SUPPLEMENTARY DATA

Information for Supplemental Materials and Tables

Torcetrapib dose determination

Torcetrapib (CP-529,414) was generously provided by Pfizer. Dosing of torcetrapib was determined using pharmacokinetic parameters previously defined according to Prakash et al.(1) and information from human studies conducted by Clark et al.(2). We chose to use a primed, constant intravenous (IV) infusion because mice rapidly metabolize the drug ($t_{1/2} \approx$ 1.8hrs), and finer control of plasma drug concentration would therefore be more easily achieved using this method. In humans, the EC_{50} of torcetrapib is approximately 40nM, and is achieved by an oral dose of approximately 1.2mg/kgBW (2). However, IV infusions of the drug in our animals required a slightly higher dose to achieve significant inhibition, possibly because of the higher level of CETP activity in our mice. As a consequence, based on our pilot data (not shown) and the above equations, we chose a dose of 150ng/gBW/min for the prime and 15ng/gBW/min for the steady-state infusion.

Torcetrapib vehicle determination

Previous intravenous infusions of torcetrapib utilized Cremophor EL (also called Kolliphor EL) as the excipient. However, data from our laboratory (not shown) and others have shown this substance produces many toxic effects which include abnormal lipoprotein patterns, causing all lipoproteins to co-elute with VLDL when separated by density or electrophoresis (reviewed by Gelderblom et al.(3)). Pilot data in our laboratory showed high solubility of the drug in dimethyl sulfoxide (DMSO) and reduced comparative toxicity of this excipient in our mice at low doses, with minimal effects on lipoproteins (data not shown). This prompted us to use a vehicle of 5:5:90 (v/v/v) ethanol:DMSO:PBS (phosphate-buffered saline). Torcetrapib was first dissolved in ethanol and DMSO, and then PBS was added drop-wise, with gentle mixing, until final vehicle proportions were achieved.

Acute torcetrapib infusion studies

On the day of studies, mice were fasted for 5 hours. At t = -2 hours, red blood cells were infused to maintain hematocrit (Supplemental Figure 1A). For control animals, vehicle volume rate was matched to the theoretical drug infusion rate for the mass of the mouse. At t = 0 hours, a priming infusion of torcetrapib (150ng/gBW/min) or volume rate-matched vehicle began. At t =15min, the rate was changed to the steady state infusion rate (15ng/gBW/min), and this rate was continued until end of study (Supplemental Figure 1A). Arterial blood samples were collected in an EDTA tube at t =0, 15min, 1, 2, and 3 hours and plasma was separated by centrifugation and kept on ice. At t = 3 hours, mice were euthanized and their tissues were collected and frozen in liquid nitrogen. Plasma and tissues were kept in -80 ^{o}C for future analysis.

mRNA levels of genes in adipose tissues

Total RNA was isolated from approximately 100mg of snap-frozen adipose tissues using QIAzol Lysis Reagent (Qiagen) and then purified with the RNeasy Lipid Tissue Mini Kit (Qiagen) per manufacturer's instructions. Purified RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher).

cDNA synthesis and real-time PCR were performed as described in the section for liver mRNA quantification. TaqMan Gene Expression assays for determination of mRNA levels of genes were from Life Technology (Supplemental Table 2).

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CETP enzyme activity in hepatocytes and serum

Hepatocyte CETP activity: To test whether CETP is produced in the liver in mice and whether the intracellular activity would be affected by anacetrapib *in vivo*, hepatocytes were isolated from CETP mice treated with anacetrapib or vehicle (n=4 per group). One portion was used for *ex vivo* fatty acid oxidation (see Research Design and Methods). Hepatocytes from age and diet matched WT mice were used as negative control. One portion of the cells were frozen at -80 ^{o}C immediately. Later, cells were lysed in T-PER tissue protein extraction reagent (Thermal Scientific) with protease and phosphatase inhibitors. 80μg of proteins from each sample were used for CETP enzyme activity with the kit (Roar Biomedical).

Serum CETP activity: 0,5 μl of serum from WT male mice (n=5), CETP male mice (n=4) and human serum from Vanderbilt Lipid Clinic Core (n=4) were subjected for CETP activity assay using the kit from Roar Biomedical.

Euglycemic-hyperinsulinemic clamp study for WT DIO mice

To study whether anacetrapib affects insulin sensitivity in WT mice, age matched male WT DIO (diet induced obese) mice were purchased from Jackson Laboratory. One week after arrived at MMPC at Vanderbilt Medical Center, mice were treated with anacetrapib or vehicle for 5 doses every 12 hours and subjected to euglycemic-hyperinsulinemic clamp as described in Research Design and Methods.

Euglycemic-hyperinsulinemic clamp Study for CETP vs WT littermates

To study the difference of insulin sensitivity between CETP and WT mice under the same condition, age matched male CETP mice and their WT male littermates were fed a HFD for 3 months. Then, mice were subjected to euglycemic-hyperinsulinemic clamp as described in Research Design and Methods.

