Evaluation of CETP activity in vivo under non-steady-state conditions: influence of anacetrapib on HDL-TG flux

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Abstract  Studies in lipoprotein kinetics almost exclusively rely on steady-state approaches to modeling. Herein, we have used a non-steady-state experimental design to examine the role of cholesteryl ester transfer protein (CETP) in mediating HDL-TG flux in vivo in rhesus macaques, and therefore, we developed an alternative strategy to model the data. Two isotopomers ([1H11] and [13C18]) of oleic acid were administered (orally and intravenously, respectively) to serve as precursors for labeling TGs in apoB-containing lipoproteins. The flux of a specific TG (52:2) from these donor lipoproteins to HDL was used as the measure of CETP activity; calculations are also presented to estimate total HDL-TG flux. Based on our data, we estimate that the peak total postprandial TG flux to HDL via CETP is ~13 mg·h⁻¹·kg⁻¹ and show that this transfer was inhibited by 97% following anacetrapib treatment. Collectively, these data demonstrate that HDL TG flux can be used as a measure of CETP activity in vivo. The fact that the donor lipoproteins can be labeled in situ using well-established stable isotope tracer techniques suggests ways to measure this activity for native lipoproteins in free-living subjects under any physiological conditions.—McLaren, D. G., S. F. Previs, R. D. Phair, S. J. Stout, D. Xie, Y. Chen, G. M. Salituro, S. S. Xu, J. M. Castro-Perez, G. J. Opiteck, K. O. Akinsanya, M. A. Cleary, H. M. Dansky, D. G. Johns, and T. P. Roddy. Evaluation of CETP activity in vivo under non-steady-state conditions: influence of anacetrapib on HDL-TG flux. J. Lipid Res. 2016. 57: 398–409.

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Nichols and Smith (1) were the first to observe that cholesteryl esters (CEs) could be transferred from HDL to apoB-containing lipoproteins in exchange for TG in incubated human serum, a finding later confirmed by Hopkins and Barter (2). Subsequent investigations by Morton et al. (3, 4) identified a single lipid transfer protein in human plasma that was capable of carrying out this apparent heterolipid exchange and that is known today as cholesteryl ester transfer protein (CETP). In these and other reports, the exchange of HDL-CE for apoB-lipoprotein-TG was evaluated in vitro using lipoproteins containing radiolabeled CE and/or TG. Using this approach, the effects of donor and acceptor lipid composition (4), hypercholesterolemia (5), and many other parameters on CETP-mediated neutral lipid exchange have been assessed. The CETP-mediated exchange of neutral lipids between lipoproteins in vivo has also been studied (6–9). In such cases, the experimental protocol typically involves injection of lipoprotein particles, radiolabeled with CE either in vitro or in vivo, into subjects followed by collection of blood samples for further analysis. Transfer activity is then evaluated by measuring the loss of enrichment from the donor particle and increase in enrichment of acceptor particles that were isolated from the postinjection blood samples.

We hypothesized that the movement of TG from apoB lipoproteins to HDL could serve as a quantifiable activity measurement in vivo that would not require prior isolation and reinjection of donor particles. As noted, although the protein is commonly referred to as “cholesteryl ester transfer protein,” the transfer of TG also occurs; this should allow an experimental protocol to be designed based on well-characterized methods for labeling TGs in free-living subjects. Lipoprotein TG kinetics have been studied for at least 30 years using both radioisotopes and stable isotopes to label the fatty acid precursors or the glycerol backbone. In the majority of cases, the goals of such studies have been determination of the flux (mass per unit

Abbreviations: BW, body weight; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; FSD, fractional standard deviation; TPGS, tauro-cocopherol polyethylene glycol succinate.

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time) of TG in specific lipoprotein compartments at one or more physiological steady states (10–13). Using these methods as a starting point, we previously demonstrated the techniques necessary to characterize TG transfer between lipoprotein compartments in vivo using C57Bl/6 wild-type mice (which lack CETP) and natural flanking region (NFR)-CETP-transgenic mice (14). Clear differences in the kinetics of TG appearance in HDL were observed between these two extreme conditions (i.e., in the absence and presence of CETP).

The goals of the present study were 2-fold: 1) to develop and test a mathematical model to describe HDL-TG flux as a measure of neutral lipid exchange between particles in a non-steady state and 2) to determine whether this approach would be useful to assess pharmacological inhibition of CETP activity in vivo. In order to achieve these goals, we sought to develop the simplest possible model that allows in vivo quantification of CETP-mediated TG flux and to use this to measure inhibition by anacetrapib. While development of a more complex model to account for other neutral lipid exchanges could be pursued following similar techniques, we specifically constrained our interest to measuring HDL-TG flux in order to establish a clear proof of concept on which future studies could be based.

