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Original Article

Hypocholesterolemic phospholipid transfer protein knockout mice exhibit a normal glucocorticoid response to food deprivation

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Abstract: Objectives: Glucocorticoids, adrenal-derived steroid hormones, facilitate the physiological response to stress. High-density lipoproteins (HDL) are considered the primary source of cholesterol used for glucocorticoid synthesis in mice. Phospholipid transfer protein (PLTP) is a key player in HDL formation. In the current study we tested the hypothesis that HDL deficiency associated with genetic lack of PLTP negatively impacts the adrenal steroid function. Methods: We determined the glucocorticoid response to overnight food deprivation stress and the adrenal lipid and genetic phenotype of wild-type and PLTP knockout mice. Results: Basal plasma corticosterone levels, adrenal weights, and adrenocortical neutral lipid stores were not different between wild-type and PLTP knockout mice. Strikingly, plasma corticosterone levels were also equally high in the two groups of mice under fasting conditions (two-way ANOVA genotype effect: $P>0.05$). However, compensatory mechanisms were active to overcome adrenal lipid depletion, since gene expression levels of cholesterol synthesis, acquisition and mobilization proteins were ~2-fold higher in PLTP knockout adrenals versus wild-type adrenals. In support of an overall similar glucocorticoid stress response, hepatic relative mRNA expression levels of the glucocorticoid receptor target/glucocorticoid-sensitive genes PEPCK, ANGPTL4, FGF21, TDO2 and HMGCS2 were also not different. Conclusions: We have shown that hypocholesterolemic PLTP knockout mice exhibit a normal glucocorticoid response to food deprivation. These novel data (1) highlight that the effect of HDL deficiency on adrenal glucocorticoid output in mice is model dependent and (2) imply that other (lipoprotein) cholesterol sources than HDL can also generate the pool utilized by adrenocortical cells to synthesize glucocorticoids.

Keywords: Phospholipid transfer protein, adrenal, glucocorticoid, steroidogenesis, lipoprotein metabolism, gene expression

Introduction

The lipid transfer/lipopolysaccharide binding protein family member phospholipid transfer protein (PLTP) is a significant player in the life cycle of high-density lipoprotein (HDL) particles. During lipolysis of triglyceride-rich very-low-density lipoproteins (VLDL), PLTP facilitates the transfer of phospholipids from VLDL and low-density lipoproteins (LDL) to HDL particles to accommodate the formation of both smaller and larger HDL subspecies [1]. As a result, lack of functional PLTP proteins in PLTP knockout mice is associated with a significant decrease in levels of both pre-beta and alpha-HDL particles and a concomitant >60% decrease in plasma HDL-cholesterol levels [2].

Previous studies have suggested that, in mice, cholesterol associated with HDL particles is used by adrenocortical cells as substrate for the production of glucocorticoids, steroid hormones that facilitate the body's physiological response to stress through modulating the transcription of target genes by interacting with the nuclear glucocorticoid receptor [3, 4]. More specifically, probucol treatment-induced HDL deficiency and genetic deficiencies in the key HDL-associated proteins apolipoprotein A1 (APOA1) and lecithin-cholesterol acyltransferase (LCAT) are associated with adrenal glucocorticoid insufficiency in mice [5-7]. In addition, absence of the HDL receptor scavenger receptor BI (SR-BI) is associated with a diminished adrenal glucocorticoid output and functional

glucocorticoid insufficiency [8-10]. Based upon these findings it can be hypothesized that the HDL-cholesterol deficiency resulting from PLTP function ablation in mice may also translate into an impaired ability of the adrenals to generate glucocorticoids. To test this hypothesis, in the current study the adrenal glucocorticoid response to food deprivation stress was evaluated in age-matched wild-type and PLTP knockout mice.