Supplementary Table 1. Peptides used for MRM-MS quantification of HDL proteins

Protein	Peptide 1	Peptide 2	Peptide 3	Peptide 4
apoA1	VAPLGAELQESAR	LSPVAEEFR	SNPTLNEYHTR	ARPALEDLR
apoA2	THEQLTPLVR	TSEIQSQAK		
apoA4	NLAPLVEDVQSK	ATIDQNLEDLR	ALVQQLEQFR	SLAPLTVGVQEK
apoC1	AWFSEAFGK	EFGNTLEDK		
apoE	ELEEQLGPVAEETR	LQAEIFQAR	TANLGAGAAQPLR	LGPLVEQGR
apoM	CVEEFQSLTSCLDFK	ETGQGYQR		
PON1	IFFYDAENPPGSEVLR	IQNILSEDPK		
LCAT	DPVAALYEDGDDTVATR	VSNAPGVQIR	ILASGDNQGIPILSNIK	
LPL	ITGLDPAGPNFEYAEAPSR	LVGNDVAR		
PLTP	TLLQIGVMPLLNER	AVEPQLEDDER		
SAA1	DPNYYRPPGLPDK	EAFQEFFGR		

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Supplementary Table 2. TaqMan Gene Expression assays from Life Technologies

Gene Symbol	Gene Name	Assay Name
Endogenous control		
18S	18S ribosomal RNA	Hs99999901_s1
For anti-inflammatory function of HDL assay		
CCl2	CCl2-chemokine (C-C motif) ligand 2	Hs00234140_m1
ICAM1	Intercellular adhesion molecule 1	Hs00164932_m1
VCAM1	Vascular cell adhesion molecule 1	Hs01003372_m1
For mRNA levels in liver and adipose tissues		
CCl2	CCl2-chemokine (C-C motif) ligand 2	Mm00441242_m1
PPARα	PPARα- peroxisome proliferator-activated receptor α	Mm00440939_m1
PPARγ	PPARγ- peroxisome proliferator-activated receptor γ	Mm00440940_m1
DGAT1	DGAT1- diacylglycerol O-acyltransferase1	Mm00515643_m1
DGAT2	DGAT2- diacylglycerol O-acyltransferase2	Mm00499536_m1
CD36	CD36- scavenger receptor class B member 3	Mm00432403_m1
PPargc1	PGC1-PPARγ co-activator 1	Mm01208835_m1
UCP1	UCP1-uncoupleing protein 1	Mm01244861_m1
Lipe	HL-hepatic lipase	Mm00495359_m1
Slc2a4	Glute4-glucose transporter 4	Mm00436615_m1
Slc27a1	FATP1- fatty acid transport protein 1	Mm00449511_m1
Rps6kb1	S6K1- Ribosomal protein S6 kinase beta-1	Mm01310033_m1
SAA3	SAA3- serum amyoid A3	Mm00441203_m1
FASN	FASN-fatty acid synthase	Mm00662319_m1
Srebf1	SREBP1- sterol regulatory element-binding protein 1	Mm00550338_m1
Nr1h2	LXR-liver X receptor	Mm00437265_g1
Xbp1	Xbp1-X box binding protein 1	Mm00457357_m1

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SUPPLEMENTARY DATA

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1. C Prakash, W Chen, M Rossulek, K Johnson, C Zhang, T O'Connell, M Potchoiba and D Dalvie. Metabolism, Pharmacokinetics, and Excretion of a Cholesteryl Ester Transfer Protein Inhibitor, Torcetrapib, in Rats, Monkeys, and Mice: Characterization of Unusual and Novel Metabolites by High-Resolution Liquid Chromatography-Tandem Mass Spectrometry and 1H Nuclear Magnetic Resonance. *Drug Metabolism and Disposition*. 2008;**36:**2064-2079.
2. RW Clark, TA Sutfin, RB Ruggeri, AT Willauer, ED Sugarman, G Magnus-Aryitey, PG Cosgrove, TM Sand, RT Wester, JA Williams, ME Perlman and MJ Bamberger. Raising High-Density Lipoprotein in Humans Through Inhibition of Cholesteryl Ester Transfer Protein : An Initial Multidose Study of Torcetrapib. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2004;**24:**490-497.
3. H Gelderblom, J Verweij, K Nooter and A Sparreboom. Cremophor EL: the drawbacks and advantages of vehicle selection for drug formulation. *European Journal of Cancer*. 2001;**37:**1590-1598.

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Supplementary Figure 1. Acute torcetrapib infusion reduces CETP activity and sustains insulin levels during fasting in chow- and HFD-fed mice. A: Schematic of experimental design, arrows indicate the time points for blood samples taken. **B and E:** CETP activity over time in chow- (B) and HFD- (E) fed mice. **C-G:** Plasma insulin and glucose levels over time in chow- (C and D) and HFD- (F and G) fed mice. Significant differences were determined using two-way ANOVA for *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

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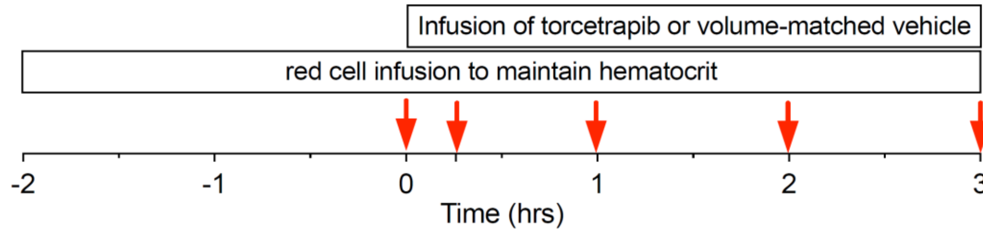