We selected rhesus macaques as the experimental model for these studies based on the facts that they endogenously express CETP and are frequently used as a translational model for pharmaceutical research. To control as much as possible for interanimal variability, we designed a protocol wherein each subject was treated alternately with vehicle or the potent, selective CETP-inhibitor anacetrapib. Others have previously shown that rates of CE transfer are accelerated in the postprandial state (15) and under conditions of hyperlipidemia (16–18). This suggested that the greatest differences in HDL-TG flux between control and anacetrapib-treated subjects would most likely occur under similar conditions. We therefore elected to study TG transfer kinetics following administration of an intravenous lipid challenge and a standardized liquid meal, each of which contained independent isotopomers of oleic acid, $[^{13}C_{18}]$ (intravenous) and $[^{2}H_{1}]$ (oral), to label lipoprotein TG, thereby creating a postprandial, hyperlipidemic non-steady state. Pioneering approaches to non-steady-state flux calculations have, until recently, been based on approximations (19) that are difficult to justify in many experimental situations (20, 21) and that could have confounded our mathematical modeling efforts here. Recent advances in kinetic modeling (22) have rendered these calculations tractable, and we therefore sought to apply these newer methods to developing a model capable of quantifying TG fluxes from plasma chylomicrons, VLDL, and LDL to plasma HDL and characterizing the effects of the CETP inhibitor anacetrapib on these parameters.

**MATERIALS AND METHODS**

**Rhesus studies**

All animals were maintained in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All experimental procedures were approved by the Institutional Animal Care and Use Committee and were in conformance with the National Research Council’s Guide for the Care and Use of Laboratory Animals. Male rhesus macaques (n = 6) ranging in age from 6 to 12 years and with an average body weight (BW) of ~11 kg were used in each study. A longitudinal, crossover design was used wherein each animal served as his own control (Fig. 1). Animals were fasted overnight and, with the exception of tracer study days, were given yogurt mixed with either 20% $[^{2}H_{1}]$-tocopherol polyethylene glycol succinate (TPGS) vehicle (day -6 through day 0) or 150 mg/kg of anacetrapib in 20% TPGS (day 1 through day 14) for self-administration. Animals were fed their normal biscuit ration once the entire yogurt mixture had been consumed (LabDiet Fiber-Plus Monkey Diet; composition by kCal, 26.5% protein, 13.9% fat, 59.6% carbohydrate).

On the day of each tracer study (day -4 and day 10) following the overnight fast, the animals were transferred to restraint chairs and intravenous catheters were placed and flushed with saline every 30 min. At $t = -1$ h animals were gavaged with 0.625 ml/kg 20% TPGS vehicle (on day -4) or 150 mg/kg of anacetrapib (0.625 ml/kg in 20% TPGS; on day 10). Thirty minutes later at $t = -0.5$ h, animals were dosed with 7.5 ml/kg of a high-fat liquid meal challenge consisting of two parts Ensure liquid meal (Abbott) and 1 part heavy cream, which also contained the deuterated isotopomer of oleic acid at a concentration of 6.7 mg/ml for a final dose of 50 mg/kg ([$^{2}H_{1}$]-oleate custom synthesized in house; the chemical structure is shown in supplementary Fig. 1). At $t = 0$ h, animals were given a 2 ml/kg intravenous bolus of Intralipid 20 (Sigma) which contained the $[^{13}C_{18}]$-labeled isotopomer of oleic acid (Cambridge Isotope Laboratories) at a concentration of 5 mg/ml for a final dose of 10 mg/kg. Both isotopomers of oleic acid were formulated from the potassium salt forms. Prior to and following dosing, blood was taken via intravenous catheter at ~1 h and during postdose intervals 20, 40, 60, 80, and 100 min and 2, 3, 4, 5, 24, 48, and 120 h. Animals were sedated with ketamine HCl for the 24, 48, and 120 h sample collections. Whole blood samples were held on ice and centrifuged at 3,000 rpm for 8 min at 8°C within 30 min of collection. Plasma

![Diagram](image-url)
was harvested and aliquoted into 96-well plates containing lipase and protease inhibitors for subsequent analysis.

Analytical methods

Chylomicrons were isolated from fresh plasma by ultracentrifugation following the method of van Heek et al. (23). Briefly, 200 μl of plasma was applied to individual polycarbonate centrifuge tubes and overlaid with 587 μl of a d = 1.006 g/ml solution. Samples were spun at 25,000 g for a 1 h run at 25°C. The top one-third of the tube was harvested as chylomicrons and subsequently frozen at −80°C prior to analysis. Lipoprint gel electrophoresis was used to separate VLDL, LDL + IDL, and HDL from a separate, 25 μl aliquot of plasma using LDL gel kits (Quantimetrix, Redondo Beach, CA). Gel bands containing the isolated lipoprotein fractions were excised and homogenized in PBS buffer for subsequent lipid extraction as previously described (14). The concentrations of all isotoptomers of TG52:2 (M₀, M₁₁, M₁₈, M₂₂, and M₃₆) were determined in each lipoprotein fraction using an LC/MS method that has been described elsewhere (24). It is worth noting that we did not observe any measurable amounts of the M₂₀ TG52:2 isotoptomer that could have been formed by incorporation of both the [²H₁₁] oral and [¹³C₁₈] intravenous oleic acid isotoptomers into a single molecule of TG52:2.

