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Lcat deficiency in mice is associated with a diminished adrenal glucocorticoid function

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Menno Hoekstra¹, Suzanne J.A. Korporaal¹², Ronald J. van der Sluis¹, Veronica Hirsch-Reinshagen³, Andrea E. Bochem⁴, Cheryl L. Wellington³, Theo J.C. Van Berkel¹, Jan Albert Kuivenhoven⁵, Miranda Van Eck¹

¹Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, Gorlaeus Laboratories, Einsteinweg 55, 2333CC Leiden, The Netherlands
²Department of Clinical Chemistry and Haematology, University Medical Center, Utrecht, The Netherlands
³Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, Canada
⁴Department of Vascular Medicine, Academic Medical Center, Amsterdam, The Netherlands
⁵Department of Pathology & Medical Biology, Molecular Genetics, University of Groningen, University Medical Center Groningen, The Netherlands
Abstract
Background
In vitro studies have suggested that high-density lipoprotein (HDL) and apolipoprotein B-containing lipoproteins can provide cholesterol for synthesis of glucocorticoids. Here we assessed adrenal glucocorticoid function in lecithin-cholesterol acyltransferase (Lcat) knockout mice to determine the specific contribution of HDL-cholesteryl esters to adrenal glucocorticoid output in vivo.

Methods and Results
Lcat knockout mice exhibit an 8-fold higher plasma free cholesterol-to-cholesteryl ester ratio (P<0.001) and complete HDL-cholesteryl ester deficiency. Apolipoprotein B-containing lipoprotein and associated triglyceride levels are increased in Lcat knockout mice as compared to C57BL/6 controls (+44%; P<0.05). Glucocorticoid producing adrenocortical cells within the zona fasciculata in Lcat knockout mice are devoid of neutral lipids. However, adrenal weights and basal corticosterone levels are not significantly changed in Lcat knockout mice. In contrast, adrenals of Lcat knockout mice show compensatory upregulation of genes involved in cholesterol synthesis (HMGCR; +516%; P<0.001) and acquisition (LDLR; +385%; P<0.001) and a marked 40-50% lower glucocorticoid response to adrenocorticotropic hormone exposure, endotoxemia, or fasting (P<0.001 for all).

Conclusion
Our studies show that HDL-cholesteryl ester deficiency in Lcat knockout mice is associated with a 40-50% lower adrenal glucocorticoid output. These findings further highlight the important novel role for HDL as cholesterol donor for the synthesis of glucocorticoids by the adrenals.
**Introduction**

The production and subsequent secretion of glucocorticoids by adrenocortical cells of the zona fasciculata is dependent on the availability of the steroidogenic precursor cholesterol. Unesterified cholesterol is converted to glucocorticoids through a series of side-chain modifications by cytochrome P450 enzymes and hydroxysteroid dehydrogenases. The intra-mitochondrial transfer of unesterified cholesterol by the enzyme steroidogenic acute regulatory protein (StAR) is considered to be the rate-limiting step in the basal synthesis of glucocorticoids. In vitro studies using isolated adrenocortical cells have suggested that both high-density lipoprotein (HDL) and apolipoprotein B (apoB)-containing lipoproteins are able to provide cholesterol as source for the synthesis of glucocorticoids. We and others have shown that under conditions where glucocorticoids are physiologically relevant, i.e. under stress, the exogenous uptake and intracellular processing of lipoprotein-associated cholesteryl esters becomes of crucial importance to maintain optimal adrenal glucocorticoid function in vivo. Probucol-induced depletion of plasma cholesterol associated with both high- (HDL) and low-density lipoproteins (LDL) in C57BL/6 wild-type mice is associated with a lower stress-induced glucocorticoid level. In addition, a defect in the hydrolysis of lipoprotein-associated cholesteryl esters in hormone-sensitive lipase (Hsl) knockout mice is associated with adrenocortical hypofunction. Furthermore, apolipoprotein A1 (Apoa1) knockout mice that virtually lack HDL particles and scavenger receptor BI (Scarb1) knockout mice that exhibit an impaired uptake of cholesteryl esters from HDL both show a parallel diminished adrenal glucocorticoid function. Combined, these findings suggest that the uptake of HDL-cholesteryl esters by the adrenals is essential to maintain optimal glucocorticoid production in vivo.

The HDL-associated enzyme lecithin-cholesterol acyltransferase (LCAT) mediates the synthesis of HDL-cholesteryl esters. Human subjects with a deleterious mutation on both alleles of the Lcat gene present with HDL deficiency, whereas heterozygotes typically have HDL cholesterol levels that are half of normal HDL cholesterol. Heterozygous and homozygous Lcat knockout mice show a similar dose dependent decrease in plasma HDL levels and thus represent a good mouse model to study the consequences of HDL-cholesteryl ester deficiency on general physiology. To delineate the quantitative contribution of HDL-associated cholesteryl esters to the adrenal glucocorticoid output, here we assessed adrenal glucocorticoid function in Lcat knockout mice.

