Adrenocortical LDL receptor function negatively influences glucocorticoid output

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Abstract

Over 50% of the cholesterol needed by adrenocortical cells for the production of glucocorticoids is derived from lipoproteins. However, the overall contribution of the different lipoproteins and associated uptake pathways to steroidogenesis remains to be determined. Here we aimed to show the importance of LDL receptor (LDLR)-mediated cholesterol acquisition for adrenal steroidogenesis *in vivo*. Female total body LDLR knockout mice with a human-like lipoprotein profile were bilaterally adrenalectomized and subsequently provided with one adrenal either expressing or genetically lacking the LDLR under their renal capsule to solely modulate adrenocortical LDLR function. Plasma total cholesterol levels and basal plasma corticosterone levels were identical in the two types of adrenal transplanted mice. Strikingly, restoration of adrenal LDLR function significantly reduced the ACTH-mediated stimulation of adrenal steroidogenesis ($P<0.001$), with plasma corticosterone levels that were respectively 44–59% lower ($P<0.01$) as compared to adrenal LDLR negative controls. In addition, LDLR positive adrenal transplanted mice exhibited a significant decrease (−39%; $P<0.001$) in their plasma corticosterone level under fasting stress conditions. Biochemical analysis did not show changes in the expression of genes involved in cholesterol mobilization. However, LDLR expressing adrenal transplants displayed a marked 62% reduction ($P<0.05$) in the transcript level of the key steroidogenic enzyme HSD3B2. In conclusion, our studies in a mouse model with a human-like lipoprotein profile provide the first *in vivo* evidence for a novel inhibitory role of the LDLR in the control of adrenal glucocorticoid production.

Key Words
- cholesterol
- lipoprotein
- LDL
- receptor
- adrenal
- steroidogenesis
- corticosterone
- transplantation
- mice

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Introduction

Cholesterol is the sole precursor for the synthesis of steroid hormones including adrenal-derived glucocorticoids, i.e., cortisol in man and corticosterone in rodents. The cholesterol used for steroidogenesis can theoretically be acquired from endogenous *de novo* synthesis of cholesterol, intracellular catabolism of stored cholesteryl esters, and the uptake of exogenous cholesterol carried by plasma lipoproteins (Kraemer 2007). It is generally assumed that the newly synthesized cholesterol pool is sufficient for basal glucocorticoid production by cells of the adrenal cortex. Upon stress-induced activation of the hypothalamic–pituitary–adrenal axis, the adrenals secrete high levels of glucocorticoids that mediate the physiological response needed to cope with stress through activation of

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the glucocorticoid receptor (GR) in target tissues (Kadmiel & Cidlowski 2013). We have previously shown that lipoprotein-associated cholesterol contributes at least 50% to the total cellular pool of cholesterol utilized for the production of glucocorticoids under high steroidogenic pressure conditions (Hoekstra *et al*. 2010).

Adrenals express high levels of scavenger receptor BI (SR-BI; Acton *et al*. 1996, Liu *et al*. 1997), the sole mediator of the selective cholesteryl ester uptake from HDLs (Out *et al*. 2004). In addition, expression of the LDL receptor (LDLR) has also been detected on glucocorticoid-producing cells of the adrenal zona fasciculata (Fong *et al*. 1989). In line with this, *in vitro* studies have suggested that both HDL and LDL fractions are capable of supplying cholesterol to the steroidogenic pool of cultured adrenocortical cells (Gwynne & Hess 1980, Higashijima *et al*. 1987, Rainey *et al*. 1992, Yaguchi *et al*. 1998).

A genetic defect in proteins involved in the formation and cellular uptake of HDL-cholesteryl esters is associated with a diminished adrenal glucocorticoid output in experimental mouse models (Cai *et al*. 2008, Hoekstra *et al*. 2008, 2009) as well as in humans (Vergeer *et al*. 2011, Bochem *et al*. 2013). HDL-cholesterol thus appears to be of crucial importance for an optimal production of glucocorticoids by the adrenals *in vivo*.

A genetic deficiency of the LDLR does not alter the basal or stimulated corticosterone production by murine adrenocortical cells *in vitro* (Kraemer *et al*. 2007). Interestingly, homozygous human carriers of a mutation in the LDLR gene, familial hypercholesterolemia (FH) patients, do display a decrease in the maximal adrenal glucocorticoid output (Illingworth *et al*. 1983). However, no significant change in adrenal steroidogenesis rates has been detected in heterozygote human LDLR mutants (Illingworth *et al*. 1984). Importantly, lowering LDL-cholesterol levels in FH patients by lovastatin (mevinolin) treatment does not further affect the adrenal glucocorticoid function (Laue *et al*. 1987). Furthermore, stimulation of the alternative LDL/LDLR route of cholesterol delivery to the adrenals by introducing human cholesteryl ester transfer protein in mice genetically lacking the HDL receptor SR-BI is also not able to reverse the associated glucocorticoid insufficiency (Hoekstra *et al*. 2009). Based on these combined findings, it can be suggested that the LDL/LDLR route does not play a major role in the supply of the steroidogenic cholesterol substrate *in vivo*. However, it should be noted that the lipoprotein profile in FH patients (hypercholesterolemic) and SR-BI-deficient mice (hyperalphalipoproteinemia) is markedly different from that of their respective normolipidemic controls, which makes proper interpretation of these data regarding the specific contribution of the adrenal LDL/LDLR route to steroidogenesis difficult. In the current study, therefore, we – by applying our adrenal transplantation technique – specifically modulated the adrenal LDLR genotype to determine the relative contribution of adrenocortical LDLR-mediated cholesterol uptake to the *in vivo* glucocorticoid function.