The total cholesterol and TG contents of whole plasma were determined using commercially available enzymatic kits (Cholesterol E, Wako; Infinity Triglycerides, Thermo Scientific); the lipid composition of distinct lipoprotein fractions was determined by fast-protein liquid chromatography as previously described (25). Ex-vivo CETP activity was determined using a radioactive transfer assay to quantify the amount of [²H]cholesteryl oleate transferred from prelabeled exogenous LDL to HDL by CETP (26). Concentrations of CETP protein in plasma were determined by sandwich ELISA (27) and are shown in supplementary Fig. 2. The concentrations of anacetrapib in plasma were determined by LC/MS using methods similar to those that have been previously reported (28); modifications are summarized in the supplementary data, and plots of the concentration vs. time curves are presented in supplementary Fig. 3.

Development of a mathematical model for HDL-TG flux

Early work on the mechanism of CETP focused on CETP as a free carrier protein and suggested that TG transfer was mediated by a stoichiometric exchange with CE, but more recent reviewers (29) favor bidirectional transfer governed by mass action. Moreover, the current widely discussed view of CETP as a hydrophobic tunnel (30) connecting lipoprotein cores is closer to Tall’s (31) original ternary complex model and appears incompatible with the stoichiometric exchange concept. The inside diameter of the tunnel is reportedly less than two neutral lipid diameters creating a nearly insurmountable problem for theories that suggest one-for-one exchange. These data suggest that concerted transfer of neutral lipid from apoB-containing particles to HDL (and vice versa) is more likely than bimolecular exchange, and we adopted this concept in developing the model presented here.

If CETP is taken as the only mechanism for delivery of TG to HDL, it is possible to provide a large number of data-derived constraints on the calculation of CETP-mediated fluxes. Throughout the meal transient and less frequently thereafter, measurements of diet-derived plasma 1) chylomicron-TG52:2 M₀, 2) chylomicron-TG52:2 M₁₁, 3) VLDL-TG52:2 M₁₁, 4) VLDL-TG52:2 M₁₈, 5) LDL-TG52:2 M₁₁, 6) LDL-TG52:2 M₁₈, 7) chylomicron-TG52:2 pool size, 8) VLDL-TG52:2 pool size, and 9) LDL-TG52:2 pool size were made as described above. Pool size refers to the total mass of all isotoptomers (including M₀). Because the animals are in a meal-induced non-steady state, pool sizes change with time. All measurements were expressed as micromoles per kilogram BW and were made in both vehicle and anacetrapib arms of the study. The resulting 18 time courses were used as constraints by treating both the tracer and pool size data as forcing functions. Nonlinear rate laws for CETP-mediated transfer were written based on standard chemical kinetic principles, and their parameters were estimated by requiring these rate laws to account simultaneously for the measured HDL-TG52:2 M₁₁, LDL-TG52:2 M₁₁, and HDL-TG52:2 pool size dynamics both in the absence and in the presence of anacetrapib. Use of forcing functions obviates the need to consider CETP-mediated fluxes among the different apoB lipoprotein classes because the net effect of all such transfers is contained in the measured experimental data and presented to the model as CETP substrates for transfer to HDL.

It is important to note that for this study a single typical TG, TG52:2 (16:0/18:1/18:1) accounting for ~7% of Rhesus plasma TG, was the focus. Although the method can readily be extended to other TG species, our estimates of total CETP-mediated TG fluxes are based on the known fractional abundance of TG52:2 in the total TG pool.

Rate laws for CETP-mediated TG transfer

A rate law is an algebraic expression that represents the flux (mass per unit time) through a biological pathway as a function of the substrates and regulators of that pathway. Taking the flux of TG from LDL to HDL as a prototype, the flux through CETP can be written as shown in equation 1.

\[ \text{Flux} = \frac{f_v \cdot V \cdot \text{CETP} \cdot S_i}{K_i + S_i} \]  
(Eq. 1)

where \( f_v \) is the flux of TG52:2 from LDL to HDL; \( f_v \) is a mole fraction representing the competition for transfer presented by neutral lipids other than TG52:2. These competition fractions were calculated as moles of TG52:2 in the source lipoprotein divided by total moles of neutral lipid in the source lipoprotein and were treated as constants for the purposes of this study; \( V \) is the maximal velocity of CETP-mediated neutral lipid transfer; \( S_i \) is the concentration or abundance of LDL-TG (the substrate for this reaction); \( K_i \) is the LDL-TG substrate constant (and similarly for the other lipoproteins); \( S_{\text{LDL}} \) is the plasma concentration of CETP inhibitor; and \( K_{\text{LDL}} \) is the anacetrapib inhibition constant. In the context of a non-steady-state physiological meal, the fluxes, \( f_v \), will be functions of time because the substrate concentrations will, in general, vary with time during the post-prandial period. Equation 1, based on classical enzyme kinetics, gives the unidirectional flux of TG52:2 through a protein-mediated process. It is a saturable, rapid-equilibrium mass action rate law with competitive inhibition. The denominator binding polynomial accounts for competition among the apoB lipoproteins for the C-terminal end of the CETP hydrophobic tunnel.

