Universiteit
Leiden
The Netherlands

Dynamic system-wide mass spectrometry based metabolomics approach for a new Era in drug research

Castro Perez, J.M.

Citation
Castro Perez, J. M. (2011, October 18). *Dynamic system-wide mass spectrometry based metabolomics approach for a new Era in drug research*. Retrieved from https://hdl.handle.net/1887/17954

Version:	Corrected Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/17954

Note: To cite this publication please use the final published version (if applicable).

Part II

Lipid modulating therapies; evaluation of animal models and siRNA mediated KD

Chapter 4

Anacetrapib a novel cholesteryl ester transfer protein inhibitor and its evaluation on the dyslipidemic Syrian golden hamster animal model

Based on: Castro-Perez J.M., Gagen K., Briand F., Wang S.P., Chen Y., McLaren D.G., Shah V., Vreeken R.J., Hankemeier T., Roddy T.P., Sulpice T., Hubbard B.K., Johns D.G. Evaluation of the dyslipidemic Syrian golden hamster as a model to study cholesteryl ester transfer protein and the novel CETP inhibitor anacetrapib. *(Submitted to the Journal of Lipid Research)*

Part II: Chapter 4

Anacetrapib a novel cholesteryl ester transfer protein inhibitor and its evaluation on the dyslipidemic Syrian golden hamster animal model

SUMMARY

Anacetrapib (ANA), a reversible inhibitor of cholesteryl ester transfer protein (CETP) raises HDL cholesterol and lowers LDL cholesterol in dyslipidemic patients. The purpose of this study was to determine the utility of a dyslipidemic hamster model to characterize compositional changes in plasma lipoproteins and fecal sterols in response to CETP inhibition, using an analytical LC/MS platform in combination with gel electrophoresis and fast protein liquid chromatograpy. Treatment of hamsters with ANA (60mg/kg/d, 2 weeks) inhibited CETP activity and increased total and HDL-cholesterol. In HDL, free cholesterol and cholesteryl esters were increased with ANA treatment, with the largest increase in cholesterol observed in large HDL compared to intermediate HDL. Major triglycerides (10 species of TG) were reduced in HDL and reciprocally increased in LDL with ANA-treatment. Fecal bulk cholesterol and cholic acid were increased with ANA treatment compared to vehicle, this finding was further substantiated by a macrophage-to-feces excretion experiment using ^{3}H-cholesterol where an increment of the levels of radioactivty were recorded for animals treated with ANA. In vitro, serum from ANA-treated hamsters also displayed greater cholesterol efflux capacity. These data indicate that ANA stimulates cholesterol efflux and cholesterol accumulation in HDL, which is associated with increased fecal cholesterol excretion. This study demonstrates the utility of the dyslipidemic Syrian golden hamster model to study regulation of lipoprotein composition by CETP and to evaluate the potential benefit of CETP inhibition on lipoprotein and cholesterol metabolism.

INTRODUCTION

Cardiovascular disease (CVD) is one of the major underlying causes for mortality in the USA and Europe (1-3) and increasingly in other geographies (4). Despite the significant number of therapies which specifically target the modulation of LDL cholesterol, cardiovascular events still remain high. Statins are the most widely prescribed drugs to treat dyslipidemia; however a large unmet medical need in the management of atherosclerosis still exists. Epidemiological studies have suggested that the presence of higher levels of HDL is associated with reduced cardiovascular risk (5-9). Therefore, alternative therapies that increase HDL cholesterol are being explored to take advantage of the inverse relationship between HDL and risk.

Cholesteryl ester transfer protein (CETP) has re-gained momentum as a possible target for treating cardiovascular disease. This is largely due to the recent discharging of the increased mortality observed with torcetrapib treatment in the ILLUMINATE study (10) as being attributed to off-target effects (11-14), and a lack of such effects with other CETP inhibitors including anacetrapib and dalcetrapib. Anacetrapib is a potent CETP inhibitor that has been shown to increase HDL cholesterol and reduce LDL cholesterol in humans in addition to statin therapy (15), and a recent phase III safety study (DEFINE) further supports the safety and tolerability of anacetrapib (16).

Preclinical animal models commonly utilized for studying CETP include rabbits and hamsters which naturally express CETP, in contrast to mice and rats which do not express CETP. The hamster model has been widely used for studying specific lipid therapies including ezetimibe and other lipid regulatory pathways (17-20). Although recent studies have reported the effects of CETP inhibition on cholesterol metabolism in normolipidemic hamsters (21), there are no reports of the effects of anacetrapib in dyslipidemic hamsters, which may better reflect human dyslipidemia.

The purpose of this study was to test the hypothesis that CETP inhibition with anacetrapib will raise HDL and lower LDL in a similar manner to what is observed in humans, and to test the effect of anacetrapib on plasma and fecal cholesterol parameters in Syrian golden hamsters fed a high fat diet.

MATERIALS AND METHODS

Animals

All animal work was performed in accordance with the Merck Research Laboratories Institutional Animal Care and Use Committee (Rahway, NJ). Male Syrian golden hamsters (weight at start of studies between100-120g) were placed on a dyslipidemic diet (Research Diets D08092301, 45% kcal from fat/lard, 0.12% cholesterol), provided *ad libitum* for 3 weeks prior to initiation of treatment. Anacetrapib was administered in the high fat diet to deliver a 60 mg/kg dosage. This dose was selected based on a pilot dose ranging study in which 60mg/kg produced maximum efficacy (effects on lipoproteins and CETP activity). Animals (n=8) were treated with Anacetrapib for 2 weeks. Control group consisted of animals (n=8) on a high fat diet with no drug treatment. A single time point terminal bleed was collected at the end of the study and serum samples were stored at -80°C until analyzed. Fecal samples were collected for 24 hrs prior to and after treatment with control and anacetrapib-containing diets. All individual fecal samples were weighed and recorded accordingly.

In a separate set of studies, the effects of anacetrapib on cholesterol absorption were examined. This was performed in two separate cohorts of dyslipidemic Syrian golden hamsters. In one group, animals were made dyslipidemic and treated with anacetrapib-containing diet or control diet as described above. In another cohort, dyslipidemic hamsters were treated with ezetimibe-containing diet (1.5mg/kg/day) or control diet for 2 weeks. For both studies, following treatment hamsters were treated with D_6-cholesterol (Sigma, St Louis, USA) at an oral dose of 12 mg/kg; feces were collected post tracer treatment for 24 hr, 48 hr, 72 hr and 96 hr. The fecal samples were frozen at -20°C until analyzed by LC/MS.

In vivo macrophage-to-feces reverse cholesterol transport

For this study, separate cohorts of animals (n= 12 vehicle and n=12 Anacetrapib 60mg/kg) were utilized and maintained at the same conditions as already mentioned. Preparation of J774 cells were performed as previously described (22). J774 cells obtained from the American Type Culture Collection (ATCC; Manassas, VA), were grown in suspension in RPMI/HEPES supplemented with 10% FBS and 0.5% gentamicin in suspension in Nalgene Teflon flasks. Cells were radiolabeled with 5 µCi/mL ^{3}H-cholesterol and cholesterol loaded with 50µg/mL oxidized LDL over 48 hours. Radiolabeled cells were then washed with RPMI/HEPES and equilibrated for 4 hr in fresh RPMI/HEPES supplemented with 0.2% BSA and gentamicin. Cells were pelletted by low speed centrifugation and resuspended in MEM/HEPES prior to injection into hamsters.

^{3}H-cholesterol–labeled and oxidized LDL-loaded J774 cells ($2.5x10^6$ cells containing $10x10^6$ dpm in 0.5 mL minimum essential medium) were injected intraperitoneally into individually caged hamsters. Animals had free access to food and water, and were maintained on diet and treatment during the 72 hr experiment. Blood was collected by from the jugular vein under isoflurane anesthesia at 24, 48 and 72 hr to measure radioactivity released into the plasma and HDL after

phosphotungstate/$MgCl_2$ precipitation (50µL of plasma or HDL counted). Hamsters were then sacrificed by cervical dislocation, exsanguinated and liver was harvested from each animal. A ≈ 50mg-piece of liver was homogenized using an ultrasound probe in 500 µL water then 100 µL were counted in a liquid scintillation counter. Feces were collected over 72 hr and were stored at 4°C before extraction of cholesterol and bile acids.

