HDL is redundant for adrenal steroidogenesis in LDLR knockout mice with a human-like lipoprotein profile

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Abstract The contribution of HDL to adrenal steroidogenesis appears to be different between mice and humans. In this current study, we tested the hypothesis that a difference in lipoprotein profile may be the underlying cause. Here, we determined the impact of HDL deficiency on the adrenal glucocorticoid output in genetically modified mice with a human-like lipoprotein profile. Genetic deletion of APOA1 in LDL receptor (LDLR) knockout mice was associated with HDL deficiency and a parallel increase in the level of cholesterol associated with nonHDL fractions. Despite a compensatory increase in the adrenal relative mRNA expression levels of the cholesterol synthesis gene, HMG-CoA reductase, adrenals from APOA1/LDLR double knockout mice were severely depleted of neutral lipids, as compared with those of control LDLR knockout mice. However, basal corticosterone levels and the adrenal glucocorticoid response to stress were not different between the two types of mice. In conclusion, we have shown that HDL is not critical for proper adrenal glucocorticoid function when mice are provided with a human-like lipoprotein profile. Our findings provide the first experimental evidence that APOB-containing lipoproteins may facilitate adrenal steroidogenesis, in an LDLR-independent manner, in vivo in mice.—Hoekstra, M., and M. Van Eck. HDL is redundant for adrenal steroidogenesis in LDLR knockout mice with a human-like lipoprotein profile. J. Lipid Res. 2016. 57: 631–637.

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Glucocorticoids are produced from the common steroidogenic precursor, cholesterol. It is generally accepted that under basal conditions sufficient amounts of cholesterol are acquired from endogenous synthesis by the adrenals. However, it has become evident from studies in genetically modified mice that lipoproteins, in particular HDLs, are important for delivering cholesterol substrate to adrenocortical cells under high steroidogenic pressure conditions, like stress. More specifically, probucol-induced lowering of both HDL-cholesterol and LDL-cholesterol levels in mice is associated with a >55% decrease in the glucocorticoid response to endotoxemia (1). Studies in LCAT and APOA1 knockout mice, respectively, have indicated that a specific decrease in plasma HDL-cholesterol levels is associated with a 25–50% decrease in the maximal adrenal glucocorticoid output (2, 3). Moreover, disruption of (adrenal-specific) HDL receptor function in mice is associated with a 40–50% decrease in the adrenocortical steroidogenic capacity (4, 5).

Male carriers of functional mutations in the HDL biogenesis genes, ABCA1 and LCAT, display a decrease in the 24 h urinary excretion rate of adrenal-derived steroids (6). However, basal and stimulated plasma cortisol levels are similar in HDL-deficient male ABCA1 and LCAT mutation carriers and their normolipidemic controls (6). Furthermore, both the urinary glucocorticoid excretion rate and cortisol response to corticotropin are unaltered in females with genetically low HDL (7). The presence of relatively low HDL-cholesterol levels is, thus, in striking contrast to what is observed in mice: not consistently associated with glucocorticoid insufficiency in humans.

In vitro studies have suggested that both HDL and APOB-containing lipoproteins, i.e., VLDL and LDL, can theoretically supply cholesterol to adrenocortical cells (8–11). Importantly, human subjects exhibit a markedly different lipoprotein profile as compared with mice. The majority of cholesterol in humans is carried by LDL, while the murine lipoprotein profile is characterized by relatively low to absent levels of cholesterol associated with VLDL/LDL in the context of normal HDL-cholesterol levels. The relative importance of HDL-associated cholesterol as steroidogenic substrate can, thus, hypothetically be different between these two specific species due to the fact that human plasma, as compared with murine plasma, contains additional potential cholesterol sources, i.e., LDL

Abbreviations: DKO, double knockout; LDLR, LDL receptor; SKO, single knockout.

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and VLDL. To provide experimental proof for this hypothesis in the current study, we determined the impact of HDL deficiency on the adrenal glucocorticoid output in genetically modified mice that contain a human-like lipoprotein profile.