Inclusion of \( f_v \) in these rate laws is equivalent to the hypothesis that all neutral lipids in the lipoprotein core are equally likely to be transported through the CETP hydrophobic tunnel. Values were approximated by combining the current data with reported normal values for cynomolgus lipoprotein lipid composition (16, 32). Both CE and TGs other than TG52:2 were assumed to compete with TG52:2 for entry into the CETP hydrophobic tunnel. Molar concentrations were calculated using average molecular weights of 650 Da for CE and 860 Da for TG. Using these molar concentrations, values of \( f_v \) were calculated as TG/(CE + TG) multiplied by the fraction of TG that is TG52:2. Numerical values for chylomicrons, VLDL, LDL (including IDL), and HDL are
0.0665, 0.0651, 0.0199, and 0.0357 mol TG52:2 per mole of neutral lipid, respectively. Because Rye et al. (33) and others have shown CETP-mediated transfer of TG from Intralipid to HDL, we included this process in the model. The competition fraction for Intralipid, $f_{\text{Intralipid}}$, is the fraction of soybean TG that is TG52:2 (0.034) (34). $V_{\text{max}}$ values were assumed equal for forward and reverse neutral lipid transfer through the CETP tunnel.

Model diagrams were constructed, experimental protocols and data were captured, and diagrams were converted to systems of nonlinear ordinary differential equations (as outlined in supplementary data) for solution, testing, and optimization using the mechanistic systems biology software platform ProcessDB® (Integrative Bioinformatics Inc.). In ProcessDB, differential equations are solved numerically using a fast customized interpreter and the standard CVODE code (35). Global parameter optimization was performed with a parallelized code that implements the particle swarm optimizer (36). Running on a desktop workstation with a 3.2 GHz 6-core CPU, 30 particles with 10 neighbors complete 7,000 iterations or 210,000 simulations in ~10 min. The simulator-optimizer combination searches a log-scaled hypervolume of parameter space between upper and lower bounds that are 100-fold above and 100-fold below the initial parameter values. The objective function minimized in this study is the usual sum of squared residuals weighted by the inverse of the measurement error variance. The error standard deviation was estimated for each time point as 20% of the measured value (fractional standard deviation [FSD] = 0.2). The point in parameter space selected by particle swarm then served as the starting point for local (gradient-based) optimization using the orthogonal distance regression package (ODRPACK) (37) modified to allow user-specified upper and lower bounds on adjustable parameter values (38). Parameter statistics including FSDs for adjustable parameters were obtained from ODRPACK.

Statistical analysis

Significant differences were evaluated using a paired $t$-test; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Runs tests were used to test for random distribution of residuals about the model solution. Mean FSDs for each adjustable parameter were estimated as the average over all animals of the FSD obtained for each individual animal.

RESULTS AND DISCUSSION

Data on plasma lipids from both studies are shown in Fig. 2. Treatment with anacetrapib in this rhesus model significantly increased HDL cholesterol by >2-fold, consistent with the effects of anacetrapib in dyslipidemic humans, whereas the effects on LDL cholesterol were slightly greater in the rhesus than those observed in humans on monotherapy (39). In addition to the effects on HDL and LDL cholesterol, we observed significant reductions in fasting plasma TGs in rhesus treated with anacetrapib. The largest change was in HDL-TG content, and this is consistent with reduced transfer from TG-rich lipoproteins such as VLDL and chylomicrons due to inhibition of CETP.

The mean enrichment profiles for TG52:2 in each lipoprotein fraction, labeled separately with the intravenous and oral isotopomers, are available in Supplementary Fig. 4. The observation that the enrichments in the VLDL and chylomicron fractions were comparable during both treatment paradigms whereas enrichments in the HDL fractions were substantially reduced following anacetrapib treatment suggested that the effects of anacetrapib on HDL-TG flux could be reasonably assessed using a strategy based on either intravenous or oral administration of tracer. For the purposes of this study, we elected to develop our mathematical model based on both the $[^3H]_{\text{II}}$ and $[^13C]_{\text{I}}$ data in order to maximally constrain the solution. Analysis of the $[^3H]_{\text{II}}$ data alone yielded somewhat smaller CETP-mediated TG fluxes.

Mean experimental data for TG52:2 $M_0$, $M_1$, and $M_18$ in chylomicrons, VLDL, and LDL (including IDL) are shown in Fig. 3. Measured nanomolar plasma concentrations were converted to plasma pool sizes using a plasma volume of 0.0364 l/kg BW (40), and forcing functions were constructed for each individual animal by linear interpolation from each measured datum to the next using the ProcessDB software.

Maximal velocities for total neutral lipid transfer via CETP might reasonably depend on its lipoprotein substrates, so we initially tested the hypothesis that $V_{\text{max}}^{\text{LDE-IDL}}$ was different from $V_{\text{max}}^{\text{LDL-IDL}}$ or $V_{\text{max}}^{\text{apoB-IDL}}$. This hypothesis was sufficient to account for the experimental data. The alternative hypothesis that all three $V_{\text{max}}$ values are the same was also tested. This change eliminates 2 degrees of freedom and fits the data nearly as well. Objective function values for the two optimized fits differed by <1%, which is much less than the increment of 4 in the Akaike information criterion. This outcome leads us to propose the simpler hypothesis that the hydrophobic tunnel of CETP defines a single $V_{\text{max}}$ that does not depend on which apoB-containing lipoprotein is tethered to HDL.