Fecal cholesterol and bile acid extraction was performed as previously described (22). The total feces collected from 0 to 72 hr were weighed and soaked in Millipore water (1 mL water per 100 mg feces) overnight at 4°C. The following day, an equal volume of absolute ethanol was added, and the mixtures were homogenized. To extract the ^{3}H-cholesterol and ^{3}H-bile acid fractions, 1 mL of the homogenized samples was combined with 1 mL ethanol and 200 µL NaOH. The samples were saponified at 95°C for 1 hr and cooled to room temperature, and then ^{3}H-cholesterol was extracted 2 times with 3 mL hexane. The extracts were pooled, evaporated, resuspended in toluene, and then counted in a liquid scintillation counter. To extract ^{3}H-bile acids, the remaining aqueous portion of the feces was acidified with concentrated HCl and then extracted 2 times with 3 mL ethyl acetate. The extracts were pooled together, evaporated, resuspended in ethyl acetate, and counted in a liquid scintillation counter.

Results were expressed as % of the radioactivity injected recovered in plasma, HDL and liver and feces. The plasma volume was estimated as 3.5% of the body weight.

Serum CETP activity

CETP activity was determined by measuring the transfer of ^{3}H- cholesteryl oleate and ^{3}H- triolein from pre-labeled exogenous LDL to HDL by CETP in serum as previously described (23). Briefly, the assay was performed by incubating the serum with ^{3}H- labeled exogenous LDL for 90 min at 37°C. The transfer reaction was terminated by precipitation of LDL with 20% w/v PEG 8000 (1:1 vol.). The samples were centrifuged and an aliquot of the HDL-containing supernatant was counted for radioactivity measurement by liquid scintillation.

Ex-vivo cholesterol efflux assay

Ex-vivo cholesterol efflux capacity of hamster serum was measured as described by Fournier *et al* (24) and Mweva *et al* (25) (VascularStrategies LLC, Wynnewood PA). ABCA1-mediated cholesterol efflux was determined in J774 mouse macrophages and SR-BI-mediated cholesterol efflux was assessed in Fu5AH rat hepatoma cells.

ApoB measurement by LC/MS

Serum samples (2µL) were diluted with 50 mM ammonium bicarbonate pH 8.0 followed by addition of GFEPT-[$^2H_{10}$]L-EALFGK (Bachem, Torrance, CA). The following peptide sequence was measured GFEPTLEALFGK for quantitation

purposes. 10 µL of a 10% sodium de-oxycholate solution was added to delipidate the lipoproteins, followed by reduction with dithiothreitol for 30 min at 60 °C, alkylation with iodoacetamide for 60 min at 25 °C in the dark, and protein digestion with trypsin overnight. A final volume of 200 µL 20% formic acid was added to stop digestion and precipitate sodium de-oxycholate. This was immediately followed by centrifugation at 15,800 rpm for 15 min. The supernatant was removed for LC/MS analysis using a Waters Acquity UPLC and Xevo triple quadrupole mass spectrometer (Xevo TQ, Waters, Manchester, UK). The gradient employed was 95% A (0.1% formic acid in water)/5% B (0.1% formic acid in acetonitrile) ramped to 80% A at 1 minute, 65% A at 4 min, 5% A at 5 min. The chromatographic separation was achieved by the use of a reverse phase column (Phenomenex Kinetex C18 50 x 2.1mm 1.7µm) operated at 50 °C with a column flow rate of 0.7 mL/min. The mass spectrometer was operated in electrospray positive ion mode.

Serum lipoprotein analysis by Fast Protein Liquid Chromatography and Gel Electrophoresis

Both fast protein liquid chromatography (FPLC) and gel electrophoresis were employed in this study to quantitate the cholesterol component in VLDL, LDL and HDL fractions.

For the FPLC method, EDTA serum was filtered and treated with lipase inhibitor (Paraoxan, Sigma, St Louis, USA) prior to analysis. The method comprised of a system equipped with fast performance liquid chromatography (FPLC) gel filtration using a Superose-6 size exclusion column (~24 ml, GE LifeSciences, Inc.) attached to a Dionex HPLC system. 25µl of serum was injected onto the column and eluted with PBS -/-buffer containing 1mM EDTA at a flow rate of 0.4 ml/min. The column effluent was mixed with a commercially available enzymatic colorimetric cholesterol detection reagent (total cholesterol E, Wako USA) that was delivered at a rate of 0.2 ml/min. After passing through a 1,500ul knitted reaction coil (~15 min) that was maintained in a 37°C heated chamber, the reaction mixture was read at 600nm absorbance using a photodiode array (PDA) detector. The first peak of cholesterol eluted from the column was attributed to VLDL, the second peak to IDL/LDL and the third to HDL. The area under each peak was calculated using Chromeleon software™ provided with the HPLC (Dionex, Inc). To calculate the cholesterol concentration for each lipoprotein fraction, the ratio of the corresponding peak area to total peak area was multiplied by the total cholesterol concentration measured in the sample as determined using the Total Cholesterol E Kit by Wako (Richmond, VA).

Gel electrophoresis was carried out using a commercially available kit according to the manufacturer's instructions (Lipoprint®, Quantimetrix, Redondo Beach, CA). Briefly, 25 µL of serum was loaded in the gel followed by staining with Sudan black B for cholesterol and cholesterol esters quantitation in the lipoprotein bands. The gels were scanned and cholesterol measurements were conducted by entering the total cholesterol levels previously measured by the Wako E cholesterol kit (Richmond, VA).

To assess lipid composition in lipoprotein particles, VLDL, LDL and HDL lipoprotein bands were cut from Lipoprint gels. The gel fragments were placed in homogenization tubes, 400 µL phosphate buffered saline (PBS) was added to the

VLDL and HDL bands and 1000 µL of PBS was used for the IDL and LDL bands. The gel fragments were homogenized at 5,000 rpm for 15 secs at room temperature. Homogenates were centrifuged at a speed of 20,000 rpm 10°C, for 10 min. And the resulting supernatant was extracted for lipids using the Bligh and Dyer method (26).

Lipid analysis by LC/MS was conducted using non endogenous internal standards [(1,2-diheptadecanoyl-sn-glycero-3-phosphocholine (PC 17:0/17:0), 1-heptadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine (LysoPC 17:0), triheptadecanoin (TG 17:0/17:0/17:0) , cholesteryl ester (CE 17:0) (Avanti Lipids, Alabaster, AL) and D_6-cholesterol (Sigma, St Louis, MO)] standards were added to samples at a final concentration of 1 µg/mL. In addition to this, external lipid calibrants (cholest-5-en-3β-yl hexadecanoate (CE 16:0), cholest-5-en-3β-yl octadecanoate (CE 18:0), cholest-5-en-3b-yl (9Z-octadecenoate) (CE 18:1), cholest-5-en-3β-yl (9Z,12Z-octadecadienoate) (CE 18:2), cholest-5-en-3β-yl (9Z,12Z,15Z-octadecatrienoate) (CE 18:3), cholest-5-en-3β-yl (5Z,8Z,11Z,14Z-eicosatetraenoate) (CE 20:4), and cholest-5-en-3β-yl (4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoate) (CE 22:6) and free cholesterol (Avanti Lipids, Alabaster, AL)) in buffer ranging from 0.01 - 2 µg/mL were used for free cholesterol and cholesteryl ester lipid quantitation.

The subsequent lipid extracts were analyzed on a hybrid orthogonal quadrupole time of flight mass spectrometer (Synapt G2 HDMS, Waters, Manchester, UK). Positive electrospray ionization (ESI) mode was utilized for the CE and TG analysis and atmospheric pressure photoionization (APPI) mode was utilized for the measurement of free cholesterol in the lipoprotein fractions as described by Castro-Perez *et al* (27). The mass spectrometer was coupled to an inlet system comprised of an Acquity UPLC (Waters, Milford, MA, USA). The lipid extracts were injected (10µL) onto a 1.8 µm particle 100 x 2.1 mm id Waters Acquity HSS T3 column (Waters, Milford, MA, USA); the column was maintained at 55 °C. The flow rate used for these experiments was 0.4 mL/min. A binary gradient system consisting of acetonitrile (Burdick & Jackson, USA) and water with 10 mM ammonium formate (Sigma-Aldrich, St Louis, MO) (40:60, v/v) was used as eluent A. Eluent B, consisted of acetonitrile and isopropanol (Burdick & Jackson, USA) both containing 10 mM ammonium formate (10:90, v/v). The sample analysis was performed by using a linear gradient (curve 6) over a 15 min total run time; during the initial portion of the gradient, it was held at 60% A and 40% B. For the next 10 min the gradient was ramped in a linear fashion to 100% B and held at this composition for 2 min hereafter the system was switched back to 60% B and 40% A and equilibrated for an additional 3 min.