Materials and methods

Adrenal transplantation

Total body LDLR knockout mice crossed back to the C57BL/6 background (greater than eight generations) and C57BL/6 WT mice were bred in house. Adrenalectomy and subsequent adrenal transplantations were carried out as previously described (van der Sluis *et al*. 2012). At postnatal day 10, adrenal glands were isolated from female donor LDLR knockout or C57BL/6 pups to serve as transplant donors (Karpac *et al*. 2005). Eight- to 10-week-old female recipient LDLR knockout mice were bilaterally adrenalectomized under isoflurane inhalation anesthesia through a dorsal midline skin incision and lateral retroperitoneal incisions. Subsequently, one donor adrenal per recipient was placed under the kidney capsule through a slit in the renal capsule made by tweezers. Skin wounds were closed using michel suture clips. All mice were group-housed with three to five mice per cage and continuously supplied with normal chow diet, a 0.9% NaCl solution, and normal water *ad libitum*. During the first week after surgery, a part of the bottom of each cage surface was heated by a heating mattress for optimal recovery from the operations. Based on our previous studies, adrenal transplants were given 8 weeks to become fully mature before subsequent transplant function and morphology analyses were executed. Fifteen of the 33 C57BL/6 and 12 of the 16 LDLR knockout recipient mice effectively completed the adrenal recovery period. At the end of the study, mice were subjected to overnight fasting to induce physiological stress, anesthetized, and killed. Subsequently, the arterial tree was perfused *in situ* with PBS (with the pressure of 100 mmHg) for 10 min via a cannula in the left ventricular apex, and organs were harvested, weighed, and either snap–frozen (RNA measurements) or stored in 3.7% formalin (tissue sectioning). No signs of endogenous adrenal regeneration were macroscopically visible in any of the transplanted mice. Animal experiments were performed in a temperature and light cycle (12 h light:12 h darkness) controlled room at the Gorlaeus Laboratories of the Leiden Academic Centre for Drug Research in

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accordance with the National Laws. All experimental protocols were approved by the Ethics Committee for Animal Experiments of Leiden University.

Adrenal transplant glucocorticoid function tests

Ad libitum-fed adrenal transplanted mice were bled at 0900 h via tail cut to measure basal plasma corticosterone levels on the lowest point of the circadian rhythm. Subsequently, the mice were administered a single dose of 200 μg tetracosactide (adrenocorticotropic hormone (ACTH) fragment 1–24 human, rat; Sigma) intraperitoneally, followed by hourly blood draws up till 3 h post tetracosactide injection. To obtain a second measure of the maximal glucocorticoid output, adrenal recipient mice were bled by tail cut after food restriction from 1700 to 2100 h the next morning. During blood draws mice were handled identically and restrained for a maximum of 30 s to exclude an impact on blood corticosterone levels.

Adrenal transplant histology

Formalin-fixed cryosections (8 μM) of adrenal transplants were prepared on a Leica CM3050-S cryostat (Leica Microsystems, Eindhoven, The Netherlands). Cryosections were routinely stained with hematoxylin and eosin or Oil Red O to visualize neutral lipids.

Plasma measurements

Corticosterone levels were determined using the Corticosterone Kit from MP Biomedicals (Irvine, CA, USA). Concentrations of cholesterol were determined in blood of *ad libitum*-fed and overnight fasted mice using enzymatic colorimetric assays (Roche Diagnostics). The distribution of cholesterol over the different lipoprotein fractions in overnight fasting plasma was determined using a Superose 6 column (3.2×30 mm, Smart System; Pharmacia), taking the efficiency of recovery from the column into account.