If the N- and C-terminal openings of the CETP tunnel both traverse lipoprotein surface monolayers as proposed by Zhang et al. (30), it is conceivable that interaction with core neutral lipids is completely independent of the identity of the lipoprotein. To test this hypothesis, all substrate constants, $K_{S}^{\text{VLDL}}$, $K_{S}^{\text{LDL}}$, $K_{S}^{\text{apoB}}$, and $K_{S}^{\text{HDL}}$ (the substrate constant for HDL-TG characterizing reverse TG flux from HDL to apoB lipoproteins), would be constrained to be equal to one another. We did not pursue this hypothesis in the present study because $K_{S}^{\text{apoB}}$ was consistently found to be much larger than any of the other substrate constants. It remains possible that the much larger value of $K_{S}^{\text{apoB}}$ results from the presence of Intralipid TG in the chylomicron fraction. The simplest hypothesis we have found that is quantitatively consistent with all 24 data sets in each individual animal (144 data sets, total) is the single $V_{\text{max}}$ individual $K_{S}$ paradigm. The system diagram for this model is shown in Fig. 4. Forward rate laws for CETP-mediated TG52:2 transfer to HDL are as follows, for LDL-TG:

\[
F_{\text{LDL-IDL}}^{\text{apoB}} = \frac{f_{\text{LDL}}^{\text{apoB}} V_{\text{CETP}}^{\text{apoB}} S_{\text{LDL}}^{\text{apoB}}}{K_{S}^{\text{apoB}} + S_{\text{LDL}}^{\text{apoB}} + S_{\text{HDL}}^{\text{apoB}} + S_{\text{max}}^{\text{apoB}}} \tag{1}
\]
Fig. 2. The effects of anacetrapib on lipoprotein total cholesterol and TG. Treatment with anacetrapib significantly increased total and HDL-cholesterol and decreased VLDL and LDL-cholesterol. HDL-TG content was also significantly reduced. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

for VLDL-TG:

$$F_{\text{VLDL-HDL}}^{\text{VLDL}} = \frac{f_{\text{VLDL-HDL}}^{\text{VLDL}} \cdot \frac{S_{\text{VLDL}}}{K_{\text{VLDL}}}}{1 + \frac{S_{\text{VLDL}}}{K_{\text{VLDL}}} + \frac{S_{\text{HDL}}}{K_{\text{HDL}}} + \frac{S_{\text{ANA}}}{K_{\text{ANA}}}} \quad (\text{Eq. 2})$$

for chylomicron-TG:

$$F_{\text{Chylo-HDL}}^{\text{Chylo}} = \frac{f_{\text{Chylo-HDL}}^{\text{Chylo}} \cdot \frac{S_{\text{Chylo}}}{K_{\text{Chylo}}}}{1 + \frac{S_{\text{Chylo}}}{K_{\text{Chylo}}} + \frac{S_{\text{HDL}}}{K_{\text{HDL}}} + \frac{S_{\text{ANA}}}{K_{\text{ANA}}}} \quad (\text{Eq. 3})$$

for Intralipid-TG:

$$F_{\text{Intralipid-HDL}}^{\text{Intralipid}} = \frac{f_{\text{Intralipid-HDL}}^{\text{Intralipid}} \cdot \frac{S_{\text{Intralipid}}}{K_{\text{Intralipid}}}}{1 + \frac{S_{\text{Intralipid}}}{K_{\text{Intralipid}}} + \frac{S_{\text{HDL}}}{K_{\text{HDL}}} + \frac{S_{\text{ANA}}}{K_{\text{ANA}}}} \quad (\text{Eq. 4})$$

where $K_{\text{ANA}}$ is the Intralipid-specific inhibition constant. Other symbols are as defined in Materials and Methods.

The corresponding reverse rate laws are as follows:

$$F_{\text{HDL-VLDL}}^{\text{HDL}} = \frac{f_{\text{HDL-VLDL}}^{\text{HDL}} \cdot \frac{S_{\text{HDL}}}{K_{\text{HDL}}}}{1 + \frac{S_{\text{HDL}}}{K_{\text{HDL}}}} \quad (\text{Eq. 5})$$
where apoBLp represents any of the lipoproteins, LDL, VLDL, or chylomicrons. The remaining process is TG removal from HDL by all other means. This is treated as a simple mass action rate law, and this equation is presented in the supplementary data.

Particle swarm global optimization was carried out as detailed in Materials and Methods. Table 1 contains the optimized values for the nine adjustable lipoprotein-related parameters for each individual animal, along with the mean for all animals. Also included in Table 1 are the FSDs for each optimized parameter. Of the 54 FSDs in Table 1, only 4 are greater than the usual identifiability standard of 0.3. Three of these four are for estimates of $K_{\text{Ana-Intralipid}}$, which indicates that this parameter could not be identified in three of the six animals.

