Anacetrapib promotes reverse cholesterol transport and bulk cholesterol excretion in Syrian golden hamsters

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Abstract Cholesteryl ester transfer protein (CETP) transfers cholesteryl ester (CE) and triglyceride between HDL and apoB-containing lipoproteins. Anacetrapib (ANA), a reversible inhibitor of CETP, raises HDL cholesterol (HDL-C) and lowers LDL cholesterol in dyslipidemic patients; however, the effects of ANA on cholesterol/lipoprotein metabolism in a dyslipidemic hamster model have not been demonstrated. To test whether ANA (60mg/kg/day, 2 weeks) promoted reverse cholesterol transport (RCT), 3H-cholesterol-loaded macrophages were injected and 3H-tracer levels were measured in HDL, liver, and feces. Compared to controls, ANA inhibited CETP (94%) and increased HDL-C (47%). 3H-tracer in HDL increased by 69% in hamsters treated with ANA, suggesting increased cholesterol efflux from macrophages to HDL. 3H-tracer in fecal cholesterol and bile acids increased by 90% and 57%, respectively, indicating increased macrophage-to-feces RCT. Mass spectrometry analysis of HDL from ANA-treated hamsters revealed an increase in free unlabelled cholesterol and CE. Furthermore, bulk cholesterol and cholic acid were increased in females from ANA-treated hamsters. Using two independent approaches to assess cholesterol metabolism, the current study demonstrates that CETP inhibition with ANA promotes macrophage-to-feces RCT and results in increased fecal cholesterol/bile acid excretion, further supporting its development as a novel lipid therapy for the treatment of dyslipidemia and atherosclerotic vascular disease.—Castro-Perez, J. C., F. Briand, K. Gagen, S-P. Wang, Y. Chen, D. G. McLaren, V. Shah, R. J. Vreeken, T. Hankemeier, T. Sulpice, T. P. Roddy, B. K. Hubbard, and D. G. Johns. Anacetrapib promotes reverse cholesterol transport and bulk cholesterol excretion in Syrian golden hamsters. J. Lipid Res. 2011. 52: 1965–1973.

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Cardiovascular disease continues to be a major contributor to morbidity and mortality throughout the world.

Despite therapies such as statins, which reduce circulating levels of low density lipoprotein cholesterol (LDL-C), cardiovascular event rates remain high. Numerous epidemiological studies (e.g., the Framingham Heart Study) indicate that high density lipoprotein cholesterol (HDL-C) levels are inversely correlated with cardiovascular risk (1–6). Therefore, therapies that increase HDL-C have gained recent attention as possible treatments for dyslipidemia and atherosclerosis.

Cholesteryl ester transfer protein (CETP) mediates transfer of cholesteryl ester (CE) and triglyceride (TG) between HDL and apoB-containing lipoproteins such as LDL and therefore, represents an attractive target for increasing HDL-C and reducing LDL-C. Indeed, initial clinical trials with torcetrapib established the validity of CETP inhibition as a mechanism for elevation of HDL-C (7, 8). However, the phase III outcome trial ILLUMINATE demonstrated that torcetrapib treatment was associated with an increase in cardiovascular events and overall mortality, possibly due to off-target effects on blood pressure and circulating adrenal hormones (9). A series of preclinical studies further corroborated that torcetrapib had compound-specific off-target activity that was unrelated to CETP inhibition (10–12).

Anacetrapib (ANA) is a potent CETP inhibitor that has not demonstrated the off-target activities of torcetrapib in preclinical or clinical studies (10, 13–15). ANA treatment increases HDL-C by over 100% and lowers LDL-C by 30–40% as a monotherapy and when coadministered with statins (13–15). In a recent 1.5 year safety study in ~1,600 patients with cardiovascular disease (15), ANA treatment

Abbreviations: ANA, anacetrapib; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; FC, free cholesterol; FCR, fractional catabolic rate; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; RCT, reverse cholesterol transport; TC, total cholesterol; TG, triglyceride.

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had no effect on blood pressure, electrolytes, or aldosterone, and the distribution of cardiovascular events suggested that ANA treatment would not be associated with an increase of cardiovascular risk that was observed with torcetrapib.

