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VLDL/LDL serves as the primary source of cholesterol in the adrenal glucocorticoid response to food deprivation

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ABSTRACT

The contribution of individual lipoprotein species to the generation of the adrenal cholesterol pool used for the synthesis of anti-inflammatory glucocorticoid species remains unknown. Here we examined the impact of specific lowering of very low-density lipoprotein (VLDL) and low-density (LDL) levels on adrenal cholesterol and glucocorticoid homeostasis. Hereto, lethally-irradiated hypercholesterolemic apolipoprotein E (APOE) knockout mice received APOE-containing bone marrow from wild-type mice ($n = 6$) or APOE knockout control bone marrow ($n = 10$) and were subsequently fed a regular chow diet. Transplantation with wild-type bone marrow was associated with a 10-fold decrease in VLDL/LDL-cholesterol levels. No changes were observed in adrenal weights, adrenal cholesterol content, or basal plasma corticosterone levels. However, food deprivation-induced corticosterone secretion was 64% lower ($P < 0.05$) in wild-type bone marrow recipients as compared to APOE knockout bone marrow recipients, in the context of similar plasma adrenocorticotropic hormone (ACTH) levels. A parallel 19–29% decrease in adrenal relative mRNA expression levels of ACTH-responsive genes SR-BI ($P < 0.01$), STAR ($P < 0.05$), and CYP11A1 ($P < 0.05$) was detected. In support of relative glucocorticoid insufficiency, blood lymphocyte and eosinophil concentrations were respectively 2.4-fold ($P < 0.01$) and 8-fold ($P < 0.001$) higher in wild-type bone marrow recipients under food deprivation stress conditions.

In conclusion, we have shown that a selective lowering of VLDL/LDL levels in APOE knockout mice through a transplantation with APOE-containing wild-type bone marrow is associated with a decreased maximal adrenal glucocorticoid output. Our studies provide experimental support for the hypothesis that, in vivo, VLDL/LDL serves as the primary source of cholesterol used for glucocorticoid synthesis during food deprivation stress.

1. Introduction

Steroid producing cells such as adrenocortical cells have a dual need for cholesterol. Besides their use of cholesterol in general cellular processes like the maintenance of membrane functionality [1,2], adrenocortical cells need to acquire sufficient levels of cholesterol to serve as precursor for the synthesis of steroid hormones, i.e. glucocorticoids [3–6].

Although it is well established that cholesterol is the sole precursor for the synthesis of glucocorticoids such as cortisol in humans and corticosterone in rodents, the exact contribution of different sources to the pool of cholesterol used for adrenal steroidogenesis remains unknown to date. Statin treatment does not impact on cortisol levels in humans [7,8], which argues against an essential role for de novo cholesterol synthesis in the generation of the cholesterol substrate. In contrast, low-density lipoprotein (LDL) deficient human subjects that carry functional mutations in the apolipoprotein B100 (APOB100) gene display a reduced capacity of the adrenals to produce cortisol [9]. Moreover, subjects carrying a deleterious mutation in the high-density lipoprotein (HDL) receptor scavenger receptor class B type I (SR-BI) gene also display signs of glucocorticoid insufficiency [10]. It thus appears that, in humans, both LDL and HDL supply the adrenals with a significant amount of the cholesterol substrate that is needed for steroidogenesis. In accordance with the assumption that lipoproteins also act as essential cholesterol source in rodents, an estradiol treatment-induced reduction in plasma cholesterol levels is associated with a diminished adrenal glucocorticoid response to an adrenocorticotropic hormone (ACTH) challenge [3]. A specific disruption of HDL-mediated adrenal cholesterol acquisition generally replicates the lipoprotein deficiency-associated reduction in adrenal cholesteryl ester stores and maximal glucocorticoid output in normolipidemic mice [5,6,11] Notably, studies in cultured adrenocortical cells and gene targeted mice by Kraemer et al. and Li et al. have indicated that hormone-sensitive lipase (HSL)-mediated breakdown of lipoprotein-derived cholesteryl esters is