MATERIALS AND METHODS

Animals

APOA1 knockout mice lacking a functional APOA1 protein (12) were provided on a hyperlipidemic LDL receptor (LDLR) knockout background (13) by Dr. J. A. Kuivenhoven from the Amsterdam Medical Center (Amsterdam, The Netherlands). These APOA1/LDLR double knockout (DKO) mice were subsequently inbred to maintain an in-house colony. Male APOA1/LDLR DKO (N = 14) and control LDLR single knockout (SKO) mice (N = 14) were maintained on a regular chow diet. Throughout the experiment, both types of mice were housed in the same climate-controlled stable with a 12 h/12 h light-dark cycle and handled identically. At 4 months of age, ad libitum-fed age-matched mice (N = 6 per genotype) were bled at 9:00 AM from the tail to obtain a basal plasma corticosterone measurement. Subsequently, these six mice per genotype were injected intraperitoneally with a sublethal dose (50 mg/kg) of lipopolysaccharide from *Salmonella minnesota* R595 and euthanized 2 h later to measure the maximum endotoxemia-related plasma corticosterone response (1, 14). The remaining mice (N = 8 per genotype) were subjected to overnight fasting by food deprivation from 5:00 PM onwards. At 9:00 AM the next morning, mice were bled via the tail for fasting plasma corticosterone and blood glucose measurements. After anesthesia by subcutaneous injection with a mix of 70 mg/kg bodyweight xylazine, 1.8 mg/kg bodyweight atropine, and 350 mg/kg bodyweight ketamine, mice were bled via retro-orbital bleeding, euthanized, and subjected to whole body perfusion with ice-cold PBS. Adrenals were collected free of surrounding fat, weighed, and stored at −20°C or fixed overnight in 3.7% neutral-buffered formalin solution (Formalinfixx; Shandon Scientific Ltd, UK). All animal work was approved by the Leiden University Animal Ethics Committee and performed in compliance with the Dutch government guidelines and Directive 2010/63/EU of the European Parliament.

Blood and plasma analyses

Corticosterone levels in tail blood plasma were determined using the corticosterone 3H RIA kit from ICN Biomedicals according to the protocol from the supplier. Plasma concentrations of free cholesterol and cholesterol esters were determined using enzymatic colorimetric assays. The cholesterol distribution over the different lipoproteins in plasma was analyzed by fractionation on a Superose 6 column (3.2 × 30 mm, Smart-system; Pharmacia). Total cholesterol content of the effluent was determined using enzymatic colorimetric assays. Blood glucose levels were routinely measured using a calibrated Accu-Check glucometer (Roche Diagnostics, Almere, The Netherlands).

Adrenal lipid composition and histology

Lipids from adrenals were extracted using the method of Bligh and Dyer (15). After dissolving the lipids in 1% Triton X-100, the contents of free cholesterol and cholesterol esters were determined using enzymatic colorimetric assays and expressed as micrograms per milligram of protein. Seven micrometer cryosections were prepared on a Leica CM9505-S cryostat. Cryosections were routinely stained with hematoxylin (Sigma) and Oil red O (Sigma) for lipid visualization.

RESULTS

Regular chow diet-fed LDLR knockout mice exhibit a highly similar lipoprotein profile to that observed in normolipidemic humans (13). To investigate whether the contribution of HDL to adrenal glucocorticoid output is different in mice with a human-like lipoprotein profile, we determined the impact of genetic HDL deficiency in mice on a LDLR knockout genetic background. Hereto, HDL-deficient APOA1 knockout mice were crossed with LDLR SKO mice to generate the respective APOA1/LDLR DKO mice.

