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Original Article

MOLECULAR METABOLISM

Adropin: An endocrine link between the biological clock and cholesterol homeostasis

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ABSTRACT

Objective: Identify determinants of plasma adropin concentrations, a secreted peptide translated from the *Energy Homeostasis Associated* (*ENHO*) gene linked to metabolic control and vascular function.

Methods: Associations between plasma adropin concentrations, demographics (sex, age, BMI) and circulating biomarkers of lipid and glucose metabolism were assessed in plasma obtained after an overnight fast in humans. The regulation of adropin expression was then assessed *in silico*, in cultured human cells, and in animal models.

Results: In humans, plasma adropin concentrations are inversely related to atherogenic LDL-cholesterol (LDL-C) levels in men (n = 349), but not in women (n = 401). Analysis of hepatic *Enho* expression in male mice suggests control by the biological clock. Expression is rhythmic, peaking during maximal food consumption in the dark correlating with transcriptional activation by RORα/γ. The nadir in the light phase coincides with the rest phase and repression by Rev-erb. Plasma adropin concentrations in nonhuman primates (rhesus monkeys) also exhibit peaks coinciding with feeding times (07:00 h, 15:00 h). The ROR inverse agonists SR1001 and the 7-oxygenated sterols 7-β-hydroxysterol and 7-ketocholesterol, or the Rev-erb agonist SR9009, suppress *ENHO* expression in cultured human HepG2 cells. Consumption of high-cholesterol diets suppress expression of the adropin transcript in mouse liver. However, adropin over expression does not prevent hypercholesterolemia resulting from a high cholesterol diet and/or LDL receptor mutations.

Conclusions: In humans, associations between plasma adropin concentrations and LDL-C suggest a link with hepatic lipid metabolism. Mouse studies suggest that the relationship between adropin and cholesterol metabolism is unidirectional, and predominantly involves suppression of adropin expression by cholesterol and 7-oxygenated sterols. Sensing of fatty acids, cholesterol and oxysterols by the RORα/γ ligand-binding domain suggests a plausible functional link between adropin expression and cellular lipid metabolism. Furthermore, the nuclear receptors RORα/γ and Rev-erb may couple adropin synthesis with circadian rhythms in carbohydrate and lipid metabolism.

Keywords Cholesterol; LDL; Cardiovascular disease; Obesity; Adropin; Sex

1. INTRODUCTION

Secreted peptides are involved in signaling metabolic status at the organismal and cellular levels to maintain cardiovascular and metabolic homeostasis. Studies in male C57BL/6J (B6) mice suggest the secreted peptide adropin provides such a signal of metabolic condition [1]. Adropin is a product of the *Energy Homeostasis Association* (*ENHO*) gene, comprised of two-exons on human chromosome 9p13.3. A highly conserved open reading frame in exon 2 encodes the full-length peptide (adropin$^{1-76}$). Adropin$^{1-33}$ is a secretory signal peptide [1]; adropin$^{34-76}$ is biologically active when administered to mice and cultured cells. For example, adropin$^{34-76}$ alters whole body glucose and lipid metabolism when administered to mice [1,2], rats [3,4], and also activates signaling pathways in mammalian cell lines [5]. Adropin$^{34-76}$ may also function to preserve the circulatory system, regulating endothelial function and activity of endothelial nitric oxide

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synthase [5–8]. The *Enho* transcript is however widely expressed, with high levels of expression in the nervous system relative to other tissues in B6 mice [1,5,9]. Analysis of male B6 mice that are adropin-deficient, over express adropin or are administered pharmacological doses of synthetic peptide suggest adropin suppresses fat oxidation and enhances oxidative glucose disposal and glucose tolerance [1,2,10].

Studies investigating plasma adropin concentrations in humans and nonhuman primates have observed associations with diet [11–14], with indices of insulin resistance [6,11,15,16], and with risk for cardiovascular disease [17,18]. However, determinants of plasma adropin concentration in humans are still poorly defined.

Here we report that plasma adropin concentrations are inversely related to plasma levels of low-density lipoprotein cholesterol (LDL-C) in males, but not in females. We also report that the biological clock may be plausible focal point linking *Enho* transcription with nutrient intake and cellular metabolic condition.

2. MATERIALS AND METHODS

2.1. Human studies

Plasma adropin concentrations were measured in the EDTA plasma fraction of blood samples collected after an overnight fast in five studies:

- 389 participants of a previously unpublished and unregistered Reference Range Study (RRS);
- Two NIH funded studies examining the impact of sugar consumption over 2 wk (n = 182) (DRS, NCT01103921) [19] or 10 wk (n = 31) (IPOP, NCT01165853) [20] on cardiometabolic risk factors;
- A randomized intervention trial examining weight loss on cardiometabolic risk factors (Caloric Restriction, Exercise, and Glucoregulation in Humans; NCT00777621) (n = 68) [21]; and
- Samples from participants of the HERITAGE study (NCT00005137) examining the interactions between genetics and exercise on cardiometabolic risk factors (n = 80) [22].

The relationship between plasma adropin concentrations and atherogenic cholesterol was assessed in data pooled from the RRS, DRS, IPOP, CREG and HERITAGE studies [11,12,15]. These data sets were selected based on collection of common demographic and blood chemistry data (**supplemental data**, Table S1). The studies are cross sectional, using plasma samples collected at baseline prior to dietary and/or behavioral interventions, and incorporated previously published data from smaller studies [11,12,15].

2.1.1. Reference range study

The original study involved 395 participants (190 men, 205 women) aged 21–75y recruited by investigators at the University of California, Davis, CA. EDTA-plasma samples were available from 187 male and 202 female volunteers for a study designed to validate a commercial assay for small diameter LPL. Study participants were recruited through an Internet listing (craigslist.com) and local postings of flyer. Subjects aged <21 and >75 years, used drugs of abuse, used drugs affecting lipoprotein metabolism (statins, fibrates, bile acid sequestrants or nicotinic acid), taking drugs for treating diabetes, are on hormone replacement therapy, had current or recent cardiovascular/coronary heart disease, or with current or recent cancer were excluded. Body mass index (BMI) ranged from 16.3 to 43 kg/m^2 (11 underweight), BMI <18.5 kg/m^2; 210 normal/healthy body weight, BMI 18.5–24.9 kg/m^2; 113 overweight (BMI 25.0–29.9 kg/m^2; 55 obese, BMI > 30 kg/m^2). The study was conducted in accordance with an experimental protocol reviewed and approved by the UC Davis Institutional Review Board. Participants provided written informed consent.

2.1.2. Dietary sugar studies

Participants in these studies are subgroups of NIH-funded investigations involving 182 participants (DRS) [19] and 31 participants (IPOP) [23,24]. DRS participants (92 men, 90 women) were recruited through internet listings (craigslist.com) and local postings of flyers, underwent telephone and in-person interviews with medical history, complete blood count, and serum biochemistry panel to assess eligibility. Inclusion criteria included age 18–40 y and BMI 18–35 kg/m^2 with a self-report of stable body weight during the prior 6 months. Exclusion criteria included diabetes (fasting glucose >125 mg/dL), evidence of renal or hepatic disease, fasting plasma triglyceride >400 mg/dL, hypertension (>140/90 mm Hg), hemoglobin <8.5 g/dL, and surgery for weight loss. Individuals who smoked, habitually ingested >2 alcoholic beverages/d, exercised >3.5 h/wk at a level more vigorous than walking, or used thyroid, lipid-lowering, glucose-lowering, antihypertensive, antidepressant, or weight loss medications were also excluded. The study was conducted in accordance with an experimental protocol that was approved by the UC Davis Institutional Review Board, and participants provided written informed consent.

Participants for IPOP were recruited through newspaper advertisements and underwent a telephone and an in-person interview with medical history, a complete blood count, and a serum biochemistry panel to assess eligibility. Inclusion criteria included age from 40 to 72y and BMI of 25–35 kg/m^2 with a self-report of stable body weight during the prior 6 months. Women were considered postmenopausal based on a self-report of no menstruation for at least 1 year. Exclusion criteria included evidence of diabetes, renal disease, or hepatic disease; fasting serum TG concentrations greater than 400 mg/dL; hypertension (>140/90 mmHg); and history of surgery for weight loss. Individuals who smoked, reported exercise of more than 3.5 h/wk at a level more vigorous than walking, or reported having used thyroid, lipid-lowering, glucose-lowering, antihypertensive, antidepressant, or weight-loss medications were also excluded. Diet-related exclusion criteria included habitual ingestion of more than 1 sugar-sweetened beverage per day or more than 2 alcoholic beverages per day. The UC Davis Institutional Review Board approved the experimental protocol, and subjects provided informed consent for participation in the study.