In order to comprehensively understand the impact of the robust changes in lipoprotein-associated cholesterol and cholesterol homeostasis induced by ANA, multiple approaches must be used. Macrophage-to-feces reverse cholesterol transport (RCT) is widely studied in mice to examine pathways that affect the egress of cholesterol from peripheral tissues to the feces (16). However, because mice do not inherently express CETP, mouse models are of little use without transgenic overexpression. The Syrian golden hamster expresses CETP endogenously and both normallipidemic and dyslipidemic versions of this model have been used to study RCT in response to lipid-modifying therapies (for review, see Ref. 17). In most cases using the approach described by Rader and Rothblat (16–21). Further, a comprehensive analysis of lipid metabolism including the study of RCT and monitoring lipoprotein lipid composition and bulk cholesterol and bile acid excretion is critical to more completely understand the effects of CETP inhibition on how lipoproteins handle or “traffic” cholesterol. The information gleaned from comprehensive profiling of lipoprotein metabolism and cholesterol trafficking in response to anacetrapib treatment will inform the CETP field on the mechanisms by which CETP inhibition with anacetrapib might prove beneficial in the clinic. The purpose of this study was to test the hypothesis that CETP inhibition with ANA will promote macrophage-to-feces RCT and cholesterol excretion in a dyslipidemic hamster model.

METHODS

Animals

All animal protocols were reviewed and approved by the Merck Research Laboratories Institutional Animal Care and Use Committee (Rahway, NJ). Male Syrian golden hamsters (weight ~100 g at beginning of study) were given free access to food and water. For tracer-based RCT/ fractional catabolic rate (FCR) experiments and for bulk cholesterol experiments, hamsters were placed on a high-fat diet [45% kcal from fat (lard), 35% kcal from carbohydrate, 20% kcal from protein, 0.12% cholesterol; Research Diets, New Brunswick, NJ]. This diet was chosen based upon pilot studies, which determined that this level of dietary cholesterol would produce dyslipidemia relative to normal hamster diet without inducing liver steatosis. After 4 weeks of induction of dyslipidemia, hamsters were maintained on the high-fat diet and administered ANA, admixed into the diet to deliver 60 mg/kg, or control high-fat diet (no ANA). Prior to treatment, blood samples were taken for pretreatment biochemical analysis (see below). The dose of ANA was identified in pilot dose-ranging studies that indicated this dose produced maximal inhibition of CETP and maximal changes in lipoproteins (e.g., increase in HDL-C; data not shown). Animals were treated with ANA for 2 weeks. Plasma ANA concentrations were determined by standardized LC/MS methods by the Merck Research Laboratories Drug Metabolism/Pharmacokinetic department.

RCT and FCR studies

Experiments studying RCT and FCR of HDL were performed at Physiogenex (Labege, France). For these studies, each treatment group (control, ANA) was divided into two groups of 12 animals; one for RCT studies and one for FCR experiments. Animals were maintained on the same diet and treatment regimen during the RCT and FCR procedures.

Biochemical analysis. For RCT and FCR studies, total cholesterol (TC) and TGs were assayed using commercial kits (Biorneux, Marcy l’Etoile, France). HDL-C was determined using the phosphotungstate/MgCl2 precipitation method. Non-HDL-C levels were then determined by subtracting HDL-C values from total plasma cholesterol. Plasma CETP activity was measured by fluorescence using commercial kits (Roarbiomedical, New York, NY).

In vivo macrophage-to-feces RCT. Preparation of J774 cells and in vivo RCT study 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 0.5 µCi/ml 3H-cholesterol and cholesterol loaded with 50 µg/ml oxidized LDL over 48 h. Radiolabeled cells were then washed with RPMI/HEPES and equilibrated for 4 h in fresh RPMI/HEPES supplemented with 0.2% BSA and gentamicin. Cells were pelleted by low speed centrifugation and resuspended in MEM/HEPES prior to injection into hamsters.

