SUPPLEMENTAL MATERIAL

METHODS:

Human rHDL clinical trial

*rHDL constituents and preparation*

rHDL consists of apolipoprotein AI (apoAI) isolated from human plasma and phosphatidylcholine (PC) from soy bean. ApoAI and PC are combined in the presence of sodium cholate in a molar ratio of 1:150 and form disc-shaped, non-covalently associated particles resembling nascent high density lipoproteins (HDL). Although this preparation does not fully reflect a complex composition of mature HDL, upon infusion rHDL undergoes rapid remodelling and/or interaction with host HDL making it likely that its composition becomes similar to that of mature HDL. This preparation produces biological responses analogous to native HDL in previous studies. Protein electrophoresis demonstrated that apoAI was the dominant protein in both rHDL and the native HDL used to treat cell cultures, and that these preparations did not contain any other proteins (leptin, insulin, adiponectin) likely to induce a metabolic response (data not shown).

*Plasma analyses*

Blood from participants was collected into appropriate anti-coagulant, plasma immediately separated by centrifugation and snap frozen. HDL-cholesterol was measured by colorimetric assay (WAKO Pure Chemical Industries, Osaka, Japan), apoAI protein by ELISA (Alerchek, Portland, USA), glucose by mass-spectroscopy (LC-MS) and insulin by RIA (Millipore, Billerica, MA, USA). Beta cell function and insulin resistance index were calculated using the homeostasis model assessment (HOMA).
Muscle biopsy analyses

Biopsy samples were immediately snap frozen in liquid nitrogen. Before analysis, frozen samples were placed in lysis buffer and immediately homogenised and clarified by centrifugation. Protein analysis was performed as described below.
Human primary skeletal muscle cell culture studies

Lipoprotein isolation

Human HDL (d=1.12-1.21g/mL) and LDL (d=1.063-1.085g/mL) were isolated from whole, pooled plasma from healthy individuals by ultracentrifugation, as previously described.6 Lipid-free apoAI was isolated and purified from HDL by size exclusion chromatography following delipidation with chloroform-ethanol, according to the method described previously.7 Presence of phospholipid in the isolated apoAI was not detectable using the Bartlett phospholipid assay8 after repeated chloroform-methanol extraction. The concentrations of all lipoproteins and their derivatives added to cultures were based on protein content.

AMPKα and ACCβ phosphorylation

Skeletal muscle cells were serum deprived for 4hrs then treated for designated times with either phenformin, insulin, HDL or apoAI. Cells were washed and lysed in protein lysis buffer and western blotting performed as previously described.9

AMPK activity

Cells were serum deprived for 4hrs then treated with either HDL or apoAI for 5mins or phenformin for 30mins. AMPK activity was determined in the presence of 200µM AMP as described previously.9

LKB1 activity

Cells were serum deprived for 4hrs then treated with either HDL or apoAI for 5mins. LKB1 activity was determined as described previously.10

Intracellular Ca$^{2+}$

Skeletal muscle cells were incubated in the presence of either the calcium ionophore, A23187 (10µM) to directly elevate Ca$^{2+}$ levels, or the sarcoplasmic reticulum (SR) Ca$^{2+}$ pump inhibitor,
cyclopiazonic acid (CPA, 10µM). CPA inhibits the SR Ca^{2+} pump resulting in increased cytoplasmic Ca^{2+}. Cells were plated onto glass coverslips and grown and treated as described above. Intracellular Ca^{2+} concentration was estimated by fluorescence spectroscopy using Fura-2 AM (Molecular Probes) and a ratiometric fluorescence imaging system (Ionoptix, Boston, MA), with excitation performed at 340 and 380 nm and emission monitoring at 505 nm. Fluorescence data were expressed as the 340/380 ratio after subtraction of background auto-fluorescence.

**Antibodies**

Anti-human apoAI monoclonal antibodies were generated at the Baker IDI Heart and Diabetes Institute, Melbourne, Victoria, Australia. Anti-phospho Thr-172 AMPKα, Ser-221 ACCβ, AMPKα1/2 and LKB1 polyclonal antibodies were generated as previously described. Anti-leptin antibody was from R&D systems and anti-insulin and anti-adiponectin antibodies were from Chemicon. Anti-total CaMKK (α and β) was from BD transduction laboratories. Anti-β-actin was from Cell Signaling Technologies. ABCA1 antibodies were generated at the Baker IDI Heart and Diabetes Institute. Both anti-mouse and anti-rabbit HRP conjugated secondary antibodies were from Amersham Biosciences (Buckinghamshire, UK).
References


Figure Legends

Figure 1; Effects of the ABCA1 blocking antibody (4C2) on cholesterol efflux from skeletal muscle cells.

Cholesterol efflux was measured as the amount of $^3$H-cholesterol effluxed into media as a percentage of total cholesterol in both media and cells. Treatments, HDL (10µg/ml from pooled plasma) or rHDL (10µg/ml), were applied after incubation with antibodies (Isotype control or ABCA1 blocking, 4C2) for 18hrs. Background values (i.e. the efflux in the absence of HDL) were subtracted from all values. n=5.

Figure 2; Schematic diagram of the mechanisms by which HDL is postulated to modulate plasma glucose.

HDL is postulated to increase peripheral glucose uptake and reduce plasma glucose by both increasing plasma insulin (possibly by increased insulin secretion) and via the ABCA1 receptor and stimulation of CaMKK to activate the AMPK pathway in skeletal muscle.
Figure 1

The bar chart shows cholesterol efflux (%) for different conditions:

- **Cont**: Isotype Control Antibody
- **HDL**: ABCA1 Blocking Antibody
- **rHDL**: ABCA1 Blocking Antibody

The chart compares the cholesterol efflux across these conditions, with error bars indicating variability.