Effects of HDL Elevation with CETP Inhibition on Insulin Secretion

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Running title: HDL, CETP Inhibition and Insulin Secretion

Subject codes:
[90] Lipid and lipoprotein metabolism
[112] Lipids
[190] Type 2 diabetes

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In April 2013, the average time from submission to first decision for all original research papers submitted to Circulation Research was 13.5 days.
ABSTRACT

Rationale: High-density lipoprotein (HDL) cholesterol elevation via cholesteryl ester transfer protein (CETP) inhibition represents a novel therapy for atherosclerosis, which may also have relevance for type 2 diabetes.

Objective: The current study assessed the effects of a CETP inhibitor (CETPi) on postprandial insulin, ex vivo insulin secretion and cholesterol efflux from pancreatic β-cells.

Methods and Results: Healthy participants received a daily dose of CETPi (n=10) or placebo (n=15) for 14 days in a randomized, double-blind study. Insulin secretion and cholesterol efflux from MIN6N8 β-cells was determined following incubation with treated plasma. CETP inhibition increased plasma HDL cholesterol, apoAI and postprandial insulin. MIN6N8 β-cells incubated with plasma from CETPi-treated individuals (vs placebo) exhibited an increase in both glucose-stimulated insulin secretion (GSIS) and cholesterol efflux over the 14 day treatment period.

Conclusions: CETP inhibition increased postprandial insulin and promoted ex vivo β-cell GSIS, potentially via enhanced β-cell cholesterol efflux.

Keywords: Cholesterol Homeostasis, HDL cholesterol, insulin secretion, lipoproteins, oxidized low-density lipoprotein (LDL), type 2 diabetes mellitus

Non-standard Abbreviations and Acronyms:

ABCA1  ATP binding cassette transporter A1
ABCG1  ATP binding cassette transporter G1
AMREP  Alfred Medical Research and Education Precinct
ApoAI  Apolipoprotein AI
CETPi  Cholesteryl ester transfer protein inhibitor
GIP  Gastric inhibitory polypeptide
GLP-1  Glucagon-like peptide 1
GSIS  Glucose-stimulated insulin secretion
KRBB  Krebs-Ringer Bicarbonate buffer
LXR  Liver-X-receptor
oxLDL  Oxidized low-density lipoprotein
rHDL  Reconstituted high-density lipoprotein
TBARS  Thiobarbituric acid reactive substances

INTRODUCTION

Recent clinical studies indicate that high-density lipoprotein (HDL) cholesterol exerts beneficial actions beyond cardiovascular protection, which may extend to type 2 diabetes. We have previously shown that reconstituted HDL (rHDL) infusion in individuals with type 2 diabetes can increase plasma insulin and lower circulating glucose. The actions of HDL and its major protein, apolipoprotein AI (apoAI), to enhance insulin secretion and to stimulate glucose uptake in skeletal muscle suggest that HDL-raising agents may have efficacy in improving β-cell function and glucose homeostasis. These
findings raise the possibility that HDL-elevating agents currently under development for treatment of atherosclerotic coronary disease may have therapeutic relevance for the management of type 2 diabetes.7,8

Pancreatic β-cell dysfunction is a hallmark of late stage type 2 diabetes.9 Underlying mechanisms include lipid accumulation with subsequent inflammation,10 oxidative stress11 including lipid peroxidation12 and endoplasmic reticulum stress.13 HDL may protect against β-cell dysfunction and increase glucose-stimulated insulin secretion (GSIS) via cellular cholesterol removal2,4,14-16 and/or direct signaling actions.3 Evidence for the emerging role of HDL in glucose metabolism has been recently reviewed.17-19

Previous cellular, animal and human studies suggest that raising plasma HDL cholesterol may modulate insulin secretion.5,20-22 Cholesteryl ester transfer protein (CETP) inhibition raises plasma HDL cholesterol via a reduction in the transfer of neutral lipids (triglycerides and cholesteryl esters) between HDL and triglyceride-rich lipoprotein particles. The efficacy of this strategy to improve cardiovascular outcomes has not yet been determined and there is controversy regarding whether CETP inhibition might produce a dysfunctional form of HDL cholesterol, which is less efficient at promoting reverse cholesterol transport than native HDL.23 This controversy has arisen in part due to the early termination of two CETP inhibitor trials due to adverse events in the case of torcetrapib24 and more recently, lack of efficacy with dalcetrapib.25 Despite this, the CETP inhibitor (CETPi) class of HDL-elevating agents remains the most advanced therapeutic strategy for long-term HDL elevation with two agents currently in late phase clinical development (Phase III) for the management of coronary artery disease.8,26

While the primary focus of CETP inhibition has been on cardiovascular outcomes, a sub-study of the Investigation of Lipid Level Management to Understand its Impact in Atherosclerotic Events (ILLUMINATE) trial reported favorable effects on glucose metabolism in patients with type 2 diabetes when treated with torcetrapib.20 While the mechanisms leading to improved glycemic control were not investigated, we hypothesize that enhanced β-cell function is a likely contributor.

The current study investigated whether raising HDL cholesterol via CETP inhibition can promote insulin secretion in humans. In a randomized, placebo-controlled parallel design clinical trial of a 14 day CETPi intervention, plasma indices of glycemic control were measured. Subsequent ex vivo analyses of MIN6N8 pancreatic β-cell function were performed, including insulin secretion and cholesterol efflux following treatment with plasma from CETPi vs placebo-treated individuals.

METHODS

Human CETPi trial.
To examine the effects of HDL-elevation on insulin secretory function, a randomized, placebo-controlled parallel design clinical trial was performed. The CETPi intervention period was 14 days. Twenty-five healthy males (n=10 CETPi, n=15 placebo) gave written informed consent to participate in the study, which was approved by the Ethics Committee of the Two Basels (Switzerland). A fasting blood sample was taken prior to a standardized breakfast (10.4g fat, 18.5g protein, ≤80g carbohydrate; total energy 1989kJ) and a 15min postprandial blood sample was also taken immediately before a once daily oral dose (420mg) of CETPi (RG7232, PCT #WO2007090748) or matching placebo.