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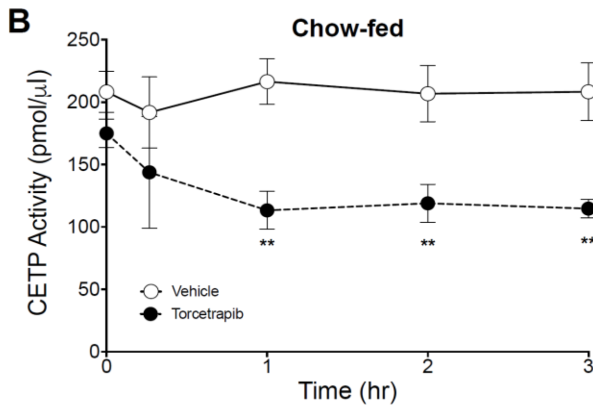

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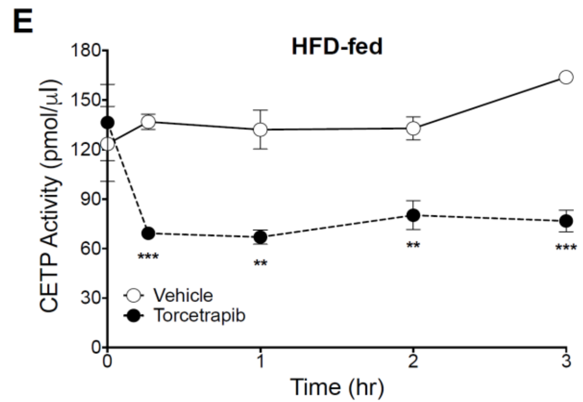

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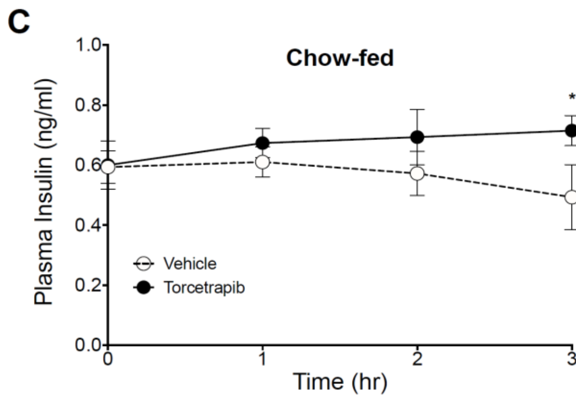

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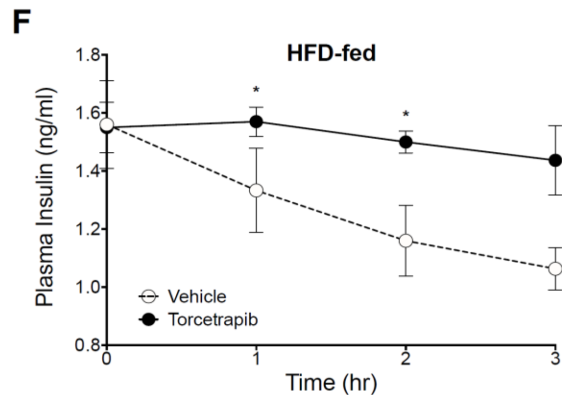

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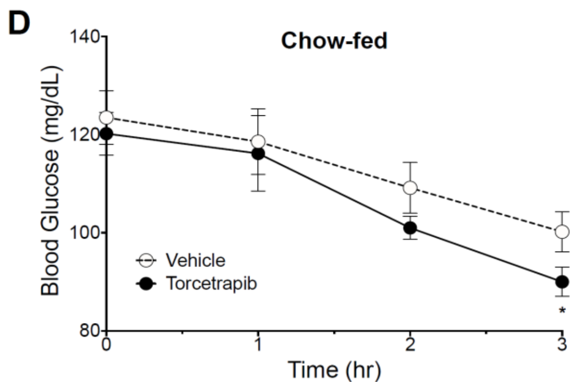

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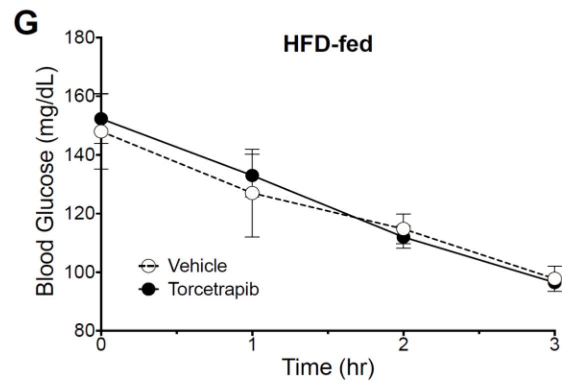