Fecal cholesterol and bile acid composition analysis by LC/MS

Fecal samples from each time point were weighed and homogenized as follows; 10 mL of 80% methanol 20% water was added to the fecal samples in 50mL Falcon tubes. The volume was increased to 20 mL if the weight of feces per animal exceeded 2g. The samples were then allowed to stand at room temperature for approximately 30 min. After this process was completed, the fecal matter was homogenized at a speed of 30,000 rpm for 3 min. For neutral lipid extraction 200µL of the homogenate was transferred to a 1.5 mL eppendorff tube, and 320 µL of dichloromethane containing 10ug/mL D_6-cholesterol (ISTD) and 5 µg/mL D_6-cholesterol ester 18:2 internal standard was added to all the samples. For cholesterol

absorption, instead $^{13}C_{18}$ CE 18:1 was used as the internal standard. Samples were vortexed for 60 secs and 80µL of H_2O was added to each sample followed by another vortex cycle of 60 secs. In order to create a two-phase liquid layer, the samples were centrifuged at 20,000 rpm, 10ºC for 10 min. 75µL of the lower organic layer was transferred to a deep 96-deep well plate and diluted with 300 µL of injection solvent (65% Acetonitrile: 30% Isopropanol: 5 % water). The plate was then centrifuged at 4,000 rpm for 10 minutes to pellet any residual solids. 100µL of the supernatant was transferred to a new 96-well plate which was then sealed before analysis by LC/MS. An external cholesterol calibration curve (0.01-20 µg/mL) in buffer was used to quantify D_6-cholesterol.

For bile acid extraction from the fecal samples, 10µL of the fecal homogenate was transferred to a 1.5mL eppendorff tube and diluted with 490µL of 80% methanol containing 500nM D_4-cholic acid, D_4-glycocholic acid, D_4-chenodeoxycholic acid & D_5-lithocholic acid as the internal standards. The resulting mixture was then vortexed for 60 seconds. This was followed by centrifugation at 10,000 RPM for 10 minutes at 10ºC. Finally, the samples were transferred (100µL) of the supernatant to a 96 well plate and centrifuged again for 10 minutes to pellet any residual solids. The supernatant was transferred to a new 96-well plate and care was taken to avoid disturbance of any pellet for analysis. For quantitation of bile acids present in the feces, external calibrants in buffer were utilized in the range of 0.02µM -4µM for Lithocholic acid (LCA) , Glycholic acid (GCA) , Taurocholic acid (TCA), Deoxycholic acid (DCA), Cholic acid (CA), Chenodeoxycholic acid (CDCA), Glycochenodeoxycholic acid (GCDCA) and Taurochenodeoxycholic acid (TCDCA) (Sigma, St Louis, MO). Resulting analyte concentrations for the samples were computed against each calibration line for the corresponding bile acid.

Data processing and statistical analysis

Data processing was conducted using the instrument manufacturer software for the lipoprotein analysis using FPLC and GGE. Lipid composition and quantitation analysis work by LC/MS was carried out using MassLynx (Waters, Milford, USA). For statistical analysis; all the figures are presented as ± standard error mean (SEM). Differences between groups were computed by unpaired Student's *t-test* with two-tailed p-values statistical analysis (GraphPad Prism, La Jolla, CA). A p-value of <0.05 was considered as being statistically significant.

Lipid nomenclature

The lipid nomenclature utilized throughout the manuscript is the same as described by Fahy *et al.* (28). For instance, CE 18:1 denotes cholesteryl ester containing 18 hydrocarbons and 1 double bond as the fatty acyl substituent. Equally the same applies to triglycerides cited in the manuscript. For example TG 54:3, translates to a triglyceride containing 54 hydrocarbons attached to the glycerol back-bone and a total of 3 double bonds, which forms part of the 3 fatty acyl substituents.

RESULTS

Anacetrapib treatment of dyslipidemic Syrian golden hamsters results in reduced CETP activity

Anacetrapib-treated hamsters showed a reduction in serum CETP activity, manifest as a reduction in both cholesteryl ester and triglyceride transfer (46% and 47% reduction respectively) compared to the vehicle treated animals (Figure 1A, B).

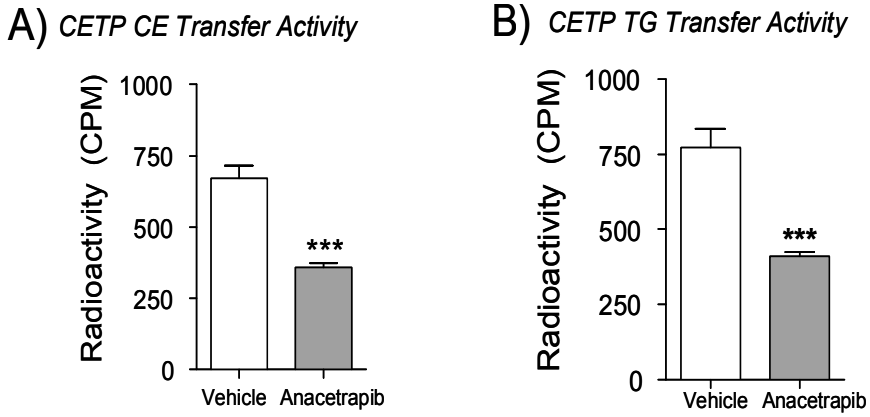

Figure 1. Anacetrapib treatment (60mg/kg, 2 weeks) inhibits serum CETP transfer activity for (A) cholesterol esters (B) and triglyceride ***P<0.001 vs. vehicle.

FPLC analysis of hamster serum (Figure 2A) showed a 43% increase in HDL-cholesterol (Figure 2B) and 32% reduction in LDL-cholesterol (Figure 2C) compared to vehicle-treated controls. Figure 3A depicts the analysis of the same samples by GGE showing a very comparable increase in the level of HDL-cholesterol (53% increase compared to vehicle), but no change in LDL-cholesterol (Figure 3B and C respectively).

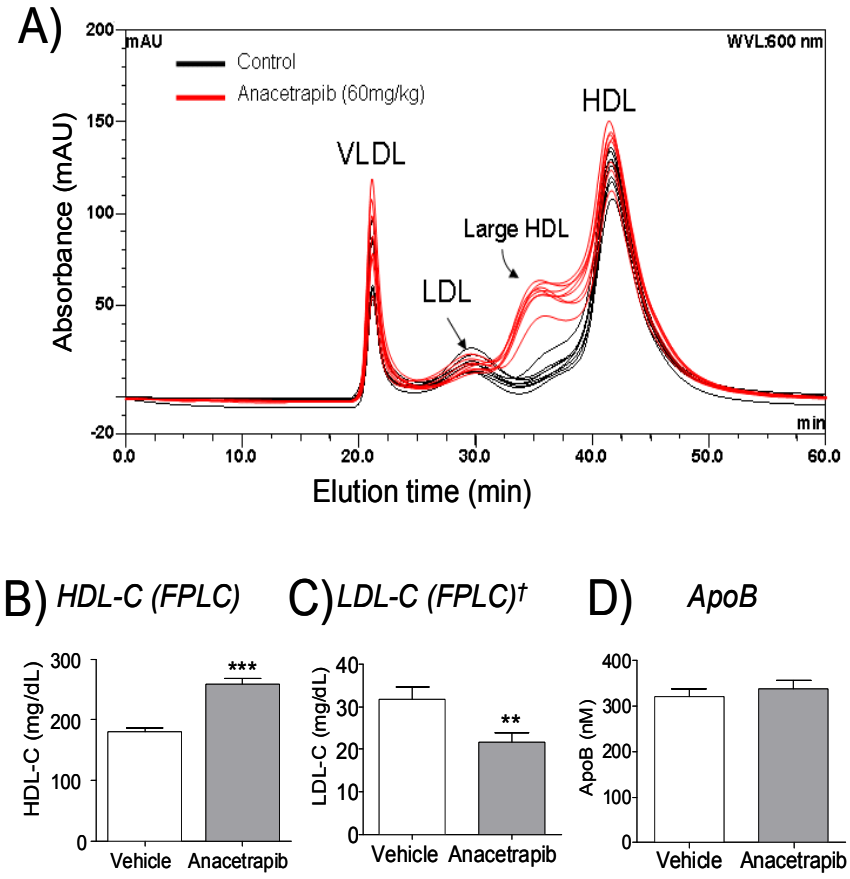

Figure 2. Anacetrapib treatment (60mg/kg, 2 weeks) results in (A) formation of large HDL particles, (B) increased HDL-cholesterol, (C) apparent reduction in LDL from FPLC analysis and (D) no change in plasma apoB ***P*<0.01, ****P*<0.001 vs. vehicle. †*presence of large HDL confounds resolution of LDL peak*