Plasma analyses.
Fasting and 15min postprandial blood was collected on the first (day 1) and last day (day 14) of the study and the plasma was stored at -80°C until analysis. Total, HDL and low-density lipoprotein (LDL) cholesterol and triglyceride concentrations were measured in fasting plasma samples on a Roche Modular
System using either enzymatic methods or direct homogeneous assays (Modular Analytics D2400 module, Roche Diagnostics, IN). Apolipoprotein AI and apoAII were measured using an automated nephelometer (Dade Behring BNII, Siemens Healthcare Diagnostics, IL) in fasting plasma after depletion of apoB-containing lipoproteins by a precipitation method using 0.1% 1.1 dextralip/MgCl₂ (Sigma-Aldrich, Sydney, NSW, Australia). HDL-associated free cholesterol and triglyceride concentration were measured using an enzymatic colorimetric assay on a chemistry immuno-analyzer (Olympus AU 2700, Olympus, PA). Human ELISA kits were used to measure plasma insulin (EZHIASF-14K, Millipore Australia, Kilsyth, VIC, Australia), gastric inhibitory polypeptide (GIP; EZHGIP-54K, Millipore Australia) and glucagon-like peptide 1 (GLP-1; EZGLP1T-36K, Millipore Australia) concentrations in postprandial samples taken on day 1 and day 14. Postprandial C-peptide, fasting and postprandial glucose concentrations were all measured on an automated analyzer (Architect ci16200, Abbott Diagnostics, IL).

To evaluate the efficacy of the CETP inhibitor, plasma CETP activity was measured using a homogeneous, fluorometric assay kit (Roar Ex-vivo CETP Activity Assay, Roar Biomedical, NY).

**Plasma preparation for ex vivo analyses.**

To study the effects of HDL, plasma was prepared for ex vivo studies by precipitating out apoB-containing lipoproteins via incubation with 0.1% 1.1 dextralip/MgCl₂ (Sigma-Aldrich) followed by centrifugation at 20,817 x g for 30min to remove very low-density lipoprotein (VLDL) and LDL particles.¹ Supernatant was dialyzed against PBS/EDTA using tube-o-dialyzers (G biosciences; St Louis, MO) to remove all molecules less than 15,000Da, including insulin and glucose and subsequently referred to as CETPi-plasma or placebo-plasma.

**LDL oxidation.**

LDL (density = 1.019-1.063g/ml) was isolated from the plasma of healthy individuals by sequential ultracentrifugation, as previously described.¹ The lipoproteins were then dialyzed overnight against PBS/EDTA at 4°C. For the ex vivo cholesterol efflux and insulin secretion assays, LDL was oxidized by incubating 1.5mg/ml LDL protein with 10μmol/L CuSO₄ for 110min at RT in the dark. Oxidation was stopped by adding EDTA at 60μmol/L at RT. The extent of lipoperoxide formation was quantitated by measuring thiobarbituric acid reactive substances (TBARS) in a validated assay.²⁷ Duplicate standards and oxLDL samples were mixed with two volumes of ice-cold 10% (w/v) trichloroacetic acid (Sigma-Aldrich) and 3 volumes of 1% (w/v) thiobarbituric acid (Sigma-Aldrich) and incubated at 95°C for 30min. Samples were centrifuged (16,200 x g, 5min) at RT before 200μl of the supernatant was transferred to a 96-well plate and absorbance measured at 532nm using a Bio-Rad plate reader (Bio-Rad Laboratories, Gladesville, NSW, Australia). An 8-point calibration curve was prepared using 1,1,3,3-tetramethoxypropane (0-100μM) and results expressed as nmol of malondialdehyde (MDA) equivalents/mg LDL protein. The oxLDL used for the ex vivo assays was not fully oxidized as indicated by the TBARS assay (27nmol MDA equivalents/mg LDL protein) and compared to previous studies.²⁸, ²⁹

**Ex vivo insulin secretion from MIN6N8 pancreatic β-cells.**

Insulin secretion was assessed in murine MIN6N8 pancreatic β-cells provided by Prof Jun-ichi Miyagaki (Osaka University, Osaka, Japan)³⁰ using a modified protocol.³¹ MIN6N8 pancreatic β-cells were grown in 5mmol/L glucose DMEM supplemented with 10% FCS plus 1% penicillin/streptomycin (37°C, 95% O₂/5% CO₂) to sub-confluence then placed in 5mmol/L glucose DMEM with 2% FCS. Cells were incubated with 3.5% CETPi-plasma, placebo-plasma, lipoprotein-free plasma, HDL (1.8mmol/L)-spiked plasma, DMSO or 100ng/ml CETPi for 72h along with oxidized low-density lipoprotein (oxLDL) (50μg/ml) to simulate the pathophysiology of type 2 diabetes.², ³², ³³ This is a well-validated technique to cholesterol-load cells²⁶, ³⁴ and an appropriate model to examine whether plasma HDL elevation in individuals treated with the CETPi increased GSIS and cholesterol efflux. Treatment of MIN6N8 pancreatic β-cells with oxLDL (50μg/ml) had no adverse effect on cell morphology, culture confluence or key apoptotic (Bax, Casp3, Bclx, Ptpn2, Bim & Puma) or anti-apoptotic (Bcl2) gene markers involved in different cell death pathways (data not shown). Basal insulin secretion was determined after incubation in

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DOI: 10.1161/CIRCRESAHA.113.300689
low (2.8mmol/L) glucose Krebs-Ringer Bicarbonate buffer (KRBB) for 30min whereas first phase (20min) and second phase (40min) GSIS were measured in response to separate 20mmol/L glucose boluses. Cells were lyzed in water and sonicated for 15min at 4°C to determine intracellular insulin. Secreted and intracellular insulin were measured using a rat/mouse insulin ELISA kit (EZRMI-13K, Millipore Australia). Total insulin was calculated as the sum of insulin secreted during the basal, first and second phases, plus intracellular insulin at the end of the experiment. Insulin secretion data are expressed as either a percentage change in absolute insulin secretion at day 14 compared to day 1 (Figure 1) or insulin secretion rate (ng/ml/hr; Table 4).