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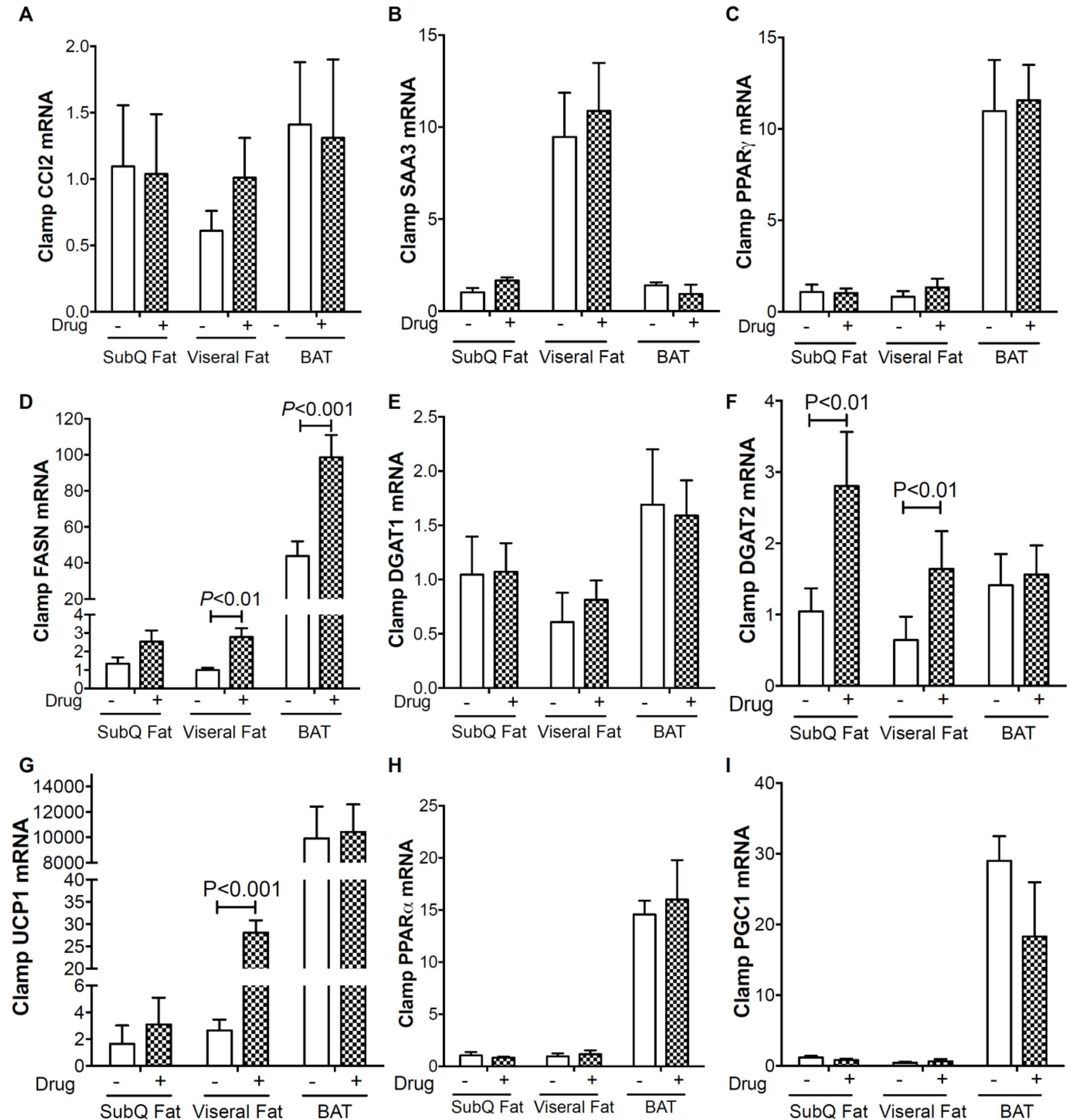
Supplementary Figure 2. Effect of CETP inhibition on FASN, DGAT2 and UCP1 mRNA in adipose tissues. Total RNA was isolated from adipose tissues (SubQ: subcutanouse; BAT: brown adipose tissue) from the 2nd cohort mice after insulin clamp. cDNA was then synthesized and mRNA levels of CCl2 (A), SAA3 (B), PPARγ (C), FASN (D), DGAT1 (E), DGAT2 (F), UCP1 (G), PPARα (H) and PGC1 (I) were determined by qPCR. 18s RNA was used as endogenous control, mRNA levels from subcutanouse fat (subQ) in vehicle treated mice were set as "1". n>=5, significant differences were determined using 2-way ANOVA. Differences between vehicle and anacetrapib treatment were marked.