As can be appreciated from Fig. 1A, plasma free and total cholesterol levels did not significantly differ between regular chow diet-fed male DKO and SKO mice. However, lipoprotein distribution analysis on pooled plasma (Fig. 1B) revealed that DKO mice exhibited a highly similar reduction in plasma HDL-cholesterol levels (−65%), as previously noted in APOA1 knockout mice on a wild-type background (3). DKO mice showed a parallel 89% increase in levels of cholesterol associated with VLDL particles, as compared with their respective APOA1-containing SKO controls (Fig. 1B). As a result, the plasma nonHDL-cholesterol over HDL-cholesterol ratio was, thus, markedly higher in DKO mice as compared with SKO mice (Fig. 1C).

Previous studies by the group of Dr. Mary Sorci-Thomas have suggested that, after a short-term (4 h) fasting period, the adrenals of DKO mice are severely depleted of cholesterol esters, despite the fact that DKO mice still carry ~30% of the normal amount of HDL-associated cholesterol in APOE-enriched HDL particles (17). Quantification of the adrenal lipid stores revealed that both free cholesterol (−21%; P = 0.002) and cholesterol ester (−51%; P = 0.005) levels were also markedly lower in the adrenals of our DKO mice, as compared their SKO controls, after an
neutral lipids in their adrenal cortex. In contrast, an equally low extent of lipid accumulation was microscopically detected within cortical cells of DKO adrenals (Fig. 2B), as previously noted in glucocorticoid insufficient LCAT knockout mice and probucol-treated C57BL/6 mice (1, 2).

Fig. 2. A: Adrenal free cholesterol and cholesterol ester levels in APOA1/LDLR DKO and LDLR SKO mice. B: Representative images of Oil red O-stained adrenal sections showing neutral lipid depletion in the cortex of DKO mice. C: Adrenal relative gene expression levels as measured by quantitative PCR. **P < 0.01, ***P < 0.001 versus SKO. HMGCR, HMG-CoA reductase.

Fig. 1. Plasma free and total cholesterol levels (A), the cholesterol distribution over the different lipoprotein fractions (B), and the plasma nonHDL- to HDL-cholesterol ratio (C) in age-matched male APOA1/LDLR DKO and LDLR SKO mice.
An efficient feedback system exists that modulates the expression of genes involved in cholesterol synthesis and uptake in response to changes in intracellular cholesterol levels [reviewed by Sato and Takano (18)]. Quantitative real-time PCR was employed to uncover possible compensatory gene regulation. No change was noted, as compared with SKO adrenals, in the relative mRNA expression level of the HDL receptor, SR-BI, in DKO adrenals (Fig. 2C). In addition, genetic APOA1 deficiency was not associated with a difference in relative mRNA expression levels of hormone-sensitive lipase (HSL), ACAT-1, and steroidalogenic acute regulatory protein (STAR) that are respectively involved in the de- and re-esterification of cholesterol and intracellular mobilization of cholesterol to the steroidalogenic pathway (Fig. 2C). However, we did observe a marked increase (425%; \( P < 0.001 \); Fig. 2C) in the mRNA expression of the enzyme, HMG-CoA reductase, in DKO adrenals. It thus appears that, in a human-like lipoprotein context, HDL deficiency in mice is associated with depletion of adrenal cholesterol stores despite a compensatory increase in intra-adrenal cholesterol synthesis.