Materials and methods

Animals

Colonies of PLTP knockout mice, originally generated by Jiang et al. [2] and acquired from the Jauhiainen lab [11], and C57BL/6 wild-type mice (obtained from the Jackson Laboratory) were bred in house at the Gorlaeus Laboratories. Mice were group-housed with 3-5 littermates per cage and fed a standard laboratory diet. At 8 to 11 weeks of age, groups of age-matched male C57BL/6 mice (N=8) and PLTP knockout mice (N=8) were transferred to the same climate controlled room with a stable 12 h/12 h dark-light cycle. From this point onwards, all mice were handled identically. After one week of acclimatization, all mice were bled at 9:00 AM in the ad libitum fed state via tail chop for baseline plasma measurements. The following afternoon (at ~5:00 PM), the mice were put in new cages and deprived of food. At 9:00 PM the next morning, i.e. after ~16 hours of fasting, mice were subjected to an additional blood draw to measure plasma parameters in the stressed state. Subsequently, mice were killed through cervical dislocation. Tissues were collected, weighed, and stored at -20°C or fixed overnight in formalin. All procedures were performed in accordance with the national guidelines for animal experimentation and complied with the ARRIVE guidelines. The experimental protocol #10217 was approved by the Ethics Committee for Animal Experiments of Leiden University.

Plasma analyses

Plasma concentrations of free cholesterol, cholesteryl esters, and triglycerides were determined using enzymatic colorimetric assays. Corticosterone levels in plasma samples were measured using the Corticosterone Competitive ELISA kit from ThermoFisher according to the protocol from the supplier.

Adrenocortical neutral lipid visualization

Seven micrometer cryosections from formalin-fixed adrenals (N=2 per genotype) were prepared on a Leica CM3050-S cryostat. Cryosections were routinely stained with Oil red O for neutral lipid visualization.

Real-time quantitative PCR

Total RNA was isolated from liver and adrenal specimens using a standard phenol/chloroform extraction protocol (N=8 per genotype). Equal amounts of RNA were reverse transcribed and subsequently real-time quantitative PCR analysis was executed on the cDNA using an ABI Prism 7500 apparatus (Applied Biosystems, Foster City, CA, USA) using SYBR Green technology according to the manufacturer's instructions. Acidic ribosomal phosphoprotein P0 (36B4), peptidylprolyl isomerase A/cyclophilin A (PPIA), beta-actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and ribosomal protein L27 (RPL27) were used as reference genes for normalization.

Western blotting

Total protein lysates were generated by homogenizing adrenals in Nonidet P-40 buffer (N=6 per genotype). The protein concentration was measured using BCA assay (Sigma-Aldrich). Proteins were separated using 7.5% SDS-PAGE gels and then transferred onto methanol-activated polyvinylidene difluoride (PVDF) membranes (Millipore). Subsequently, the LDL receptor (LDLR), SR-BI and tubulin were detected with the following primary antibodies: LDLR - Ab52818 (Abcam); SR-BI - Ab52629 (Abcam); Tubulin - T-9026 (Sigma-Aldrich). HRP-coupled secondary antibodies were used. Images were generated through enhanced chemiluminescence (Thermo Fisher Scientific) using an Amersham Imager 600 (GE Healthcare Life science). Band intensities were quantified and normalized against the tubulin expression levels using ImageJ software.

Data analysis

Statistical analysis was performed using Graphpad Instat software (San Diego, USA, http://www.graphpad.com). Normality of the data between experimental groups was confirmed using the method of Kolmogorov and Smirnov. The significance of differences was

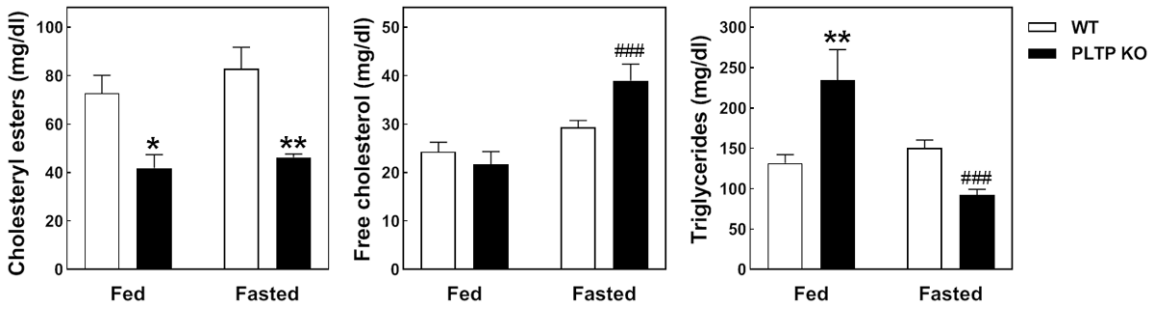

Figure 1. The effect of PLTP deficiency on plasma lipid levels is influenced by the feeding status. Plasma cholesteryl ester, free cholesterol, and triglyceride levels in male wild-type (WT) and PLTP knockout (PLTP KO) mice as measured in the ad libitum fed (Fed) and overnight food deprived (Fasted) state. *P<0.05, **P<0.01 versus WT; ###P<0.001 versus Fed.