**Ex vivo cholesterol efflux from MIN6N8 pancreatic β-cells.**

Cholesterol efflux assays were performed using a modified protocol as described previously. MIN6N8 pancreatic β-cells were grown in 5mmol/L glucose DMEM with 10% FCS to sub-confluence then labelled with [1α,2α(n)-3H]cholesterol (1μCi/ml; GE Healthcare, UK) in ethanol vehicle (<0.4%) and oxLDL (50μg/ml) to facilitate cholesterol loading for 48h at 37°C (95% O2/5% CO2). Cells were washed with PBS then treated with the liver-X-receptor (LXR) agonist (TO-901317, 1μmol/L) to stimulate ABCA1 expression in serum-free DMEM for 18h. Human HDL (10, 25 and 50μg/ml) and apoAI (20μg/ml) were used as positive controls. Experimental treatments included CETPi-plasma (n=5) and placebo-plasma (n=5) collected on day 1 and day 14 of the treatment period, at a concentration of 3.5% v/v. This plasma dilution was selected on the basis of preliminary experiments showing that 3.5% plasma elicited cholesterol efflux of a magnitude similar to 50μg/mL of HDL; a concentration in the mid-range of the cholesterol efflux dose-response curve. This was the same dilution used in the insulin secretion experiments. [3H]cholesterol was measured in quadruplicate samples after a 3h cholesterol efflux assay into serum-free DMEM, which was centrifuged at 15,700 x g for 5min. Cells were lyzed in 0.1M NaOH/0.1% SDS and assayed for protein content. [3H]cholesterol in the medium and cells was quantified by liquid scintillation counting, from which the percentage of cholesterol released was determined. Cholesterol efflux data are expressed as the proportion of [3H]cholesterol transferred from cells to the medium.

**Statistical analysis.**

Data were analyzed using unpaired two-tailed Student’s t-tests when only two experimental groups were compared and for more than two groups by one-way ANOVA or repeated measures one-way ANOVA as appropriate and where significant (p<0.05), comparison of individual means was performed with a Tukey HSD test. Correlation analysis was performed using the Pearson coefficient. Results are expressed as mean ± SD. All analyses were conducted using SPSS (v16) and Sigmastat (v3.5). p<0.05 was deemed significant.

**RESULTS**

**Effect of CETPi on human plasma parameters.**

There were no differences in age (CETPi: 32±9 vs placebo: 35±9 years) or body mass index (CETPi: 23.0±1.0 vs placebo: 24.5±0.5kg/m²) between groups. There were also no pre-dosing differences in CETP activity at baseline between the treatment groups (Table 1). The efficacy of the CETPi was confirmed with more than 80% inhibition of plasma CETP activity achieved for up to 12h post-dose on both day 1 and day 14 of the treatment period (Table 1).

CETP inhibition increased plasma HDL cholesterol by 46%, HDL-associated apoAI by 22%, apoAII by 10% (p<0.05) and free cholesterol by 70%, and decreased plasma LDL cholesterol by 32% (p<0.0001 vs placebo unless otherwise stated, Table 2). Circulating triglycerides were also lower after
CETPi treatment (*p*<0.05), but not as a constituent of HDL (Table 2). The increase in postprandial insulin (+75%) after the 14 day CETPi intervention was greater than with placebo (+42%) treatment (*p*<0.05, Figure 1A). The change in plasma C-peptide concentration mirrored that of insulin, though failed to reach statistical significance (*p* = 0.08; Figure 1B). Despite the increase in insulin, there was no difference between groups in fasting or postprandial glucose measured 15min after a standardized breakfast (Table 2). Importantly, there was no effect of the CETPi on the change in postprandial plasma GIP (Figure 1C) or GLP-1 (Figure 1D) concentrations.

Over the 14 day intervention period there was a strong correlation between the change in plasma HDL cholesterol and apoAI (*r*=0.90, *p*<0.001). The change in both plasma HDL and apoAI correlated significantly with the change in postprandial insulin (HDL *r*=0.53, *p*<0.01; apoAI *r*=0.41, *p*<0.05, Table 3).

Effect of CETPi-plasma on insulin secretion from mouse pancreatic β-cells.

There was no difference between groups (CETPi-plasma vs placebo-plasma) in the absolute rate (Table 4) or percentage change (day 14 - day 1) in basal insulin secretion (Figure 2A). Glucose-stimulated insulin secretion was higher after treatment with the CETPi-plasma. The percentage change (from day 1) in both first phase (+41±91%) and second phase (+32±49%) GSIS was greater after exposure to CETPi-plasma compared with placebo-plasma (*p*<0.05, Figure 2B & C). Additionally, when the data were expressed as a rate, the absolute change (day 14 - day 1) in first phase (*p* = 0.05, Table 4) and second phase (*p*<0.05, Table 4) GSIS were higher after treatment with CETPi-plasma compared with placebo-plasma. The change in total insulin remaining in lysed pancreatic β-cells after 14 days of treatment was correspondingly lower after incubation with CETPi-plasma compared with placebo-plasma (*p*<0.05, Table 4). The change in GSIS between day 1 and 14 in the placebo group was not significant. There was no significant correlation between the change in either plasma HDL or apoAI and the change in first or second phase GSIS (Table 3).

To test whether HDL can stimulate insulin release, MIN6N8 pancreatic β-cells were treated with lipoprotein-free plasma and plasma spiked with human HDL at a concentration (1.8mmol/L) equivalent to that achieved after CETPi treatment in study participants. The data show a 7.6±0.6-fold increase in first phase (n=6, *p*<0.0001) and a 2.7±0.3-fold increase in second phase insulin secretion (n=6, *p*<0.001) in the HDL-spiked plasma relative to lipoprotein free plasma.

Importantly, direct incubation with the CETPi drug at concentrations equivalent to those found in plasma following the 14 day treatment period resulted in no change in either basal insulin secretion or first and second phase GSIS (Table 5). There was also no change in total insulin remaining in the lysed cells following direct incubation with the drug (Table 5).

Effect of CETPi-plasma on cholesterol efflux from mouse pancreatic β-cells.