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a perquisite to effectively generate the steroidogenic cholesterol pool used under high steroidogenic pressure conditions [12,13]. In agreement with the notion that HDL is an effective cholesterol donor in mice carrying a human-like lipoprotein profile, we have recently observed an anticipated depletion of the adrenal cholesteryl ester stores in hypercholesterolemic LDL receptor knockout mice that genetically lack HDL [14]. Interestingly, these HDL deficient APOA1 x LDL receptor double knockout mice did – however – not display the expected glucocorticoid insufficiency phenotype. More specifically, they exhibited a normal plasma corticosterone response to both overnight food deprivation and the induction of endotoxemia [14]. From these latter observations, it can be hypothesized (1) that the adrenal cholesteryl ester pools are not the primary determinant for the overall steroidogenesis rate in mice or (2) that mice can compensate the loss of HDL-derived cholesterol pool by using the non-HDL lipoprotein fractions as source of the steroidogenic cholesterol substrate. Apolipoprotein E (APOE) knockout mice lacking a functional APOE gene exhibit relatively high cholesterol levels associated with non-HDL species, i.e. very low-density lipoprotein (VLDL)- and LDL particles, in the context of normal plasma HDL-cholesterol levels [15], which is paralleled by hypercorticosteronemia [16]. To uncover the contribution of VLDL/LDL to the adrenal glucocorticoid output, in the current study we investigated the effect of a specific lowering of VLDL/LDL-cholesterol levels on adrenal cholesterol and glucocorticoid homeostasis in APOE knockout mice.

2. Materials and methods

2.1. In vivo setup

Animal experiments were performed at the Leiden Academic Centre for Drug Research within the Gorlaeus Laboratories of Leiden University. All animal work was approved by the Dutch Ethics Committee and regulatory authority at Leiden University and was carried out in compliance with Dutch government guidelines and the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

All experimental mice were held in a 12 h light/12 h dark light-cycle and temperature controlled room. From one week prior until two weeks after the bone marrow transplantation, male APOE knockout recipient mice were provided with autoclaved drinking water supplemented with antibiotics (83 mg/L ciprofloxacin, 67 mg/L polymyxin B sulfate, 6.5 g/L sucrose). Bone marrow aplasia was induced in recipient mice by exposing them to 2 × 4.5 Gy total body X-ray irradiation (0.19 Gy/min, 200 kV, 4 mA) using an Andrex Smart 225 Röntgen source (YXLON International, Copenhagen, Denmark) with a 6-mm aluminium filter. The next day, bone marrow from the tibias and femurs of C57BL/6 wild-type and APOE knockout donor mice was harvested, filtered to create a unicellular suspension and counted. A total amount of $5 * 10^6$ bone marrow cells were subsequently intravenously injected into the tail vain of the irradiated recipient mice. Mice that had received the bone marrow were individually housed in sterilized filter top cages and fed sterilized chow diet (RM3 (E) DU; Special Diet Services, Witham, England) ad libitum. At 9 weeks after bone marrow transplantation, all mice were bled via a tail cut at 09:00 in the “non-stressed” ad libitum fed state for plasma corticosterone analysis. Food deprivation overnight, i.e. during the regular active eating phase, is considered a stressful event for mice and consistently associated with a rise in adrenal glucocorticoid output and blood glucocorticoids levels (unpublished data and [6,11]). At ten weeks after the bone marrow transplantation, mice were therefore subjected to overnight food deprivation from 05:00 PM and tail bleeding at 09:00 for plasma “fasting stress” corticosterone analysis. Subsequently, mice were injected with a mix of xylazine (70 mg/kg), ketamine (350 mg/kg) and atropine (1.8 mg/kg), bled via the eye for biochemical plasma analysis and hematological analysis, and sacrificed. Organs were perfused in situ with PBS (pressure 100 mmHg) for 10 min via a cannula in the left ventricular apex. Perfused organs were fixated for about 24 h in 3.7% neutral-buffered formalin solution (Formal-fixx®, Shandon Scientific Ltd., UK) and subsequently preserved in 0.1% sodiumazide in PBS solution or snap-frozen and stored at -20 °C for further analysis.

2.2. Plasma measurements

Plasma corticosterone levels were determined in tail blood using a ^{125}I-corticosterone kit (MP Biomedicals, Santa Anna, USA) following the manufacturer's protocol. Plasma adrenocorticotropic hormone (ACTH) levels were measured in tail blood using a ^{125}I-radiolabeled kit following the manufacturer's protocol (Phoenix pharmaceuticals, Burlingame, CA, USA). Plasma free cholesterol and cholesteryl ester levels were determined using colorimetric assays. The cholesterol distribution over the different lipoproteins was determined in pooled plasma via fast protein liquid chromatography-based fractionation (FPLC; Superose 6 column; 3.2–30 mm; Smart-System, Pharmacia, Uppsala, Sweden). FPLC fractions 1–13 represent the non-HDL species (VLDL and LDL), while fractions 13–19 represent HDL.

2.3. Adrenal lipid analysis

One adrenal per mouse was used for tissue lipid quantification using the extraction method developed by Bligh and Dyer [17]. Free cholesterol and cholesteryl ester levels in the lipid extract were quantified using colorimetric assays. Tissue lipid levels were corrected for the protein input.