Analysis of gene expression by real-time quantitative PCR

Quantitative gene expression analysis on isolated adrenal transplants that were intact and free of surrounding fat and livers was performed as described (Hoekstra *et al.* 2003). In short, total RNA was isolated using a standard phenol/chloroform extraction method and reverse transcribed with RevertAid Reverse Transcriptase (Life Technologies). Gene expression analysis was performed using real-time SYBR Green Technology (Eurogentec, Maastricht, The Netherlands). Primers were validated for identical efficiencies. Primer sequences can be found in Table 1. Beta-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase, hypoxanthine phosphoribosyltransferase (HPRT), ATP synthase, H^+ transporting, mitochondrial Fo complex, subunit F6 (ATP5J), ribosomal protein L27 (RPL27), and acidic ribosomal phosphoprotein P0 (36B4) were used as the standard housekeeping genes. Relative expression levels were calculated using by subtracting the cycle threshold (*C*t) number from the gene of interest from the average housekeeping *C*t and raising 2 to the power of this difference. No significant difference in the housekeeping *C*t values was observed between the two adrenal transplant recipient groups.

Table 1 Primers used for real-time PCR analysis

Gene	GenBank accession no.	Forward primer	Reverse primer
36B4	NM007475	5′-CTGAGTACACCTTCCCACTTACTGA-3′	5′-CGACTCTTCCTTTGCTTCAGCTTT-3′
ACTB	NM007393	5′-AACCGTGAAAAGATGACCCAGAT-3′	5′-CACAGCCTGGATGGCTACGTA-3′
ACAT1	NM144784	5′-AGCTGTTTCTCTGGGCCATCCAAT-3′	5′-GAACTCTCCTGGCTTCAGGGCAT-3′
ATP5J	NM016755	5′-CAGGGCCGGAAGTAGAACGG-3′	5′-AAGGACAGAGGAGAGCCTGAAGA-3′
CD14	NM009841	5′-GGCGCTCCGAGTTGTGACT-3′	5′-GTACCTGCTTCAGCCCAGTGA-3′
CD68	NM001291058	5′-TGCCTGACAAGGGACACTTCGGG-3′	5′-GCGGGTGATGCAGAAGGCGATG-3′
CD8	NM009858	5′-GTGACTTCTACTTCTGCGCGAC-3′	5′-AGGGTAGTCTTCTTGGTTGGGG-3′
CYP11A1	NM019779	5′-AGAACATCCAGGCCAACATTACCGAG-3′	5′-AGGACTTCAGCCCGCAGCATC-3′
CYP11B1	NM001033229	5′-GCCTGACCCGATGGACAA-3′	5′-CAGAGATGAAATCCCAGGACTCTAA-3′
CYP21A1	NM009995	5′-GGGAACTGCCCAGCAAGTT-3′	5′-AGGATGGTGTTCTGGGATTCTTC-3′
HPRT	NM013556	5′-TACAGCCCCAAAATGGTTAAGG-3′	5′-AGTCAAGGGCATATCCAACAAC-3′
HSD3B2	NM153193	5′-AGCCTTCCTGTGCCCCTACT-3′	5′-CAGGAGGAAGCTCACAGTTTCC-3′
HSL	NM010719	5′-CTGACAATAAAGGACTTGAGCAACTC-3′	5′-AGGCCGCAGAAAAAAGTTGAC-3′
PEPCK	NM011044	5′-TTGAACTGACAGACTCGCCCT-3′	5′-GATATGCCCATCCGAGTCATG-3′
RPL27	NM011289	5′-CGCCAAGCGATCCAAGATCAAGTCC-3′	5′-AGCTGGGTCCCTGAACACATCCTTG-3′
STAR	NM011485	5′-GGGCATACTCAACAACCAGGAAGG-3′	5′-CTACCACCACCTCCAAGCGAAAC-3′
SR-BI	NM016741	5′-GGCTGCTGTTTGCTGCG-3′	5′-GCTGCTTGATGAGGGAGGG-3′
TDO2	NM019911	5′-ATGGCCATGTCAGGGATGA-3′	5′-AAGATGACCACCACACGATGC-3′

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Statistical analysis

Statistical analysis was performed using GraphPad Instat Software (San Diego, CA, USA, http://www.graphpad.com). Normality testing of the experimental groups 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 value <0.05 was considered significant.

Results

Our previous studies regarding the role of the HDL receptor SR-BI in the adrenal glucocorticoid function have indicated the value of our whole adrenal transplantation technique for studies regarding the contribution of adrenocortical cell-derived transcripts to glucocorticoid homeostasis (Hoekstra *et al.* 2013). To determine the impact of the LDLR located on adrenocortical cells for glucocorticoid production *in vivo*, in this study, we therefore transplanted either one C57BL/6 adrenal carrying a functional LDLR (LDLR positive (LDLR+)) or one adrenal genetically lacking the LDLR (LDLR negative (LDLR−)) under the renal capsule of recipient mice. We anticipate that the importance of the LDL/LDLR pathway of cholesterol delivery in adrenals of normolipidemic WT mice – as opposed to humans – is limited as virtually no LDL-associated cholesterol can be detected in plasma of these mice due to rapid clearance of LDL particles by the LDLR located on hepatocytes within the liver. In the current experimental setup we therefore specifically chose to transplant adrenals of either genotype into adrenalectomized total body LDLR knockout recipient mice that exhibit a human-like plasma lipoprotein profile, i.e. with relatively high LDL-cholesterol levels, due to an impaired hepatic clearance of LDL particles (Ishibashi *et al.* 1993).