Following 2 weeks of ANA treatment (described above), 3H-cholesterol-labeled and oxidized LDL-loaded J774 cells (2.5 × 106 cells containing 10 × 106 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 h experiment. Blood was collected by from the jugular vein under isoflurane anesthesia at 24, 48, and 72 h to measure radioactivity released into the plasma and HDL after phosphotungstate/MgCl2 precipitation (50 µl of plasma or HDL counted). Hamsters were then euthanized by cervical dislocation, exsanguinated, and the liver was harvested from each animal. For total liver 3H-tracer determination, a 50 mg piece of liver was homogenized using an ultrasound probe in 500 µl water then 100 µl were counted in a liquid scintillation counter. Liver and fecal 3H-labeled cholesterol and bile acids were determined as described (21). Briefly, for 3H-cholesterol and 3H-bile acid determination from liver, a 50 mg piece of liver was homogenized in 0.5 ml distilled water. To extract the 3H-cholesterol and 3H-bile acid fractions, each homogenized sample was combined with 1 ml ethanol and 200 µl NaOH. The samples were saponified at 95°C for 1 h and cooled to room temperature, and then 3H-cholesterol was extracted twice with 3 ml hexane. The extracts were pooled, evaporated, resuspended in toluene, and then counted in a liquid scintillation counter. To extract 3H-bile acids, the remaining aqueous portion of the feces was acidified with concentrated HCl and then extracted twice with 3 ml ethyl acetate. The extracts were pooled together, evaporated, resuspended in ethyl acetate, and counted in a liquid scintillation counter. Feces were collected over 72 h and were stored at 4°C before extraction of cholesterol and bile acids. Fecal 3H-cholesterol and 3H-bile acids were measured as described above for liver, with the exception that 1 ml of distilled water was used per 100 mg of feces for homogenization. Results were expressed as a percent of the radioactivity injected recovered in plasma, HDL, liver, and feces. The plasma volume was estimated as 3.5% of the body weight.

In vivo 3H-cholesterol oleate-HDL kinetics (FCR). A plasma pool was obtained from normocholesterolemic hamsters maintained on a control diet. Hamster HDLs (d = 1.07–1.21) were then isolated by ultracentrifugation as previously described (22).
After extensive dialysis against saline, HDL was labeled with $^{3}H$-cholesterol olate in the presence of lipoprotein-deficient serum collected from rabbit plasma. The labeled HDLs were reisolated by ultracentrifugation ($d = 1.07-1.21$) then extensively dialyzed prior to injection.

The day before the in vivo experiment, a catheter was inserted into the jugular vein under isoflurane anesthesia for both injection and blood collection. Catheters were kept with NaCl 0.9%. To prevent blood circulation and coagulation inside the catheter, a small volume of heparin (500 IU/ml) and glycerol (1 g/ml) was injected at the catheter extremity.

The day of the experiment, hamsters were weighed and placed into individual cages. Animals had free access to food and water and were kept treated over the 48 h experiment. The $^{3}H$-cholesterol olate-labeled HDLs ($\sim 2-3$ million dpm) were injected intravenously and blood samples (150 $\mu$L) were collected at time $t = 5$ min, 1 h, 3 h, 6 h, 24 h, and 48 h after injection. Plasma and HDL (phosphotungstate/MgCl$_2$ precipitation) were immediately isolated and stored at 4°C prior radioactivity measurement in a liquid scintillation counter (10 $\mu$L plasma or HDL counted). Fece samples were collected over 48 h and were stored at 4°C before cholesterol and bile acids extraction as described above.

Plasma and HDL decay curves were normalized to radioactivity at the initial 5 min time point after $^{3}H$-cholesterol olate-labeled HDL injection. Plasma and HDL FCR was then calculated from the area under the plasma disappearance curves fitted to a bicompartamental model using the SAAM II software.

Ex vivo cholesterol efflux assay. Ex vivo cholesterol efflux capacity of hamster serum was measured as described by Fournier et al. (23) and Mwewa et al. (24) (VascularStrategies LLC, Wynnewood, PA). ABCA1-mediated cholesterol efflux was determined in J774 mouse macrophages. Scavenger receptor class B type I (SR-BI)-mediated cholesterol efflux was assessed in Fu5AH rat hepatoma cells. ABCG1-mediated efflux was assessed in baby hamster kidney cells overexpressing human ABCG1.