The mean for $K_{\text{Ana}}$ was found to be 22 nM, which is >100-fold less than the plasma concentration of anacetrapib measured in this study (supplementary Fig. 3). This is in excellent agreement with reported IC$_{50}$ values: ~17 nM (isolated lipoproteins) and ~50 nM (95% human serum) (26), which convert to $K_{\text{Ana}}$ values of 8 nM and 25 nM.

With these parameter values, the 18 measured forcing functions in Fig. 3 yield fits of the six HDL-TG52:2 data sets (pool size, M$_{11}$ and M$_{18}$, vehicle and anacetrapib-treated) shown in Fig. 5. The points in Fig. 5 represent the mean values at each time point, and the smooth lines represent the model fits obtained using the same methods used for the individual animals. Fits for the individual animals are included in supplementary Fig. 5.
A method for quantifying CETP-mediated TG transfer in vivo is an immediate consequence of these results. Shown in Fig. 6 are mean TG:52:2 fluxes in μmol TG52:2·h⁻¹·kg BW⁻¹ for the 120 h following the test meal and intravenous lipid challenges. Additionally, fluxes for each individual animal at the postprandial peak, on vehicle and anacetrapib, are tabulated and compared in Table 2. Interestingly, in the vehicle control studies, flux from VLDL to HDL (blue) is generally 4-fold greater than TG flux from LDL to HDL (red), consistent with the estimate that \( f_{VLDL} > f_{LDL} \) (29). Furthermore, the dominance of VLDL-TG transfer is abolished in the presence of anacetrapib (Fig. 6B). Because \( V_{CETP}^{\text{max}} \) can be written as \( k_{\text{cat}}'N_{CETP} \), it becomes possible to estimate \( k_{\text{cat}}' \), the in vivo turnover number of CETP, using the measured plasma concentration of CETP of 1.75 mg CETP/l plasma.

**TABLE 1.** Optimized parameter values and FSDs for the individual animals studied

| Parameter | 1    | Value | FSD  | 2    | Value | FSD  | 3    | Value | FSD  | 4    | Value | FSD  | 5    | Value | FSD  | 6    | Value | FSD  | Mean | Value | FSD  |
|-----------|------|-------|------|------|-------|------|------|-------|------|------|-------|------|------|-------|------|------|-------|------|------|-------|------|
| \( K_{VLDL} \) (nM) | 10.2  | 0.111 | 15.7 | 0.08 | 23.5  | 0.081 | 22.4 | 0.308 | 20.8 | 0.081 | 37.1  | 0.199 | 21.6 | 0.143 |
| \( K_{LDL} \) (nM) | 249   | 0.061 | 188  | 9.644 | 246   | 0.629 | 213  | 1.599 | 204  | 0.101 | 184   | 0.295 | 214  | 2.055 |
| \( k_{HDL-TG} \) (h⁻¹) | 0.574 | 0.056 | 0.368 | 0.157 | 0.316 | 0.922 | 0.455 | 0.239 | 0.374 | 0.052 | 0.328 | 0.080 | 0.402 | 0.101 |
| \( K_{CHYLO} \) (μmol TG52:2·kg BW⁻¹) | 0.742 | 0.101 | 0.644 | 0.06 | 0.764 | 0.928 | 0.809 | 0.141 | 1.017 | 0.036 | 0.768 | 0.085 | 0.791 | 0.075 |
| \( K_{VLDL} \) (μmol TG52:2·kg BW⁻¹) | 0.139 | 0.031 | 0.512 | 0.093 | 0.376 | 0.098 | 0.424 | 0.124 | 0.263 | 0.066 | 0.380 | 0.058 | 0.349 | 0.078 |
| \( K_{CHYLO} \) (μmol TG52:2·kg BW⁻¹) | 0.354 | 0.049 | 0.364 | 0.043 | 0.239 | 0.086 | 0.362 | 0.139 | 0.322 | 0.033 | 0.200 | 0.153 | 0.307 | 0.084 |
| \( K_{VLDL} \) (μmol TG52:2·kg BW⁻¹) | 0.965 | 0.089 | 1.10 | 0.033 | 2.41  | 0.014 | 2.48 | 0.411 | 1.36  | 0.057 | 2.48  | 0.107 | 1.80  | 0.118 |
| \( K_{CHYLO} \) (μmol TG52:2·kg BW⁻¹) | 225   | 0.084 | 293  | 0.075 | 262   | 0.045 | 286  | 0.244 | 277  | 0.025 | 266   | 0.149 | 268  | 0.104 |
| \( V_{\text{CETP}}' \) (μmol neutral lipid-h⁻¹·kg BW⁻¹) | 22.2  | 0.054 | 14.3 | 0.031 | 16.5  | 0.019 | 15.5 | 0.176 | 14.8  | 0.005 | 16.8  | 0.151 | 16.7  | 0.073 |
and taking 74 kDa as its molecular weight. This calculation reveals that each molecule of CETP can, if saturated with substrate, transfer ~323 molecules of neutral lipid per minute in vivo.