2.1.3. CREG study

Selection criteria, interventions, and outcomes were reported previously [11,21]. The study involved sedentary, men and women with excess weight aged 45–65y. The study was reviewed and approved by the Institutional Review Boards (IRB) of Washington University and Saint Louis University.

2.1.4. HERITAGE study

Plasma from 40 participants (20 males, 20 females) showing no response ("nonresponders") and 40 participants (20 males and 20 females) exhibiting high training-induced gains in VO_2max ("responders") were selected by ranking participants from minimal to maximal response, stratified by sex. The current study uses baseline data.

2.1.5. Blood chemistries and measurement of circulating hormones

For the RRS, lipids were measured on a Hitachi 911 analyzer (Hitachi Inc, Tokyo, Japan) at Tufts University. For the DRS and IPOP, lipid

concentrations were measured with a Polychem Chemistry Analyzer (PolyMedCo Inc.) with reagents from MedTest DX. Glucose concentrations were measured with an automated glucose analyzer (YSI) and insulin by radioimmunoassay (Millipore).

In the CREG study, sera were analyzed in a Clinical Laboratory Improvement Amendments (CLIA)-certified clinical laboratory for concentrations of total, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C), and glycerol-blanked triglyceride concentrations using automated enzymatic/colorimetric assays (Roche/Hitachi Modular Analytics System, Roche Diagnostics Corporation, Indianapolis, IN). Plasma glucose was measured using the glucose oxidase method (YSI STAT Plus; YSI Life Sciences, Yellow Springs, OH); insulin was measured using IMMULITE Chemiluminescence Kits (Diagnostics Products Corporation, Los Angeles, CA).

Very low density lipoprotein (VLDL) was calculated by subtracting (HDL-C + LDL-C) from total cholesterol.

Plasma adropin concentrations were determined using a commercially available enzyme immunoassay (Peninsula Laboratories, Inc., San Carlos, CA) previously validated and extensively used by our laboratory [11–13,15,25]. The intra-assay coefficient of variation is <5%, the inter-assay coefficient of variation is 25–30%. Plasma adropin concentrations in the RRS and DRS were measured in singletons in one batch. Measurements of plasma adropin in the CREG and IPOP study samples were duplicates, as previously described [11,15]. All samples used were EDTA-plasma collected from individuals fasted overnight.

2.2. Regulation of *Enho* expression in cultured cells and mice

2.2.1. In silico analysis of the *Enho* promotor

RAW sequencing reads files (SRA format) downloaded from the NIH SRA (short reads archive) server were converted to the FASTQ format using 'fastq-dump' program (version 2.3.5) and aligned to the mouse genome (mm10) using STAR (version 2.4.2a). The aligned reads were analyzed using HOMER (version 4.8) [26]. Peaks were determined using "findPeaks" with default parameters as FDR $\leq$0.001, fold enrichment over input $\geq$4, Poisson p $\leq$ 0.0001. Motif analysis, annotation, and quantification of the binding sites were performed using "findMotifsGenome.pl" or "annotatePeaks.pl". Visualization of ChIP-Seq signals were in IGV genome browser (version 3.0) following fcompression of the BAM files into the TDF format.

2.2.2. Measurement of *Enho* gene expression in mouse liver

Rhythmicity in liver *Enho* expression was analyzed by qRT-PCR as previously described [1,10,25] using total RNA previously collected from male AKR/J mice (The Jackson Laboratories, Bar Harbor, ME) [27]. In brief, mice were acclimated to a regular Chow diet (Purina 5015) *ad libitum* under a strict 12-h-light/12-h-dark cycle for 2 wk. Staff handled the mice frequently to reduce stress of human contact. After acclimation period, animals were killed in groups of three every 4 h over a 48-h period. *Enho* expression was then measured using qRT-PCR, as previously described [1]. This experiment was reviewed and approved by the IACUC at the Pennington Biomedical Research Center, LSU System in Baton Rouge, LA.

The effect of dietary cholesterol on hepatic *Enho* expression was investigated in three experiments. In the first experiment, total RNA isolated from male B6 mice fed chow or high cholesterol diet (Teklad TD.05305) treated with diluent (control) or an RORα/γ inverse agonist (SR1001) [28] or Rev-erb agonist (SR9009) [29,30]. These experiments were reviewed and approved by the IACUC at Saint Louis University School of Medicine in St. Louis, MO.

The second experiment examined *Enho* expression in liver of six 4-wk old mice for five of the eight founder strains of the Diversity Outbred (DO) mouse population [31] fed a synthetic chow diet (AIN-76A, Research Diets). After 2 wk, 3 mice from each founder strain were switched to an atherogenic high-fat, high-cholesterol diet with cholic acid (HFCA) diet for 16 wk, while the others remained on the synthetic diet. Livers from each mouse were flash frozen and homogenized before RNA was extracted and used for RNA sequencing on the Illumina HiSeq platform. RNA sequencing reads were aligned using MapSplice 2 to pseudo-genomes for each founder strain. Pseudo-genomes were built by using the coordinates of known variants that differ between C57BL/6 and each of the other 7 strains to convert the mm10 reference genome. RNA sequencing by maximization estimation (RSEM) was used to quantify reads, and DESeq2 was used for normalization and differential gene expression analysis.

The third experiment examined the effect of dietary cholesterol on *Enho* expression in liver of mice from DO mouse population developed from eight inbred strains of mice [31]. Full details on animal handling, feeding, measurement of phenotypes, and microarray analysis were previously published [32,33]. Briefly, a cohort of 288 DO mice were fed either an atherogenic high-fat, high-cholesterol diet with cholic acid (HFCA, n = 144) or a calorie-matched non-atherogenic high protein (HP, n = 144) diet for 18 wk. After diet treatment, blood was drawn to measure lipids, including total cholesterol and HDL-C. LDL-C values were calculated by subtracting HDL-C from total cholesterol for each mouse. Livers from 263 mice were flash frozen and homogenized before total RNA was extracted and used for microarray analysis of gene expression using the Affymetrix MoGene 2.1 array. For microarray analysis, robust multiarray average (RMA) method was used for normalization, and probes with known SNPs from the eight founder strains of the DO population were masked.

2.2.3. Regulation of *ENHO* expression in cultured HepG2 cells

HepG2 (ATCC-HB-8065) cells were maintained in advanced MEM (Advanced MEM, Life Technologies Inc., MD) supplemented with 10% FBS (Gemini Bio Products, CA) at 37 °C in a humidified incubator under 5% CO_2. Cells were seeded in 6-well plates and treated with DMSO (Sigma–Aldrich), SR9009 (10 μM) or SR1001 (10 μM) for 24 h prior to analysis of gene expression. For these experiments, triplicates of each conditions were done, and each experiment was repeated at least two times, giving similar results.

The effects of cholesterol and 7-oxygenated sterols (7β-hydroxysterol, 7-ketocholesterol) on *ENHO* expression in HepG2 cells were examined as previously described [34] using quadruplicates. Cells were maintained in charcoal stripped FBS (Thermo Fisher) only (control) for 24 h, or with charcoal stripped FBS plus 10 μM cholesterol (Sigma, C3045), 10 μM 7β-hydroxysterol (Sigma, H6891) or 10 μM 7-ketocholesterol (Sigma, C2394). One experiment is shown for each study; each point represents the averages for triplicates or quadruplicates.

2.3. Analysis of cholesterol metabolism in transgenic mice over expressing adropin

The impact of adropin overexpression on plasma cholesterol was investigated in adropin transgenic (AdrTG) male and female mice on the B6 background [1,10] fed chow or high-cholesterol diets. To examine whether adropin may be protective against the development of atherosclerosis, lesions in aorta of LDL receptor knock-out

(B6.129S7-*Ldlr*^{tm1Her}/J referred to hereafter as *Ldlr*−/−; purchased from The Jackson Laboratory, Bar Harbor, ME) mice was induced by exposing the mice to atherogenic diet for 12 wk. AdrTG mice were crossed onto the *Ldlr*−/− background. Male AdrTG;*Ldlr*−/− mice were mated with female *Ldlr*−/− female mice producing AdrTG;*Ldlr*−/− and *Ldlr*−/− mice used for these studies.