2.4. Analysis of gene expression by real-time quantitative PCR

Total RNA was isolated for quantitative gene expression analysis as previously described [18]. In short, total RNA was isolated using a standard phenol/chloroform extraction method and reverse transcribed using RevertAid Reverse Transcriptase. Gene expression analysis was performed by using SYBR-Green technology (Eurogentec) on a 7500 Fast Real time PCR system (Applied Biosystems, Foster city, US). Primer sequences are available upon request. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ribosomal protein lateral stalk subunit P0 (36B4) and beta-actin (β-actin) were used as the housekeeping genes.

2.5. Statistical analysis

Statistical analysis was performed using GraphPad Instat Software (San Diego, CA, USA, http://www.graphpad.com). Normality testing was performed using the method of Kolmogorov and Smirnov. Significance was calculated using a two-tailed Student's *t*-test or two-way ANOVA with Bonferroni's post-test where appropriate. *P* values < 0.05 were considered significant.

3. Results

Reintroduction of APOE through transplantation of wild-type bone marrow is an effective means to induce a specific, long-term, reduction in plasma VLDL/LDL-cholesterol levels and, as a result, lower the associated atherosclerosis susceptibility in APOE knockout mice [19–21]. To determine the relative impact of VLDL/LDL on glucocorticoid output in vivo, we therefore evaluated adrenal cholesterol and glucocorticoid homeostasis in male APOE knockout mice transplanted with either bone marrow from APOE containing wild-type donor mice or with control bone marrow from age- and sex-matched APOE knockout donors.

As evident from Fig. 1A, successful recovery of APOE, i.e. in hepatic macrophages (Kupffer cells), could be verified on the gene expression level in livers of mice that were transplanted with wild-type bone marrow. No functional APOE transcript was detected in livers of APOE knockout bone marrow recipients, as expected. The effective

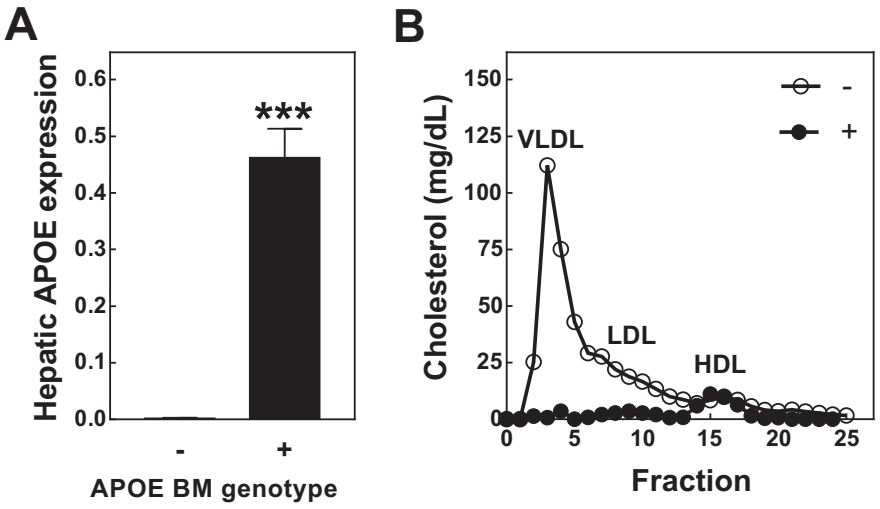

Fig. 1. Transplantation of wild-type bone marrow (APOE BM+) into lethally irradiated APOE knockout mice restores APOE mRNA expression levels within the liver (A) and lowers plasma very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) as compared to APOE knockout bone marrow (APOE BM-) recipients. Data in panel A represent means + SEM of 10 (APOE BM-; white bars/symbols) and 6 (APOE BM+; black bars/symbols) mice per group. ***$P < 0.001$. HDL, high-density lipoprotein.

Table 1
Plasma cholesterol levels in chow diet-fed lethally irradiated APOE knockout mice transplanted with wild-type (BM+) or APOE knockout (BM-) bone marrow.

	Ad libitum fed			Overnight food deprivation		
	BM-	BM+	P value	BM-	BM+	P value
Free cholesterol (mg/dL)	152 ± 8	27 ± 1	< 0.001	147 ± 9	27 ± 3	< 0.001
Cholesteryl esters (mg/dL)	746 ± 58	130 ± 9	< 0.001	717 ± 51	90 ± 6	< 0.001

restoration of APOE functionality was associated with a 5- to 7-fold decrease ($P < 0.001$) in plasma free cholesterol and cholesteryl ester levels (Table 1). FPLC-based lipoprotein fractionation indicated that the decrease in cholesterol levels was due to a 10-fold decrease in cholesterol associated with VLDL/LDL particles (Fig. 1B). Importantly, no relevant change in plasma HDL-cholesterol levels was observed in response to APOE reintroduction (Fig. 1B).