Incubation of cholesterol-loaded MIN6N8 pancreatic β-cells with human HDL stimulated net cholesterol efflux with a linear dose-dependent relationship (Figure 3A). ApoAI also acted as a cholesterol acceptor, resulting in a greater than two-fold increase in cholesterol efflux even at a relatively low concentration (20μg/ml) (Figure 3A). There was a nine-fold increase in cholesterol efflux at the highest dose of HDL (50μg/ml) when compared to control with no acceptor (Figure 3A), which is equivalent to the magnitude of efflux achieved with 3.5% plasma (Figure 3B). The absolute change (day 14 - day 1) in cholesterol efflux to CETPi-plasma (1.23±0.39%) was 12-fold greater than for placebo-plasma (0.10±0.13%, *p*<0.001, Figure 3B). Both the change in plasma HDL cholesterol and apoAI concentrations correlated strongly with the change in cholesterol efflux (r=0.78 and 0.70 respectively, *p*<0.05, Table 3).
DISCUSSION

We report here for the first time that CETP inhibition in healthy humans increases postprandial insulin and ex vivo GSIS. These observations provide clinical context to previous work linking HDL with insulin secretion in cell and animal models,\textsuperscript{1, 3, 5} as well as human studies of both a single HDL infusion\textsuperscript{*} as well as HDL elevation over 6 months.\textsuperscript{20} These data are consistent with the hypothesis that HDL elevation via CETP inhibition may improve β-cell function and postprandial insulin secretion in individuals with type 2 diabetes to mitigate harmful postprandial glycemic excursions.\textsuperscript{36, 38, 39} This possibility requires testing in large CETPi clinical trials involving patients with type 2 diabetes.

Following CETP inhibition for 14 days, postprandial insulin and C-peptide increased more than two-fold when compared to placebo. Furthermore, the change in plasma HDL cholesterol and apoAI positively correlated with the change in postprandial insulin over that same treatment period. The postprandial sample was taken 15 minutes after a standardized meal when glucose concentrations in the placebo and CETPi-treated groups were equivalent. This suggests that for a given postprandial rise in glucose (2-3mmol/L in this study) in these healthy individuals, CETPi treatment resulted in a greater increase in plasma insulin. Importantly, this was not due to an effect on the major incretins, GIP and GLP-1.

It is anticipated that the higher circulating insulin would enhance postprandial glucose clearance after CETPi-induced HDL elevation, though blood was not sampled at later time points. Increased postprandial insulin could benefit those with established type 2 diabetes, who typically have high fasting insulin, but impaired β-cell sensitivity and insulin secretory response to a glucose load. This latter effect generally manifests itself as a blunted postprandial first phase insulin response resulting in a greater plasma glucose excursion and delayed post-meal recovery.\textsuperscript{37} CETP inhibition thus has the potential to improve postprandial glycemic control without disturbing the incretin response.

In a previous study we reported that HDL at a concentration of 50μg/ml, which equates to an apoAI concentration of approximately 40μg/ml, rescues oxLDL-induced reductions in MIN6N8 pancreatic β-cell GSIS, but has no effect alone.\textsuperscript{1} The current study develops a therapeutic context, showing that apoB-depleted, dialyzed plasma taken from CETPi-treated (vs placebo-treated) individuals, increases first and second phase GSIS from MIN6N8 pancreatic β-cells pre-treated with oxLDL. Importantly, this effect was shown to be specific to glucose-stimulated insulin responses as basal insulin secretion was not altered.

The insulin secretory effect is most likely due to the HDL elevation induced by the CETPi since there was no direct effect of the CETPi on insulin secretion and lipoprotein free plasma spiked with HDL at the concentration (1.8mmol/L) equivalent to that achieved after CETPi treatment in study participants stimulated significant insulin secretion. HDL-elevating therapies, such as CETP inhibition may therefore augment β-cell sensitivity to glucose, resulting in enhanced GSIS and improved glucose tolerance without exacerbating the characteristic fasting hyperinsulinemia observed in those individuals with type 2 diabetes.\textsuperscript{20}

While plasma HDL cholesterol and apoAI positively correlated with the change in postprandial insulin, the weaker relationships between the change in first and second phase GSIS and plasma HDL/apoA-I would be anticipated given the more complex and variable nature of the insulin secretion assay, compared with direct measures of plasma insulin. Specifically, the insulin secretion assay includes variables related to the MIN6 cell culture including variability in cell density, culture conditions, cell viability and response to oxLDL treatment. In addition, the plasma preparation used to stimulate insulin secretion involves multiple preparation steps including ultracentrifugation, dialysis and dilution, all of which could introduce further variability.
Mechanisms.

There are a number of mechanisms that may explain the observed increase in GSIS from MIN6N8 pancreatic β-cells after incubation with plasma from individuals treated with the CETPi (CETPi-plasma). Given the higher plasma HDL cholesterol concentrations after CETP inhibition and the important role of HDL and apoAI in reverse cholesterol transport, a reduction in β-cell lipid content is one possible explanation. Our results show functional cholesterol efflux from MIN6N8 pancreatic β-cells to human plasma. Most importantly, efflux to CETPi-plasma was increased over the 14 day treatment period with no change in efflux to placebo-plasma. Interestingly, the change in plasma HDL cholesterol and apoAI across the treatment period strongly correlated with the change in ex vivo cholesterol efflux. Consistent with previous literature linking HDL to insulin secretion from β-cells,2, 4, 14-16 our observations suggest that CETP inhibition may modulate insulin secretion via changes in net cholesterol efflux. Cholesterol accumulation has been identified as an important precursor to β-cell dysfunction and failure, particularly in the context of type 2 diabetes.3, 16, 40, 41 These pathologies can be largely rescued in vitro by reducing β-cell cholesterol content with statins or methyl-β-cyclodextrin.41, 42 Combined, these data suggest that abnormalities in cholesterol homeostasis may contribute to impaired β-cell function typical of type 2 diabetes and that this may be rescued by CETP inhibition.