Levels of the primary glucocorticoid, corticosterone, were measured in plasma under basal and stressed conditions to verify whether the depletion of adrenal cholesterol esters also executed a negative impact on the overall steroid output. In line with the general notion that lipoprotein-derived cholesterol is not required for the synthesis of glucocorticoids under low steroidalogenic conditions, plasma corticosterone levels were similar in nonstressed ad libitum-fed SKO and DKO mice (Fig. 3A). Food deprivation is a powerful inducer of an adrenal glucocorticoid response in mice (19, 20). Overnight fasting resulted in a significant 6.6-fold increase \( (P < 0.001 \text{ vs. basal}) \) in circulating corticosterone levels in SKO mice, as anticipated. Strikingly, corticosterone levels were virtually identical in both groups of fasted mice (256 ± 21 ng/ml for DKO vs. 269 ± 15 ng/ml for SKO; \( P > 0.05 \)). HDL deficiency thus does not seem to be associated with glucocorticoid insufficiency in mice with a human-like lipoprotein. In agreement with a normal metabolic glucocorticoid action in HDL-deficient mice, DKO mice did not display hypoglycemia, as compared with SKO mice under fasting conditions (Fig. 3B). The induction of endotoxemia is associated with a concomitant rise in the plasma level of glucocorticoids (21, 22). In further support of a similar maximal steroidalogenic capacity of the adrenals in the two types of mice, equally high levels of corticosterone (~250 ng/ml; Fig. 3A) were detected in the plasma of SKO and DKO mice after induction of endotoxemia through injection of a sublethal dose of lipopolysaccharide.

**DISCUSSION**

In the current study, we tested the hypothesis that a difference in lipoprotein profile between mice and humans can explain the relative importance of HDL-cholesterol as substrate for adrenal steroidalogenesis. A 70% reduction in plasma HDL-cholesterol levels in APOA1 SKO mice is associated with a severe depletion of adrenal cholesterol esters and a concomitant impairment of the adrenal glucocorticoid response to stress (3). The APOE-rich HDL particles remaining in these mice (23) are apparently not able to compensate for the lack of

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**Fig. 3.** A: Plasma corticosterone levels in APOA1/LDLR DKO and LDLR SKO mice measured in the basal state, in response to overnight food deprivation (fasting), and after injection with lipopolysaccharide (endotoxemia). B: Plasma glucose levels as measured in the fasted state. *\( P < 0.001 \) versus respective basal values.
cholesterol supplied by APOA1-containing particles for steroidogenesis. Although it cannot be excluded that the APOE-rich HDL is a poor substrate for adrenal cholesterol delivery, we anticipate that the adrenal cholesterol insufficiency observed in APOA1 SKO mice is primarily the result of an overall too low amount of HDL particles being present in the circulation.

Genetic variations in the APOA1 gene have also been associated with HDL deficiency in humans (24–26). However, due to the limited number of subjects with genetic APOA1 deficiency, the specific contribution of APOA1-containing HDL particles to adrenal steroidogenesis remains to be determined in the human setting. In the current study, we observed that APOA1 deficiency in mice with a human-like lipoprotein profile alleviates the glucocorticoid insufficiency observed in APOA1 knockout mice on a wild-type (normolipidemic) background. In contrast, APOA1/LDLR DKO mice do not suffer from glucocorticoid insufficiency, as their maximal glucocorticoid output in vivo is dependent on the adrenal LDLR controls. It thus appears that the presence of a human-like lipoprotein profile alleviates the glucocorticoid insufficiency associated with APOA1 deficiency in mice.

Studies by Plump et al. (3) have suggested that adrenals from APOA1 knockout mice are still able to respond to stress, although to a minor extent as compared with those of wild-type mice, due to compensatory upregulation of pathways that are normally of minor importance, such as cholesterol uptake by the LDLR and de novo cholesterol synthesis. A 5-fold increase in the gene expression of HMG-CoA reductase in the adrenals of DKO mice was detected under fasting stress conditions, which suggests that de novo cholesterol synthesis is stimulated to compensate for the loss of HDL-cholesterol. In contrast to our DKO mice, HDL-deficient LCAT knockout mice do display a diminished adrenal glucocorticoid function despite a marked 6-fold increase in adrenal HMG-CoA reductase expression (2). From these combined findings, it can be concluded that such a 5- to 6-fold increase in adrenal HMG-CoA reductase expression is, by itself, not sufficient to overcome adrenal glucocorticoid insufficiency. All mice used in the current study did not express a functional LDLR, which excludes a compensatory role for LDLR-mediated cholesterol acquisition by adrenals in DKO mice. In vitro studies by Kraemer et al. (27) have suggested that the LDLR is of negligible importance for acute steroidogenesis by adrenocortical cells. Furthermore, the levels of corticosterone measured in the fasting state in male LDLR knockout mice in the current experiment are almost identical to those found in male wild-type mice in our previous studies (28). We therefore consider it highly unlikely that the impact of APOA1/HDL deficiency on glucocorticoid output in vivo is dependent on the adrenal LDLR genotype.