In accordance with accumulation of cholesterol in the blood compartment as a result of defective clearance by the liver, total cholesterol levels were significantly higher in plasma of both types of *ad libitum*-fed LDLR knockout recipient mice (~200–250 mg/dl; Fig. 1) as compared to those generally found in WT mice (60–100 mg/dl; Van Eck *et al.* 2003, Out *et al.* 2007). Subjecting mice to food deprivation stimulates a stress response that involves an ACTH-mediated activation of adrenal glucocorticoid production (Chida *et al.* 2007). Fasting the mice overnight had no significant effect on plasma total cholesterol levels (Fig. 1; two-way ANOVA: $P>0.05$). Fasting free cholesterol levels were unchanged by the adrenal genotype (110±12 mg/dl for LDLR+ adrenal recipients vs

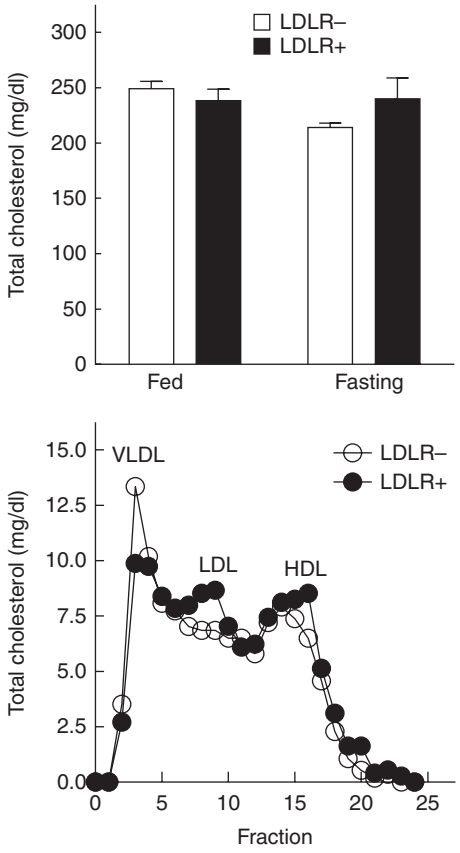

Figure 1
Effect of the adrenal LDL receptor (LDLR) genotype on plasma cholesterol levels. Mice were bled in the *ad libitum*-fed state (fed) or after overnight food deprivation (fasting). Data represent means±S.E.M. of 12 LDLR knockout mice that genetically lack the LDLR in their adrenal transplants (LDLR−) and 15 LDLR knockout mice that were transplanted with WT adrenals containing functional LDLRs (LDLR+). Pooled plasma of the different adrenal recipients was used to generate the lipoprotein distribution profiles. VLDL, very-LDL.

90±3 mg/dl for LDLR− adrenal recipients; $P>0.05$). In addition, the adrenal transplant genotype did not impact on total cholesterol levels either in the fasting or *ad libitum*-fed state (Fig. 1; two-way ANOVA: $P>0.05$). Two-way ANOVA also did not reveal a significant interaction ($P>0.05$) between the adrenal transplant genotype and feeding status. As evident from Fig. 1, the distribution of cholesterol over the different lipoprotein fractions was also virtually identical in the two groups of recipients under fasting stress conditions, with the majority of cholesterol being transported in the very-LDL (VLDL) and LDL fractions. These primary findings suggest i) a negligible contribution of LDLR-mediated uptake of cholesterol by the adrenals to the regulation of plasma cholesterol levels and ii) that a possible difference in adrenocortical function

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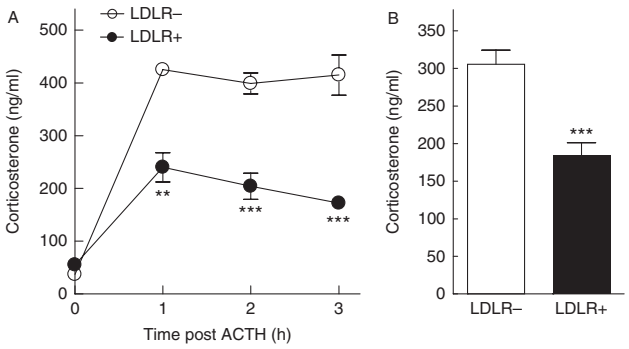

Figure 2
Effect of the adrenal LDL receptor genotype on plasma glucocorticoid levels. (A) Time profile of plasma corticosterone levels upon i.p. injection of ACTH. Two-way ANOVA: $P<0.001$ for time; $P<0.001$ for adrenal genotype; and $P<0.001$ for interaction. (B) Corticosterone levels in overnight fasted mice. Data represent means±S.E.M. of four to nine mice (ACTH response) and 13–15 mice (fasting) per group. $^{**}P<0.01$, $^{***}P<0.001$ vs LDLR−.

between the experimental groups is not secondary to a shift in lipoprotein-associated cholesterol (substrate) availability.