It is also possible that HDL and apoAI may increase insulin secretory capacity via direct signaling actions independent of lipid removal. Using apoAI at a concentration approximately 20-fold higher (864μg/ml) than our earlier study,1 Fryirs et al. reported an increase in basal insulin secretion and GSIS from mouse pancreatic β-cells, in the absence of cholesterol loading with oxLDL.3 This effect was observed without depletion of intracellular cholesterol, although functional cholesterol efflux was not measured. These results suggest that at these higher doses, apoAI may act as an insulin secretagogue, perhaps acting via direct signaling pathways linked to the HDL transporters, ATP binding cassette transporter A1 (ABCA1) and ABCG1.3, 14, 15, 17 A critical role for ABCA1 and cellular cholesterol has recently been reported for regulating insulin granule fusion events in pancreatic β-cells.43

The facilitative role of ABCA1 in β-cell insulin secretion, whether via cholesterol removal or direct signaling, is supported by the observation that insulin secretory response is defective in mice with a β-cell specific deletion of the ABCA1 transporter. This is also true for patients with loss of function mutations in the ABCA1 gene with low circulating HDL.14, 40 Furthermore, the absence of both ABCA1 and ABCG1 induces greater defects in β-cell function than deficiency of either transporter individually, suggesting that these transporters provide important and complementary contributions to β-cell function by maintaining islet cholesterol homeostasis in vivo.45 At a cellular level, cholesterol content can affect membrane fluidity and permeability,46 as well as cell signaling via membrane microdomains or sterol regulatory element-binding proteins.3, 47

Clinical context.

While no large clinical trials have directly examined the long-term effects of HDL-raising agents on postprandial glycemic control, circumstantial evidence of a beneficial effect is emerging. Post-hoc analyses following the ILLUMINATE trial showed that patients treated with torcetrapib in conjunction with atorvastatin had lower fasting glucose (-0.34mmol/L) and HbA1c (-0.33%) at 3 months compared with those on the statin alone.20 This was observed in addition to conventional management of type 2 diabetes with hypoglycemic agents and despite lower fasting insulin in the CETPi-treated group.20 The improved glucose control in these patients may be due to a combination of factors including increased circulating HDL cholesterol and apoAI with a concomitant reduction in LDL cholesterol, improvement in β-cell function or enhanced insulin sensitivity and skeletal muscle glucose uptake, as we and others have shown previously.1, 6 As glycemic control was not the primary endpoint in the ILLUMINATE trial, no
postprandial glucose or insulin data were collected. Current large clinical trials examining the effects of potent second generation CETP inhibitors, including anacetrapib and evacetrapib on cardiovascular events provide an ideal opportunity to study the effects of HDL elevation on glucose homeostasis in more detail.

Limitations.

Ideally, a more comprehensive time-course study of CETPi actions on circulating insulin and glucose would have been undertaken. In vivo assessment of insulin secretion would also have been informative in these individuals. However, this clinical trial was not designed to assess these parameters and the necessary investigations were either not performed or the plasma was not available. Despite these shortcomings, the current study provides evidence that HDL elevation via CETP inhibition can influence postprandial insulin responses and therefore supports a rationale for more detailed investigations.

Conclusion.

The current study links HDL cholesterol to insulin secretion via novel actions of CETP inhibition in raising postprandial insulin in healthy individuals in association with enhanced ex vivo cholesterol efflux and GSIS from MIN6N8 pancreatic β-cells. These data provide clinical context for investigation of HDL-raising agents as potential therapies for the management of type 2 diabetes. Of particular interest is the possibility that CETP inhibition, via effects on β-cell insulin secretion, may reduce harmful postprandial glycemic excursions and associated vascular complications.

ACKNOWLEDGMENTS

F. Hoffman La Roche (Basel, Switzerland) provided plasma from their clinical trial using the CETPi (RG7232, Patent Cooperation Treaty #WO2007090748), but provided no funding for the study.

SOURCES OF FUNDING

This work was supported by the National Health and Medical Research Council of Australia (NHMRC ID: 586626); The CASS Foundation (SM/10/3026); Perpetual Philanthropic Services (#442); and the Victorian Operational Infrastructure Support Program. DS, JMF and BAK are supported by NHMRC Fellowships.

DISCLOSURES

None

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Table 1. Human CETPi trial – Mean CETP activity

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n=15)</th>
<th>CETPi (n=10)</th>
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<tr>
<td><strong>CETP activity</strong> (pmol/ml/min)</td>
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<tr>
<td><strong>Day 1 – 0hr</strong></td>
<td>11.1±5.9</td>
<td>12.9±5.6</td>
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<tr>
<td><strong>Day 1 – 12hr</strong></td>
<td>16.1±6.2</td>
<td>0.1±0.4*</td>
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<td><strong>Day 14 – 0hr</strong></td>
<td>9.3±5.4</td>
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<td><strong>Day 14 – 12hr</strong></td>
<td>16.4±7.6</td>
<td>0.8±1.2*</td>
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Data are presented as mean±SD; Measures on Day 1 – 0hr are baseline data; *denotes significant (p<0.05) difference vs Placebo.
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<tr>
<th></th>
<th>Placebo day 1 (n=15)</th>
<th>Placebo day 14 (n=15)</th>
<th>Change</th>
<th>CETPi day 1 (n=10)</th>
<th>CETPi day 14 (n=10)</th>
<th>Change</th>
<th>p-value Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.8±0.8</td>
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<td>4.4±0.8</td>
<td>4.1±0.5</td>
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<td>HDL cholesterol (mmol/L)</td>
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<td>1.2±0.2</td>
<td>-</td>
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<td>LDL cholesterol (mmol/L)</td>
<td>2.9±0.8</td>
<td>3.0±0.6</td>
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<td>1.0±0.3</td>
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<td>Fasting glucose (mmol/L)</td>
<td>4.9±0.3</td>
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<td>4.8±0.5</td>
<td>4.5±0.3</td>
<td>-0.29±0.29</td>
<td>0.081</td>
</tr>
<tr>
<td>Postprandial glucose (mmol/L)</td>
<td>7.1±1.2</td>
<td>7.3±1.0</td>
<td>0.15±0.23</td>
<td>7.8±1.2</td>
<td>7.8±1.0</td>
<td>-0.03±0.18</td>
<td>0.705</td>
</tr>
</tbody>
</table>