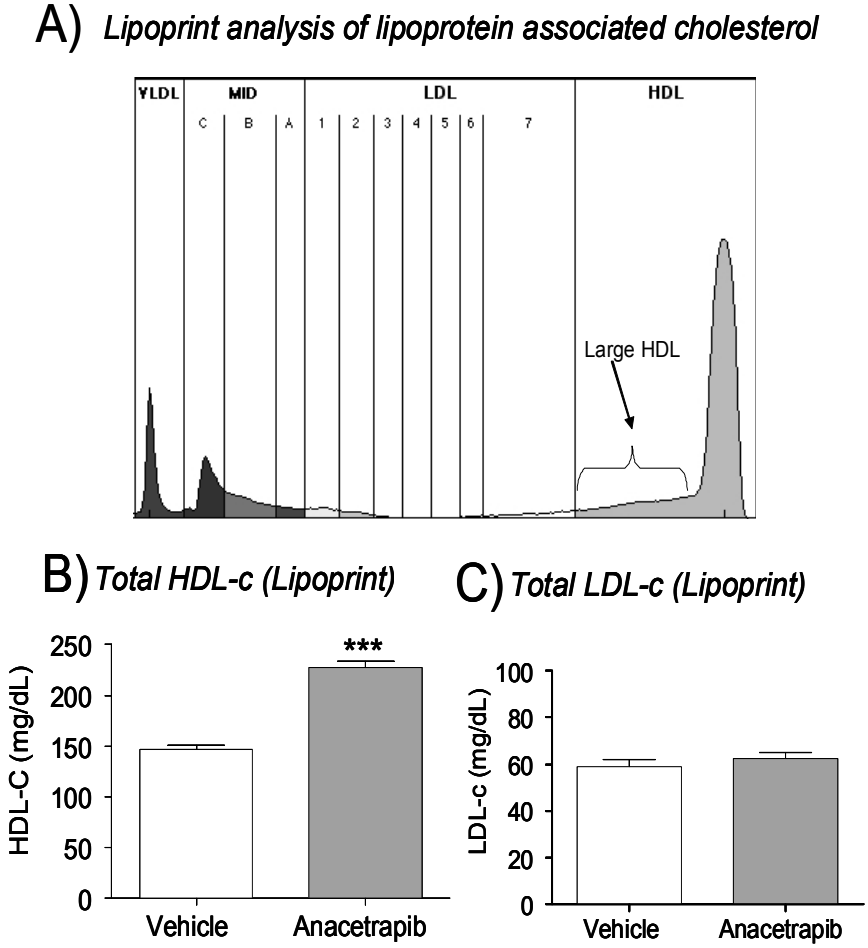

Figure 3. Gradient gel electrophoresis separation of lipoproteins (A) Trace from Lipoprint® scan following gradient gel electrophoresis of Ana-treated hamster serum (60mg/kg, 2 weeks) (B) Anacetrapib treatment increases total HDL-cholesterol with (C) no effect on total LDL-cholesterol ***P<0.001 vs. vehicle

For analysis of lipid composition in the VLDL and LDL fraction (IDL and LDL bands from gradient gel electrophoresis subfractions were combined as one fraction) CE and FC were measured by LC/MS. These measurements did not reveal any significant changes between the ANA and control groups (Figure 4A, B and C). HDL subfractions were also measured using an HDL specific lipoprotein electrophoresis kit. In this case, the large HDL from anacetrapib-treated hamsters contained 89% more cholesterol than in the vehicle group ($p<0.0001$) and the intermediate HDL subfraction 29% more cholesterol than in the vehicle group ($p<0.01$) (Figure 5A)

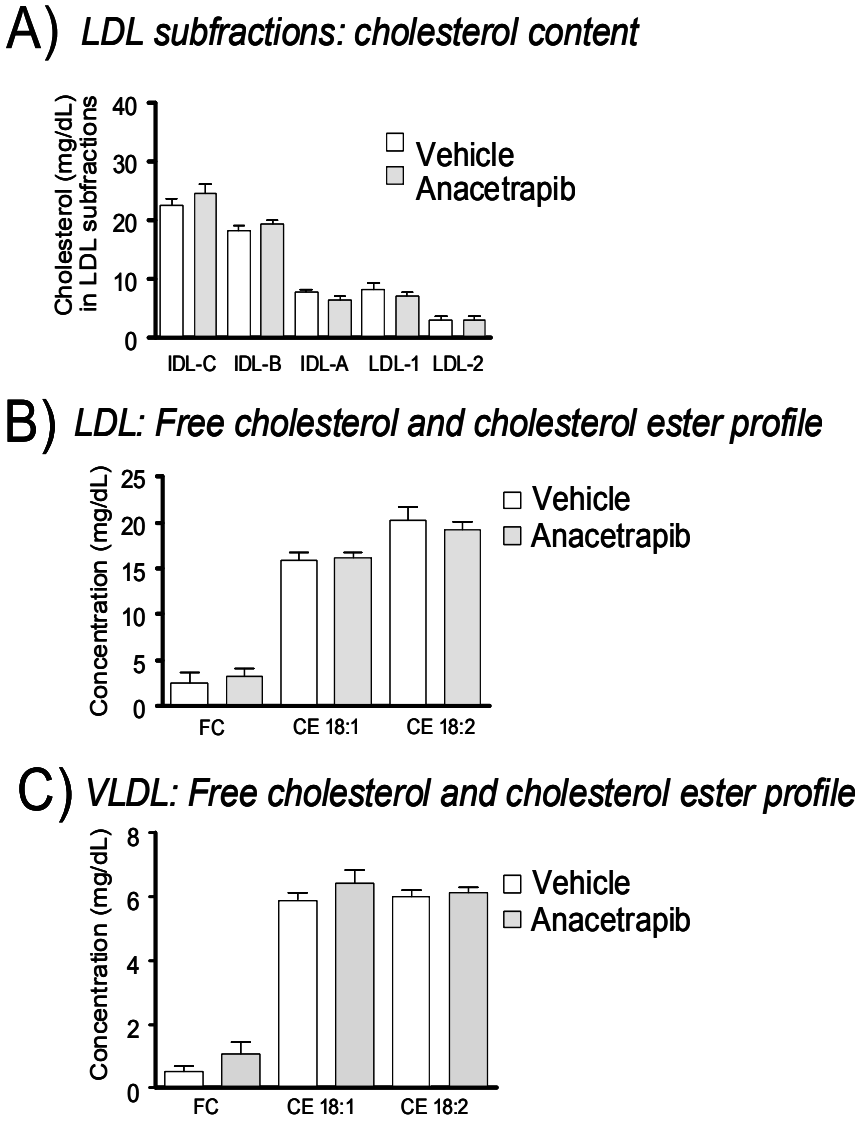

Figure 4. Gradient gel electrophoresis LDL subfraction analysis. (A) Distribution of cholesterol in subfractions of LDL. VLDL and LDL cholesterol composition analyzed by LC/MS. (B) Free cholesterol and major cholesteryl ester species present in total LDL and (C) Free cholesterol and major cholesteryl ester species present in total VLDL