In line with the general notion that the lipoprotein/lipoprotein receptor system is not of quantitative importance for the control of basal adrenal steroidogenesis, plasma levels of corticosterone were not different between the two adrenocortical LDLR genotypes in the *ad libitum* fed non-stressed state (Fig. 2). LDLR− adrenal transplant recipient mice displayed a rapid increase in circulating levels of corticosterone upon i.p. injection of a synthetic analog of the pituitary-derived steroidogenic activator ACTH (tetracosactide), indicative of an efficient recovery of the adrenal steroid function after adrenal transplantation. Importantly, as can be appreciated from Fig. 2, restoration of adrenal LDLR function in total body LDLR knockout mice led to a marked reduction in the ACTH-mediated stimulation of adrenal steroidogenesis (two-way ANOVA: $P<0.001$ for genotype), with plasma corticosterone levels that were respectively 44–59% lower ($P<0.01$) as compared to adrenal LDLR− controls. In addition, LDLR+ adrenal transplanted mice exhibited a significant decrease (−39%; $P<0.001$) in their plasma corticosterone level under fasting stress conditions. Normalization of the adrenal LDLR function was thus paralleled by a diminished glucocorticoid output both in response to ACTH stimulation and under fasting stress conditions.

Glucocorticoids activate their cognate GR to execute immunosuppressive effects in lymphoid organs, while stimulating the synthesis and utilization of glucose in metabolic tissues (Kadmiel & Cidlowski 2013). In our study, thymus weights did not differ between the two adrenocortical genotypes under fasting stress conditions (Fig. 3), suggesting the presence of an equally effective glucocorticoid response to induce acute apoptosis in thymocytes. In contrast, we did note an increase in relative spleen weights (+34%; $P<0.05$; Fig. 3) pointing to a long-term impairment of overall glucocorticoid action in splenocytes in response to normalization of the adrenocortical LDLR function. In further support of general glucocorticoid insufficiency upon replenishment of adrenal LDLR function, hepatic transcript levels of the glucocorticoid-responsive genes phosphoenolpyruvate carboxykinase (PEPCK) and tryptophan 2,3-dioxygenase (TDO2) were respectively 53% ($P<0.01$) and 33% ($P<0.01$) lower in LDLR+ as compared to LDLR− adrenal transplanted mice under fasting stress conditions (Fig. 4).

We performed gene expression analysis on the six LDLR+ adrenal transplants and five LDLR− adrenal transplants that could be isolated microscopically intact and virtually free of adjacent kidney tissue to uncover the (molecular) mechanism underlying the diminished glucocorticoid output under fasting stress conditions. To exclude that the reduction in steroidogenic capacity was secondary to a host vs graft response directed against the LDLR that was introduced on adrenocortical cells through transplantation, we measured relative mRNA expression level of inflammatory cell markers (Fig. 5A). Basal mRNA expression levels of the respective cytotoxic T cell, monocyte, and macrophage markers CD8 (*Ct*: 32.9±1.2), CD14 (*Ct*: 29.6±1.1), and CD68 (*Ct*: 28.0±0.8) were low in control LDLR− adrenal transplants and not significantly higher in transplants derived from LDLR

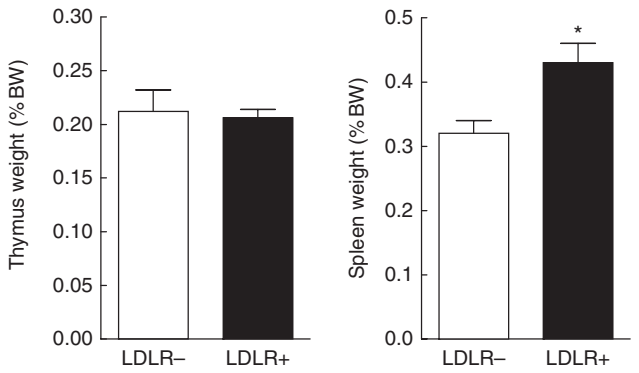

Figure 3
Effect of the adrenal LDL receptor genotype on thymus and spleen weights. Respective tissue weights are derived from mice subjected to overnight fasting and expressed as percentage of the total body weight (%BW). Data represent means±S.E.M. of 12–14 mice/group. $^{*}P<0.05$ vs LDLR−.