Adrenals were isolated from the two types of bone marrow recipient mice for morphological and biochemical analysis. No significant difference in absolute adrenal weights was observed (Fig. 2A). Notably, lipid extraction and subsequent quantification revealed that tissue levels of free cholesterol (Fig. 2B) and cholesteryl esters (Fig. 2C) were also not different between the two groups of adrenals.

The bone marrow APOE-induced reduction in plasma VLDL/LDL did not significantly impact on baseline plasma corticosterone levels (Fig. 3A). The bone marrow genotype did, however, affect the plasma corticosterone response to food deprivation-associated stress (Fig. 3A). Corticosterone levels increased 8-fold in APOE knockout bone marrow recipient controls after overnight food deprivation (260 ± 54 ng/mL in the fasted state versus 32 ± 5 ng/mL in ad libitum fed state, respectively; $P < 0.001$). In contrast, food deprivation was only associated with a 2-fold rise ($P > 0.05$) in plasma corticosterone levels in wild-type bone marrow recipients. As a result, plasma corticosterone levels were 64% lower (92 ± 11 ng/mL; $P < 0.05$) in wild-type bone marrow recipients as compared to APOE knockout bone marrow recipients in the fasting state (Fig. 3A).

Relatively high plasma glucocorticoid levels are associated with immunosuppression as a result of anti-inflammatory actions directed by the glucocorticoid receptor on immune cells, i.e. lymphocytes [22,23]. Food deprived wild-type bone marrow recipients exhibited a relatively high inflammatory status as measured by routine hematological analysis, which suggests that these mice also exhibited a reduced immunosuppressive glucocorticoid function as compared to APOE knockout bone marrow recipient mice. The significant increase in total

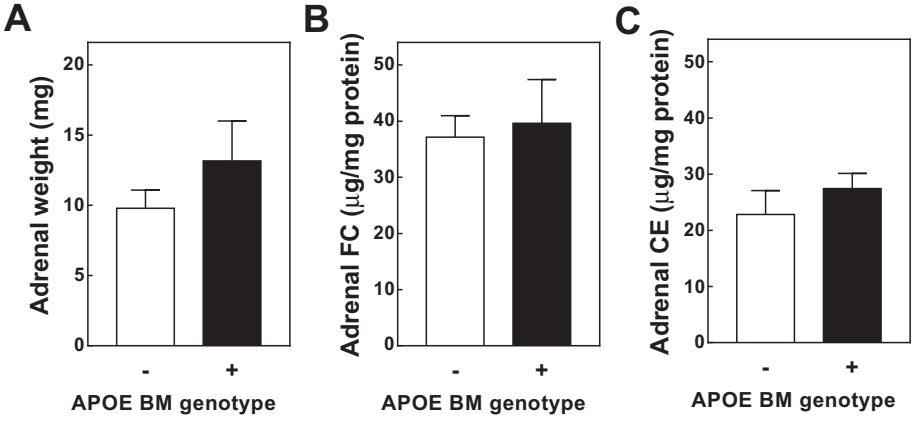

Fig. 2. Reintroduction of APOE into APOE knockout mice via bone marrow transplantation did not affect the adrenal weight (A) or the adrenal free cholesterol (B) and cholesteryl ester (C) content. Data represent means + SEM of 10 (APOE BM-; white bars) and 6 (APOE BM+; black bars) mice per group.