Mice were fed atherogenic diet (Teklad 88137, 42% from fat) starting at 6 wk of age. Body weight was measured weekly. After 12 wk of atherogenic diet, mice were euthanized, perfused with saline and blood and aorta samples collected. Aorta were stored in 10% neutral buffered formalin for 48 h, then transferred to ice-cold saline. Aorta were cleaned by removing adventitious tissues, cut open, pinned and lesions quantified using Image-Pro Plus software. Isolated plasma fractions were sent to the Metabolic Core Facility at the The Scripps Research Institute in Florida for analysis. Total cholesterol, triglyceride, LDL (low density lipoproteins), HDL (high density lipoproteins), and liver transaminases using a Cobas c311 Clinical Chemistry Analyzer (Roche Diagnostics). Plasma lipoprotein fractions were collected by FPLC using Bio-Rad Biologic DuoFlow System with a BioFrac Fraction Collector and total cholesterol measured in each lipoprotein fractions in 96-well plates (Cholesterol E Enzymatic Kit, WAKO Pure Chemical Industries) [35].

These experiments were reviewed and approved by the IACUC at Saint Louis University School of Medicine in St. Louis, MO.

2.4. Measurement of 24-hour plasma adropin concentrations in rhesus monkeys

Adult male rhesus monkeys were individually housed at the California National Primate Research Center at the University of California, Davis. The animals were fed a standard grain based primate commercial primate diet (Lab Diets 5047, Advance Protocol Old World Primate; 30% energy as protein, 11% energy as fat, and 59% energy as carbohydrate) *ad libitum* with fresh food provided two times per day at feeding times of 7 am and 3 pm. The monkeys were trained to present an arm for venipuncture blood collections and samples were collected for measurement of adropin every 2 h over a 24-hour period, during which the animal consumed their main meals at the normally accustomed times, although food was available for consumption at other times. The experimental protocol was approved by the Animal Use and Care Committee of the University of California, Davis

2.5. Statistical analysis

Data were analyzed using SPSS Statistics Version 23 (IBM). Assessment of meal effects in the rhesus macaque data used a 1-way ANOVA with repeated measures. Data was missing from 1 time point (ZT0); analysis excluding data from that time point or estimating the missing value using two methods (regression, group average at ZT0) did not affect the outcome. Relationships between plasma adropin concentrations, demographics (sex, age, BMI, waist circumference, diastolic and systolic blood pressure) and blood chemistries assessing cardiometabolic risk (lipid panel, glucose, insulin and HOMA-IR) were initially investigated using correlation (Spearman's rho or controlling for age, BMI). For linear regression, standardized b coefficients are reported with *p* values when significance was observed. The Bonferroni correction was used for *post hoc* analyses involving multiple comparisons. Associations were then further explored by separating data into quantiles ranked by plasma adropin concentration. For comparisons of demographic and blood chemistry data between quantiles, univariate analysis used sex and quantile as fixed factors; covariates controlling for potential confounding effects on dependent variables were applied as needed.

3. RESULTS

3.1. Inverse association between adropin and LDL-C in men

As previously reported [11], plasma adropin concentrations exhibited a unimodal distribution with a rightward skew and kurtosis in males and females (concentrations in ng/ml for males; mean, 4.23; median, 3.11; std. deviation, 3.91; skewness, 3.12; kurtosis, 11.94; for females, mean, 3.25; median, 2.72; std. deviation, 2.66; skewness, 4.45; kurtosis, 29.68, effect of sex, $p < 0.001$) (Figure 1A). Correlation analysis using log-transformed data identified an inverse association between plasma adropin concentration and HOMA-IR (Spearman's $rho = -0.077$, $p < 0.05$), BPsys ($rho = -0.072$, $p < 0.05$), and indices of cholesterol metabolism: total cholesterol ($rho = -0.118$, $p = 0.001$), LDL-C ($rho = -0.123$, $p = 0.001$), non-HDL-C ($rho = -0.099$, $p < 0.01$). However, when adjusted for BMI and age, the only correlations observed were with indices of cholesterol metabolism (total cholesterol, $r = -0.188$, $p = 0.001$; LDL-C, $r = -0.194$, $p = 0.001$; nonHDL-C, $r = -0.177$, $p = 0.001$). We therefore focused on examining relationships between adropin and cholesterol metabolism.

The relationship between plasma adropin concentrations and cholesterol is sex-dependent, and is only observed in males (Figure 1B and C). Limiting the correlation analysis to men increased correlation coefficients (total cholesterol, $rho = -0.246$, $p = 0.001$; LDL-C ($rho = -0.224$, $p < 0.001$) and non-HDL-C ($rho = -0.213$, $p < 0.001$). These correlations remained significant when adjusted for age and BMI (total cholesterol, $r = -0.171$, $p < 0.005$; LDL-C, $r = -0.1794$, $p < 0.001$; non-HDL-C, $r = -0.177$, $p = 0.001$). Separating the data into quartiles ranked low to high by LDL-C levels revealed the predicted decline in plasma adropin concentrations with increasing LDL-C in men, but not women (Figure 1D).

Obesity is a risk factor for coronary heart disease, at least in part due to increased LDL-C [36]. To determine whether plasma adropin concentrations interact with obesity to determine LDL-C levels in men, data were separated into tertiles ranked by either BMI. Each tertile ($n = 116$) was then separated into quartiles ranked by plasma adropin concentration ($n = 29$). Plasma adropin data shown in Table 1 demonstrate the skewed distribution; plasma adropin concentrations were markedly higher in the 4th adropin quartile owing to the cluster of men with extreme values (Figure 1A).

The cholesterol data shown in Figure 2 were analyzed using age and BMI as covariates, as both variables exhibited the well-known positive effect on cholesterol levels (data not shown). There was a significant effect of adropin quartile on LDL-C, nonHDL-C and total cholesterol ($p < 0.005$), but not for HDL-C (Figure 2A,C, E, G); average concentrations of LDL-C, nonHDL-C and total cholesterol were significantly lower in the 3rd and 4th quartile relative to 1st quartile.

Analysis of the deviation from the mean for cholesterol suggests that the effects of BMI tertiling and adropin quartiling are not equal for LDL-C, nonHDL-C and total cholesterol (Figure 2B,D, F). In general terms, plasma cholesterol levels adjusted for age and BMI appeared to be highest in overweight men with low adropin, and lowest in overweight men with higher plasma adropin concentrations. This difference is not evident for HDL-C (Figure 2H).

Indices of insulin sensitivity adjusted for BMI, ethnicity, age, and study, of men and women grouped into quartiles ranked by plasma adropin concentration were not significantly different between adropin quartile (Table 1). Differences in BMI and blood pressure were also not observed between adropin quartile (supplemental data, Table S2).

Differences in average age between BMI tertile were also consistent with the known association between aging and weight gain (age in

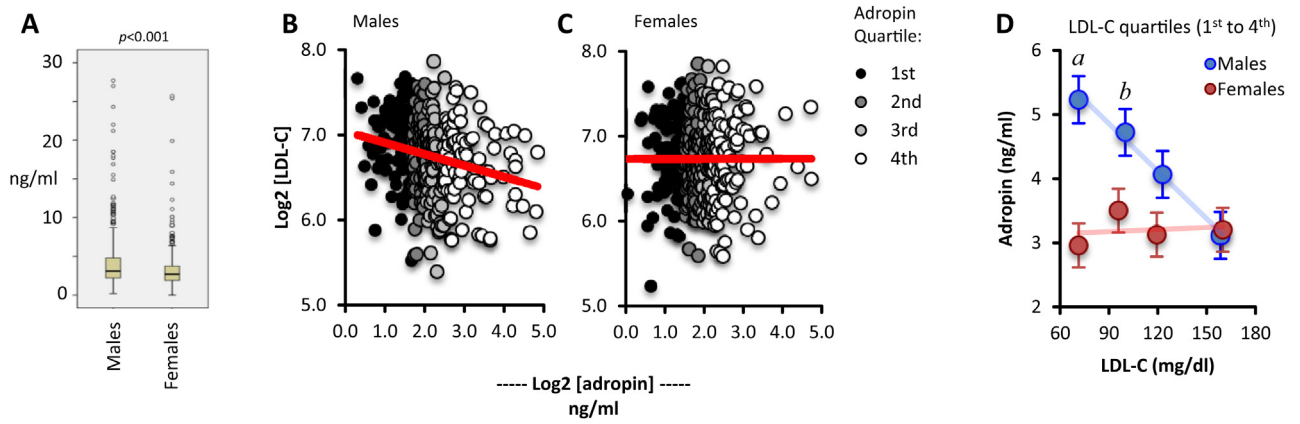


Figure 1: Inverse association between plasma adropin concentrations LDL-C levels in data pooled from the RRS, CREG, DRS, IPOP and HERITAGE studies. (**A**) Box and whisker plot showing distribution and range of plasma adropin concentrations in males and females. Extreme values are shown as circles. Relationship between fasting plasma adropin concentration and LDL-C (Log2-transformed) in males (**B**) and females (**C**). In panels B and C shading is used to indicate separation of data into quartiles ranked by plasma adropin concentration. (**D**) Plasma adropin concentrations in data pooled into quartiles by ranking LDL-C levels low to high (1st through 4th quartile read left to right in the panel). Mean plasma adropin concentrations decline with increasing LDLc in men, but not woman. LDL-C levels in D are estimated marginal means ± SEM adjusted for age and BMI. *a* $p < 0.01$ vs. the 4th LDL-C quartile for males, $p < 0.05$ vs. females all quartiles; *b* $p < 0.05$ vs. females in the 1st quartile.