In our experimental setup, APOB-containing lipoproteins could not be cleared by the LDLR, which is normally suggested to be the primary route of cholesterol delivery by these circulating lipid/protein complexes. Considerable evidence is, however, present that SR-BI is also able to facilitate the uptake of cholesterol from APOB-containing lipoproteins. Initial in vitro studies by Swarnakar et al. (29) and Stangl, Hyatt, and Hobbs (30) showed that murine SR-BI is able to mediate the selective uptake of cholesterol esters from human LDL. Subsequent cell culture studies by Webb et al. (31) verified a similar interaction of SR-BI with autologous mouse LDL. In support of a parallel role for SR-BI in APOB-containing lipoprotein cholesterol delivery in vivo, the removal of the blood circulation and tissue uptake of β-migrating VLDL particles, LDL, and chylomicron-remnants has been shown to be significantly lower in mice lacking functional SR-BI expression (32–34). As a result, SR-BI knockout mice not only display increased plasma levels of HDL-cholesterol, but also exhibit an increase in the amount of cholesterol carried by APOB-containing lipoproteins (35), while plasma levels of APOB-containing lipoproteins are markedly lower in mice upon transgenic or adenoviral overexpression of SR-BI (36, 37). Adrenal glucocorticoid output is diminished in human subjects carrying a functional mutation in the SR-BI gene (38). Several heterozygote SR-BI P297S carriers actually show signs of adrenal dysfunction in spite of markedly increased plasma HDL-cholesterol levels (38). Given that, in the human situation, disruption of SR-BI function appears to be associated with a more extreme effect on the adrenal steroidogenic capacity than genetic lowering of HDL-cholesterol levels, it can be suggested that, in our current human-like lipoprotein setting, the impaired acquisition of cholesterol from APOA1-containing HDL particles can be fully compensated by enhanced cholesterol synthesis combined with SR-BI-mediated delivery of cholesterol from APOB-containing lipoproteins to the adrenals.

Novel intervention strategies to reduce cardiovascular disease risk, such as proprotein convertase subtilisin/kexin type 9 (PCSK9) antibody treatment and statin/ezetimibe combination therapies, are aimed at reaching extremely low plasma LDL-cholesterol levels. No remarkable adrenal-associated events have been reported in meta-analyses of anti-PCSK9 antibody (39) and statin/ezetimibe trials (40). This may, at first sight, argue against our current working hypothesis that APOB-containing lipoproteins serve as primary cholesterol donors for steroidogenesis. However, one should take into account that: 1) adrenal dysfunction may only become evident under stress conditions; and 2) in-depth adrenal function testing is not common within these cardiovascular-oriented clinical trials. As such, inclusion of the adrenocorticotropic hormone (ACTH) stimulation test, the standard method to assess the maximal adrenal cortisol response, in trial protocols may aid in validating our hypothesis in the human setting. In light of our challenging concept, it is of interest to note that several case studies by Illingworth and colleagues (41–43) have indicated that genetic LDL deficiency (abetalipoproteinemia) in humans is associated with subclinical adrenal insufficiency, as evident from an impaired ACTH-induced cortisol response and a lower urinary (free) cortisol excretion rate.
In conclusion, we have shown that HDL is not critical for proper adrenal glucocorticoid function in mice with a human-like lipoprotein profile. Our findings contribute to a better understanding of the adrenal glucocorticoid function under human-like lipoprotein conditions and provide the first experimental evidence that APOB-containing lipoprotein fractions may facilitate adrenal steroidogenesis, in an LDLR-independent manner, in vivo.

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