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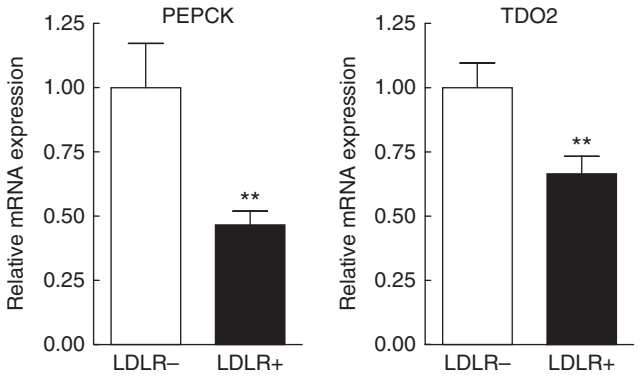

Figure 4
Effect of the adrenal LDL receptor genotype on hepatic relative expression levels of glucocorticoid receptor target genes. Gene expression was measured in livers isolated from mice that had been subjected to overnight fasting. Data represent means±S.E.M. of 8–12 mice/group. $^{**}P<0.01$ vs LDLR−.

expressing adrenal recipients. Combined with the fact that hematoxylin and eosin-stained slides (Fig. 5B) of both adrenal transplant subtypes did not show the immune cell infiltrates that are typically seen in graft vs host specimens, it is suggested that an immune response directed against the LDLR likely does not explain the inhibitory effect on adrenal steroidogenesis. In accordance with a stress-induced stimulation of the steroidogenic machinery, expression of rate-limiting enzymes involved in the mobilization of cholesterol, such as SR-BI (*C*t: 21.4±1.1) and STAR (*C*t: 23.2±1.1), and subsequent conversion into corticosterone, i.e., CYP11A1 (*C*t: 20.9±0.9), HSD3B2 (*C*t: 26.3±0.9), CYP21A1 (*C*t: 21.6±0.7), and CYP11B1 (*C*t: 25.4±1.3), could be readily detected in LDLR− adrenal transplants under fasting conditions. Stimulation of the expression of the HDL receptor SR-BI is an obligatory event in the ACTH-mediated adrenal stress response to maintain functional adrenal cholesterol stores (Rigotti *et al.* 1996, Hoekstra *et al.* 2008, 2009, 2013). Interestingly, as can be seen in Fig. 6, the LDLR genotype did not impact on the adrenal transplant relative mRNA expression levels of SR-BI. It can therefore be suggested that the rate of HDL-associated cholesterol ester uptake into adrenals and the efficacy of the adrenocortical cell ACTH signaling was not different in the two groups of adrenal recipients. The expression of genes associated with the respective formation and catabolism of cholesteryl esters, acetyl-CoA acetyltransferase 1 (ACAT1) and hormone-sensitive lipase (HSL), was also not affected by the adrenal LDLR genotype (Fig. 6). In addition, we detected a similar relative mRNA expression level of the STAR protein that delivers cholesterol to the inner mitochondrial membrane for subsequent use in the steroidogenic pathway (Fig. 6). It thus appears that the change in maximal steroidogenesis rate in response to a change in adrenal LDLR genotype could not be attributed to an altered (intracellular) mobilization of cholesterol. In accordance, Oil Red O lipid neutral lipid staining revealed that the two adrenal transplant types both contained high amounts of cholesterol under fasting stress conditions (Fig. 7). No significant change in the relative mRNA expression levels of mitochondrial cholesterol side-chain cleavage enzyme (CYP11A1), steroid 21-hydroxylase (CYP21A1), or steroid-11-beta-hydroxylase (CYP11B1) was noted. In contrast, LDLR+ adrenal transplants displayed a marked 62% reduction ($P<0.05$; Fig. 6) in the transcript level of 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase type 2 (HSD3B2) that executes the second step in the steroidogenesis pathway, i.e., the catabolism of pregnenolone into progesterone. These combined findings suggest that the reduced fasting glucocorticoid response associated with restoration of adrenocortical LDLR function may be secondary to a diminished adrenal HSD3B2 activity.

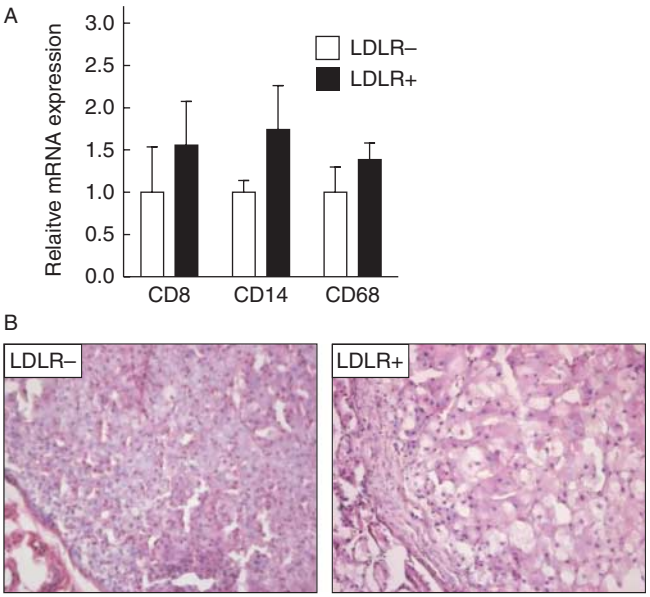

Figure 5
Effect of the adrenal LDL receptor genotype on adrenal transplant inflammation status. (A) Gene expression of inflammatory cell markers was measured in adrenal transplants isolated from mice that had been subjected to overnight fasting. Data represent means±S.E.M. of five to six mice per group. (B) Representative adrenal transplant sections were stained with hematoxylin and eosin. Clear immune cell infiltrates are absent in both adrenal transplant groups. Original magnification, 200×. A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-15-0023.