Table 1 — Plasma adropin concentrations and indices of insulin sensitivity in pooled data grouped by sex and adropin quartile. Laboratory measurements are adjusted for age and BMI. N is shown in parenthesis.

Demographic, laboratory measurement	Sex	Group averages by sex	Group averages by sex, adropin quartile			
			1st	2nd	3rd	4th
Blood chemistries						
Adropin (ng/ml)	M	4.26 ± 0.13 (348)	1.43 ± 0.27 (87)	2.72 ± 0.27 (87)	3.75 ± 0.27 (87)	9.24 ± 0.27 (87)
	F	3.24 ± 0.13 (400)	1.31 ± 0.25 (100)	2.31 ± 0.25 (100)	3.27 ± 0.25 (100)	6.07 ± 0.25 (100)
Statistics		Sex, $p < 0.000$		Quartile, $p < 0.000$.; Interaction, $p < 0.000$		
Insulin (μU/ml)	M	12.80 ± 0.30 (347)	13.19 ± 0.59 (87)	12.00 ± 0.59 (87)	12.76 ± 0.60 (86)	13.25 ± 0.59 (87)
	F	13.01 ± 0.28 (390)	13.36 ± 0.55 (99)	13.04 ± 0.55 (98)	12.75 ± 0.56 (97)	12.88 ± 0.56 (96)
Glucose (mg/dL)	M	92.7 ± 0.4 (348)	93.7 ± 0.9 (87)	91.7 ± 0.9 (87)	92.9 ± 0.9 (85)	92.3 ± 0.9 (87)
	F	90.8 ± 0.4 (398)	90.7 ± 0.8 (100)	91.3 ± 0.8 (99)	90.8 ± 0.8 (99)	90.3 ± 0.8 (100)
Statistics		Sex, $p < 0.01$				
HOMA-IR	M	2.96 ± 0.08 (346)	3.07 ± 0.16 (87)	2.74 ± 0.16 (87)	2.96 ± 0.16 (86)	3.09 ± 0.16 (86)
	F	2.98 ± 0.08 (388)	3.02 ± 0.15 (99)	3.02 ± 0.15 (97)	2.96 ± 0.15 (96)	2.91 ± 0.15 (96)

years for BMI 1st tertile, 33.3 ± 1.46; 2nd tertile, 37.6 ± 1.45; 3rd tertile, 44.2 ± 1.36; effect of BMI tertile, $p < 0.001$). Males in the BMI 3rd tertile were significantly older relative to the 1st and 2nd tertiles ($p < 0.005$). Surprisingly, while observing an inverse association between age and plasma adropin concentrations, the average age of the adropin quartiles was not significantly different (data not shown). However, there was a significant interaction between BMI tertile and age quartile (BMI tertile × adropin quartile interaction, $p = 0.001$).

To further examine the relationship between plasma adropin concentration, aging and obesity we repeated the analysis separating the data into tertiles ranked by age, and then quartiling ranked by plasma adropin concentration. Aging-related increases in BMI are evident in all men (effect of age tertile, $p < 0.001$) (Figure 3A,B). However, high plasma adropin concentrations appears to associate with a phenotype characterized by leanness early in youth, but increased risk of obesity in the mid-to late-stages of life (Figure 3C,D) (age tertile × adropin quartile interaction, $p < 0.05$).

3.2. Regulation of adropin expression by cholesterol, Rev-Erb and RORα/γ

Adropin expression and circulating levels appear to be nutritionally regulated in mice and humans [1,10—13,25,37,38]. The current study suggests an important link between adropin and cholesterol metabolism. However, the mechanisms that associate metabolic control with adropin expression are unclear. Measurement of *Enho* expression in liver collected at 4 h intervals over 48 h from male AKR/J mice fed *ad libitum* shows rhythmicity (Figure 4A). *Enho* expression peaks between ZT20-ZT4 (4 h before and after lights-on at 06:00 h), coincident with the last phase of peak nutrient consumption in nocturnal mice.

Plasma adropin concentrations in humans relate to macronutrient composition of the diet [11,13]. However, evidence for rapid meal-related responses is less clear, perhaps due to heterogeneity between individuals [11,13,15]. In nonhuman primates (male rhesus macaques, n = 6) maintained on a standard low-fat chow diet, evidence for a rhythm in plasma adropin concentrations in samples collected at 2 h interval is observed (effect of time, $p < 0.05$), with peaks centered around the timing of food presentation (Figure 4B—D). Peak *Enho* expression in mouse liver coincides with maximal lipid production driven by the nuclear receptor RORα/γ [39]. Inspection of the *ENHO* gene sequence revealed a multitude of potential upstream transcription factor binding sites including members of the Sp family (Sp1, Sp2 and Sp5), estrogen-related receptors alpha and beta (Erra, Esrrb), several members of the Homeobox A family involved in

