolated from whole, pooled plasma by ultracentrifugation, as previously described.² Lipid-free apolipoprotein AI (apoAI) was isolated and purified from HDL as previously described.² For phospholipid (PL) preparation soy bean phosphatidylcholine (Sigma Aldrich, St Louis, MO) solution in chloroform was dried under nitrogen after which PBS was added. Samples were sonicated and then multilamellar micelles precipitated by ultracentrifugation at 100,000 rpm for 1 hour at 4°C. The supernatant was removed and kept at 4°C for experimental use. PL was used at a 150:1 molar ratio PL:apoAI as per rHDL. Methyl-β cyclodextrin (CD) was diluted to a working solution (10mM) using PBS. To prepare a CD-cholesterol (CDchol) complex, cholesterol was dissolved in methanol/chloroform (1:1; vol/vol), and dried under nitrogen. CD was added and then the sample was vortexed and sonicated in a water bath for 2 minutes, producing a sample of 100% saturated cyclodextrin:cholesterol. This solution was incubated at 37°C in a rotating wheel overnight and sterilised through a 0.44μm filter prior to experimental use.
**Platelet preparation**

The small volume of blood available from the clinical trial necessitated reduced centrifugal speeds.

**Aggregation assays**

Platelet aggregation in response to ADP (Sigma, St Louis, MO), Horm type I collagen (Kollagen reagens, Horm Suspension; Nycomed, Austria) and collagen-related peptide (Dept Matrix Biochemistry, Cambridge University) were performed as previously described\(^1,3\). For receptor blocking studies, platelets were incubated with an SR-BI blocking antibody (Novus Biologicals; Littleton, CO) at 37°C for 15 minutes prior to incubation with rHDL or PBS.

**Integrin \( \alpha_{IIb}\beta_3 \) activation**

Integrin \( \alpha_{IIb}\beta_3 \) activation was assessed by quantification of oregon green-conjugated fibrinogen (20µg/mL; Molecular Probes, Eugene, OR) binding in response to ADP (5µM) or collagen-related peptide (1µg/mL) as described previously.\(^1\)

**P-selectin expression**

Washed platelets (5x10\(^7\)/mL) treated with PBS or rHDL for 2 hours at 37°C were stimulated with thrombin (1U/mL; Parke David, New York, NY) for the indicated times in the presence of anti-P-selectin antibody (AK4; 1µg/mL; Santa Cruz Biotechnology, Santa Cruz, CA) as described previously.\(^3\)
**In vitro platelet thrombus formation**

Human anti-coagulated whole blood (800U/mL lepirudin; Pharmion, Summit, NJ) was incubated with rHDL at a dose equivalent to the final concentration of apoAI at the conclusion of the rHDL infusion (1.6mg/ml) at 37°C for approximately 1 hour. Thrombi were formed under flow conditions as described previously. Thrombus formation was visualised (DiOC₆; 1µg/mL) and analysed for volume and height in a given field (covering 25,058.89µm²) using an inverted Leica DMIRB confocal microscope (Leica Microsystems, Germany) with Metamorph 6.0 software (Molecular Devices, Downingtown, PA).

**Western Blotting**

THP-1 cells were incubated for 18 hours in the absence or presence of the liver X receptor agonist, TO-901317, known to increase ATP-binding cassette transporter (ABCA1) expression. Platelets and THP-1 cells were resuspended in 5mM cold Tris-HCl (pH 7.5) containing protease inhibitors. Samples were lysed, clarified by centrifugation and membranes isolated by ultracentrifugation (100,000rpm, 1 hour, 4°C). Pellets were resuspended in sample buffer and analysed by 8% SDS-PAGE gel. ABCA1 expression was detected using H10 antibody (1:2000; Abcam, UK). Scavenger receptor BI (SR-BI) expression was detected using an anti-SR-BI antibody (1:2000; Novus Biologicals; Littleton, CO). Antibody binding was detected using HRP-conjugated secondary antibodies following by enhanced chemiluminescence.
**Cholesterol Efflux**

Washed platelets were loaded with 1α,2α(n)-[^3]H]-cholesterol (1µCi/mL; GE Healthcare, UK) for approximately 3 hours. Samples were washed and subsequently incubated with treatments then samples were pelleted, supernatant removed and platelets lysed. Aliquots of supernatant and lysed platelets were counted in liquid scintillation on a beta counter in triplicate (Beckman Coulter, Fullerton CA). Cholesterol efflux was expressed as radioactivity in supernatant as a percentage of total radioactivity (supernatant plus platelets).
REFERENCES


Supplementary Figure 1: Platelet response to rHDL in diabetic subjects. Oregon-green labelled fibrinogen binding as a marker of $\alpha_{\text{IIb}}\beta_3$ activation in response to rHDL (50µg/mL); D=diabetes; Data expressed as mean±SEM; *p<0.05 vs untreated; n=11/group.