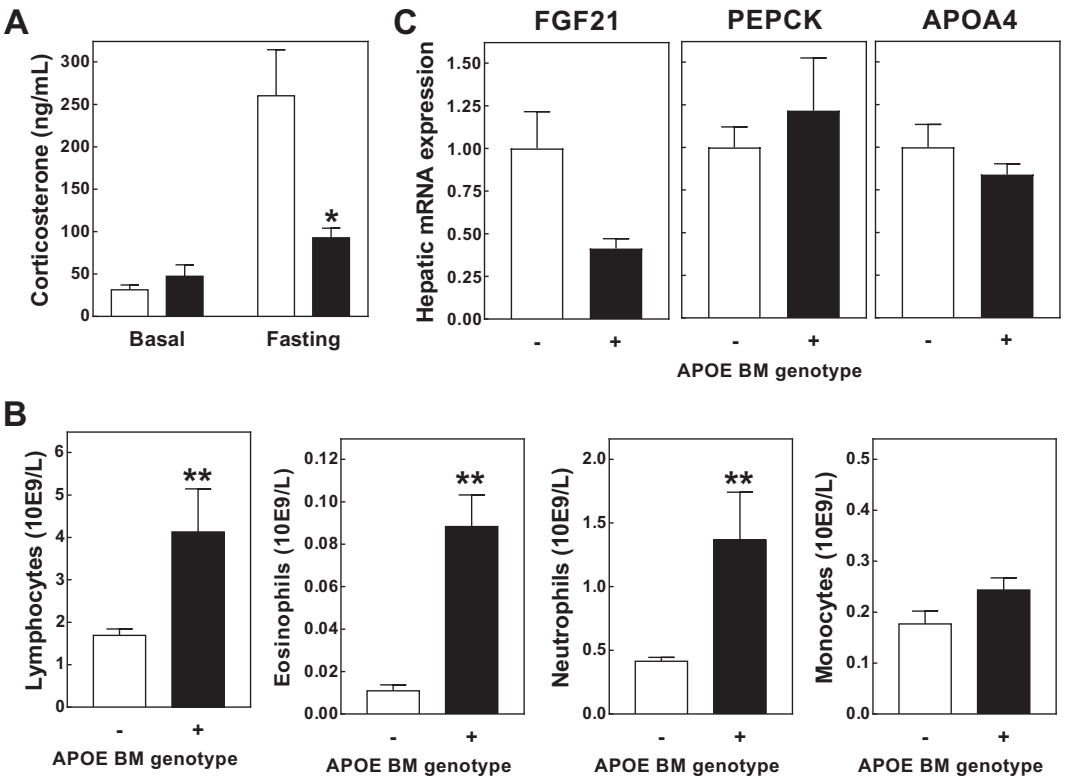

Fig. 3. Reintroduction of APOE into APOE knockout mice via bone marrow transplantation reduced the fasting-induced plasma corticosterone response while it did not impact the basal adrenal corticosterone output (A). Restoration of APOE functionality in APOE knockout mice is associated with increased blood lymphocyte, eosinophil and neutrophil concentrations, without a change in blood monocyte counts (B). No significant difference in hepatic relative mRNA expression levels of the glucocorticoid response genes FGF21, PEPCK and APOA4 was detected under food deprivation-associated stress conditions (C). Data represent means + SEM of 10 (APOE BM-; white bars) and 6 (APOE BM+; black bars) mice per group. *$P < 0.05$, **$P < 0.01$.

blood leukocyte concentrations (5.8 ± 1.4 × 10E9/L versus 2.5 ± 0.1 × 10E9/L; $P < 0.01$) was primarily driven by a 2.4-fold increase ($P < 0.01$) in the blood lymphocyte count (Fig. 3B). The lymphocytosis was paralleled by a striking 8-fold rise ($P < 0.001$) in the number of eosinophils as well as a 3.3-fold increase ($P < 0.01$) in blood neutrophil concentrations, while no significant change in blood monocyte counts was observed (Fig. 3C, D, and E).

The liver is a primary target organ of glucocorticoids under fasting conditions as glucocorticoids through their interaction with the glucocorticoid receptor located on hepatocytes modulate lipid and glucose metabolism, i.e. stimulate gluconeogenesis [24]. As can be appreciated from Fig. 3C, no significant difference in the relative mRNA expression levels of fibroblast growth factor 21 (FGF21), phosphoenolpyruvate carboxykinase (PEPCK) and apolipoprotein A4 (APOA4) was detected in livers of the two bone marrow recipients groups. It thus seems that, unexpectedly, the reduction in plasma glucocorticoid levels upon APOE re-introduction was not paralleled by a concomitant change in the hepatic expression levels of glucocorticoid receptor target genes. However, it should be acknowledged that the global effect of APOE function replenishment on total body (lipid) metabolism may have overshadowed the individual effect of the lowered plasma glucocorticoid levels on the liver gene expression profile.

The extent of adrenal glucocorticoid output not only depends on the adrenocortical cell cholesterol availability, but also on the level of activation of intracellular signaling pathways that stimulate the transcription of genes involved in the intracellular mobilization of cholesterol and the conversion cholesterol to corticosterone. The steroidogenic trigger was equally high in the two experimental groups as judged from the observation that APOE-mediated reversal of the hypercholesterolemia was not associated with a change in the fasting plasma levels of the primary steroidogenic activator ACTH (Fig. 4A). Strikingly, as can be appreciated from Fig. 4B, we did – however – measure a significant decrease in the adrenal relative mRNA expression levels of the established ACTH-responsive genes SR-BI [25,26], steroidogenic acute regulatory protein (STAR) [27], and cholesterol side-chain cleavage enzyme (CYP11A1) [28]. Gene expression levels of SR-BI, STAR, and CYP11A1 were respectively 29% ($P < 0.01$), 19% ($P < 0.05$), and 27% ($P < 0.05$) lower in wild-type bone marrow recipients as compared to APOE knockout bone marrow controls (Fig. 4B). In accordance with the findings of Ho et al. that, in rats, the 11-beta-hydroxylase (CYP11B1) transcription rate is increased by ACTH treatment [29], CYP11B1 mRNA expression levels also tended to be lower in adrenals of wild-type bone marrow recipients (−32%; $P > 0.05$; Fig. 4B). No parallel change was observed in the mRNA expression levels of HMG-CoA reductase (HMGCR), the LDL receptor (LDLR), acetyl-Coenzyme A acetyltransferase 1 (ACAT-1), hormone-sensitive lipase (HSL), hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2 (HSD3B2), and 21-hydroxylase (CYP21A1) that represent other genes crucially involved in respectively adrenocortical cell cholesterol mobilization and storage and steroidogenesis. It can therefore be anticipated that the observed changes in SR-BI/STAR/CYP11A1/CYP11B1 gene expression were not resulting from a general change in cellular cholesterol metabolism but rather due to a specific effect on the cellular ACTH response.