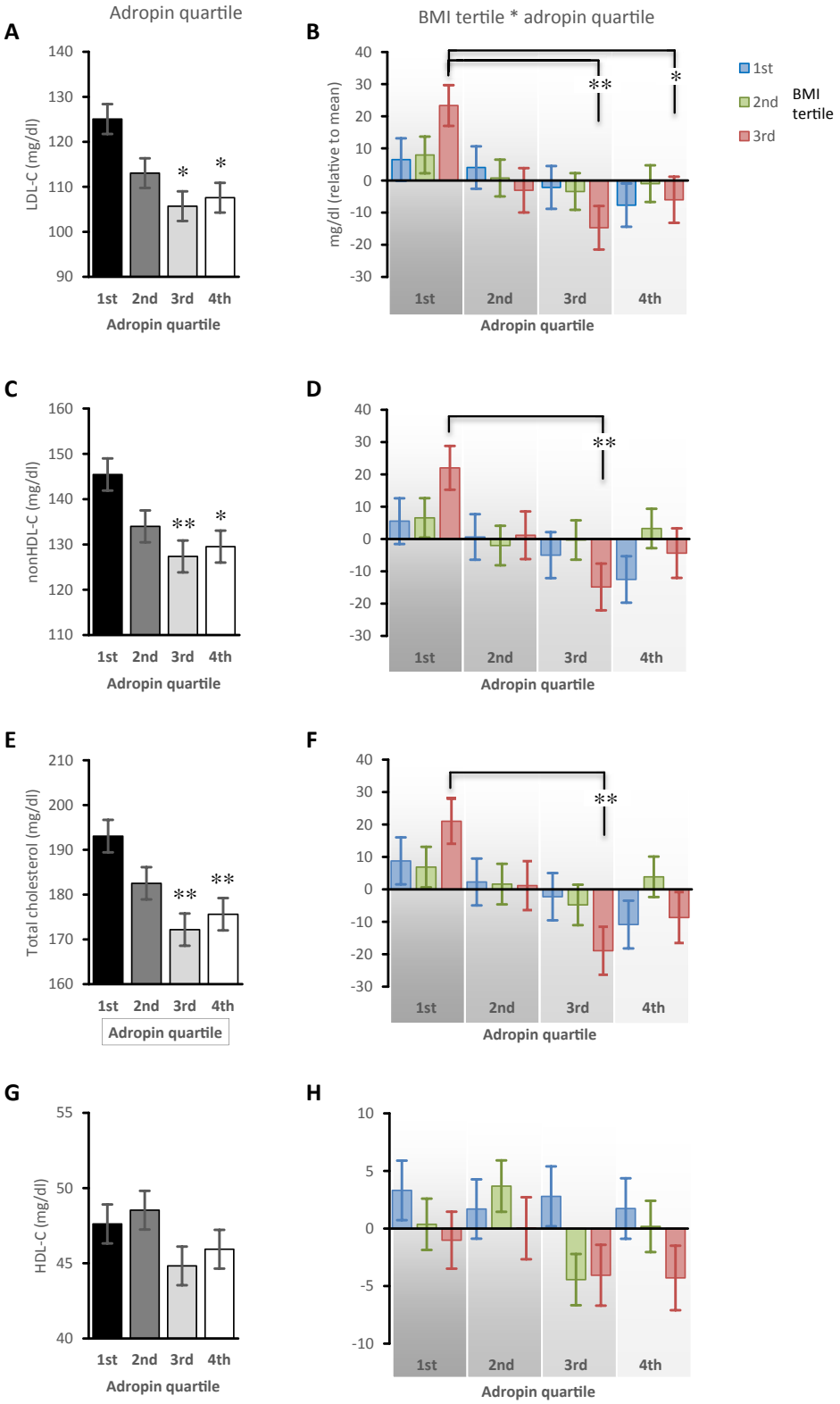


Figure 2: Significantly lower age- and BMI-adjusted levels of atherogenic cholesterol in men with high plasma adropin concentrations. Men were separated into tertiles (n = 116) ranked by BMI (low to high), and then further ranked within BMI tertile into quartiles ranked by plasma adropin concentration. Average concentrations of LDL-C (**A**), nonHDL-C (**C**), total cholesterol (**E**) and HDL-C (**G**) within adropin quartile (n = 87/group). Relative difference (plus or minus) to the mean of all samples for LDL-C (**B**), nonHDL-C (**D**), total cholesterol (**F**) and HDL-C (**H**) are shown within groups defined by BMI tertile and adropin quartile (n = 29). *$p < 0.05$ vs. adropin 1st quartile or groups indicated by line; **$p < 0.01$ vs. adropin 1st quartile or groups indicated by line. Values are estimated marginal means ± SEM.

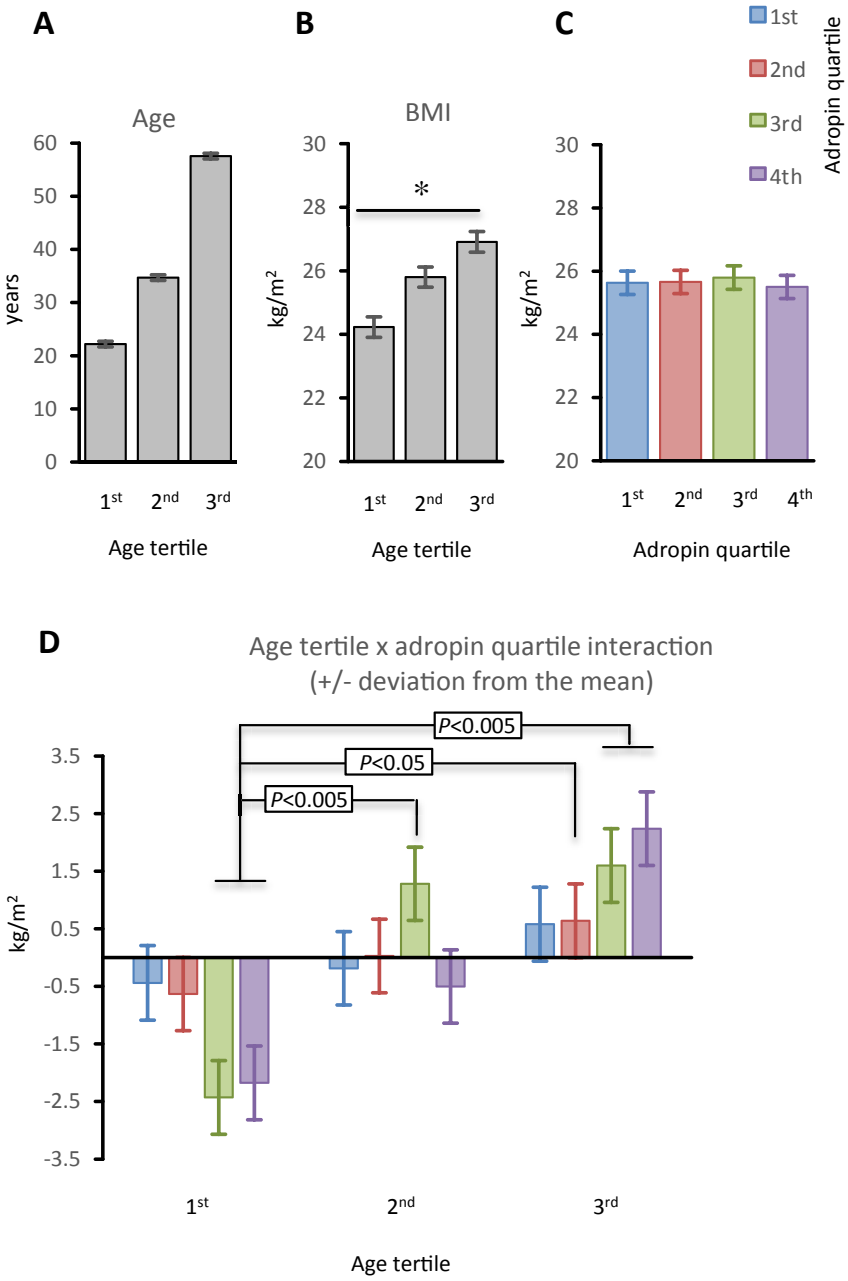


Figure 3: An interaction between age and adropin quartile on BMI in men. Men were separated into tertiles (n = 116) ranked by age (youngest to oldest), and then further ranked within age tertile into quartiles ranked by plasma adropin concentration. (**A**) Average age in years within each age tertile. (**B**) Average BMI within age tertile demonstrating the predicted increase observed with aging. * all groups are significantly different, 2nd vs 3rd tertile, $p < 0.05$; $p < 0.005$ for other comparisons. (**C**) Average BMI within adropin quartile (n = 87). (**D**) Average difference from the mean for BMI within groups defined by BMI tertile and adropin quartile (n = 29). The interaction between age tertile and adropin quartile is significant ($p < 0.05$). Men in the youngest age tertile generally have a lower BMI compared to the mean for all participants. Conversely, men in the oldest age tertile generally have a higher BMI. The largest difference in BMI between age tertile observed in men with higher plasma adropin concentrations. On the other hand, gains in BMI between age groups is modest in men with low plasma adropin concentrations.

embryonic development, Nr4a1 (Nur77), Srebf1a, Bmal1, members of the Krueppel-like factor (KLF) family, thyroid hormone receptor (THRb) and vitamin D receptor (VDR) (data not shown).

The Srebf1 binding site is of interest, given its role in regulating lipogenesis [40]. However, hepatic adropin expression in liver is suppressed by high sugar diets compared to low-fat, low-sugar chow diets [11]. Hepatic adropin expression is also rapidly stimulated by high-fat diets [1,25]. Finally, hepatic adropin expression is suppressed in mouse models of obesity in which hepatic lipogenesis is increased by the insulin-Srebpf1 pathway [1,41,42]. These observations are not consistent with a major direct role for adropin in hepatic lipogenesis. ROR and Rev-erb on the other hand drive rhythms in the expression of genes involved in lipid and carbohydrate metabolism [39,43]. Analysis of ROR, Rev-Erb, H3K9ac ChIP-Seq and GRO-Seq results derived from liver [39] indicated two overlapping RORα/γ and Rev-Erb binding sites upstream (−339 bp) and down-stream (+2952 bp) of the *Enho*