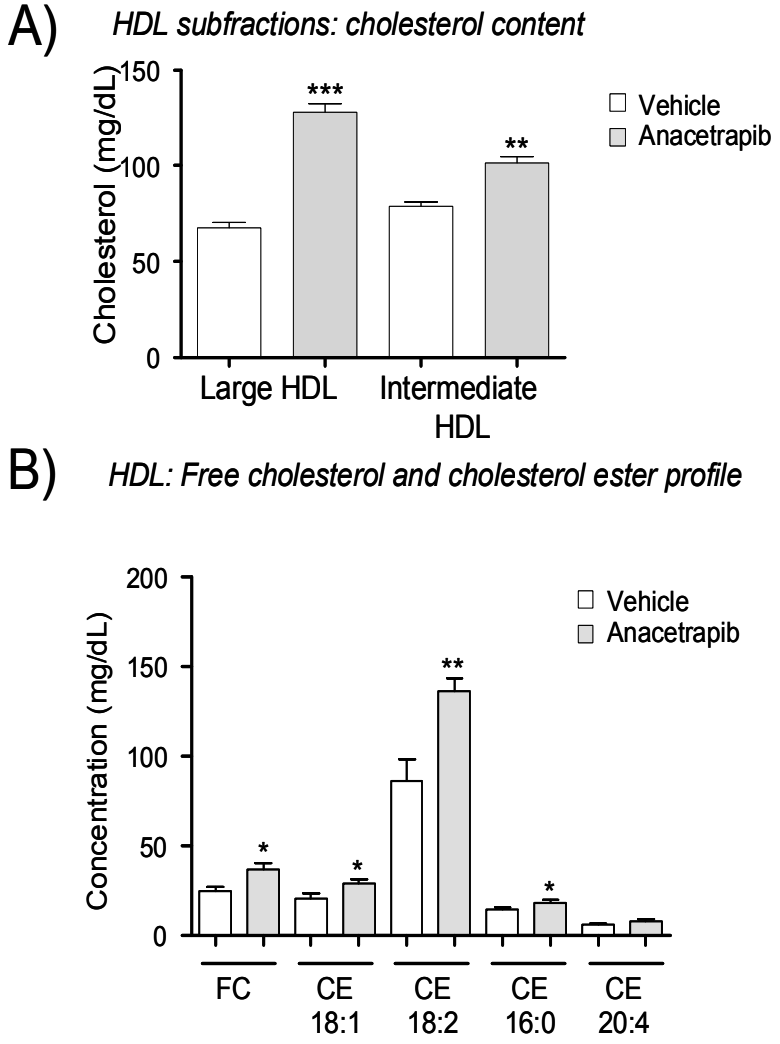

Figure 5. HDL cholesterol composition analyzed by LC/MA (A) Distribution of cholesterol in subfractions of HDL, (B) Free cholesterol and major cholesteryl ester species present in total HDL.*P<0.05, **P<0.01, ***P<0.001 vs. vehicle

The added specificity of LC/MS permitted investigation of the lipid composition for different CE's and FC in the VLDL, LDL and HDL particles. The HDL fraction measurement by LC/MS (Figure 5B) showed statistically significant increases of FC (33% anacetrapib vs. vehicle ($p<0.05$), CE 18:1 (41% anacetrapib vs. vehicle ($p<0.05$), CE 18:2 (57% anacetrapib vs. vehicle ($p<0.01$), CE 16:0 (26% anacetrapib vs. vehicle ($p<0.05$) and CE 20:4 (35% anacetrapib vs. vehicle ($p=0.0523$) in the anacetrapib treated animals vs. the vehicle group. When triglyceride levels in the VLDL, LDL and HDL lipoprotein fractions were measured by LC/MS, it was observed that for the HDL fraction the level of the TG was significantly reduced ~180% (Figure 6C) in the anacetrapib treated group vs. the control group. For the VLDL and LDL

fraction TG levels were ~ 80-100% higher (Figure 6A and B respectively) than in the vehicle group, supporting the notion that CETP mediated TG transfer was inhibited by anacetrapib.

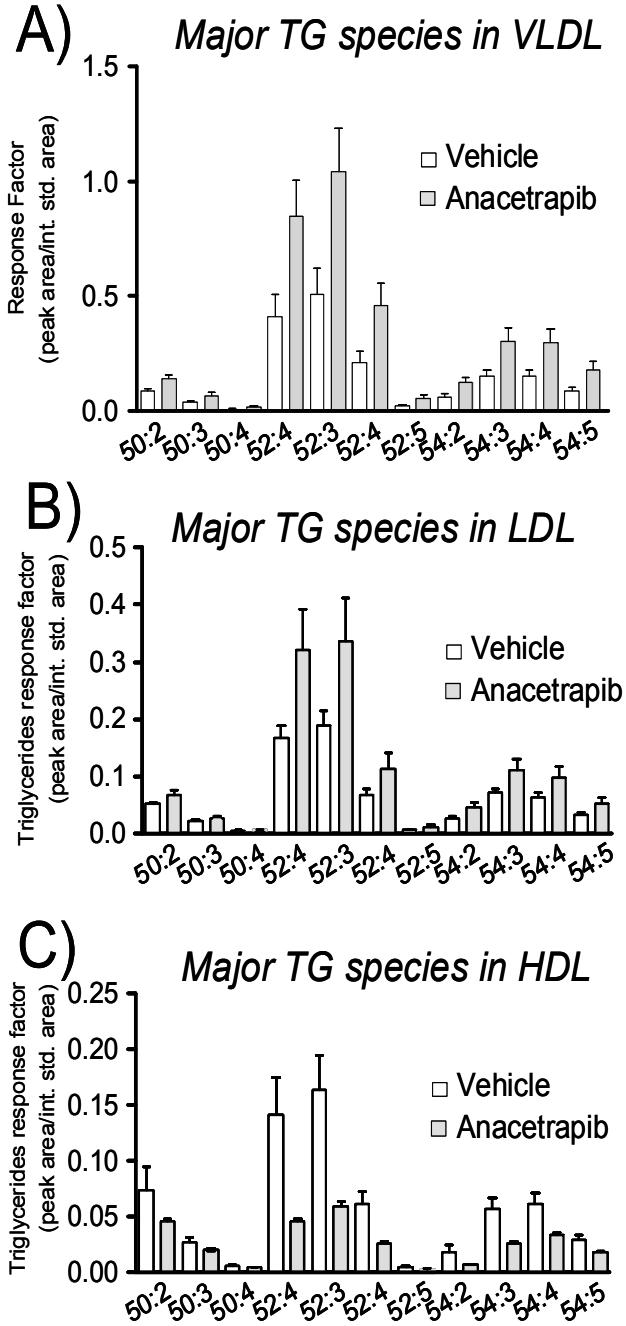

Figure 6. Triglyceride composition of (A) VLDL, (B) LDL and (C)) HDL, isolated by gradient gel electrophoresis and analyzed by LC/MS.

Fecal bulk cholesterol excretion and promotion of reverse cholesterol transport following CETP inhibition

Treatment with anacetrapib resulted in an increased concentration of cholesterol in the feces by 29.6% $p<0.05$ (Figure 7A) with respect to the vehicle groups after the initial 2 week period in which the animals were equilibrated with the HF diet. Cholic acid excretion (Figure 7B) showed a very similar pattern as the cholesterol excretion, reaching steady excretion state at 2 weeks following the HFD and with anacetrapib treatment a net increase on the excretion of CA of 28.5% $p<0.05$.

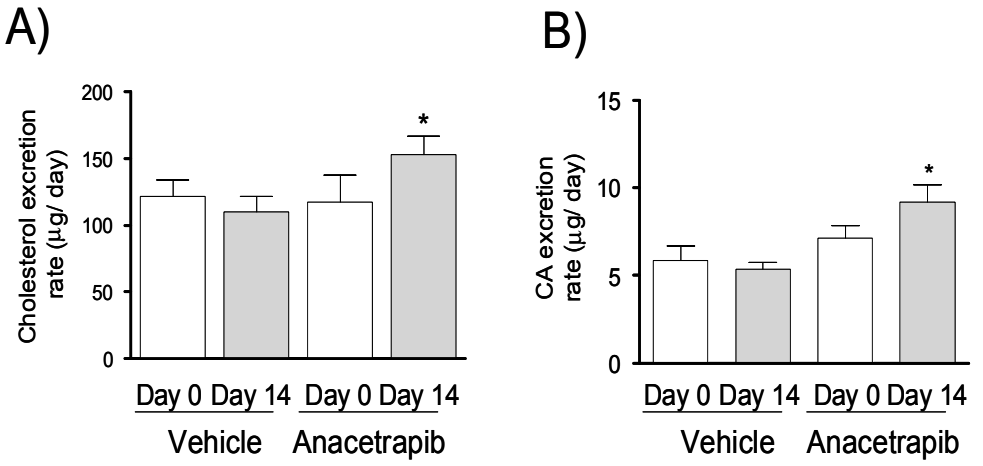

Figure 7. Anacetrapib treatment increases concentration of (A) fecal cholesterol, and (B) fecal cholic acid after 14 days of treatment (24hr collection of feces post-treatment). * *P*<0.05 vs. day 0

In order to evaluate whether anacetrapib treatment influenced RCT, radiolabeled cholesterol-loaded macrophages were injected in hamsters following treatment with either control or anacetrapib-containing diets as described in the materials and methods section. Figure 8A shows the appearance of radio-tracer in plasma was 20% higher in animals treated with anacetrapib compared to controls at 72h. HDL plasma (figure 8B) resulted in an increment of 69% of radio- tracer for anacetrapib-treated animals than that of control animals at 72h. Liver radioactivity data (figure 8C) resulted in a reduction of 29% for animals treated with anacetrapib in comparison to the control group. Fecal excretion of ^{3}H-cholesterol and radiolabeled bile acids for the anacetrapib treatment showed a higher degree of injected radioactivity than in the control group (figure 8D).