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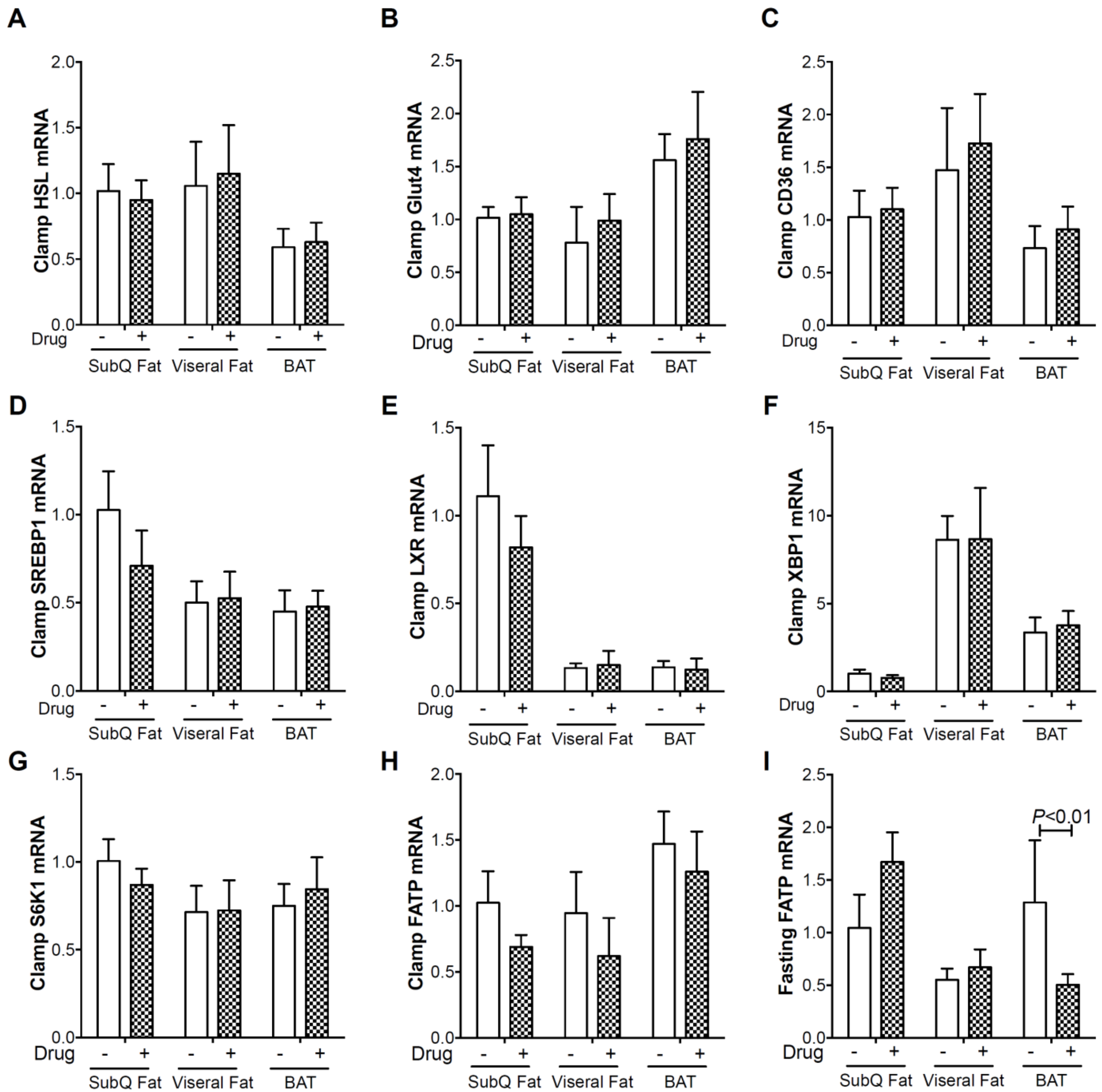
Supplementary Figure 3. mRNA levels adipose tissues during insulin clamp and fasting. Total RNA was isolated from adipose tissues (SubQ: subcutanouse; BAT: brown adipose tissue) from the 2nd cohort mice after insulin clamp. cNDA was then synthesized and mRNA levels of HSL (A), Glue4 (B), CD36 (C), SREBP1 (D), LXR (E), XBP1 (F), S6K1 (G), and FATP (H) were determined by qPCR. I: FATP mRNA levels in adipose tissues from the 1st cohort mice. 18s RNA was used as endogenous control, mRNA levels from subcutanouse fat (subQ) in vehicle treated mice were set as "1". Significant differences were determined using 2-way ANOVA. Differences between vehicle and anacetrapib treatment were marked.



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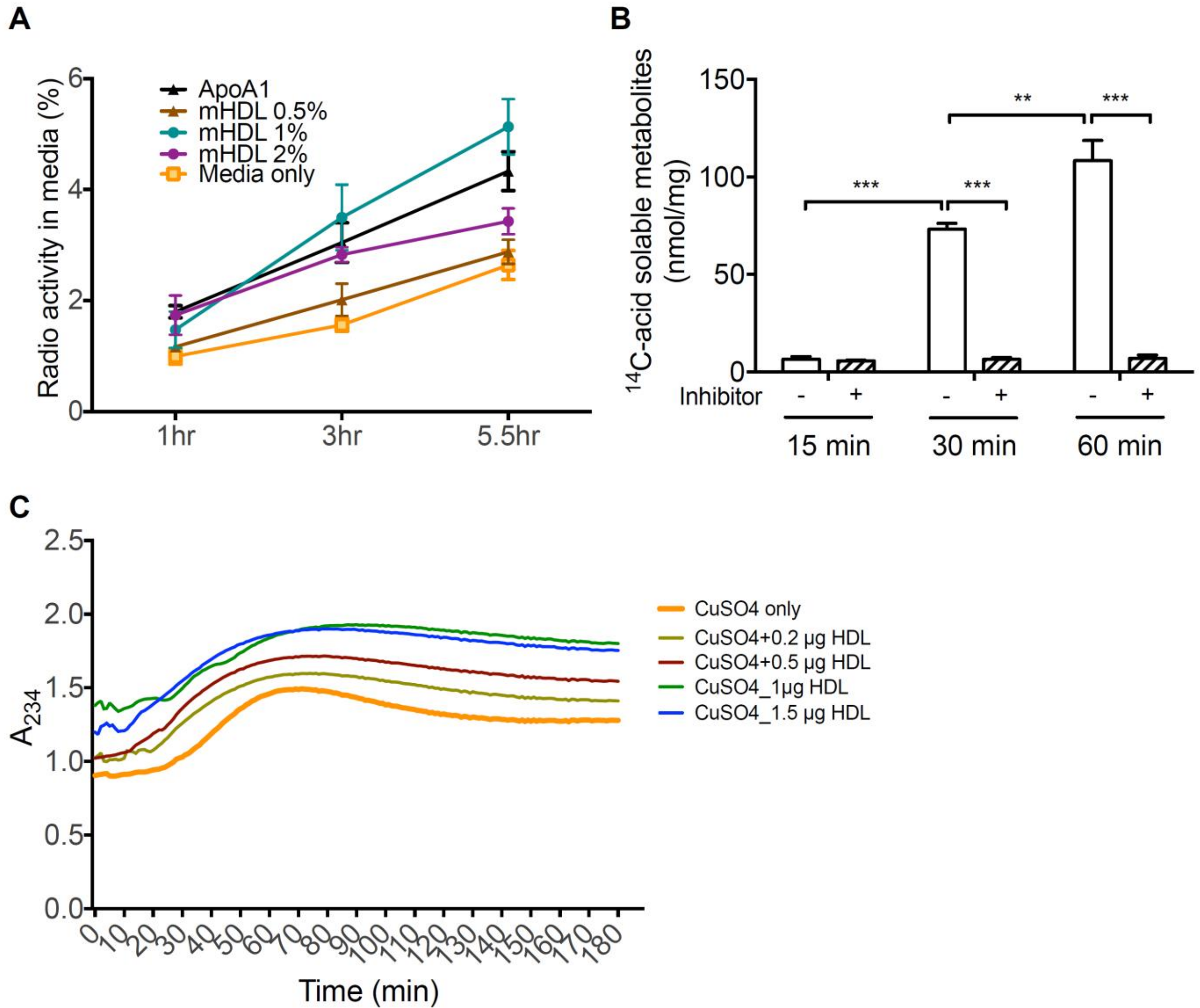
Supplementary Figure 4.