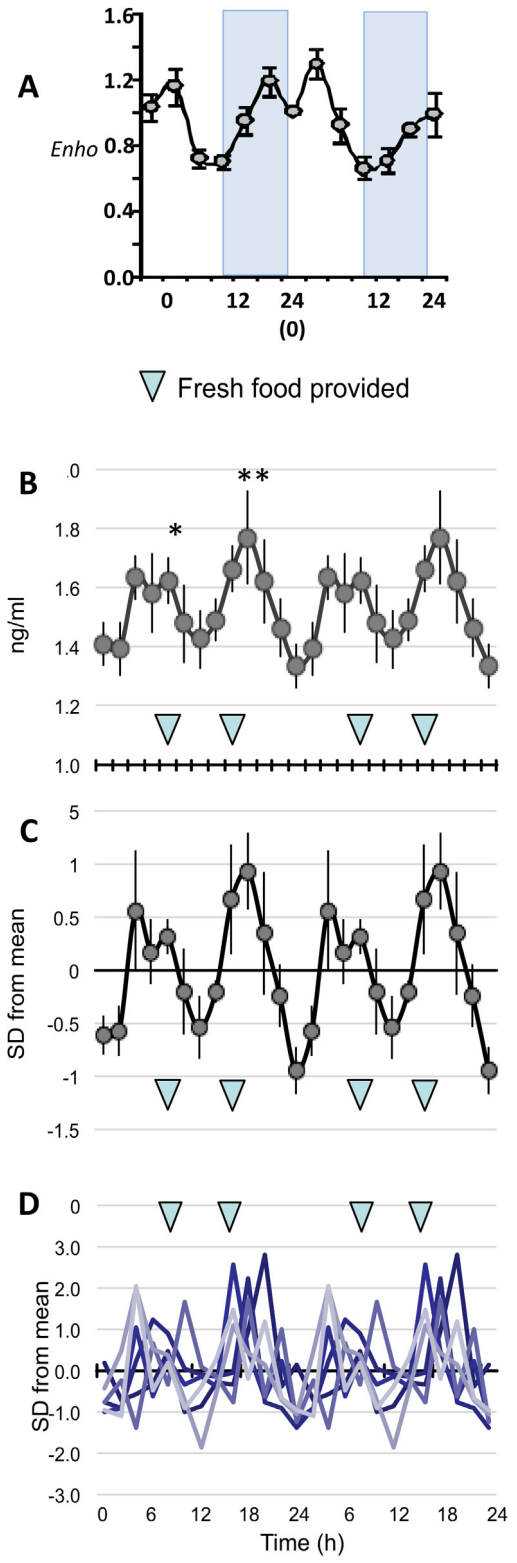


Figure 4: Rhythmicity in hepatic *Enho* expression in mice (A) and in plasma adropin concentration in nonhuman primates (B–E). (**A**) Liver *Enho* expression at 4 h intervals over 48 h in AKR/J mice (n = 3/group). Peak expression occurs between ZT20 and ZT4, corresponding to a window 4 h prior to and after lights on at 06:00 h. (**B**) Averaged plasma adropin concentrations (n = 6/group). (**C**) Averaged deviation from the 24 h mean. (**D**) Plasma adropin concentration data shown for the individual monkeys. *, $p < 0.05$ vs. 12:00 h and 24:00 h; **, $p < 0.05$ vs. 06:00 h, 12:00 h and 24:00 h. Data shown in panels B-D are double plotted.

transcription start site (Figure 5A). Comparison of GRO-Seq signals at ZT10 and ZT22 indicate *Enho* is transcriptionally repressed at ZT10, a time point when Rev-Erb is highly expressed. *Enho* transcription is activated at ZT22, when Rev-Erb is not expressed. Importantly, these dynamic, time-dependent repression and activation events correlated with similar changes in H3K9 acetylation at the *Enho* gene locus. As a nuclear receptor responsive to cholesterol and oxysterols [39,43], ROR could provide a link between cholesterol metabolism with *Enho* expression in the liver. These results also suggest an ability of Rev-erb to dominantly repress *Enho* gene transcription. ROR may play a positive role, given its ability to bind to overlapping Rev-Erb sites and the reported constitutive activation function of ROR.

The potential role of cholesterol in regulating *Enho* expression was then investigated in male B6 mice fed chow or high cholesterol diet (Teklad TD.05305); some of these mice were also treated with an RORα/γ inverse agonist (SR1001) [28] or Rev-erb agonist (SR9009) [29,30]. If RORα/γ activates *Enho* transcription; an inverse agonist would be hypothesized to suppress expression. Agonists enhancing Rev-erb repressor activity would also suppress expression. Twice-daily injections of 100 mg/kg ip. of the Rev-erb agonist SR9009 for 10 days [29] reduced liver *Enho* expression by 70%; however treatment with RORα/γ inverse agonist SR1001 had no effect (Figure 5B). Feeding male B6 mice a high cholesterol diet for 31 d suppressed hepatic *Enho* expression (Figure 5B); SR9009 treatment for 21 d did not affect *Enho* expression, while SR1001 treatment resulted in further suppression of expression (Figure 5B).

We then examined liver *Enho* expression in 5 inbred strains (A/J, WSBEiJ, B6, 129S1 and NODShiLtJ) used for the Diversity Outbred (DO) population [31]. In all strains maintained on the high cholesterol diet, hepatic *Enho* expression was reduced by 1.3–4.8-fold compared to controls (Figure 5C). In the mixed background DO population, *Enho* expression in liver was reduced by 1.5-fold in mice maintained on HFCA diet related to controls (RMA values for controls fed a non-atherogenic high protein diet, mean, 5.34; SD, 0.52; n = 139; for HFCA-fed mice, mean, 4.78, SD, 0.64, n = 123; $p < 0.000$).

The lack of response to SR1001 in chow-fed mice, and of SR9009 in mice fed the cholesterol diet, may reflect ceiling/floor effects on *Enho* expression in the liver and/or result from indirect metabolic consequences of chronic treatment. To more directly examine regulation of *ENHO* expression, HepG2 cells were treated with SR1001 or SR9009. The Rev-erb agonist SR9009 reduced *ENHO* expression by 50% in HepG2 cells (Figure 5D), while the ROR inverse agonist SR1001 also suppressed *Enho* expression (Figure 5E). The 7-oxygenated sterols 7-β-hydroxysterol (7β-OHC) and 7-ketocholesterol (7-KC) are high affinity ligands for RORα, and are thought to function as RORα/γ inverse agonists [34]. In HepG2 cells maintained in charcoal-treated FBS to reduce lipids, coincubation with 7β-OHC and 7-KC for 24 h markedly suppressed *ENHO* expression, along with genes involved in cholesterol and fatty acid metabolism (Figure 5F).

3.3. Adropin does not reduce cholesterol levels or prevent atherosclerosis in mice

We next examined responses of transgenic mice over expressing adropin controlled by a human β–actin promotor (AdrTG) [1,10] to high cholesterol diets (HCD). Weight gain, atherosclerosis and hypercholesterolemia observed in AdrTG;*Ldlr*–/– mice fed a HCD for 12 wk was similar to that observed in *Ldlr*–/– mice (Figure 6A–C). Total cholesterol levels in chow-fed AdrTG;*Ldlr*–/– and *Ldlr*–/– mice was not significantly different (Figure 6D). Analysis of liver gene expression indicated a significant increase in *Enho* in the AdrTG (Figure 7). Expression of some enzymes involved in cholesterol biosynthesis were

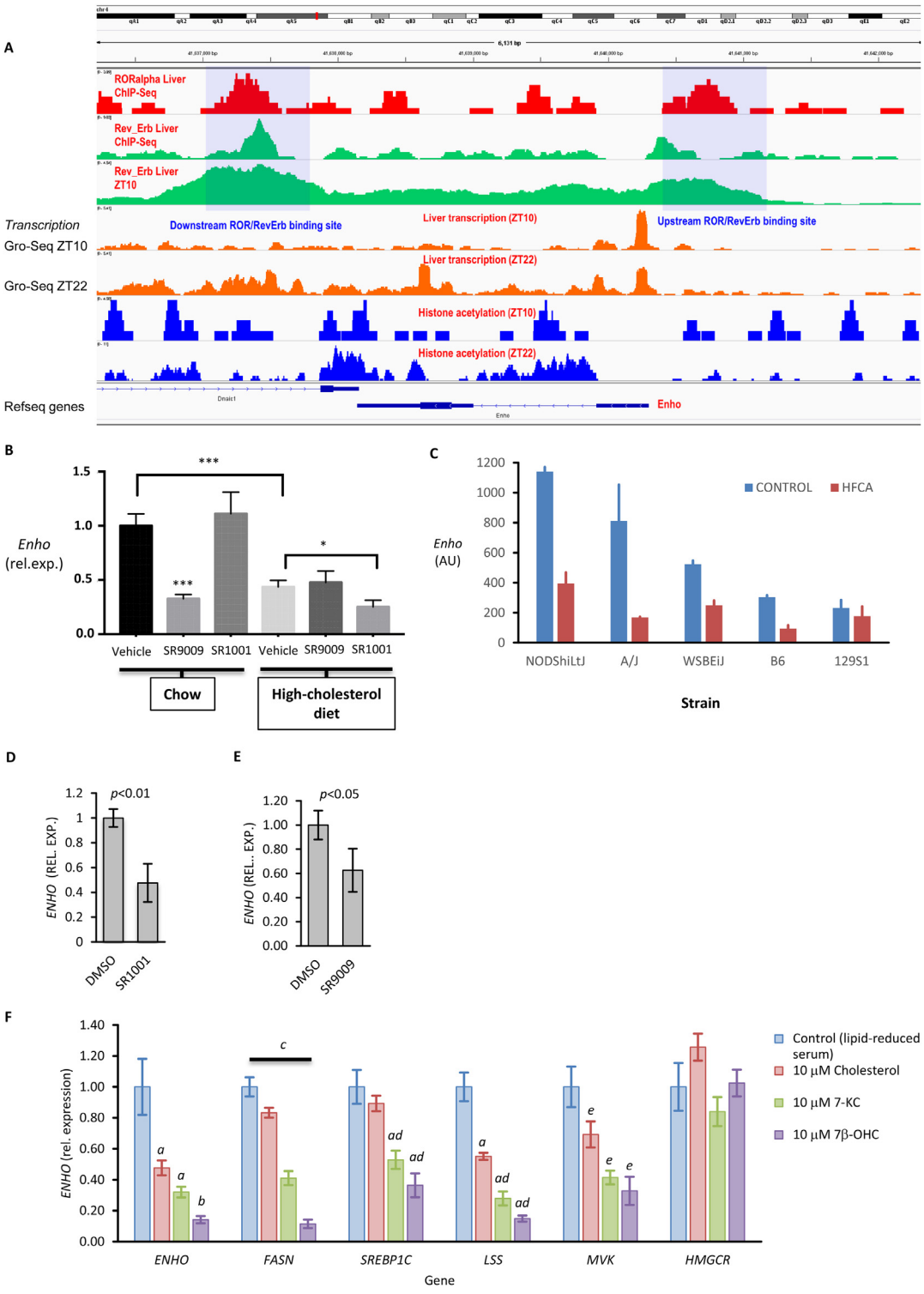
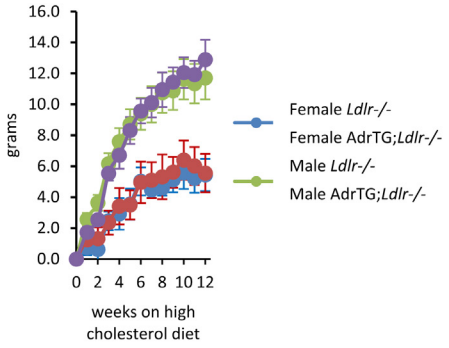
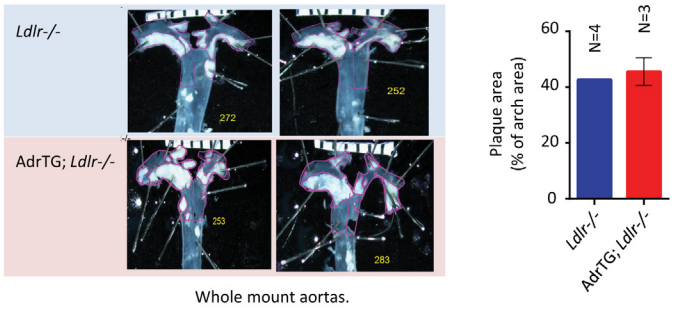