calculated using a two-tailed unpaired t-test or two-way analysis of variance (ANOVA) with Bonferroni post-test where appropriate. Probability values less than 0.05 were considered significant.

Results

Total cholesterol levels were significantly lower in plasma samples from PLTP knockout mice as compared to those from wild-type controls: 63±8 mg/dl versus 97±9 mg/dl in ad libitum fed mice and 88±4 mg/dl versus 112±9 mg/dl after overnight food deprivation (P<0.05 for both), as expected [2, 12]. The effect of genetic PLTP deficiency on total cholesterol levels could primarily be attributed to an ~45% reduction in plasma cholesteryl ester concentrations (**Figure 1**). Non-fasting and fasting plasma free cholesterol levels were either not different or mildly increased due to lack of a functional PLTP protein (**Figure 1**). Notably, the effect of PLTP deficiency on plasma triglyceride levels was dependent on the feeding status (**Figure 1**). In accordance with the assumption that PLTP activity is essential for the metabolism of triglyceride-rich chylomicron particles [13], triglyceride concentrations were significantly elevated in PLTP knockout mice as compared to wild-type mice under ad libitum feeding conditions (+78%; P<0.01). In contrast, plasma triglyceride concentrations tended to be lower in PLTP knockout mice than in wild-type controls after overnight food deprivation, which can probably be attributed to a reduced hepatic generation and secretion of triglyceride-rich VLDL particles [14].

Plasma levels of the major glucocorticoid species corticosterone were determined as quantitative measure for the adrenal glucocorticoid output. Although non-stressed, ad libitum fed plasma corticosterone values appeared to be somewhat lower in PLTP knockout mice as compared to wild-type mice, this effect did not reach significance (P>0.05; **Figure 2A**). In accordance with findings from previous studies [8-10, 15], food deprivation was associated with a significant increase in plasma corticosterone levels in wild-type mice (+95%; P<0.01 versus ad libitum fed values; **Figure 2A**). Strikingly, PLTP knockout mice were able to increase their plasma corticosterone levels to the same extent in response to food deprivation as observed in wild-type mice (P<0.001; two way ANOVA - feeding effect: P<0.001; genotype effect: P>0.05). As a result, plasma corticosterone levels were nearly identical under fasting conditions in the two experimental groups (394±139 mg/dl for PLTP knockout mice versus 380±144 ng/ml for wild-type mice; P>0.05; **Figure 2A**). It thus appears that the marked reduction in plasma cholesteryl ester levels in PLTP knockout mice does not translate into a diminished adrenal glucocorticoid output.

Glucocorticoid insufficiency resulting from decreased adrenal acquisition of HDL-associated cholesterol is generally thought to be secondary to the depletion of adrenal cholesteryl ester stores. In accordance with the apparently normal capacity of the PLTP knockout adrenals to generate glucocorticoids, the adrenal neutral lipid distribution profile was also very similar in both groups of mice. The cortex of both the wild-type and PLTP knockout adrenals stained intensively positive for the presence of cholesteryl esters (**Figure 2B**).