4. Discussion

In the current study we aimed to provide proof for the concept that mice use non-HDL lipoprotein fractions, i.e. VLDL and LDL, as primary source of cholesterol substrate for the production of glucocorticoids. Here we show that selective depletion of VLDL/LDL in APOE knockout mice is associated with significant decreases in the adrenal expression

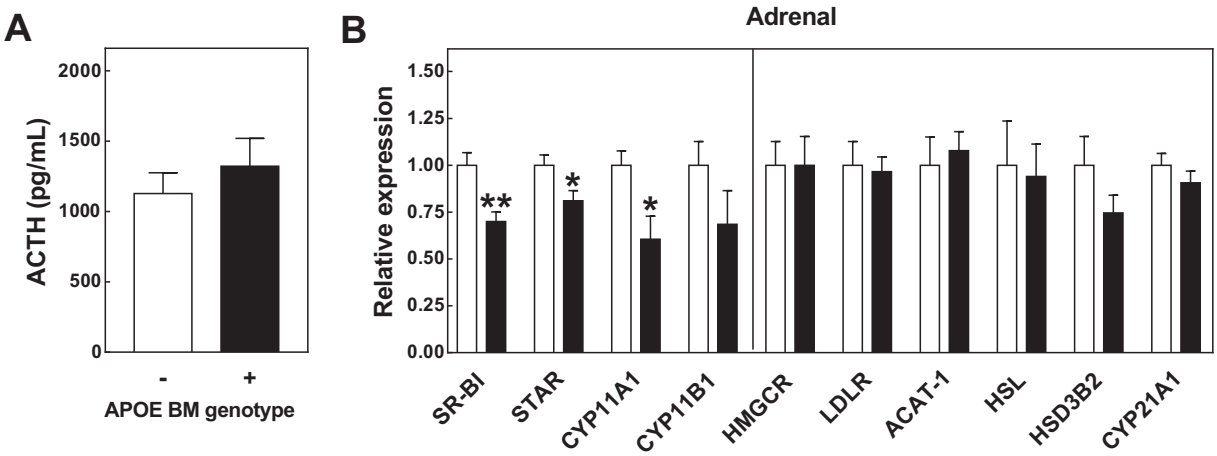

Fig. 4. Reintroduction of APOE into APOE knockout mice via bone marrow transplantation did not impact fasting plasma ACTH levels (A), but lowered adrenal relative mRNA expression levels of the ACTH-responsive steroidogenic genes SR-BI, STAR and CYP11A1 without altering transcript levels of other key proteins involved in adrenal cholesterol handling and steroidogenesis (B). Data represent means + SEM of 10 (APOE BM-; white bars) and 6 (APOE BM+; black bars) mice per group. *P < 0.05, **P < 0.01.

of genes involved in cholesterol mobilization and steroidogenesis and a reduced maximal adrenal glucocorticoid output under food deprivation (fasting) stress conditions.

The marked decrease in the fasting stress-induced adrenal glucocorticoid output upon bone marrow APOE repopulation in APOE knockout mice was not paralleled by a change in adrenal cholesterol stores. This finding provides support for the hypothesis, originally derived from our studies in hypercholesterolemic LDL receptor knockout mice [14], that the extent of adrenal cholesteryl ester storage is not causally related to the ability of adrenals to synthesize glucocorticoids. Furthermore, from this finding it can be concluded that in a combined HDL and VLDL/LDL setting, such as in APOE and LDL receptor knockout mice as well as in humans, VLDL/LDL is not contributing significantly to the generation of the intra-adrenal cholesteryl ester stores. In accordance, our previous studies in HDL deficient APOA1 x LDL receptor knockout double knockout mice have suggested that rather cholesterol acquired from HDL particles is stored in the adrenocortical cell cholesteryl ester pool [14]. In further support of an important contribution of HDL to adrenal cholesteryl ester accumulation, Plump et al. have shown that a genetic lack of APOA1 and the associated HDL deficiency is also accompanied by depletion of neutral lipids from adrenals in APOE knockout mice [30].