The principal objective of this study was derivation of formulas (rate laws) and parameter values for the in vivo flux of CETP-mediated TG transfer from each of the apoB-containing lipoproteins to HDL. Equations 1–4 plus the results in Figs. 5 and 6 demonstrate that this is possible. It should be emphasized that this result is only possible because the $M_0$, $M_{11}$, and $M_{18}$ signals were separately recorded and standardized. In the field of metabolic tracer kinetics, standard practice has, for decades, emphasized the calculation of tracer-tracee ratios or enrichments. Forming these ratios is undeniably convenient, but just as surely, it discards valuable information. Especially for non-steady-state experiments, such as the meal study reported here, we suggest that the various GC/MS or LC/MS peaks be quantified, calibrated, reported, and modeled separately whenever possible.

Fig. 5. Model solutions compared with mean experimental data. Solid lines are the corresponding model solutions using the same model used for the individual model fits. Filled circles are the means of the measured HDL-TG52:2 experimental data. A: Total HDL-TG52:2 pool size (red, vehicle; blue, anacetrapib). B: HDL-TG52:2 $M_{11}$ (red, vehicle; blue, anacetrapib). C: HDL-TG52:2 $M_{18}$ (red, vehicle; blue, anacetrapib). All measurements and model solutions are expressed as micromoles of TG per kilogram BW.
The hydrophobic tunnel hypothesis for CETP-mediated neutral lipid transfer among lipoproteins falls in a class of biophysical transport processes based on diffusion. Consequently, some readers will expect the rate laws for CETP-mediated transfer to involve the difference between, say, VLDL neutral lipid composition and HDL neutral lipid composition. These differences, seen in textbook diffusion equations, actually represent the difference between independent “forward” and “reverse” fluxes. In the work reported here, we have formulated the forward and reverse rate laws separately. The forward rate laws have been presented as equations 1–4, and the corresponding reverse rate laws as equation 5. Because the forward and reverse processes are modeled separately, each flux depends only on the neutral lipid content of the starting (or donor) lipoprotein. Equation 5 does not account for apoB-containing lipoproteins competing with HDL for the N-terminal ends of CETP. Future work could usefully explore this feature of the system in the context of neutral lipid exchanges among the apoB lipoproteins.

Because the experimental data do not distinguish Intralipid TG from chylomicron TG, it was challenging to dissect the relative contributions of CETP-mediated transfer from chylomicrons and from Intralipid. The model’s ability
to resolve these two fluxes depends on 1) the separately quantified pool size and tracer data and 2) the absence of labeled TG52:2 in Intralipid TG. The magnitude of the Intralipid flux has little impact on the transfer of M11 or M18 TG, but the pool size data in the presence of anacetrapib (especially the peak) could not be fitted in some animals unless the Intralipid flux was included. Interestingly, the optimized substrate constant, $K^{\text{chylomicon}}$, for chylomicron TG is found to be 5- to 6-fold greater that the substrate constants for either VLDL-TG or LDL-TG, and the optimized value of $K^{\text{apoB}}$ is larger still. Both results are consistent with the hypothesis that Intralipid-TG is a relatively poor CETP substrate compared with native lipoproteins. These findings are somewhat in contrast to the work of others performed in vitro. Granot et al. (41) demonstrated significant exchange of human plasma LDL CE for emulsion TG when incubated with Intralipid in the presence of lipoprotein-poor plasma. Weinberg and Scanu (42) also showed that Intralipid TG could be exchanged for human plasma HDL CE in vitro, but did note that HDL2 was evidently a better acceptor than HDL3, which was not resolved in our study. These differences between what has been documented in vitro and our own in vivo data are not necessarily at odds, however. In our in vivo protocol, the Intralipid emulsion must compete with other, native lipoproteins for transfer of TG to HDL via CETP, a condition that, to the best of our knowledge, has not been directly tested in vitro. This competition is captured in the Intralipid-CETP rate law presented as equation 4. The Intralipid-specific substrate and inhibition constants, $K_{i}^{\text{apoB-Intralipid}}$ and $K_{i}^{\text{apoB-Intralipid}}$, respectively, were both found to be >10-fold greater than their lipoprotein counterparts. This $K_{i}$ value implies that transfer from Intralipid to HDL is ~10-fold less sensitive to anacetrapib inhibition suggesting that some molecular feature of the lipoprotein substrates is a significant determinant of anacetrapib function. Given the optimized $K_{i}$ values, we cannot rule out the possibility that chylomicrons are, themselves, a poor substrate for CETP or that this might be a rhesus-specific phenomenon. The former question could potentially be resolved with an experimental protocol that does not include intravenous Intralipid.

