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

Running title: Adrenal function in APOA1 / LDLR double knockout mice

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ABSTRACT

The contribution of high-density lipoprotein (HDL) to adrenal steroidogenesis appears to be different between mice and humans. In the current study we tested the hypothesis that a difference in lipoprotein profile may be the underlying cause. Hereto, 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 apolipoprotein A1 (APOA1) in low-density lipoprotein receptor (LDLR) knockout mice was associated with HDL deficiency and a parallel increase in the level of cholesterol associated with non-HDL lipoprotein 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 to 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 a proper adrenal glucocorticoid function when mice are provided with a human-like lipoprotein profile. Our findings provide the first experimental evidence that apolipoprotein B-containing lipoproteins may facilitate adrenal steroidogenesis, in an LDL receptor-independent manner, in vivo in mice.

KEYWORDS

Steroid hormones, Lipoproteins, Cholesterol, HDL, LDL, Gene expression, Adrenal, Corticosterone, Mice, LDL receptor
INTRODUCTION

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 high-density lipoproteins (HDL), 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 low-density lipoproteins (LDL)-cholesterol levels in mice is associated with a >55% decrease in the glucocorticoid response to endotoxemia (1). Studies in respectively lecithin:cholesterol acyltransferase (LCAT) and apolipoprotein A1 (APOA1) knockout mice 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 ATP-binding cassette transporter A1 (ABCA1) and LCAT display a decrease in the 24-hour 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 apolipoprotein B (APOB)-containing lipoproteins, i.e. very-low-density lipoprotein (VLDL) and LDL, can theoretically supply
cholesterol to adrenocortical cells (8-11). Importantly, human subjects exhibit a markedly different lipoprotein profile as compared to 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 to murine plasma contains additional potential cholesterol sources, i.e. LDL 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
Apolipoprotein A1 (APOA1) knockout mice lacking a functional APOA1 protein (12) were provided on a hyperlipidemic LDL receptor knockout background (13) by Dr. J.A. Kuivenhoven from the Amsterdam Medical Center (Amsterdam; NL). These APOA1 / LDL receptor double knockout mice were subsequently inbred to maintain an in-house colony.

Male APOA1 / LDL receptor double knockout (N=14) and control LDL receptor single knockout 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 12h/12h dark-light 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 6 mice per genotype were injected intraperitoneally with a sub-lethal dose (50 µg/kg) of lipopolysaccharide from Salmonella Minnesota R595 and sacrificed 2 hours 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 body weight xylazine, 1.8 mg/kg bodyweight atropine and 350 mg/kg body weight ketamine, mice were bled via retro-orbital bleeding, sacrificed, 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 (Formalfixx; 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 the Directive 2010/63/EU of the European Parliament.
Blood and plasma analyses

Corticosterone levels in tail blood plasma were determined using the corticosterone \(^3\)H 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 of 30 µl pooled plasma of each mouse genotype using a Superose 6 column (3.2 x 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, 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 CM3050-S cryostat. Cryosections were routinely stained with hematoxylin (Sigma) and Oil red O (Sigma) for lipid visualization.

Real-time quantitative PCR

Gene expression analysis was performed essentially as described (16). 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) according to the manufacturer’s instructions. Acidic ribosomal phosphoprotein P0 (36B4), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), succinate dehydrogenase complex, subunit A, flavoprotein (SDHA), peptidylprolyl isomerase A (PPIA), beta-2-microglobulin
(B2M), transferrin receptor (TFRC), and beta–actin (ACTB) were used as housekeeping genes for normalization.

**Data analysis**

Statistical analysis was performed using Graphpad Instat software (San Diego, USA, http://www.graphpad.com). Normality of the experimental groups was confirmed using the method of Kolmogorov and Smirnov. The significance of differences was calculated using a two-tailed unpaired t-test or two-way analysis of variance (ANOVA) where appropriate. Probability values less than 0.05 were considered significant.
RESULTS

Regular chow diet-fed LDL receptor 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 LDL receptor knockout genetic background. Hereeto, HDL deficient APOA1 knockout mice were crossbred with LDL receptor single knockout mice (SKO) to generate the respective APOA1 / LDL receptor double knockout (DKO) mice.

As can be appreciated from Figure 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 (Figure 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 to their respective APOA1-containing SKO controls (Figure 1B). As a result, the plasma non-HDL-cholesterol over HDL-cholesterol ratio was thus markedly higher in DKO mice as compared to SKO mice (Figure 1C).