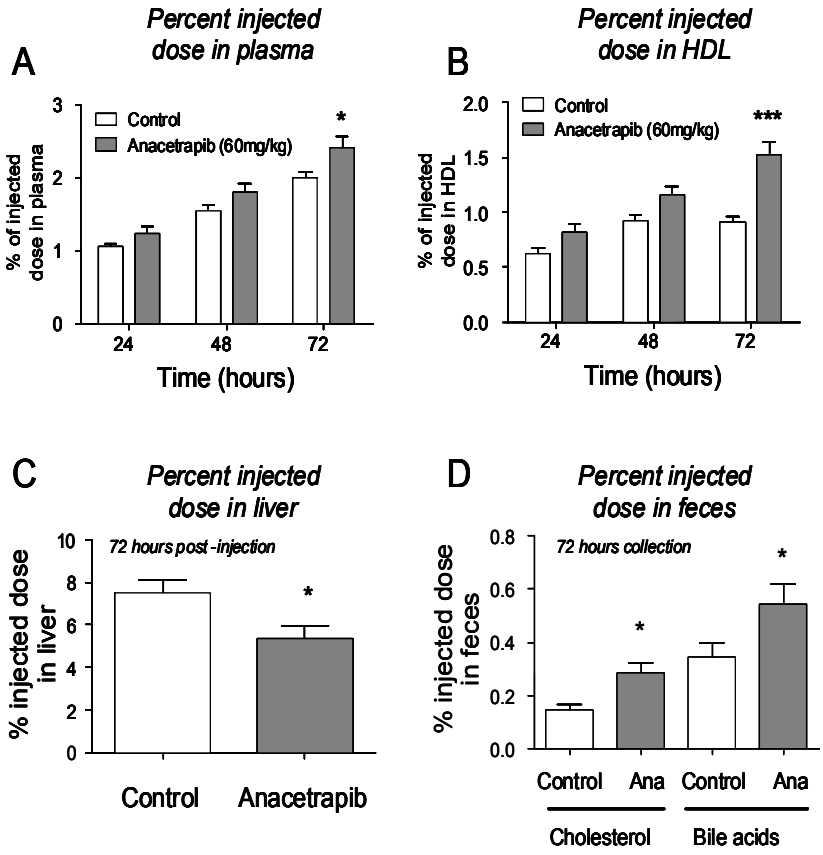

Figure 8.Anacetrapib treatment (60mg/kg, 2 weeks) of dyslipidemic hamsters and effects on macrophage-to-feces RCT. A) ^{3}H-tracer recovery in plasma, B) ^{3}H-tracer recovery in HDL fraction of plasma, C) ^{3}H-tracer recovery in liver tissue, D) ^{3}H-tracer recovery in fecal cholesterol and bile acid fraction. ***P<0.001, *P<0.05 vs. control. Open bars = control, solid bars = anacetrapib treated

To determine whether the increase in fecal cholesterol content was possibly due to effects on absorption, D_6-labeled cholesterol was used in two experiments to track removal of cholesterol from the gastrointestinal tract, by measurement of changes in D_6-labeled cholesterol in the feces after oral dosing. In both experiments, D_6-cholesterol appeared in the feces over the course of 48hr for control and treated groups (Figure 8A and C), and were undetectable beyond 48hr (data not shown). It should be noted that the ezetimibe and anacetrapib experiments were performed in separate cohorts of animals, which may explain the differences in the absolute values for fecal and plasma D_6-cholesterol between vehicle groups from both experiments.

In the experiment using ezetimibe, treated animals, showed significantly higher levels of D_6 cholesterol in the feces at both 24 and 48 hr compared to control (Figure 9A). In the anacetrapib experiment, animals treated with anacetrapib showed no significant differences in the level of D_6-cholesterol in the feces compared to control at 24 and 48hr (Figure

9C). Plasma circulating levels of D_6-cholesterol were determined by LC/MS measurements. For animals treated with anacetrapib (Figure 9D) there was a slight increase (15%) in plasma D_6-cholesterol compared to vehicle (AUC for all time points) while for ezetimibe-treated animals (Figure 9B) there was a significant drop (99.6% reduction in AUC) in plasma D_6-cholesterol compared to vehicle.

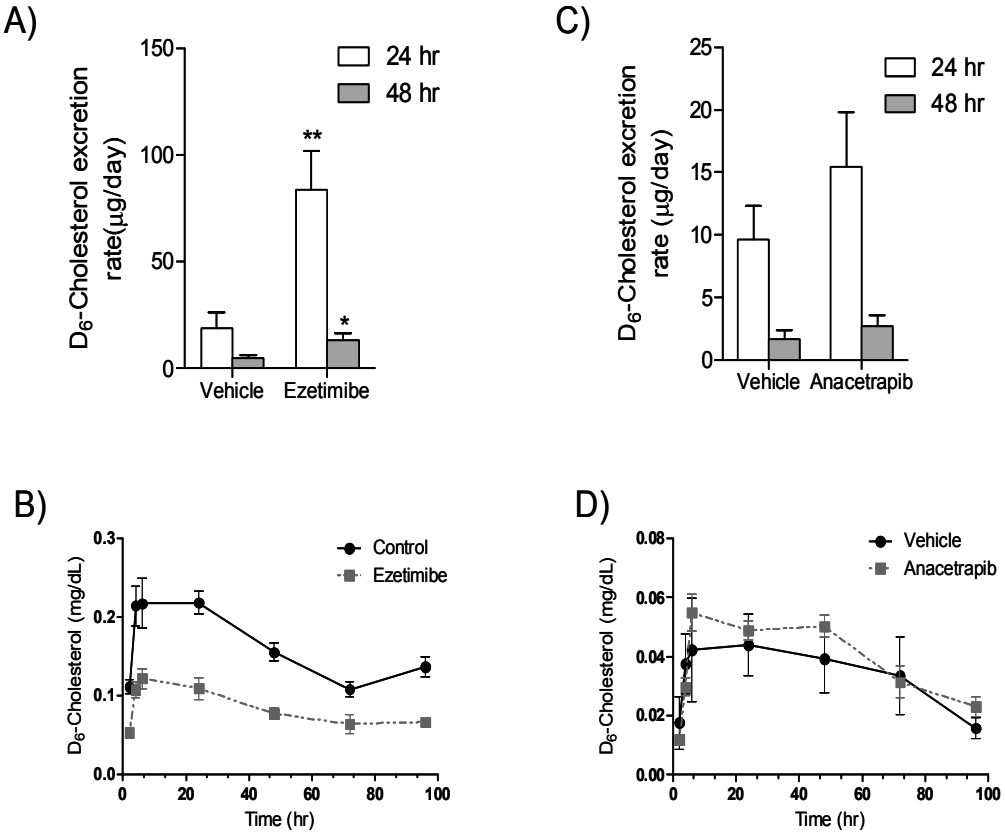

Figure 9. Anacetrapib does not affect cholesterol absorption (2 weeks treatment at 60mg/kg in-feed). (A) Fecal D6-cholesterol excretion over 48hr for vehicle and ezetimibe treated hamsters. (B) Plasma levels of D6-cholesterol in plasma for vehicle and ezetimibe treated hamsters. (C) Fecal D6-cholesterol excretion over 48hr for vehicle and anacetrapib treated hamsters. (D) Plasma levels of D6-cholesterol in plasma for vehicle and anacetrapib treated hamsters **P<0.01 ezetimibe 24 hr vs. vehicle 24 hr * P<0.05 ezetimibe 48 hr vs. vehicle 48 hr

HDL from dyslipidemic hamsters treated with anacetrapib exhibited increased cholesterol efflux capacity

A statistically significant increase in SR-BI mediated efflux was observed in HDL from anacetrapib treated animals group compared to the vehicle-treated animals (Figure 10A). In the J774 efflux assay, ABCA1-mediated efflux was increased in HDL from anacetrapib-treated hamsters compared to vehicle. In the absence of cAMP (which stimulates ABCA1 expression in these cells), there was no difference in efflux values, indicating the specificity for ABCA1-mediated efflux (Figure 10B).