Figure 5: Rhythms in adropin expression are regulated by the interaction of RORα/γ and Rev-erb with binding sites upstream of the *Enho* gene; dietary cholesterol suppresses hepatic *Enho* expression. (A) An analysis of transcriptional activity of the mouse *Enho* gene in liver at ZT10 and ZT22 using ChIP-Seq and Gro-SEQ. Histone acetylation is also shown. (B) Liver *Enho* expression in male B6 mice fed chow (n = 6 for vehicle and SR9009, n = 5 for SR1001) or high cholesterol diet treated with the RORα/γ inverse agonist SR9009 or Rev-erb agonist SR1001 (n = 9/group). *, $p < 0.05$; ***, $p < 0.000$. (C) Liver *Enho* expression in livers of 5 inbred strains used for the Diversity Outbred population maintained on HFCA or control diet (n = 3/group). *ENHO* expression in HepG2 cells treated with a Rev-erb agonist (D) or RORα/γ inverse agonist (E). (F) Suppression of *ENHO* expression in HepG2 cells cultured in reduced lipid serum treated with 10 μM 7-beta-hydoxysterol (7β-OHC), 7-Ketocholesterol (7-KC) and cholesterol for 24 h. With the exception of *HMGCR*, treatment had a significant effect on gene expression ($p < 0.005$). Columns marked *a* are significantly different from control, $p < 0.005$; *b* from cholesterol ($p < 0.05$); *c* the values between all groups were significantly different ($p < 0.05$ control vs. cholesterol; $p < 0.001$ for the other groups); *d* from cholesterol ($p < 0.01$); *e* from control ($p < 0.05$ for cholesterol, $p < 0.001$ for 7β-OHC and 7-KC).

A. Weight gain on high cholesterol diet



B. Atherosclerosis in *Ldlr-/-* mice is not affected by adropin over expression.



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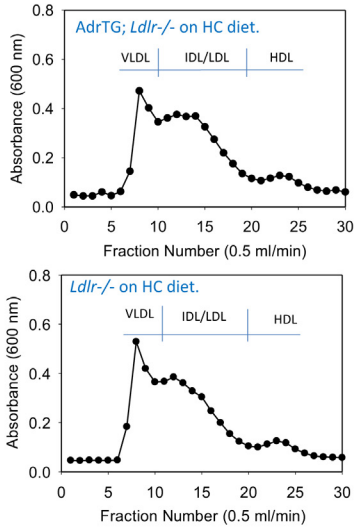
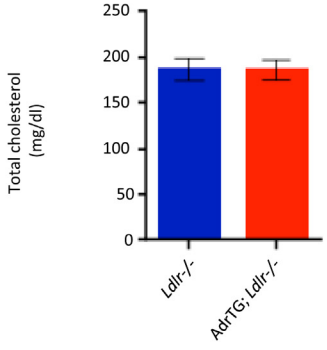
C. Hypercholesteromia in *Ldlr-/-* mice is not affected by adropin over expression.

Measurement	*Ldlr-/-*	AdrTG; *Ldlr-/-*	
Total cholesterol	1026.732	1041.513	mean
	88.079	75.262	std error
	846.591	887.584	95% CI-upper
	1206.874	1195.442	95% CI-lower
HDL	247.492	267.637	mean
	9.613	8.214	std error
	227.832	250.838	95% CI-upper
	267.152	284.436	95% CI-lower
LDL	880.764	902.227	mean
	79.569	67.991	std error
	718.028	763.17	95% CI-upper
	1043.5	1041.283	95% CI-lower

Estimated marginal means (sex, age). *Ldlr-/-* N=14 (8 M, 6 F). AdrTG;*Ldlr-/-* N=19 (10 M, 9 F).



D. Hypercholesterolemia in male chow-fed *Ldlr-/-* mice is not affected by adropin over expression.



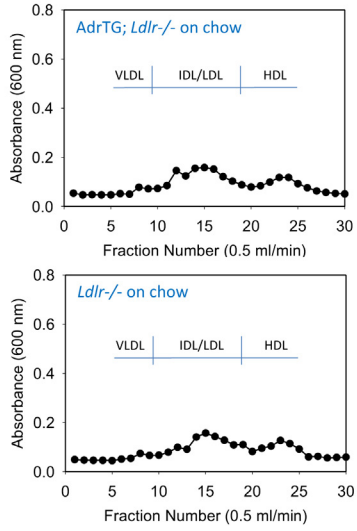


Figure 6: Adropin overexpression does not reduce cholesterol levels or prevent atherosclerosis in mice. (**A**) Body weight data for male and female *Ldlr*−/− or AdrTG;*Ldlr*−/− mice. (**B**) Atherosclerotic lesions in *Ldlr*−/− and AdrTG;*Ldlr*−/− mice maintained on a high cholesterol (HC) diet are similar. (**C**) Measurements of plasma cholesterol (total, HDL, LDL) are similar in *Ldlr*−/− and AdrTG;*Ldlr*−/− mice maintained on a high cholesterol (HC) diet are similar. (**D**) Total cholesterol levels and the distribution of cholesterol between VLDL, IDL/LDL and HDL fractions are similar in chow-fed *Ldlr*−/− and AdrTG;*Ldlr*−/− mice.

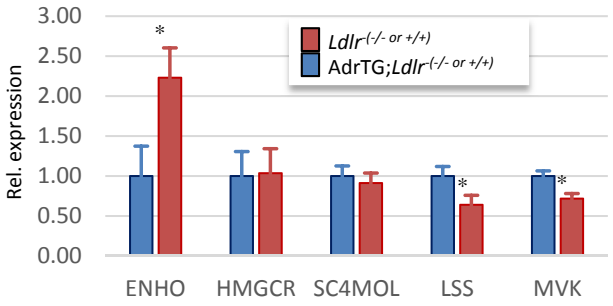


Figure 7: Adropin overexpression does not suppress 3-Hydroxy-3-Methylglutaryl-CoA Reductase (Hmgcr) expression in the mouse liver. Expression of the adropin transcript is significantly increased in mice expressing the adropin transgene (AdrTG). While some genes exhibit a modest reduction in expression, the expression of HMGCR which is rate limiting for cholesterol synthesis is normal. The data shown are estimated marginal means of genes measured using qRT-PCR. *$p < 0.05$ compared to mice not expressing the adropin transgene. LSS: lanosterol synthase, catalyzes the first step in the biosynthesis of cholesterol. MVK: mevalonate kinase, catalyzes the second step in the biosynthesis of cholesterol. SC4MOL, a sterol-C4-methyl oxidase that is localized to the endoplasmic reticulum membrane thought to function in cholesterol synthesis.

suppressed in mice carrying the adropin transgene, irrespective of *Ldlr* genotype. However, expression of HMG-coA Reductase (Hmgcr) which is the rate controlling enzyme of the mevalonate pathway producing cholesterol, was normal in AdrTG (Figure 7).