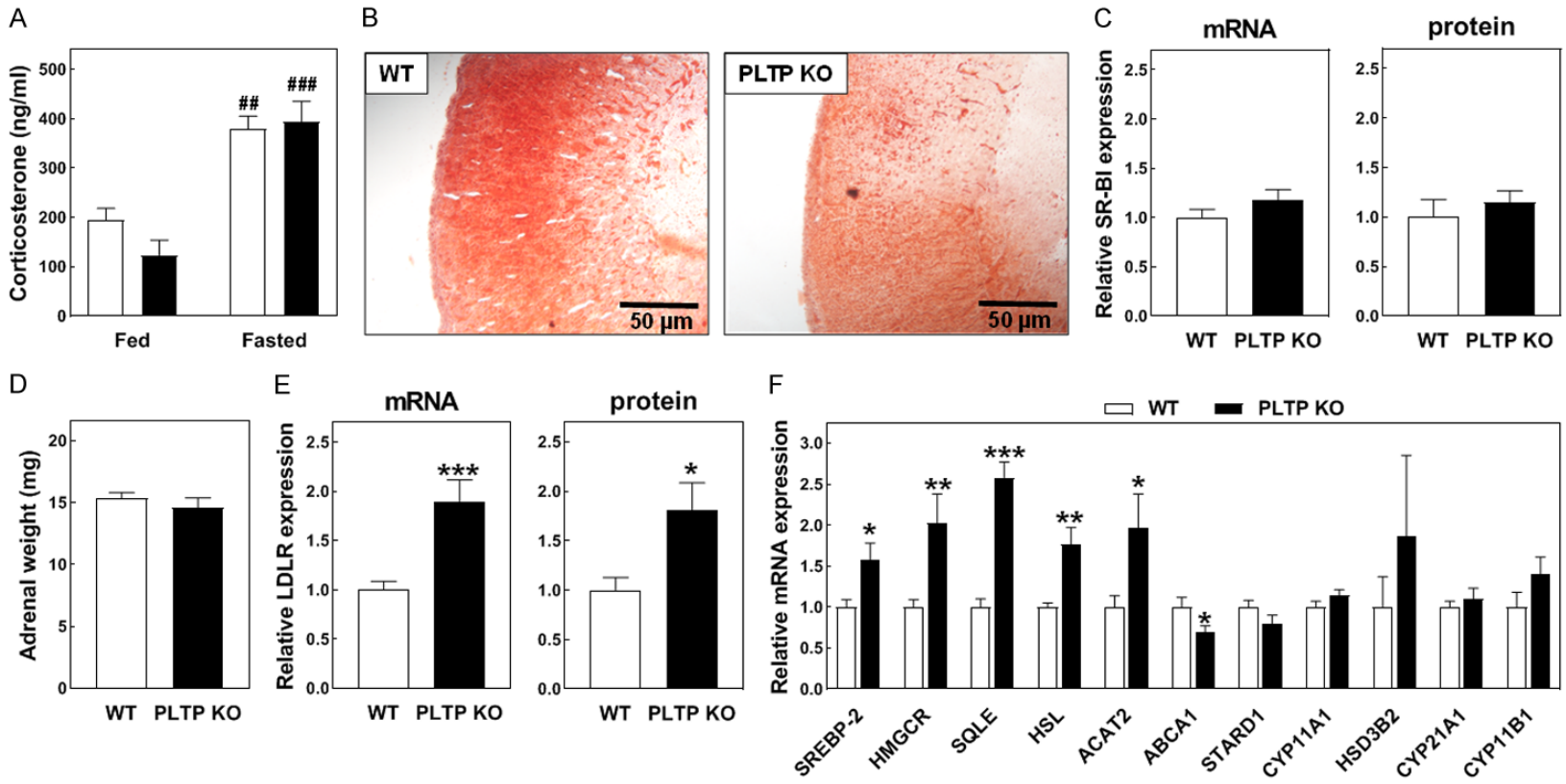

Figure 2. PLTP deficiency is associated with a normal adrenocortical neutral lipid deposition and glucocorticoid output in the context of potentially compensatory upregulation of genes involved in adrenal cholesterol synthesis and acquisition. (A) Plasma corticosterone levels in male wild-type (WT) and PLTP knockout (PLTP KO) mice as measured in the ad libitum fed (Fed) and overnight food deprived (Fasted) state. (B) Representative pictures of Oil red O-stained cryosections from adrenals showing neutral lipid deposition within the adrenal cortex of both groups of overnight fasted mice. Adrenal relative mRNA and protein expression levels of the HDL receptor SR-BI (C) and LDLR (E) and transcript levels of genes involved in cholesterol acquisition and mobilization and the conversion of cholesterol into corticosterone (F). Values are expressed relative to those in WT mice. (D) Absolute adrenal weights. *P<0.05, **P<0.01, ***P<0.001 versus WT. ##P<0.01, ###P<0.001 versus Fed.