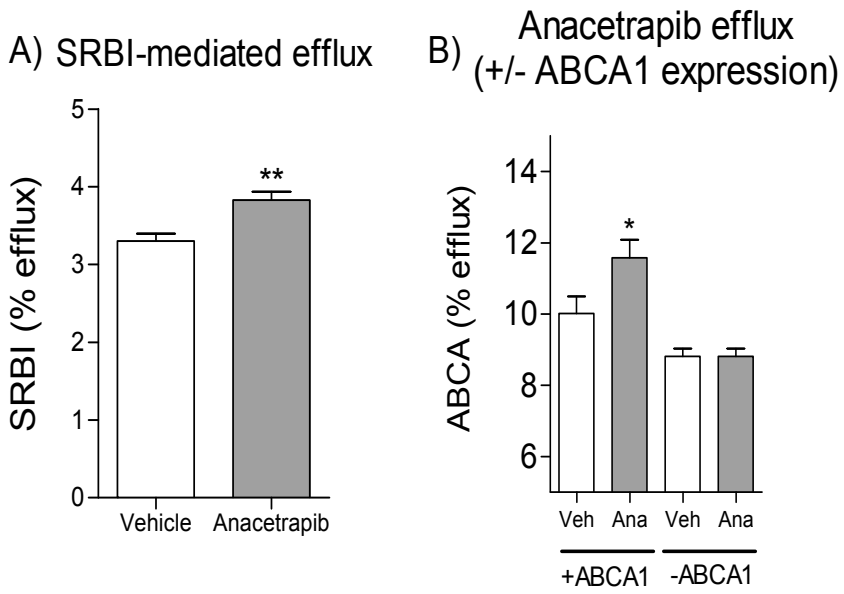

Figure 10. *In vitro* cholesterol efflux was increased by hamsters treated with anacetrapib in comparison with the control. (A) SR-BI mediated cholesterol efflux. (B) Cholesterol efflux +/- ABCA1 expression.

**P*<0.05, ***P*<0.01 vs. vehicle

DISCUSSION

In order to fully understand the mechanism of action of CETP inhibitors such as anacetrapib, a multi-faceted approach in an appropriate animal model should be used. The purpose of this study was to evaluate the dyslipidemic Syrian golden hamster treated with anacetrapib as a model to study CETP biology. By comprehensively analyzing plasma lipoprotein-associated cholesterol, detailed lipoprotein composition, and parameters related to fecal cholesterol excretion, this study uncovered clear utility in the use of dyslipidemic hamsters for studying HDL cholesterol handling, but also raised some discrepancies which may affect its use in studying integrated lipoprotein metabolism.

One aim of this study was to characterize the cholesterol and triglyceride composition of lipoproteins in response to CETP inhibition. In the case of HDL, LC/MS analysis of the HDL band excised from gradient gels showed that all sterols (free cholesterol and cholesteryl esters) were up-regulated in the HDL fraction, with CE 18:2 (cholesteryl linoleate) being the most abundant cholesteryl ester in HDL. This observation is consistent with studies in rodents that lecithin/cholesteryl acyl transferase (LCAT) preferentially hydrolyses phosphatidylcholines containing linoleate as the fatty acyl motif in the *sn-2* position for esterification of cholesterol on HDL (29, 30). Whether this substrate preference of LCAT for linoleate-containing phosphatidylcholines contributes to the predominance of cholesteryl linoleate in CETP-inhibited hamster HDL requires further study**.**

The increase in HDL free cholesterol is supportive of the notion that cholesterol efflux to HDL is improved with CETP inhibition. Indeed, it was observed *in vitro* that HDL efflux from cultured macrophages was increased in HDL from anacetrapib-treated animals. This observation is in line with studies where HDL from humans treated with anacetrapib (31) or CETP-deficient humans (32) display greater efflux capacity from cultured macrophages, and supports the use of this model to study cholesterol flux regulated by CETP, and to test the effects of CETP inhibition and movement of cholesterol into the feces for excretion.

It was observed that the fecal concentration of cholesterol and cholic acid (in a 24hr collection following 2 weeks of treatment) was increased in hamsters treated with anacetrapib, an effect that could not be attributed to alterations in cholesterol absorption from the intestine. An increase in both fecal neutral sterols and bile acids with CETP inhibition, taken together with the observation of increased free cholesterol and cholesterol ester in HDL and improved HDL efflux capacity, indicates that anacetrapib may be promoting reverse cholesterol transport, shifting cholesterol through the HDL pool and into the feces through biliary excretion. The current study utilized dyslipidemic hamsters, where it is possible that more cholesterol is being exchanged by CETP and could better reflect the human dyslipidemic state. While the combination of changes in lipoprotein composition, fecal cholesterol/bile acid, and HDL efflux capacity all point towards improvement of a net increase in cholesterol excretion, examination of true "macrophage-to-feces reverse cholesterol transport" provides more direct evidence. In this research, it was observed a marked increased of radio-tracer for both 'hot' cholesterol and bile acids in the anacetrapib treatment group, suggesting promotion of macrophage-to-feces reverse

cholesterol transport using standard methods as described by Rader and Rothblat (33-36). In combination, these studies support the hypothesis that anacetrapib promotes reverse cholesterol transport in a hamster model of dyslipidemia. Taken together with the *in vitro* cholesterol efflux data from the current study, which recapitulated observations from anacetrapib-treated and CETP-deficient humans, the dyslipidemic hamster appears to have significant utility in studying HDL-dependent cholesterol excretion.

In the case of LDL-cholesterol, due to the presence of enlarged HDL particles, the apparent reduction of LDL cholesterol observed with FPLC analysis was concluded to be artifactual, by (i) a lack of effect of treatment on serum apoB levels (Figure 2D), and (ii) a lack of effect of treatment on LDL-cholesterol analyzed by gradient gel electrophoresis and LC/MS analysis. From this analysis, gradient-gel electrophoresis with post-hoc LC/MS analysis of cholesterol species was used for more detailed characterization of lipoprotein composition as it appeared to achieve better resolution between LDL and HDL peaks with CETP inhibition. This analysis further supported of a lack of an LDL cholesterol-lowering effect by anacetrapib in this model, with the observation of no change in cholesterol species (free cholesterol, cholesteryl ester) in any of the LDL subfractions or VLDL isolated from gradient gels. Regardless, the reduction of major triglyceride species in HDL and reciprocal increase of triglycerides in LDL and in VLDL showed that inhibition of transfer activity of CETP in the hamster does affect the lipid composition. It is possible that CETP in hamsters plays a greater role in triglyceride transfer out of LDL into HDL rather than movement of cholesterol into LDL, but this requires a more in-depth investigation. Although hamsters express CETP endogenously, they do still carry the majority of their cholesterol on HDL. Placement of hamsters on a high-fat supplemented with cholesterol, as in the current study, has been shown to increase LDL cholesterol (37), but this still did not unmask an effect of CETP inhibition on LDL. Other CETP-containing species, such as nonhuman primates, in which the distribution of cholesterol across lipoproteins is more similar to humans, may be of significant utility in recapitulating the LDL reduction observed in human subject with anacetrapib.

In summary, this study reported changes in lipoprotein composition with CETP inhibition by anacetrapib in the dyslipidemic hamster. The lack of an LDL-lowering effect by anacetrapib in this model, which is not a high-LDL-cholesterol model, limits its utility. However, the changes in HDL lipid composition, coupled with increased fecal sterol excretion and increased HDL cholesterol efflux capacity, indicate that anacetrapib is promoting cholesterol excretion in this model, possibly via reverse cholesterol transport.