4. DISCUSSION

The current study began as an extension of previous investigations of associations between plasma adropin concentrations and indices of obesity and metabolic control in humans [11–13,15]. Earlier studies using relatively small samples observed associations between plasma adropin concentrations and sex [11,15], obesity [15], age [15], and indices insulin resistance and dyslipidema [11]. The current study suggests that, in a larger sample with less defined inclusion criteria, sex and cholesterol levels are major determinants of plasma adropin concentrations.

The current study also suggests a relationship between plasma adropin concentrations and indices of obesity that is more complex than previously reported. In young men, high plasma adropin concentrations are associated with a lean phenotype. By the mid-30's and later in life, this association is lost and may in fact be reversed. Given that adropin associates with increased relative saturated fat intake [11,13] and suppresses fat oxidation [2,10], weight instability over time might be a predicted outcome. However, this hypothesis is not supported by the lean phenotype observed in male B6 transgenic mice over expressing adropin. Prospective studies examining the relationship between aging, weight gain and plasma adropin concentrations are clearly needed, both in humans and in laboratory models. In addition, new studies in nonhuman primate models with controlled feeding paradigms, liver biopsies, and importantly investigation of the metabolic/endocrine effects of exogenous adropin administration that cannot be performed in humans are likely to help clarify the physiological role of adropin in intermediary metabolism.

4.1. Plasma adropin concentrations may provide a biomarker for risk of atherogenic hypercholesterolemia in obesity

The current experiments identify a relationship between plasma adropin concentrations and indices of atherogenic hypercholesterolemia that appears limited to men, indicating a major sex-difference in relationship between adropin and cholesterol homeostasis. Moreover, the relationship appears to be most pronounced in overweight to obese participants, suggesting an interaction between plasma adropin concentrations and obesity in determining LDL-C levels. There was however no association between plasma adropin concentrations and VLDL-C or HDL-C. Plasma adropin concentrations may thus associate with differences in the equilibrium between cholesterol synthesis and processing of circulating lipoprotein particles.

Experiments using mice over expressing adropin do not support a significant role for adropin in regulating cholesterol uptake from the diet, clearance from the circulation or cholesterol biosynthesis. Rather, these data suggest a relationship heavily biased towards suppression of adropin synthesis by cholesterol. Evidence for suppression of the expression of several genes involved in cholesterol synthesis was evident in the liver of AdrTG. While suggesting the possibility of a role in regulating cholesterol biosynthesis, *Hmgcr* expression which is rate-limiting in cholesterol biosynthesis is normal in AdrTG. Any changes in gene expression also appear to be insufficient to reduce circulating cholesterol levels. The current results are not comprehensive, and cannot rule out a role for adropin in regulating cholesterol biosynthesis. However, the potential impact appears minor and does not significantly impact hypercholesterolemia induced by diet and/or Ldlr-deficiency in mice.

Cholesterol biosynthesis is regulated through a sterol-sensing mechanism involving sterol regulatory element binding protein 2 (Srepb2) [44]. Srebp2 is a transcriptional activator that controls expression of enzymes involved in cholesterol biosynthesis. Inhibition of Srebp2 activity by oxygenated derivatives of cholesterol is a feedback mechanism linking cholesterol biosynthesis with demand. In mice, dietary cholesterol also suppresses *Enho* expression. Participation of adropin in a simple feedback mechanism where cholesterol regulates adropin expression and its own synthesis in parallel, while attractive, may not explain the observed association. For example, increased endogenous cholesterol production is a major factor contributing to hypercholesterolemia in obesity. Plasma adropin concentrations also appear to be lower in situations of obesity-related hypercholesterolemia, a condition in which cholesterol synthesis is likely increased.

The relationship between plasma adropin concentrations and atherogenic cholesterol levels may not be causal in nature. Rather, circulating adropin may provide an indirect biomarker of a metabolic phenotype that predisposes men to changes in LDL-C. Suppression of adropin by cholesterol may however be relevant to risk of vascular disease. Low adropin has been associated with vascular dysfunction, at least partially through effects on endothelial nitic oxide synthase activity [5,6,45,46].

It is important to note some of the deficiencies in the current study that limit our ability to draw conclusions. Rodents lack cholesterol ester transfer protein (CETP), which is involved in the heteroexchange of cholesterol esters and TG between HDL and VLDL [47]. It is also not clear that plasma adropin concentrations in humans or mice are hepatic in origin.

4.2. Adropin expression is regulated by the core elements of the biological clock

Adropin was originally identified as a secreted factor signaling metabolic conditions that are responsive to dietary macronutrients and energy balance [1]. The initial studies in male B6 mice suggest that energy sufficiency is a requirement for adropin expression in liver [1]. The degree to which feeding stimulates adropin expression is influenced by dietary macronutrients; high fat diets have a more pronounced stimulatory effect on expression, while diets rich in simple

sugars are less effective during re-feeding and may be inhibitory when compared to chow-fed conditions [1,11,25].

In the current study, peak adropin expression in the mouse associates with activation of transcription by ROR, while the nadir involves the transcriptional Rev-erb. In human HepG2 cells, small molecules affecting the repressor activity of Rev-erb and transcriptional activation by RORα/γ rapidly changed *ENHO* expression. Interestingly, high fat diets which increase hepatic adropin expression [1] have also been reported to increase RORα expression in the liver [48]. The involvement of these nuclear receptors also provides plausible nutrient sensing mechanisms in the regulation of adropin expression. The ligand-binding domain of ROR binds to cholesterol, oxysterols and fatty acid [43]. Oxygenated sterols have an inhibitory effect of transcriptional activity of RORα and RORγ in cell-based assays, functioning as inverse agonists [34]. Cholesterol, 7β-hydroxycholesterol and 7-ketocholesterol suppressed *ENHO* expression in cultured HepG2 cells. In mice, suppression of liver *Enho* expression by exogenous (dietary) cholesterol was evident in a genetically-diverse population, and is not strain-dependent.

Adropin may function as a secreted output of the biological clock that coordinate rhythms in metabolism with circadian time and metabolic condition. The exact physiological role of adropin in the liver is unknown. However, adropin has been shown to regulate fuel selection in skeletal muscle to reduce fat oxidation while enhancing glucose oxidation [2,10].

The relationship between plasma adropin concentrations, diet and meal time is less clear in humans [11–13]. In the current study, a nonhuman primate model (rhesus macaques) was used to examine whether plasma adropin concentrations exhibit a diurnal pattern that may be influenced by feeding. Evidence for two peaks during the daytime were observed, with a clear decline during the resting nighttime phase when the animals are not eating. This suggests that circulating adropin levels are labile in responding to nutrient intake. As an experimental model, rhesus monkeys have clear advantages when compared with both mice and humans. In terms of evolution and body size, the outbred rhesus monkeys maintained in primate colonies are more relevant to human physiology than inbred mouse strains. Compared to humans, experiments using rhesus are more controlled in terms of dietary history, ethnicity and health background.

5. CONCLUSIONS

The outcomes from these experiments suggest that adropin is an output of the biological clock. Peak adropin synthesis and presumably activity in the liver correlate with periods of maximal nutrient processing and lipid production. While adropin appears to prevent steatosis in male B6 mice fed high fat diets [1,25], the exact functional relationship between adropin and hepatic carbohydrate lipid metabolism requires further investigation. Further studies examining whether adropin expression in non-hepatic tissues exhibits rhythmicity are also required. Plasma adropin concentrations may provide a useful biomarker for identifying men at risk for elevated LDL-C with obesity.

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DISCLOSURE

The authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

SG, JRS, CB, CG, SS, AL, DR, JSS, TR, CB, MN, KS, DH, AD, JZ, EW, AC, BB, DP, PS, TB and PH contributed materials and reviewed the manuscript. MS assisted with statistical analysis. AB initiated and oversaw the project, collated data, contributed materials for data collection, performed the initial analysis and interpreted data, and prepared the first draft of the manuscript. AB had full access to the data, and takes responsibility for the integrity and accuracy of the analysis.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at https://doi.org/10.1016/j.molmet.2017.12.002.

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