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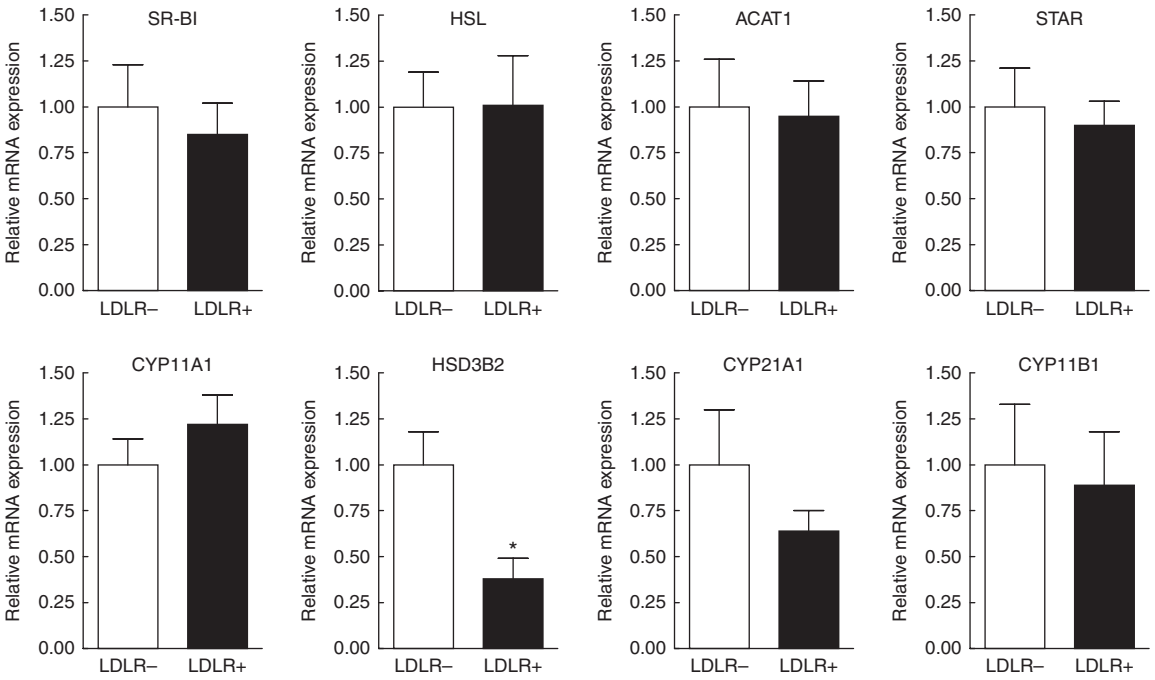

Figure 6
Effect of the adrenal LDL receptor genotype on adrenal transplant relative expression levels of genes associated with cholesterol mobilization and steroidogenesis. Gene expression was measured in adrenal transplants isolated from mice that had been subjected to overnight fasting. Data represent means ± S.E.M. of five to six mice per group. *$P<0.05$ vs LDLR−.

Discussion

Although several lines of evidence have suggested that the LDLR can supply cholesterol to the steroidogenic pathway for subsequent conversion to glucocorticoids, our current findings clearly indicate that – in mice with a human-like lipoprotein profile – the maximal glucocorticoid output is negatively influenced by the presence of the LDLR in adrenocortical cells.

Apolipoprotein E (APOE) is essential for the interaction of lipoprotein particles with the LDLR (Bradley & Gianturco 1986). Owing to a diminished LDLR-mediated clearance, APOE knockout mice display marked hypercholesterolemia, i.e., accumulation of APOB-containing lipoproteins in plasma (Zhang *et al.* 1992). Importantly, APOE knockout mice also show an increase in both basal and stress-induced glucocorticoid levels (Raber *et al.* 2000, Grootendorst *et al.* 2002, 2004, Thorngate *et al.* 2002), which provides further *in vivo* support for a causal inverse relationship between LDLR-mediated cholesterol uptake and glucocorticoid output.