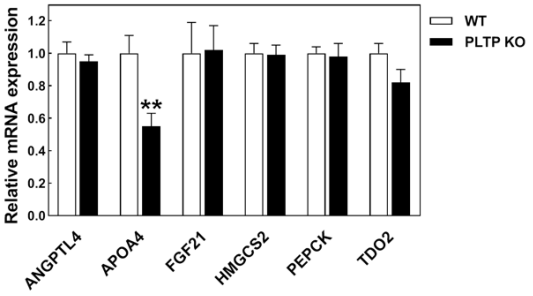

Figure 3. PLTP deficiency is not associated with a significant change in expression levels of glucocorticoid-responsive genes. Relative mRNA expression levels in livers of overnight food deprived male wild-type (WT) and PLTP knockout (PLTP KO) mice. Values are expressed relative to those in WT mice. **P<0.01 versus WT.

Adrenal mRNA and protein expression levels of SR-BI were not significantly different between PLTP knockout mice and wild-type controls (**Figure 2C**), arguing against potentially compensatory adrenocorticotropic hormone (ACTH)-driven overstimulation of adrenal HDL-cholesteryl ester uptake [16]. In further support of an overall unchanged adrenocortical (proliferative) activation by ACTH in response to PLTP deficiency, absolute adrenals weights were also similar in the two groups of mice (14.6±0.8 mg for PLTP knockout mice and 15.4±0.4 mg for wild-type mice; **Figure 2D**). Adrenals from PLTP knockout mice did - however - exhibit a potentially compensatory stimulation of cholesterol uptake from apolipoprotein B-containing lipoproteins, as judged from the 80-90% increase in relative mRNA (P< 0.001) and protein (P<0.05) expression levels of the LDLR (**Figure 2E**). LDLR gene expression levels are primarily controlled by the transcription factor sterol regulatory element-binding protein-2 (SREBP-2) that becomes active upon depletion of the cellular free cholesterol pool [17]. In accordance with stimulation of the SREBP-2 pathway in adrenals from PLTP knockout mice, relative expression levels of SREBP-2 itself (+57%; P<0.05) as well as those of the other classical SREBP-2 target genes HMG-CoA reductase (HMGCR; +102%; P<0.01) and squalene epoxidase (SQLE; +158%; P< 0.001) involved in cholesterol synthesis were significantly upregulated as compared to wild-type adrenals (**Figure 2F**). Expression levels of hormone-sensitive lipase (HSL; +76%; P<0.01) and acetyl-Coenzyme acetyltransferase 2 (ACAT2; +97%; P<0.05), enzymes involved in the catabolism of lipoprotein-associated cholesteryl esters and the re-esterification of free cholesterol for subsequent storage in lipid droplets, were similarly elevated in PLTP knockout adrenals (**Figure 2F**). Adrenal transcript levels of the key cholesterol efflux protein ATP-binding cassette transporter A1 (ABCA1) were rather decreased in response to the genetic PLTP deficiency (-31%; P=0.05; **Figure 2F**). No significant change was detected in the relative mRNA expression levels of steroidogenic acute regulatory protein (STARD1) that transports cholesterol into the mitochondria or proteins involved in the actual conversion of cholesterol into corticosterone, i.e. mitochondrial cholesterol side-chain cleavage enzyme (CYP11A1/P450SCC), 3beta-hydroxy-delta(5)-steroid dehydrogenase 2 (HSD3B2), steroid 21-hydroxylase (CYP21A1), and steroid 11-beta-monooxygenase (CYP11B1) (**Figure 2F**).

Relative expression levels of established glucocorticoid target genes were measured in livers of the different mice under food deprived conditions to validate that, in addition to the apparently normal plasma glucocorticoid response, also the downstream glucocorticoid-driven transcriptional response to fasting stress was unaffected in PLTP knockout mice (**Figure 3**). In agreement, hepatic relative mRNA expression levels of the glucocorticoid receptor target genes phosphoenolpyruvate carboxykinase (PEPCK), angiopoietin-like protein 4 (ANGPTL4) and fibroblast growth factor 21 (FGF21) were almost identical in food deprived PLTP knockout mice and wild-type controls (**Figure 3**). Relative mRNA expression levels of the glucocorticoid-sensitive transcripts tryptophan 2,3-dioxygenase (TDO2) and HMG-Co synthase 2 (HMGCS2) were also not significantly different in the two groups of livers (**Figure 3**). However, a marked 45% reduction (P<0.01) was detected in livers from PLTP knockout mice in the relative transcript levels of apolipoprotein A4 (APOA4; **Figure 3**), a gene previously also suggested to be controlled by glucocorticoids during fasting [18].