**HDL compositional analysis:**

<table>
<thead>
<tr>
<th></th>
<th>ApoAI (mg/dL)</th>
<th>ApoAII (mg/dL)</th>
<th>Free cholesterol (mg/dL)</th>
<th>Triglycerides (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>131±14</td>
<td>128±13</td>
<td>-3.3±11.9</td>
<td>9.0±2.5</td>
</tr>
<tr>
<td></td>
<td>32±3.9</td>
<td>31±5.4</td>
<td>-1.0±3.7</td>
<td>10.4±2.8</td>
</tr>
<tr>
<td></td>
<td>9.3±2.5</td>
<td>8.7±2.6</td>
<td>-</td>
<td>1.4±2.6</td>
</tr>
<tr>
<td></td>
<td>9.0±2.5</td>
<td>10.4±2.8</td>
<td>1.4±2.6</td>
<td>8.2±1.7</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD, p-values from Student’s t-test; Measures on day 1 are baseline characteristics.
Table 3. Relationship between the change in plasma HDL cholesterol/apoAI and key parameters over the 14 day treatment period

<table>
<thead>
<tr>
<th></th>
<th>Plasma HDL cholesterol</th>
<th>Plasma apoAI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
</tr>
<tr>
<td>Postprandial insulin</td>
<td>0.53</td>
<td>0.007*</td>
</tr>
<tr>
<td>First phase GSIS</td>
<td>0.46</td>
<td>0.086</td>
</tr>
<tr>
<td>Second Phase GSIS</td>
<td>0.43</td>
<td>0.110</td>
</tr>
<tr>
<td>Cholesterol efflux</td>
<td>0.78</td>
<td>0.008*</td>
</tr>
</tbody>
</table>

GSIS: Glucose-stimulated insulin secretion; \( r \) = Pearson correlation coefficient; *denotes significant \((p<0.05)\) correlation between parameters.
Table 4. Effect of CETPi-plasma on GSIS from oxDL-treated MIN6N8 β-cells

<table>
<thead>
<tr>
<th></th>
<th>Placebo-plasma (n=5)</th>
<th>CETPi-plasma (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Basal insulin secretion over 30 minutes (ng/ml/hr)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>299±148</td>
<td>357±165</td>
</tr>
<tr>
<td>Day 14</td>
<td>289±68</td>
<td>342±146</td>
</tr>
<tr>
<td>Change</td>
<td>-10±126</td>
<td>-15±106</td>
</tr>
<tr>
<td><strong>First phase GSIS over 20 minutes (ng/ml/hr)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>740±581</td>
<td>400±361</td>
</tr>
<tr>
<td>Day 14</td>
<td>512±440</td>
<td>523±406</td>
</tr>
<tr>
<td>Change</td>
<td>-228±174</td>
<td>122±337</td>
</tr>
<tr>
<td><strong>Second phase GSIS over 40 minutes (ng/ml/hr)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>205±57</td>
<td>118±24</td>
</tr>
<tr>
<td>Day 14</td>
<td>145±43</td>
<td>154±66</td>
</tr>
<tr>
<td>Change</td>
<td>-61±40</td>
<td>37±62*</td>
</tr>
<tr>
<td><strong>Intracellular insulin content (ng/ml)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>2256±865</td>
<td>2408±1111</td>
</tr>
<tr>
<td>Day 14</td>
<td>2679±973</td>
<td>2358±942</td>
</tr>
<tr>
<td>Change</td>
<td>423±176</td>
<td>-50±362*</td>
</tr>
</tbody>
</table>

GSIS: Glucose-stimulated insulin secretion; Data are presented as mean±SD; *denotes significance (p<0.05) vs Placebo-plasma.
Table 5. Direct effect of the CETPi on GSIS from oxLDL-treated MIN6N8 β-cells

<table>
<thead>
<tr>
<th></th>
<th>DMSO vehicle (n=6)</th>
<th>100ng/ml CETPi (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal insulin secretion over 30 minutes (ng/ml/hr)</td>
<td>317±35</td>
<td>343±28</td>
</tr>
<tr>
<td>First phase GSIS over 20 minutes (ng/ml/hr)</td>
<td>322±130</td>
<td>419±83</td>
</tr>
<tr>
<td>Second phase GSIS over 40 minutes (ng/ml/hr)</td>
<td>75±28</td>
<td>84±19</td>
</tr>
<tr>
<td>Intracellular insulin content (ng/ml)</td>
<td>374±17</td>
<td>360±10</td>
</tr>
</tbody>
</table>

GSIS: Glucose-stimulated insulin secretion; Data are presented as mean±SD.
FIGURE LEGENDS

**Figure 1.** Effect of CETP inhibition on postprandial insulin, C-peptide, GIP and GLP-1 concentration. Postprandial A) insulin, B) C-peptide, C) GIP and D) GLP-1 concentration before and after the CETPi (n=8-10, black bar) intervention vs placebo (n=11-15, white bar) treatment, with mean concentrations on day 1 (base of bars) and day 14 (top of bars) shown within the bars. Data are presented as mean±SD. *denotes significance (p<0.01) vs Placebo.

**Figure 2.** Effect of CETPi-plasma on basal and glucose-stimulated insulin secretion from oxLDL-treated MIN6N8 pancreatic β-cells. MIN6N8 pancreatic β-cells were incubated with oxLDL (50μg/ml) and either 3.5% placebo-plasma (n=5, white bars) or CETPi-plasma (n=10, black bars) for 72h. Insulin concentration was measured in Krebs buffer after exposure to A) 2.8mmol/L glucose for 30min (basal insulin secretion); followed by B) 20mmol/L glucose for 20min (first phase glucose-stimulated insulin secretion (GSIS)); then C) 20mmol/L glucose for a further 40min (second phase GSIS). Data are expressed as a percentage change (day 14 – day 1) in absolute insulin content (mean±SD). *denotes significance (p<0.05) between the change (day 14 – day 1) in placebo-plasma vs CETPi-plasma.

**Figure 3.** Effect of CETPi-plasma on cholesterol efflux from oxLDL-treated MIN6N8 pancreatic β-cells. MIN6N8 pancreatic β-cells were incubated with oxLDL (50μg/ml) and A) different doses of human HDL (10, 25 & 50μg/ml; black bars) and apoAI (20μg/ml; hatched bar) or B) 3.5% placebo-plasma (n=5, white bar) vs CETPi-plasma (n=5, black bar) for 3h. Data are expressed as the change (day 14 – day 1) in the percentage of cholesterol released into the media (mean±SD). *denotes significance (p<0.05) between the change (day 14 – day 1) in placebo-plasma vs CETPi-plasma. **denotes significant (p<0.001) difference compared to Control.
Novelty and Significance

What Is Known?