Bulk cholesterol/lipoprotein composition studies. Bulk cholesterol and lipoprotein composition studies were performed at Merck Research Laboratories (Rahway, NJ). For determination of the effects of ANA treatment on lipoprotein composition and bulk cholesterol excretion, hamsters were administered dyslipidemic diet and treated with ANA as described above (n = 8 per group). Following ANA treatment, blood was collected and plasma isolated by low-speed centrifugation and stored at −80°C until analysis was performed. Feces were collected over a 24 h period prior to and following 2 weeks of treatment with either vehicle or ANA. A separate cohort of hamsters was used to determine possible effects of ANA on cholesterol absorption. In this study, the diet conditions and treatment were identical as described above with the inclusion of a group of animals treated with ezetimibe (n = 8) (1.5 mg/kg admixed into the diet) as a positive control for cholesterol absorption inhibition. In this cohort, hamsters were administered D$_3$-cholesterol (Sigma, St. Louis, MO) orally at a dose of 12 mg/kg. Feces were collected over a 24 h period following administration of D$_3$-cholesterol at 24, 48, 72, and 96 h intervals. Fecal samples were frozen at −20°C until analyzed by LC/MS.

Serum lipoprotein analysis by gel electrophoresis. Gel electrophoresis was employed in this study to separate lipoproteins for quantitating the cholesterol component in VLDL, LDL, and HDL fractions. This method was selected as it permits not only in-gel quantitation of cholesterol but also isolation of lipoprotein subfraction bands and post hoc analysis of lipid composition with high-resolution mass spectrometry.
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 min to pellet any residual solids. One hundred microliters of the supernatant was transferred to a new 96-well plate which was then sealed before analysis by LC/MS. External calibration curves were used for FC and CE measurements are described in the Methods section above.

For bile acid extraction from the fecal samples, 10 μl of the fecal homogenate was transferred to a 1.5 ml eppendorff tube, and the homogenate was diluted with 490 μl of 80% methanol containing 500 nM D4-cholic acid as the internal standard. The resulting mixture was then vortexed for 60 and centrifuged at 10,000 rpm for 10 min at 10°C. One hundred microliters of the supernatant was transferred to a 96-well plate and centrifuged again for 10 min to pellet any residual solids. The supernatant was transferred to a new 96-well plate for analysis. Resulting analyte concentrations for the samples were computed against each calibration line for the corresponding bile acid.

**Data processing and statistical analysis.** Unless stated otherwise, data are presented as mean ± standard error of the mean. For comparisons between two groups, a two-tailed t-test was employed with a P-value < 0.05 being considered significant. For lipoprotein analysis using gel electrophoresis (Lipoprint), data processing was conducted using the manufacturer’s software. Lipid composition and quantitation analysis from LC/MS experiments was carried out using MassLynx (Waters).

**Lipid nomenclature.** The lipid nomenclature utilized throughout the paper is the same as described by Fahy et al. (27). For instance, CE 18:1 denotes CE containing 18 hydrocarbons and 1 double bond as the fatty acyl substituent. The same applies to TG cited in the article. For example, TG 54:3, translates to a TG containing 54 hydrocarbons attached to the glycerol backbone and a total of three double bonds, which forms part of the three fatty acyl substituents.

**RESULTS**

**RCT/FCR studies**

Two weeks of ANA treatment was associated with reduced CETP activity and increased HDL-C with no effect on LDL-C. There were no significant differences in serum TG, TC, HDL-C, or nonHDL-C between control and ANA groups prior to treatment (data not shown).

Two weeks of treatment with 60 mg/kg ANA in the diet resulted in serum ANA concentrations of 4.6 ± 0.6 μM at the time of blood collection for lipid and CETP activity analysis. Treatment with ANA was associated with 94% lower CETP activity compared with control animals (CETP activity control 43.3 ± 1.4, ANA 2.4 ± 0.4 pmol/μl/h; P < 0.001 vs. control).

As shown in Fig. 1, ANA treatment resulted in a 47% increase in HDL-C levels compared with control animals (P < 0.001). The lack of any effect on nonHDL-C with ANA treatment suggests the increase in TC observed is primarily due to the change in HDL-C. A reduction in total plasma triglyceride was also observed with ANA treatment.