Discussion

In the current study it was evaluated whether the PLTP deficiency-associated decrease in plasma cholesterol levels negatively impacts the adrenal glucocorticoid output. In contrast to the hypothesis, hypocholesterolemic PLTP

knockout mice exhibited a glucocorticoid response to food deprivation comparable to that of normolipidemic wild-type mice.

The lack of an effect of PLTP deficiency on the glucocorticoid output can highly likely be explained by the fact that adrenals from PLTP knockout mice were also able to maintain their tissue cholesteryl ester stores at a normal level, despite the genotype-associated decrease in HDL-cholesteryl ester availability. PLTP knockout adrenals exhibited a potentially compensatory ~2-fold increase in relative expression levels of genes involved cholesterol synthesis (HMGCR/SQLE), acquisition (LDLR; HSL), and storage (ACAT2). Based upon the previous observations that adrenal cholesteryl ester stores are still severely deprived in HDL deficient APOA1 and LCAT knockout mice in the context of a ~6-fold increase in adrenal HMGCR gene expression/activity levels [6, 7], the relatively minor upregulation of HMGCR, and LDLR expression levels will alone probably not have been sufficient to overcome a negative effect of the PLTP deficiency-associated hypocholesterolemia. Notably, recent studies in apolipoprotein E knockout mice have provided experimental support that, under hypercholesterolemic conditions, apoB-containing VLDL and LDL particles serve as an important source of cholesterol used for glucocorticoid synthesis during food deprivation stress [19]. In response to the genetic lack of PLTP, cholesterol-rich VLDL remnant particles are generated during lipolysis that predominantly depend on SR-BI for their subsequent removal from the blood circulation [13]. SR-BI expression levels within the adrenals are already relatively high in the basal setting [20] and even further increased during the physiological stress response, i.e. upon exposure to ACTH [21]. As such, an enhanced influx of cholesterol substrate from VLDL particles via SR-BI can also have significantly contributed to the maintenance of the adrenal cholesteryl ester stores and glucocorticoid output in PLTP knockout mice.

An interesting finding of this study is that APOA4 mRNA expression levels were significantly reduced in livers from PLTP knockout mice, while the hepatic transcript levels of five other glucocorticoid sensitive genes were not different between wild-type and PLTP knockout mice. This observation conflicts with the suggestion from Hanniman et al. [18] that the increase in plasma glucocorticoids drives the fasting-associated increase in liver APOA4 expression levels and implies that the reduction in APOA4 expression is rather secondary to the PLTP-deficiency associated decrease in plasma cholesteryl ester levels. In support of this notion, a VLDL/LDL lowering-mediated reduction in fasting glucocorticoid levels in apolipoprotein E knockout mice is also not associated with a reduction in hepatic APOA4 expression levels [19]. Notably, the activity of hepatic nuclear factor-4 alpha (HNF-4alpha) - an established regulator of APOA4 transcription [18, 22, 23] - is highly sensitive to changes in cellular levels of distinct fatty acid species that can be acquired via uptake of HDL-cholesteryl esters [24]. Based upon these combined findings, it is anticipated that the PLTP deficiency-associated decrease in plasma HDL-cholesteryl esters leads to reduced delivery of HNF-4alpha ligands to hepatocytes and a concomitant reduction in the liver APOA4 transcription rate.

In conclusion, we have shown that hypocholesterolemic PLTP knockout mice exhibit a normal glucocorticoid response to food deprivation. These novel findings (1) highlight that the effect of HDL deficiency in mice on adrenal glucocorticoid output is model dependent and (2) imply that other (lipoprotein) cholesterol sources than HDL can also generate the pool utilized by adrenocortical cells to synthesize glucocorticoids.

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Disclosure of conflict of interest

None.

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