A: Dose responses for cholesterol efflux assay that was described in Research Design and Methods. Since HDL at the concentration equaling 1% of initial serum had the best performance, we chose this concentration for the experiment for Figure 4B.

B: Time course for hepatocyte fatty acid oxidation assay. 30min assay time was chosen for Figure 6I according to this time course.

C: Dose response for the assay to evaluate HDL particle antioxdiant effect. 1 μg of HDL was used for the experiment for Figure 4C.



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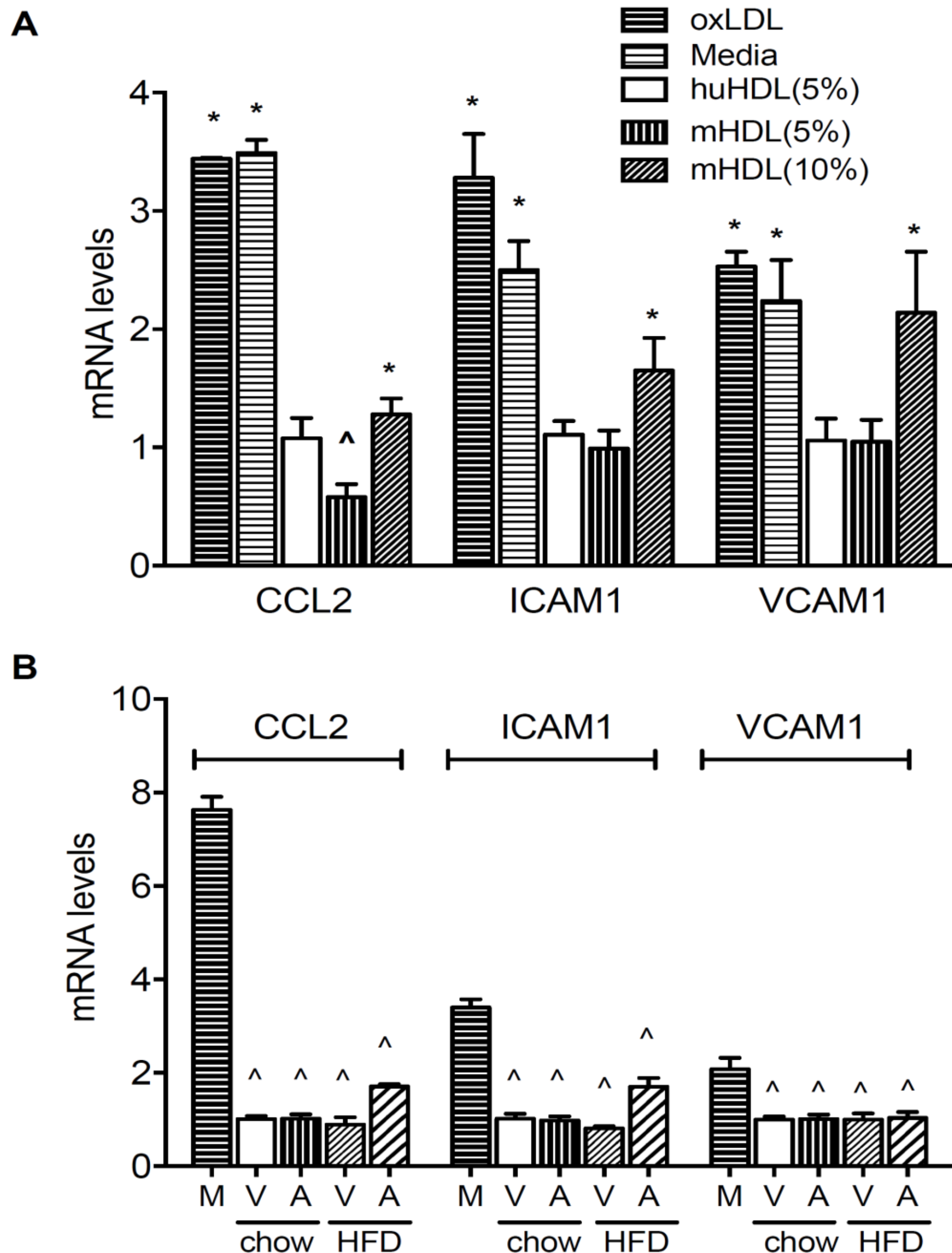
Supplementary Figure 5.