Our studies suggest that at least two distinct cholesterol mobilization routes can be discriminated within the adrenals: one in which HDL provides cholesterol to the adrenals for storage and another where VLDL/LDL delivers the cholesterol that is utilized for stress-induced adrenal steroidogenesis (Fig. 5). Previous studies using adrenal-specific and total body knockout mice have implied that the cholesterol acquisition from HDL is dependent on selective cholesteryl ester uptake from HDL particles by SR-BI [31]. Given that a genetic lack of the LDL receptor is associated with accumulation of the APOB100-containing lipoproteins VLDL and LDL in the plasma compartment, it could be assumed that these lipoprotein species deliver the majority of their cholesterol to cells via binding to and subsequent whole particle uptake via the LDL receptor. Lack of the LDL receptor does, however, not impact on the glucocorticoid production rate in adrenocortical cells in vitro [32]. In addition, total body knockout LDL receptor knockout mice display a normal ability of the adrenals to produce glucocorticoids [33,34]. The cholesterol used for steroidogenesis thus seems to be acquired from VLDL/LDL particles by adrenocortical cells in manner that is actually not dependent on the LDL receptor. The LDL receptor-related protein 1 (LRP1) is also considered to be a functional VLDL/LDL receptor, since it is able to bind and internalize APOE-containing lipoproteins [35]. LRP1 protein expression has been detected in adrenals and is not different between wild-type and APOE knockout mice [16]. Unfortunately, total body LRP1 deficiency is embryonically lethal in mice [36]. As a consequence, the relative contribution of LRP1 to the uptake of lipoprotein-associated cholesterol into the adrenals as well the impact of the impact of LRP1 deficiency on adrenal steroidogenesis remains unknown to date. Multiple studies have suggested that SR-BI can mediate selective uptake of cholesteryl esters from VLDL/LDL as well as whole particle clearance [37–40]. Studies in normolipidemic mice have shown that the relative impact of SR-BI deficiency on the adrenal glucocorticoid function (primary glucocorticoid insufficiency) is greater than that observed in response to isolated HDL deficiency (reduced adrenal glucocorticoid function), i.e. in LCAT knockout mice [11] and APOA1 knockout mice [41]. It is therefore tempting to speculate that SR-BI, next to mediating the adrenal HDL-cholesteryl ester uptake, also controls the flux of the VLDL/LDL-derived cholesterol substrate into the adrenals. Given that mutations in different domains of SR-BI impact differentially on the ability of the protein to respectively bind lipoproteins, mediate the selective uptake of cholesteryl esters, and facilitate cholesterol efflux [42], we consider it of clear interest to determine the effect of different variations in the SR-BI gene on adrenal cholesterol accumulation and steroidogenesis in mice.

An interesting observation of our studies was that bone marrow APOE-mediated lowering of plasma cholesterol levels did not only reduced the adrenal glucocorticoid output, but also negatively impacted on the mRNA expression levels of SR-BI, STAR and CYP11A1. A decrease in the expression of these three genes, that are essential for adrenal cholesterol mobilization and the subsequent conversion to corticosterone, could theoretically underlie the parallel decrease in glucocorticoid output. However, we consider it likely that the decrease in gene expression is not causally related to the decrease in glucocorticoid output. More specifically, the reduction in the fasting plasma glucocorticoid levels (−64%) was much greater than the decrease that we observed in the adrenal relative gene expression levels (−19% to −29%). Furthermore, plasma corticosterone levels are within the normal range in heterozygous CYP11A1 knockout mice that can only generate 50% of total amount of CYP11A1 protein present in wild-type controls [43]. Moreover, heterozygous STAR knockout adrenals also do not display an impaired ability to produce corticosterone [44].

ACTH is a potent activator of SR-BI, STAR, CYP11A1 and CYP11B1 transcription in adrenocortical cells in vitro and in vivo [25–28,45]. Given that we did not observe a change in plasma ACTH levels, it can therefore be suggested that restoration of bone marrow APOE and/or