Previous studies by the group of Dr. Mary Sorci-Thomas have suggested that, after a short-term (4 hour) 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 apolipoprotein E-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 adrenals of our DKO mice as compared their SKO controls after an overnight fast (Figure 2A), suggesting that the adrenal lipid depletion effect associated with APOA1 deficiency is independent of the metabolic / stress state. Oil red O staining of
adrenal cryostat sections further verified adrenal lipid depletion. Adrenals from SKO mice had abundant neutral lipids in their adrenal cortex. In contrast, a equally low extent of lipid accumulation was microscopically detected within cortical cells of DKO adrenals (Figure 2B) as previously noted in glucocorticoid insufficient LCAT knockout mice and probucol-treated C57BL/6 mice (1,2).

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 et al. (18)). Quantitative real-time PCR was employed to uncover possible compensatory gene regulation. No change was noted as compared to SKO adrenals in the relative mRNA expression level of the HDL receptor SR-BI in DKO adrenals (Figure 2C). In addition, genetic APOA1 deficiency was not associated with a difference in relative mRNA expression levels of hormone-sensitive lipase (HSL), acetyl-CoA acetyltransferase 1 (ACAT-1), and steroidogenic acute regulatory protein (STAR) that are respectively involved in the de- and re-esterification of cholesterol and intracellular mobilization of cholesterol to the steroidogenic pathway (Figure 2C). However, we did observe a marked increase (+425%; *P*<0.001; Figure 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 steroidogenic conditions, plasma corticosterone levels were similar in non-stressed ad libitum fed SKO and DKO mice (Figure 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 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 to SKO mice under fasting conditions (Figure 3B). The induction of endotoxemia is associated with an concomitant rise in the plasma level of glucocorticoids (21,22). In further support of a similar maximal steroidogenic capacity of the adrenals in the two types of mice, equally high levels of corticosterone (~250 ng/ml; Figure 3A) were detected in plasma of SKO and DKO mice after induction of endotoxemia through injection of a sub-lethal dose of lipopolysaccharide.
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 steroidogenesis.

A 70% reduction in plasma HDL-cholesterol levels in APOA1 single knockout mice is associated with a severe depletion of adrenal cholesterol ester stores 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 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 single knockout 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, i.e. on a LDL receptor knockout (hyperlipidemic) background, is associated with a similar 65% reduction in plasma HDL-cholesterol levels and adrenal cholesterol depletion as observed in APOA1 knockout mice on a wild-type (normolipidemic) background. In contrast, APOA1 / LDL receptor double knockout mice do not suffer from glucocorticoid insufficiency as their maximal glucocorticoid output is similar to that of HDL-containing single LDL receptor knockout 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. have suggested that adrenals from APOA1 knockout are still able to respond to stress, although to a minor extent as compared to those of wild-type mice, due to compensatory upregulation of pathways that are normally of minor importance such as cholesterol uptake by the LDL receptor and de novo cholesterol synthesis (3). 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 do not express a functional LDL receptor, which excludes a compensatory role for LDL receptor-mediated cholesterol acquisition by adrenals in DKO mice. In vitro studies by Kraemer et al. have suggested that the LDL receptor is of negligible importance for acute steroidogenesis by adrenocortical cells (27). Furthermore, the levels of corticosterone measured in the fasting state in male LDL receptor 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 LDL receptor genotype.

In our experimental setup APOB-containing lipoproteins could not be cleared by the LDL receptor, 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 et al. (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 from the blood circulation and tissue uptake of beta-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 that [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 et al. (41-43) have indicated that genetic LDL deficiency (abetalipoproteinemia) in humans is associated with sub-clinical 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 a 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 LDL receptor-independent manner, in vivo.
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REFERENCES


FIGURE LEGENDS

Figure 1. Plasma free and total cholesterol levels (A), the cholesterol distribution over the different lipoprotein fractions (B), and the plasma non-HDL to HDL cholesterol ratio (C) in age-matched male APOA1 / LDLR double knockout (DKO) and LDLR single knockout (SKO) mice.

Figure 2. (A) Adrenal free cholesterol and cholesterol ester levels in APOA1 / LDLR double knockout (DKO) and LDLR single knockout (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 vs SKO.

Figure 3. (A) Plasma corticosterone levels in APOA1 / LDLR double knockout (DKO) and LDLR single knockout (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 vs respective basal values.
FIGURE 1

A

Cholesterol (mg/dl)

Free
Total

B

C

non-HDL / HDL-cholesterol ratio

FIGURE 1
FIGURE 2