A: HCAEC were incubated with oxLDL (80μg/ml) for 8 hours, a group of cells were collected for baseline mRNA expression (oxLDL). The rest of cells were washed and treated with 5% (v/v) of HDLs from Cell Biolabs (huHDL), 5% (v/v) and 10% (v/v) of mouse HDLs in media containing 10% of lipoprotein deficient bovine serum (Alfa Aesar) for 36 hours. Cells growing in lipoprotein deficient media only were used as negative controls. Cells growing in huHDL were used as postive controls, and mRNA levels from these cells were set as "1". Student's *t*-test was used for statistic analysis for each gene. Significance was flagged by $P<0.05$. *, significantly increases compared to huHDL treatment; ^, significant decreases compared to huHDL treatment.

B: HCAEC were incubated with oxLDL (80μg/ml), then cells were collected were washed and treated with HDLs from experimental mice as described in Methods (V: vehicle, A: anacetrapib). HDL treatment suppressed mRNA levels of CCL2, ICAM1 and VCAM1 in comparison to media (M) containing lipoprotein deficiency bovine serum only. mRNA levels in cells treated with HDLs from chow-vehicle mice were set as "1". ^, $P<0.05$, significant decreases compared to M. Data for comparison between vehicle and anacetrpib in chow- and HFD-fed mice were graphed in Figure 4A.



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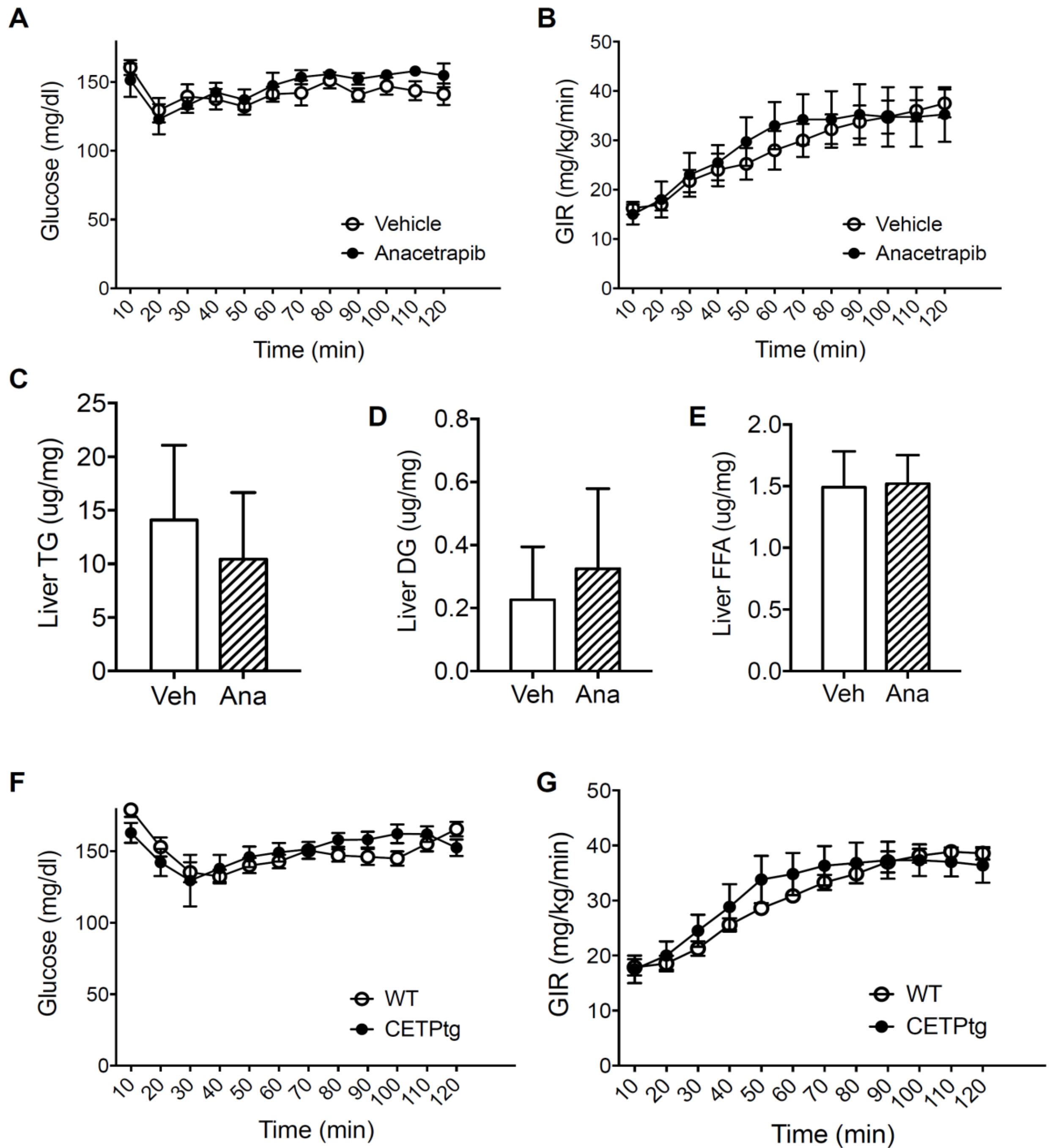
Supplementary Figure 6.