Although most studies on the effect of lipoproteins on adrenal function have focused on steroidogenesis, it should be acknowledged that cholesterol is essential for adrenal growth *in vivo* (Dominick *et al.* 1993). Based on the fact that LDLRs on adrenocortical cells are able to effectively clear cholesterol-rich LDL particles upon ACTH administration *in vivo* (Kovanen *et al.* 1980), we anticipate that the cholesterol derived from LDL is rather used for non-steroidogenic adrenal functions, including adrenocortical cell proliferation. This notion is further substantiated by the finding of Kraemer *et al.* (2007) that

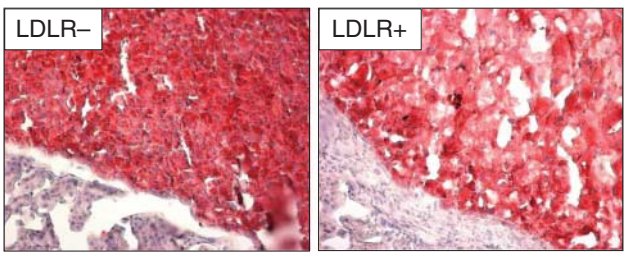

Figure 7
Effect of the adrenal LDL receptor genotype on adrenal transplant cholesterol stores. Adrenal transplants were isolated from mice that had been subjected to overnight fasting. Representative adrenal transplant sections were stained for neutral lipids using Oil Red O. Original magnification, 200×. A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-15-0023.

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the LDLR genotype of adrenocortical cells has no impact on the ACTH-stimulated steroidogenic response *in vitro*.

The decrease in maximal glucocorticoid secretion observed upon replenishment of adrenocortical LDLR function in total body LDLR knockout mice coincided with a decrease in the relative expression level of the steroidogenic enzyme HSD3B2. Importantly, genetic HSD3B2 deficiency represents an established, but rare, cause of congenital adrenal hyperplasia (CAH) in humans. Homozygous carriers of a functional mutation in HSD3B2 display significantly lower cortisol levels and salt-wasting (Alos *et al*. 2000, Jeandron & Sahakitrungruang 2012). We therefore anticipate that the glucocorticoid insufficiency phenotype associated with restoration of the LDLR function can be directly attributed to the decrease in adrenocortical HSD3B2 expression.

A literature search did not reveal a direct link between adrenocortical LDLR function, LDL-cholesterol uptake, and the regulation of HSD3B2 mRNA expression. Although other transcription factors such as GATA family members and the nuclear receptor liver receptor homolog 1 (NR5A2) are also able to modulate the promoter activity of HSD3B2, it has been established that the growth-factor-inducible immediate early gene nur/77 (NUR77) and steroidogenic factor 1 (SF1 (NR5A1)) are essential for maintaining basal HSD3B2 transcription in human adrenocortical cells (Leers-Sucheta *et al*. 1997, Martin & Tremblay 2005, Udhane *et al*. 2013). The relative mRNA expression level of NUR77 and SF1 was not significantly different between our two adrenal transplants (data not shown). The LDLR genotype thus does not directly impact on the expression level of critical upstream regulators of HSD3B2. The decrease in adrenal HSD3B2 expression due to normalization of LDLR function might, however, be secondary to a change in the activity of intracellular signaling pathways. The anti-cancer drug sunitinib lowers the secretion of cortisol by cultured human NCI-H295 adrenocortical cells, which can be attributed specifically to a decrease in the mRNA and protein expression of HSD3B2 (Kroiss *et al*. 2011). The small molecule sunitinib is a potent multi-targeted receptor tyrosine kinase inhibitor (O'Farrell *et al*. 2003, Sun *et al*. 2003), suggesting that changes in intracellular signaling pathways are able to modulate the HSD3B2 transcription rate. Studies in respectively cultured mesangial cells (Jenkins *et al*. 2000) and osteoblasts (Klein *et al*. 2006) have indicated that exposure to native LDL – the normal substrate of the LDLR – increases the activity (phosphorylation state) of MAPK and tuberin and lowers Src protein levels and Akt activity. These combined findings suggest that, by changing the activity of major intracellular signaling pathways, enhanced adrenal uptake of native LDL from the plasma compartment might ultimately decrease the HSD3B2 expression in this tissue.

In conclusion, we have shown that restoration of adrenal LDLR function in LDLR knockout mice is associated with a diminished glucocorticoid output and signaling. Our studies in a mouse model with a human-like lipoprotein profile i) provide the first *in vivo* evidence for a novel inhibitory role of the LDLR in the control of adrenal steroidogenesis and ii) highlight that the relative impact of specific lipoprotein cholesterol sources and their receptors on adrenocortical cell-mediated steroidogenesis may depend much on the overall substrate availability and complexity of the study system, i.e., *in vitro* cell cultures vs total body and tissue-specific knockout mouse models or humans.

Declaration of interest

All authors have nothing to disclose. Our grant suppliers, the Dutch Heart Foundation and Netherlands Organization for Scientific Research, are non-commercial funding agencies and were not involved in the acquisition and interpretation of the data or the decision to publish the work.

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Author contribution statement

R J v d S and M H executed the experiments and performed data analysis. M V E is supervisor of R J v d S and head of the lipid group within the Division of Biopharmaceutics and thereby facilitated all aspects of the research. M H wrote the manuscript.

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