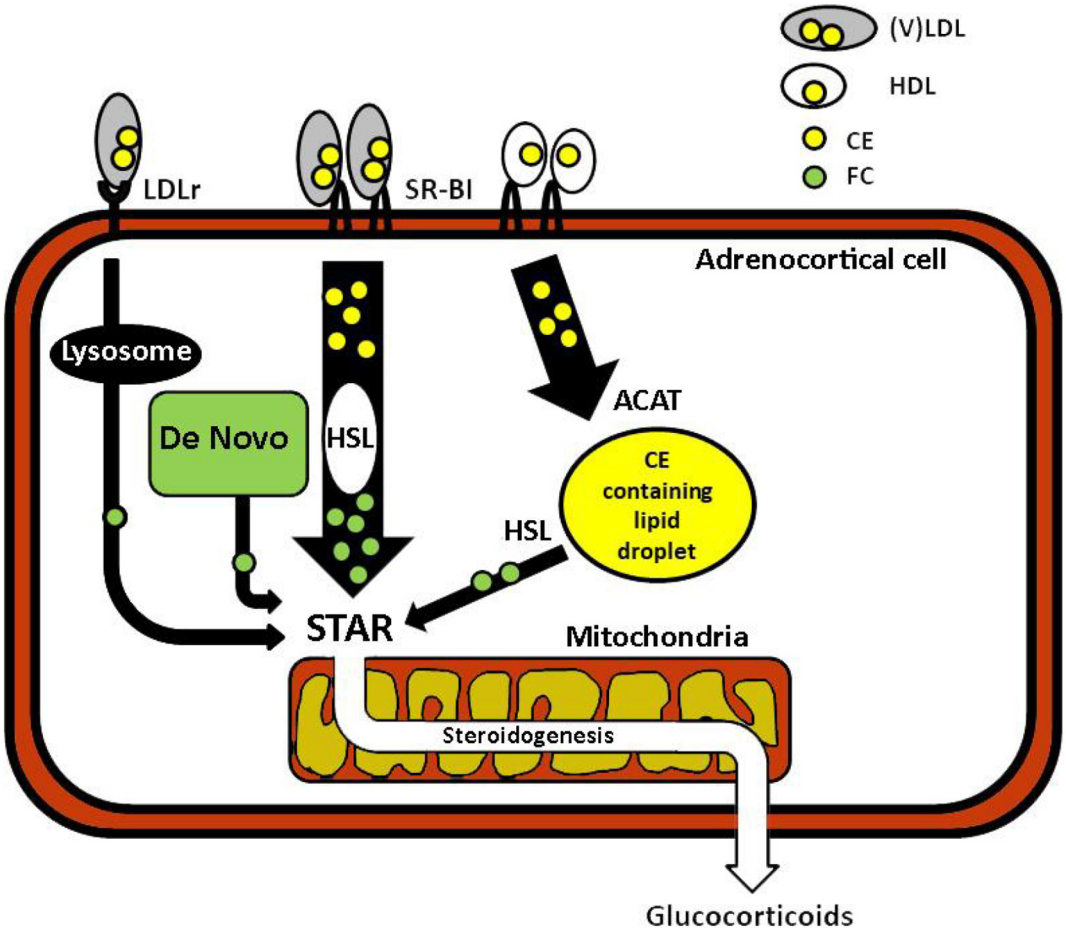

Fig. 5. A schematic overview of the suggested contributions of various cholesterol sources to the production of glucocorticoids by adrenocortical cells. (V)LDL-derived cholesterol acts as the major source of cholesterol used for adrenal glucocorticoid synthesis. HDL cholesteryl esters, acquired by SR-BI, are firstly stored in lipid droplets, but can also be regarded an important secondary cholesterol substrate source. Cholesterol acquired through LDL receptor-mediated uptake and de novo synthesis contribute for a very limited extent to steroidogenesis and therefore only become relevant under conditions that the other substrate delivery pathways are defective.

the associated decrease in plasma cholesterol levels impacts directly on adrenal ACTH signaling. Due to a lack of material we unfortunately have not been able to study in depth a possible effect on individual proteins such as the ACTH receptor/melanocortin-2 receptor and cyclic AMP response element-binding protein (CREB) and the second messenger molecule cyclic AMP that together execute the cellular response to ACTH. However, our current in vivo data complement in vitro findings from Reyland et al. which have indicated that APOE can directly inhibit steroidogenesis through blockade of cyclic AMP-mediated signal transduction in adrenocortical cells [46,47].

5. Conclusion

We have shown that a selective lowering of VLDL/LDL levels in APOE knockout mice through transplantation with APOE-containing wild-type bone marrow is associated with a decreased adrenal glucocorticoid output. Our studies provide novel experimental support for our working hypothesis that, in vivo, the cholesterol pool used for steroidogenesis under stress conditions is primarily acquired from VLDL/LDL, while HDL-derived cholesterol is mainly used for adrenal storage as cholesteryl esters.

CRediT authorship contribution statement

Ronald J. van der Sluis: Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing.**Marie A.C. Depuydt:**Data curation, Formal analysis, Investigation, Methodology, Writing - original draft.**Miranda Van Eck:**Supervision, Funding acquisition, Writing - review & editing.**Menno Hoekstra:**Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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