ANA promotes macrophage-to-feces RCT. To determine whether ANA treatment affected RCT, radiolabeled cholesterol-loaded macrophages were injected in hamsters following treatment with either control or ANA-containing diets. As shown in Fig. 2A, the appearance of tracer in plasma was 20% higher in animals treated with ANA compared with controls at 72 h. When HDL was isolated from plasma, the appearance of tracer in HDL from ANA-treated animals was 69% higher than that of control animals at 72 h (Fig. 2B). When liver radioactivity was measured, animals treated with ANA displayed 29% lower amount of tracer in the liver compared with controls (Fig. 2C). The majority of this decrease was attributable to a 33% reduction in 3H-bile acids compared with controls (Fig. 2D). Liver 3H-bile acids were not different between groups. Liver cholesterol mass (unlabeled) was also decreased in ANA-treated hamsters compared with vehicle and bile acid mass was unchanged (Fig. 2E). As shown in Fig. 2F, ANA treatment was associated with a significant increase in both 3H-cholesterol and 3H-bile acids by 90 and 57%, respectively.

To further determine the effects of ANA on cholesterol flux, hamsters were injected with 3H-cholesterol olate-labeled HDL, and the rate of 3H-tracer disappearance from plasma and HDL was determined. As shown in Fig. 3A, ANA treatment was associated with a slower rate of 3H-tracer decay in plasma over the 48 hr monitoring period. This is reflected as a 29% reduction in FCR (Fig. 3B). The same trend was observed when the degree of tracer decay was monitored in the HDL fraction (Fig. 3C, D), where ANA treatment was associated with a 48% reduction in FCR of 3H-tracer. The reductions in 3H-tracer from both plasma and HDL therefore indicate that CETP inhibition with ANA increases HDL-C levels through a reduction of FCRs. From this experiment, liver and feces radioactivity were also measured 48 h after treatment. As was observed with 3H-labeled macrophages, ANA treatment was associated with decreased...
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HDL was observed in large HDL particles (increase of 89% vs. vehicle) compared with intermediate HDL particles (increase of 29% vs. vehicle) (Fig. 4A). LC/MS analysis of FC and CE species in total HDL revealed significant increases in FC (increase of 33% vs. vehicle) and major CE species in ANA-treated hamster HDL, with the greatest increase observed with CE 18:2 (57% vs. vehicle) (Fig. 4B). No change in cholesterol or CE content was observed across the LDL subfractions (Fig. 5). This is similar to the lack of effect on LDL observed in the RCT study.

Fecal cholesterol excretion following CETP inhibition. Treatment with ANA resulted in 30% increase in fecal cholesterol content compared with vehicle (Fig. 6). Cholic acid content (Fig. 6) was also increased by 29% in ANA-treated animals compared with vehicle. To determine whether the increase in fecal cholesterol content was due to effects on intestinal absorption, the appearance of orally administered D6-labeled cholesterol in the feces was used as an index of absorption. D6-cholesterol appeared in the feces over the course of 48 h for all animal groups. Time points beyond 48 h showed levels of D6-cholesterol near the limit of detection (data not shown). The positive control ezetimibe, which inhibits cholesterol absorption as an inhibitor of intestinal NPC1L1, showed a marked increase in fecal D6-cholesterol content (344%) over vehicle samples during the first 24 h after tracer administration (Fig. 7A). Fecal D6-cholesterol concentration was not different between vehicle and ANA treated hamster fecal samples (Fig. 7C). Plasma circulating levels of D6-cholesterol were determined by LC/MS analysis. For ezetimibe-treated animals, the area under the curve for plasma D6 cholesterol was significantly reduced (Fig. 7B) (reduction of 99.6%) For animals treated with ANA (Fig. 7D), plasma D6 cholesterol

Bulk cholesterol and lipid composition analysis

For analysis of changes in bulk cholesterol and lipoprotein composition with ANA treatment, a separate cohort of hamsters were subjected to the same diet and drug treatment conditions as the RCT/FCR studies described above. Two weeks of treatment with 60 mg/kg ANA in the diet resulted in serum ANA concentrations of 7.8 ± 1.9 nmol/L. Analysis of HDL-C with gel electrophoresis (Lipoprint) showed a similar degree of increase in HDL-C with ANA treatment as was observed in the RCT study (53% increase compared with vehicle-treated). Also similar to the RCT study, no change in LDL/intermediate density lipoprotein/VLDL cholesterol was observed with ANA treatment (data not shown).

From analysis of HDL from gel electrophoresis, a greater degree of increase in cholesterol content in ANA-treated liver radioactivity (vehicle 38 ± 4%, ANA 30 ± 2% of injected dose, P < 0.05) and a nonsignificant trend toward increased fecal 3H-cholesterol and bile acid content was observed with ANA treatment (fecal 3H-cholesterol: vehicle 0.08 ± 0.01%, ANA 0.09 ± 0.02%; P = 0.4; 3H-bile acids: vehicle 0.07 ± 0.01%, ANA 0.1 ± 0.01% P = 0.09).