- Protective effects of high-density lipoprotein (HDL) against cardiovascular disease risk have been linked with increased cholesterol efflux and reverse cholesterol transport.

- HDL has more recently been shown to modulate insulin secretion and stimulate glucose uptake into skeletal muscle.

- Cholesteryl ester transfer protein (CETP) inhibitors are HDL-raising agents currently in phase 3 clinical trials, but the effects of these agents on insulin secretion have not been examined.

What New Information Does This Article Contribute?

- Raising plasma HDL cholesterol by 48% and apoAI by 24% via CETP inhibitor therapy for 14 days increases postprandial plasma insulin concentration, in comparison with placebo treatment.

- Mouse pancreatic β-cells incubated with plasma from individuals treated with a CETP inhibitor exhibited an increase in both first and second phase glucose-stimulated insulin secretion and cholesterol efflux, with no effect on basal insulin secretion.

- The effects of CETP inhibitors on glucose-stimulated insulin secretion are likely mediated by HDL elevation.

HDL-raising agents are currently being tested for efficacy in reducing the risk of atherosclerotic cardiovascular disease; however, they may be effective also in the management of type 2 diabetes, which is commonly associated with dyslipidemia. The use of one CETP inhibitor is associated with improved glycemic control in patients with type 2 diabetes; but effects on insulin secretion were not examined. The current study shows that in healthy adults HDL elevation via CETP inhibition increases postprandial plasma insulin concentration in healthy individuals and that plasma from these individuals can increase glucose-stimulated insulin secretion and cholesterol efflux from mouse pancreatic β-cells. Furthermore, the elevations in plasma HDL cholesterol and apoAI positively correlated with the change in postprandial plasma insulin. These observations provide clinical therapeutic context to previous work linking HDL with insulin secretion in cell culture and animal models, as well as human studies of both single HDL infusion and longer-term HDL elevation over months. Raising HDL via CETP inhibition may thus improve β-cell function and postprandial insulin secretion in individuals with type 2 diabetes to minimise harmful postprandial glycemic excursions.
Figure 1

A. Δ Plasma insulin (pmol/L)

B. Δ Plasma C-peptide (pmol/L)

C. Δ Plasma GP (pmol/L)

D. Δ Plasma GLP-1 (pmol/L)
Figure 2

A

Δ Basal insulin secretion (% change from day 1)

Placebo-plasma
CETPi-plasma

B

Δ First phase GSIS (% change from day 1)

C

Δ Second phase GSIS (% change from day 1)

*
Figure 3

(A) Cholesterol efflux (% cholesterol released) for different treatments: Control, HDL 10 μg/ml, HDL 25 μg/ml, HDL 50 μg/ml, and apoAI 20 μg/ml. Significant differences are indicated by **.

(B) Cholesterol efflux (% cholesterol released) for placebo and CETPi at days 0 and 14. Significant difference indicated by *.

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Effects of HDL Elevation with CETP Inhibition on Insulin Secretion

Circ Res. published online May 15, 2013;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Supplemental Material

Detailed Methods

**Human CETPi trial**

To examine the effects of high-density lipoprotein (HDL)-elevation on insulin secretory function, a randomized, placebo-controlled parallel design clinical trial was performed. The cholesteryl ester transfer protein inhibitor (CETPi) intervention period was 14 days. Twenty-five healthy males (n=10 CETPi, n=15 placebo) gave written informed consent to participate in the study, which was approved by the Ethics Committee of the Two Basels (Switzerland). A fasting blood sample was taken prior to a standardized breakfast (10.4g fat, 18.5g protein, ≤80g carbohydrate; total energy 1989kJ) and a 15min postprandial blood sample was also taken immediately before a once daily oral dose (420mg) of CETPi (RG7232, PCT #WO2007090748) or matching placebo.

**Plasma analyses**

Fasting and 15min postprandial blood was collected on the first (day 1) and last day (day 14) of the study and the plasma was stored at -80ºC until analysis. Total, HDL and low-density lipoprotein (LDL) cholesterol and triglyceride concentrations were measured in fasting plasma samples on a Roche Modular System using either enzymatic methods or direct homogeneous assays (Modular Analytics D2400 module, Roche Diagnostics, IN). Apolipoprotein AI (apoAI) and apoAII were measured using an automated nephelometer (Dade Behring BNII, Siemens Healthcare Diagnostics, IL) in fasting plasma after depletion of apoB-containing lipoproteins by a precipitation method using 0.1% 1.1 dextralip/MgCl$_2$ (Sigma-Aldrich, Sydney, NSW, Australia). HDL-associated free cholesterol and triglyceride concentration were measured using an enzymatic colorimetric assay on a chemistry immuno-analyzer (Olympus AU 2700, Olympus, PA). Human ELISA kits were used to measure plasma insulin (EZHIAF-14K, Millipore Australia, Kilsyth, VIC, Australia), gastric inhibitory polypeptide (GIP; EZHGIP-54K, Millipore Australia) and glucagon-like peptide 1 (GLP-1; EZGLP1T-36K, Millipore Australia) concentrations in postprandial samples taken on day 1 and day 14. Postprandial C-peptide, fasting and postprandial glucose concentrations were all measured on an automated analyzer (Architect ci16200, Abbott Diagnostics, IL). To evaluate the efficacy of the CETP inhibitor, plasma CETP activity was measured using a homogeneous, fluorometric assay kit (Roar Ex-vivo CETP Activity Assay, Roar Biomedical, NY).

**Plasma preparation for ex vivo analyses**

To study the effects of HDL, plasma was prepared for ex vivo studies by precipitating out apoB-containing lipoproteins via incubation with 0.1% 1.1 dextralip/MgCl$_2$ (Sigma-Aldrich) followed by centrifugation at 20,817 x g for 30min (4ºC) to remove very low-density lipoprotein (VLDL) and LDL particles. To prepare lipoprotein-free plasma, all lipoproteins were removed from whole, pooled plasma from healthy individuals by adjusting plasma density (d<1.3g/mL) with KBr followed by ultracentrifugation at 65,000 x g for 24h (4ºC). Supernatant was dialyzed against PBS/EDTA using tube-o-dialyzers (G biosciences; St Louis, MO) to remove all molecules less than 15,000Da, including insulin and glucose and subsequently referred to as CETPi-plasma or placebo-plasma.