Finally, it is of interest to extrapolate from the prototype triacylglycerol molecule (TG52:2) that was the focus of this study to estimate the total flux of CETP-mediated TG transfer from apoB-containing lipoproteins to HDL. TG52:2 molar flux is ~1.03 (peak postprandial, vehicle), and 0.035 (peak postprandial, anacetrapib) μmol·h$^{-1}$·kg BW$^{-1}$. Because TG52:2 typically makes up 3–12% of total plasma TG, we estimate (using 7%) that during postprandial chylomicronemia total TG transfer flux (using 860 Da as the molecular weight of a typical TG) is about 13 mg TG per h per kg BW. With anacetrapib present at a plasma concentration of 2 μM, peak total TG transfer flux is about 0.4 mg TG per h per kg BW. This amounts to a 97% reduction in neutral lipid transfer using this in vivo approach. When evaluated using the more common in vitro approach (26), CETP activity was found to be reduced by 71.5%. Although both measures confirm a substantial block on neutral lipid transfer following inhibition of CETP with anacetrapib in rhesus, the data available suggest that the in vivo approach measuring HDL-TG flux may provide a larger window for evaluating differences in endogenous activity. Values for individual animals are presented in Table 2.  

### Table 2. HDL-TG52:2 flux for individual animals

| Animal ID | Vehicle | Anacetrapib |
|-----------|---------|-------------|
| 1         | 1.44    | 0.0269      |
| 2         | 0.851   | 0.0284      |
| 3         | 1.07    | 0.0447      |
| 4         | 0.857   | 0.0255      |
| 5         | 0.899   | 0.0294      |
| 6         | 1.04    | 0.0500      |
| Mean      | 1.03    | 0.033       |
| FSD       | 0.217   | 0.346       |
| $P$        |         | 0.0001      |

### Future Perspectives

The model we have presented here provides a first-generation method for calculating in vivo CETP-mediated TG transfer from apoB-containing lipoproteins to HDL. We hope this will serve as a useful framework for future investigations. Some limitations of the existing model and opportunities for future development are enumerated as follows: i) Modeling of tracer data in apoB lipoproteins: In this first-generation model, the tracer data in apoB lipoproteins were used as forcing functions with linear interpolation to describe the CETP-mediated flux of neutral lipid to HDL, which was the primary focus of our investigation. While the use of forcing functions is well accepted (13, 43) there is a clear opportunity to expand the existing model to describe CETP-mediated flux between the apoB lipoproteins and/or to explore continuous forcing functions as an alternative to linear interpolation. ii) Further differentiation of substrate constants ($K_{i}$): The individual parameter values in Table 1 provide first estimates of the substrate constants for the various lipoproteins investigated. From these values, it is evident that chylomicrons and Intralipid are poorer substrates for CETP-mediated transfer than are VLDL and LDL, the latter two having $K_{i}$ values that are comparable within error. It would be valuable to repeat these determinations in additional groups of rhesus and under different experimental protocols. In particular, studies in which an oral tracer is provided in the absence of intravenous Intralipid or, conversely, where intravenous Intralipid containing a tracer is administered in the absence of a meal may be useful in further characterizing CETP-mediated transfer from chylomicron and emulsion particles in vivo. iii) Additional testing: As with any model, the one presented here cannot be proved to be unique. Although our model very successfully fits the experimental data, other models could be formulated that may do so as well. Additional testing of the model presented
in a wider variety of steady-state and non-steady-state tracer kinetic experiments is warranted. Such experiments could explore the effects of additional physiological, pathophysiological, and pharmacological perturbations on the flux of HDL-TG52:2. Conversely, additional tracers such as glycerol and, especially, CEs could be used to evaluate whether the established model can account for the analogous data obtained.

CONCLUSIONS

Classical studies in lipid and lipoprotein kinetics have almost exclusively relied on experimental designs that are constrained in order to maintain a steady-state and permit application of linear algebra and linear ordinary differential equations. The model we have presented here represents a significant departure from these traditional practices, and there are several underlying components that we feel are notable: 1) We began by developing a mechanistic, nonlinear model for studying CETP-mediated lipid exchange, which explicitly includes saturability of the CETP tunnel, the CETP turnover number, its affinities for and competition among its lipid transport substrates, and its inhibition by anacetrapib. This model is nonlinear because the underlying molecular biology and physiology are nonlinear. 2) We separately quantified and reported the labeled and unlabeled forms of TG52:2 in each lipoprotein particle. This allowed us to use both of the resulting time course data sets to test our model and constrain its parameters. 3) Doing both 1) and 2) enables a theoretically sound and physiologically relevant, non-steady-state analysis that combines the mechanistic insight of nonlinear models with the unique ability of tracers to distinguish changes in production from changes in removal. The resulting synergy provides a novel in vivo technique for measuring the flux of neutral lipid through plasma CETP, and to our knowledge, this constitutes the first report on the utility of using HDL-TG flux as a measure for CETP activity in vivo.

Finally, this approach offers an analytical paradigm that may be useful in other areas of metabolic physiology. A meal, for example, is one of the most dramatic and relevant of physiological transients. For 50 years, scientists have avoided meal studies solely because of the classic steady-state assumption of tracer kinetics. The approach we have developed removes that barrier and provides investigators with a means to study the influence of nutritional status or various dietary interventions on lipid metabolism. Each of these investigations could be readily undertaken in free-living subjects using the techniques presented here.

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