A-E: Anacetrapib treatment of male WT DIO mice. A. Euglycemia was maintained at ~150 mg/dl during the clamp. **B:** Anacetrapib did not change insulin sensitivity in WT DIO mice since the glucose infusion rate (GIR) to maintain eulgycemia was similar. **C-E**: Liver content for triglycerides (TG)**,** diacylglyerides DG, non-esterified fatty acids (NEFA) were shown in **C, D** and **E**, respectively**.**

F-G: Comparison of insulin sensitivity of 3-month HFD-fed CETP and WT male mice. F. Euglycemia was maintained at ~150 mg/dl during the clamp. **G:** GIR to maintain eulgycemia was not different between CETP mice and their WT littermates. Data shown are mean ± SD, n>=6. Statistic analysis was performed using RM 2-way ANOVA or student t test as needed.



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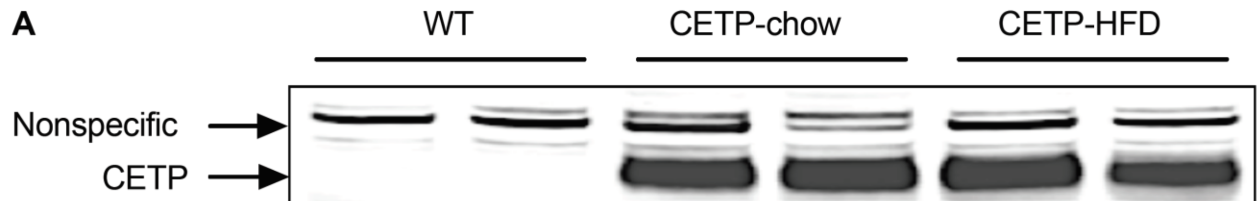
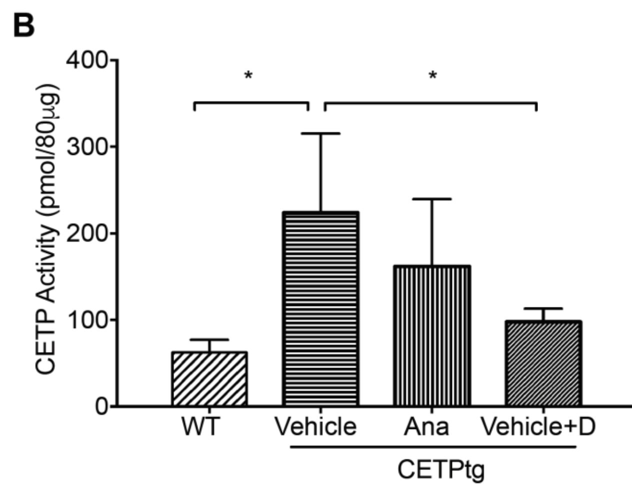
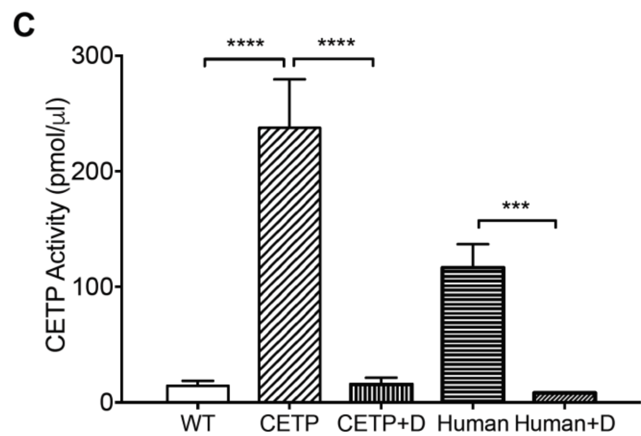
Supplementary Figure 7. A: Proteins isolated from primary hepatocytes were subjected to immunoblotting with CETP antibody. Nonspecific band (upper) and specific bands for CETP (lower) were indicated. **B:** CETP enzyme activity in primary hepatocytes isolated from WT and CETP mice treated with vehicle (Vehicle) or anacetrapibt (Ana). CETP activity was not significantly decreased in hepatocytes from CETP mice treated with anacetrapib, but was suppressed when anacetrapib was added in the enzyme activity assay reaction at the final concentration of 3μg/ml (Vehicle+D). **C:** Comparison of CETP enayme activity in serum between WT mice, CETP mice and human. Differences were determined using one-way ANOVA (n=4). *, $P < 0.05$, ***, $P < 0.001$, ****, $P < 0.0001$.







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