**LDL oxidation**

LDL (density = 1.019-1.063g/ml) was isolated from the plasma of healthy individuals by sequential ultracentrifugation. The lipoproteins were then dialyzed overnight against PBS/EDTA at 4ºC. For the ex vivo cholesterol efflux and insulin secretion assays, LDL was oxidized by incubating 1.5mg/ml LDL protein with 10μmol/L CuSO$_4$ for 110min at RT in the dark. Oxidation was stopped by adding EDTA at 60μmol/L at RT. The extent of lipoperoxide formation was quantitated by measuring thiobarbituric acid reactive substances (TBARS) in a validated assay. Duplicate standards and oxLDL samples were mixed with two volumes of ice-cold 10% (w/v) trichloroacetic acid (Sigma-Aldrich) and 3 volumes of 1% (w/v) thiobarbituric acid (Sigma-Aldrich) and incubated at 95ºC for 30min. Samples were centrifuged (16,200 x g, 5min) at RT before 200μl of the supernatant was transferred to a 96-well plate and absorbance measured at 532nm using a Bio-Rad plate reader (Bio-Rad Laboratories, Gladesville, NSW, Australia). An 8-point calibration curve was prepared using
1.1.3,3-tetramethoxypropane (0-100μM) and results expressed as nmol of malondialdehyde (MDA) equivalents/mg LDL protein. The oxLDL used for the ex vivo assays was not fully oxidized as indicated by the TBARS assay (27nmol MDA equivalents/mg LDL protein) and compared to previous studies.1,4

Ex vivo insulin secretion from MIN6N8 pancreatic β-cells
Insulin secretion was assessed in murine MIN6N8 pancreatic β-cells provided by Prof Jun-ichi Miyagaki (Osaka University, Osaka, Japan)5 using a modified protocol.6 MIN6N8 pancreatic β-cells were grown in 5mmol/L glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) plus 1% penicillin/streptomycin (37°C, 95% O2/5% CO2) to sub-confluence then placed in 5mmol/L glucose DMEM with 2% FCS. Cells were incubated with 3.5% CETPi-plasma, placebo-plasma, lipoprotein-free plasma, HDL (1.8mmol/L)-spiked plasma, DMSO or 100ng/ml CETPi for 72h along with oxidized low-density lipoprotein (oxLDL) (50μg/ml) to simulate the pathophysiology of type 2 diabetes.7,9 This is a well-validated technique to cholesterol-load cells8,10 and an appropriate model to examine whether plasma HDL elevation in individuals treated with the CETPi increased GSIS and cholesterol efflux. Treatment of MIN6N8 pancreatic β-cells with oxLDL (50μg/ml) had no adverse effect on cell morphology, culture confluence or key apoptotic (Bax, Casp3, Bclx, Ptpn2, Bim & Puma) or anti-apoptotic (Bcl2) gene markers involved in different cell death pathways (data not shown). Basal insulin secretion was determined after incubation in low (2.8mmol/L) glucose Krebs-Ringer Bicarbonate buffer (KRBB) for 30min whereas first phase (20min) and second phase (40min) GSIS were measured in response to separate 20mmol/L glucose boluses. Cells were lyzed in water and sonicated for 15min at 4°C to determine intracellular insulin. Secreted and intracellular insulin were measured using a rat/mouse insulin ELISA kit (EZRMI-13K, Millipore Australia). Total insulin was calculated as the sum of insulin secreted during the basal, first and second phases, plus intracellular insulin at the end of the experiment. Insulin secretion data are expressed as either a percentage change in absolute insulin secretion at day 14 compared to day 1 (Figure 1) or insulin secretion rat (ng/ml/hr; Table 4).

Ex vivo cholesterol efflux from MIN6N8 pancreatic β-cells
Cholesterol efflux assays were performed using a modified protocol.11 MIN6N8 pancreatic β-cells were grown in 5mmol/L glucose DMEM with 10% FCS to sub-confluence then labelled with 1α,2α(n)-[3H]cholesterol ([3H]cholesterol 1μCi/ml; GE Healthcare, UK) in ethanol vehicle (<0.4%) and oxLDL (50μg/ml) to facilitate cholesterol loading for 48h at 37°C (95% O2/5% CO2). Cells were washed with PBS then treated with the liver-X-receptor (LXR) agonist (TO-901317, 1μmol/L) to stimulate ABCA1 expression in serum-free DMEM for 18h. Human HDL (10, 25 and 50μg/ml) and apoAI (20μg/ml) were used as positive controls. Experimental treatments included CETPi-plasma (n=5) and placebo-plasma (n=5) collected on day 1 and day 14 of the treatment period, at a concentration of 3.5% v/v. This plasma dilution was selected on the basis of preliminary experiments showing that 3.5% plasma elicited cholesterol efflux of a magnitude similar to 50μg/mL of HDL; a concentration in the mid-range of the cholesterol efflux dose-response curve. This was the same dilution used in the insulin secretion experiments. [3H]cholesterol was measured in quadruplicate samples after a 3h cholesterol efflux assay into serum-free DMEM, which was centrifuged at 15,700 x g for 5min. Cells were lyzed in 0.1M NaOH/0.1% SDS and assayed for protein content. [3H]cholesterol in the medium and cells was quantified by liquid scintillation counting, from which the percentage of cholesterol released was determined. Cholesterol efflux data are expressed as the proportion of [3H]cholesterol transferred from cells to the medium.

Statistical analysis
Data were analyzed using unpaired two-tailed Student’s t-tests when only two experimental groups were compared and for more than two groups by one-way ANOVA or repeated measures one-way ANOVA as appropriate and where significant (p<0.05), comparison of individual means was performed with a Tukey HSD test. Correlation analysis was performed using the Pearson coefficient. Results are expressed as mean ± SD. All analyses were conducted using SPSS (v16) and Sigmastat (v3.5). p<0.05 was deemed significant.
References