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was slightly but not significantly increased (area under the curve increase of $\sim 15\%$).

**HDL from hamsters treated with ANA exhibited increased cholesterol efflux capacity.** A statistically significant increase in SR-BI-mediated efflux ($P = 0.003$) in the ANA group was observed (3.83% ± 0.30%) versus the vehicle group (3.30% ± 0.27%) (Fig. 8A). In the ABCA1-dependent efflux assay, treatment of cells with ANA treated plasma was associated with an increase in the average cholesterol efflux compared with vehicle in the ABCA1-upregulated group (11.58 ± 0.51% vs. 10.01 ± 0.48%, $P < 0.05$; Fig. 8B) but not in the group in which ABCA1 was not upregulated. ANA-treated plasma was also associated with an increase in ABCG1-mediated efflux compared with vehicle controls (1.49 ± 0.05% vs. 1.15 ± 0.04%, $P < 0.001$).

**DISCUSSION**

Inhibitors of CETP are being evaluated clinically for managing the treatment of cardiovascular disease, relying on the hypothesis that prevention of transfer of cholesterol ester from HDL to LDL will reduce levels of atherogenic CE associated with LDL and promote removal of cholesterol from blood vessel macrophages and excretion into the feces. The process of RCT involves efflux of cholesterol from peripheral tissues onto HDL particles, which transport the cholesterol to the liver for biliary excretion into the feces as cholesterol and bile acids (28). The method most widely used to examine effects of dietary challenge and pharmacotherapy on RCT is the labeled macrophage method described by Rader and Rothblat (16–21). This method utilizes J774 macrophages labeled in vitro with $^3$H-cholesterol and oxidized LDL and monitors the movement of $^3$H-tracer into cholesterol and bile acid pools in plasma, liver, and feces following injection into animals. This method has been used in hamsters to examine the effects of several pharmacotherapies on macrophage-to-feces RCT, including liver X receptor activation (22) and CETP inhibition with torcetrapib (29). To comprehensively assess the effects of a pharmacological intervention on cholesterol handling and excretion, it is important not only to study macrophage-to-feces RCT but also to take into account the bulk movement of cholesterol mass within lipoproteins and in the fecal compartment.

The purpose of the current study was to evaluate the effects of CETP inhibition with ANA on RCT, cholesterol mass, and lipoprotein composition using multiple
The current study was not to examine the effects of ANA in normal animals, but rather in dyslipidemic animals, it is possible that the methodological differences in how RCT was tested in each study (including the method of labeling macrophages, duration of feces monitoring) in addition to the dietary conditions used could have contributed to the different results reported. In the current study, we observed an increase in the fecal excretion of $^3$H-neutral sterols and $^3$H-bile acids (both derived from labeled macrophages) and of unlabeled cholesterol and bile acid, in two separate studies. The congruence of in vivo data from two experimental approaches in a hamster model of dyslipidemia strengthens the finding that ANA promotes cholesterol movement from the macrophage to the feces. Interestingly, in the RCT study, in both $^3$H-labeled macrophage and $^3$H-labeled HDL experiments, a lower hepatic $^3$H-tracer recovery was observed. The liver $^3$H recovery in the current study was examined at a single time point, which may not represent the intermediate role of the liver in the overall biliary flux of cholesterol from HDL to the feces. Using a hamster model of RCT, Tchoua et al. (29) demonstrated that CETP inhibition with torcetrapib promoted macrophage-to-feces RCT without increasing $^3$H-cholesterol in the liver. Although it is beyond the scope of the current study, it would be useful to determine the kinetics of the appearance and disappearance of tracer in

**Fig. 7.** Anacetrapib treatment does not affect cholesterol absorption. A: Fecal $D_6$-cholesterol excretion over the time-course of 48 hr for vehicle and ezetimibe treated hamsters. B: Plasma levels of $D_6$-cholesterol for vehicle and ezetimibe treated hamsters. C: Fecal $D_6$-cholesterol excretion over the time-course of 48 hr for vehicle and anacetrapib treated hamsters. D: Plasma levels of $D_6$-cholesterol for vehicle and anacetrapib treated hamsters. **$P < 0.01$** ezetimibe 24 h versus vehicle 24 h; *$P < 0.05$ ezetimibe 48 h versus vehicle 48 h.