REFERENCES

1. Rayner, M., Allender, S., and Scarborough, P. (2009) *Eur J Cardiovasc Prev Rehabil* **16 Suppl 2,** S43-7.

2. Menotti, A., Lanti, M., Puddu, P. E., and Kromhout, D. (2000) *Heart* **84,** 238-44.

3. Rosamond, W., Flegal, K., Furie, K., Go, A., Greenlund, K., Haase, N., Hailpern, S. M., Ho, M., Howard, V., Kissela, B., Kittner, S., Lloyd-Jones, D., McDermott, M., Meigs, J., Moy, C., Nichol, G., O'Donnell, C., Roger, V., Sorlie, P., Steinberger, J., Thom, T., Wilson, M., and Hong, Y. (2008) *Circulation* **117,** e25-146.

4. Zhang, X. H., Lu, Z. L., and Liu, L. (2008) *Heart* **94,** 1126-31.

5. Gordon, T., Castelli, W. P., Hjortland, M. C., Kannel, W. B., and Dawber, T. R. (1977) *Am J Med* **62,** 707-14.

6. Miller, N. E., Thelle, D. S., Forde, O. H., and Mjos, O. D. (1977) *Lancet* **1,** 965-8.

7. Keys, A. (1980) *Lancet* **2,** 603-6.

8. Jacobs, D. R., Jr., Mebane, I. L., Bangdiwala, S. I., Criqui, M. H., and Tyroler, H. A. (1990) *Am J Epidemiol* **131,** 32-47.

9. Kitamura, A., Iso, H., Naito, Y., Iida, M., Konishi, M., Folsom, A. R., Sato, S., Kiyama, M., Nakamura, M., Sankai, T., and et al. (1994) *Circulation* **89,** 2533-9.

10. Barter, P. J., Caulfield, M., Eriksson, M., Grundy, S. M., Kastelein, J. J., Komajda, M., Lopez-Sendon, J., Mosca, L., Tardif, J. C., Waters, D. D., Shear, C. L., Revkin, J. H., Buhr, K. A., Fisher, M. R., Tall, A. R., and Brewer, B. (2007) *N Engl J Med* **357,** 2109-22.

11. Forrest, M. J., Bloomfield, D., Briscoe, R. J., Brown, P. N., Cumiskey, A. M., Ehrhart, J., Hershey, J. C., Keller, W. J., Ma, X., McPherson, H. E., Messina, E., Peterson, L. B., Sharif-Rodriguez, W., Siegl, P. K., Sinclair, P. J., Sparrow, C. P., Stevenson, A. S., Sun, S. Y., Tsai, C., Vargas, H., Walker, M., 3rd, West, S. H., White, V., and Woltmann, R. F. (2008) *Br J Pharmacol* **154,** 1465-73.

12. Stroes, E. S., Kastelein, J. J., Benardeau, A., Kuhlmann, O., Blum, D., Campos, L. A., Clerc, R. G., and Niesor, E. J. (2009) *Br J Pharmacol* **158,** 1763-70.

13. Polakowski, J. S., King, A. J., Campbell, T. J., Nelson, R. A., Preusser, L. C., Kempf-Grote, A. J., Marsh, K. C., Gintant, G. A., Cox, B. F., and Mittelstadt, S. W. (2009) *J Cardiovasc Pharmacol* **54,** 543-51.

14. DePasquale, M., Cadelina, G., Knight, D., Loging, W., Winter, S., Blasi, E., Perry, D., and Keiser, J. (2009) *Drug Development Research* **70,** 35-48.

15. Bloomfield, D., Carlson, G. L., Sapre, A., Tribble, D., McKenney, J. M., Littlejohn, T. W., 3rd, Sisk, C. M., Mitchel, Y., and Pasternak, R. C. (2009) *Am Heart J* **157,** 352-360 e2.

16. Cannon, C. P., Shah, S., Dansky, H. M., Davidson, M., Brinton, E. A., Gotto, A. M., Stepanavage, M., Liu, S. X., Gibbons, P., Ashraf, T. B., Zafarino, J., Mitchel, Y., and Barter, P. *N Engl J Med* **363,** 2406-15.

17. Bilz, S., Samuel, V., Morino, K., Savage, D., Choi, C. S., and Shulman, G. I. (2006) *Am J Physiol Endocrinol Metab* **290,** E716-22.

18. Woollett, L. A., and Spady, D. K. (1997) *J Clin Invest* **99,** 1704-13.

19. Woollett, L. A., Spady, D. K., and Dietschy, J. M. (1992) *J Clin Invest* **89,** 1133-41.

20. Liu, G. L., Fan, L. M., and Redinger, R. N. (1991) *Comp Biochem Physiol A Comp Physiol* **99,** 223-8.

21. Niesor, E. J., Magg, C., Ogawa, N., Okamoto, H., von der Mark, E., Matile, H., Schmid, G., Clerc, R. G., Chaput, E., Blum-Kaelin, D., Huber, W., Thoma, R., Pflieger, P., Kakutani, M., Takahashi, D., Dernick, G., and Maugeais, C. *J Lipid Res* **51,** 3443-54.

22. Briand F, Treguier M, Andre A, Grillot D, Issandou M, Ouguerram K, Suplice T. *J Lipid Res* **51**:732-770, 2010.

23. Ranalletta, M., Bierilo, K. K., Chen, Y., Milot, D., Chen, Q., Tung, E., Houde, C., Elowe, N. H., Garcia-Calvo, M., Porter, G., Eveland, S., Frantz-Wattley, B., Kavana, M., Addona, G., Sinclair, P., Sparrow, C., O'Neill, E. A., Koblan, K. S., Sitlani, A., Hubbard, B., and Fisher, T. S. *J Lipid Res* **51,** 2739-52.

24. Fournier, N., Francone, O., Rothblat, G., Goudouneche, D., Cambillau, M., Kellner-Weibel, G., Robinet, P., Royer, L., Moatti, N., Simon, A., and Paul, J. L. (2003) *Atherosclerosis* **171,** 287-93.

25. Mweva, S., Paul, J. L., Cambillau, M., Goudouneche, D., Beaune, P., Simon, A., and Fournier, N. (2006) *Eur J Clin Invest* **36,** 552-9.

26. Bligh, E. G., and Dyer, W. J. (1959) *Can J Biochem Physiol* **37,** 911-7.

27. Castro-Perez, J., Previs, S. F., McLaren, D. G., Shah, V., Herath, K., Bhat, G., Johns, D. G., Wang, S. P., Mitnaul, L., Jensen, K., Vreeken, R., Hankemeier, T., Roddy, T. P., and Hubbard, B. K. *J Lipid Res* **52,** 159-69.

28. Fahy, E., Subramaniam, S., Murphy, R. C., Nishijima, M., Raetz, C. R., Shimizu, T., Spener, F., van Meer, G., Wakelam, M. J., and Dennis, E. A. (2009) *J Lipid Res* **50 Suppl,** S9-14.

29. Grove, D., and Pownall, H. J. (1991) *Lipids* **26,** 416-20.

30. Liu, M., Bagdade, J. D., and Subbaiah, P. V. (1995) *J Lipid Res* **36,** 1813-24.

31. Yvan-Charvet, L., Kling, J., Pagler, T., Li, H., Hubbard, B., Fisher, T., Sparrow, C. P., Taggart, A. K., and Tall, A. R. *Arterioscler Thromb Vasc Biol* **30,** 1430-8.

32. Matsuura, F., Wang, N., Chen, W., Jiang, X. C., and Tall, A. R. (2006) *J Clin Invest* **116,** 1435-42.

33. Zhang, Y., Zanotti, I., Reilly, M. P., Glick, J. M., Rothblat, G. H., and Rader, D. J. (2003) *Circulation* **108,** 661-3.

34. Naik, S. U., Wang, X., Da Silva, J. S., Jaye, M., Macphee, C. H., Reilly, M. P., Billheimer, J. T., Rothblat, G. H., and Rader, D. J. (2006) *Circulation* **113,** 90-7.

35. Zhang, Y., Da Silva, J. R., Reilly, M., Billheimer, J. T., Rothblat, G. H., and Rader, D. J. (2005) *J Clin Invest* **115,** 2870-4.

36. Moore, R. E., Navab, M., Millar, J. S., Zimetti, F., Hama, S., Rothblat, G. H., and Rader, D. J. (2005) *Circ Res* **97,** 763-71.

37. Briand, F. *Curr Opin Investig Drugs* **11,** 289-97.