**Fig. 8.** Cholesterol efflux was increased in HDL from anacetrapib-treated hamsters. A: SR-BI mediated cholesterol efflux. B: Cholesterol efflux ± ABCA1 upregulation with cAMP. C: ABCG1 mediated cholesterol efflux. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus vehicle.
In the current study, gel electrophoresis was used to quantify lipoprotein composition, due to the high degree of separation of different LDL, IDL, and HDL subfractions, and the ability to isolate these fractions for LC/MS analysis. Between the RCT and bulk cholesterol studies, we saw good agreement in the degree of HDL-C increase with ANA treatment between the phosphotungstate-MgCl₂ precipitation method (RCT study) and gel electrophoresis method (bulk cholesterol study). LC/MS analysis of lipoproteins showed that all sterols (FC and CEs) were upregulated in the HDL fraction with CE 18:2 being the most abundant CE component of the HDL fraction. It has been shown in rodents that LCAT preferentially hydrolyses phosphatidylcholines containing linoleate (18:2) as the fatty acyl motif in the sn-2 position (31, 32). In the current study, levels of FC were upregulated in the HDL fraction from ANA-treated animals, which could provide increased cholesterol scaffold for LCAT esterification.

The majority of the increase in HDL-C occurred in large HDL particles. This result is similar to findings by Matsuura et al. (33), who described that large spherical HDL particles are good acceptors of cholesterol from macrophages (cholesterol efflux). In that study, samples were analyzed from human subjects genetically deficient in CETP and the results described the presence of large HDL-2 particles. In these genetically deficient subjects, cholesterol efflux from macrophages was enhanced by 2- to 3-fold with respect to control HDL-2 in liver X receptor activated macrophages (34). Furthermore, Yvan-Charvet et al. (35) recently reported that plasma from humans treated with ANA showed enhanced cholesterol efflux capacity in vitro. Both of these reports are consistent with the in vitro cholesterol efflux data from the current study where an increase in efflux capacity of HDL from CETP-inhibited animals was observed.

In both experiments from the current study, the increase in HDL-C relative to CETP inhibition is similar to what has been reported in normolipidemic and dyslipidemic hamsters using both small molecules and antibody approaches (29, 30, 36). Although this increase in HDL-C is consistent with what is observed by others in similar preclinical models, no reduction in LDL-C was observed, which is in contrast to what is observed in humans. Although high-fat/high-cholesterol diet does increase LDL-C in the hamster above what is observed on normal diet (17, 22), the Syrian golden hamster still appears to carry less cholesterol on LDL. Blockade of cholesterol absorption with ezetimibe will reduce LDL-C in a dyslipidemic hamster model (37) and has been shown to promote RCT in mice due to inhibition of reabsorption of ³H-cholesterol derived from injected macrophages (38). The dyslipidemic hamster model has been used with limited success to study the LDL-lowering effects of statins (39, 40). Therefore, whereas prevention of intestinal absorption of cholesterol from the diet would directly affect LDL-C levels in a dietary model, removal of LDL via increased clearance by statins or by CETP inhibition appears more difficult to reproduce in the dyslipidemic hamster, due possibly to a greater upregulation of PCSK9 compared with LDL-receptor as reported by Dong et al. (41). Regardless, the effects of ANA on cholesterol excretion and RCT are clear from the current study, and it is tempting to speculate whether an even greater increase in cholesterol excretion might be observed in a model where ANA lowers LDL-C (i.e., in a nonhuman primate) where LDL-C and HDL-C levels are much more similar to the human.

In summary, the current study used multiple in vivo and in vitro approaches to assess the effects of anacetrapib on cholesterol metabolism in the dyslipidemic Syrian golden hamster. Taken together, the data from these independent approaches indicate that in this model, anacetrapib promotes both macrophage-to-feces RCT and the movement of the bulk cholesterol pool from the body and into the feces, with strong evidence that anacetrapib improves the ability of HDL to remove cholesterol from the system. Therefore, these results support the development of anacetrapib as a treatment for atherosclerotic cardiovascular disease.

REFERENCES