**Experimental procedures**

**Animals**

Lcat knockout mice and C57BL/6 wild-type controls were bred in house and fed a regular chow diet ad libitum. 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. Age-matched 10-12 week old C57BL/6 mice (n=10) and Lcat knockout mice (n=8) were switched to a new cage and subsequently fasted overnight (~18h) before tail chop blood draws. After an additional two weeks, these mice were injected intraperitoneally with
200 µg human ACTH analogue (ACTH(1-24); tetracosactide) followed by tail blood draws at t=1, 2, 3 hours after ACTH exposure. Six week after the start of experiment, the mice received an intraperitoneal 50 µg/kg sub-lethal dose of lipopolysaccharide (LPS; Salmonella minnesota R595) followed by tail blood draws at t=1, 2, and 3 hours after LPS exposure and sacrifice and tissue harvest at 4 hours after LPS exposure. Before all three types of stress, mice were bled through tail chop to obtain an average basal plasma corticosterone value of each separate mouse. An additional group of 12 week old Lcat knockout (n=4) and C57BL/6 mice (n=11) was subjected to overnight fasting (18h) and subsequently sacrificed for tissue harvesting. Animal care and procedures were performed in accordance with the national guidelines for animal experimentation. All protocols were approved by the Ethics Committee for Animal Experiments of Leiden University.

**Plasma lipid analyses**
Plasma concentrations of free cholesterol, cholesteryl esters, and triglycerides were determined using enzymatic colorimetric assays. The cholesterol distribution over the different lipoproteins in plasma was analyzed by fractionation of 50 µl pooled plasma of each mouse genotype using a Superose 6 column (3.2 x 30 mm, Smart-system, Pharmacia). Free cholesterol and cholesteryl ester content of the effluent was determined using enzymatic colorimetric assays.

**Adrenal neutral lipid visualization**
Seven micrometer cryosections were prepared on a Leica CM3050-S cryostat. Cryosections were routinely stained with Oil red O for neutral lipid visualization. Nuclei were detected using a hematoxylin stain.

**Plasma hormone analysis**
Corticosterone levels in plasma were determined using the corticosterone ³⁵ H RIA Kit from ICN Biomedicals according to the protocol from the supplier.

**Plasma tumor necrosis factor-α (TNF-α) analysis**
TNF-α protein levels were determined in plasma by ELISA (OptEIA kit, BD Biosciences Pharmingen, San Diego, CA) using the standard protocol.

**Real-time quantitative PCR**
Gene expression analysis was performed essentially as described. 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. Beta-actin and GAPDH 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

**Lcat knockout mice exhibit isolated HDL-cholesteryl ester deficiency**

In accordance with a prominent role for LCAT in the esterification of free cholesterol,16 *Lcat* knockout mice exhibited an almost complete absence of cholesteryl esters (-86%; $P<0.001$) in plasma with unchanged plasma free cholesterol levels (*table 1*). As a result, the free cholesterol to cholesteryl ester ratio was 8-fold higher ($P<0.001$) in plasma from *Lcat* knockout mice as compared to C57BL/6 wild-type controls (*table 1*). FPLC lipoprotein analysis revealed that both free cholesterol and cholesteryl esters levels in C57BL/6 wild-type mice were primarily associated with the HDL-fraction (65% and 78%). As anticipated, virtually no cholesteryl esters were present in the HDL fraction in plasma of *Lcat* knockout mice, while the non-HDL cholesteryl ester content was essentially unaffected (*figure 1*). The HDL-associated free cholesterol level was also markedly lower *Lcat* knockout mice as compared to C57BL/6 mice (*figure 1*). In contrast, the level of free cholesterol associated with apoB-containing lipoproteins was 2.9-fold higher in plasma of *Lcat* knockout mice (*figure 1*). In line with an increased amount of triglyceride-rich apoB-containing VLDL and LDL particles circulating in plasma of *Lcat* knockout mice, as previously already noted by Sakai et al,13 we detected a significantly higher level of plasma triglycerides (+44%; $P<0.05$) in *Lcat* knockout mice (*Table 1*).

**Lcat knockout mice show a diminished adrenal corticosterone output**

The basal secretion of glucocorticoids by adrenals in mice is relatively low and is generally assumed to be independent of the acquisition of extracellular cholesterol pools since endogenous de novo production of cholesterol from acetyl-CoA should be sufficient to maintain basal levels. However, although the effect failed to reach statistical significance ($P=0.073$), we did observe a marked decrease in basal plasma levels of corticosterone - the primary glucocorticoid circulating in rodents - in response to Lcat deficiency (96±20 ng/ml for *Lcat* knockout mice vs 148±20 ng/ml for C57BL/6 mice).

**Table 1.** Plasma lipids in wild-type C57BL/6 and LCAT knockout (LCAT KO) mice

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6</th>
<th>LCAT KO</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free cholesterol (FC; mg/dl)</td>
<td>21±1</td>
<td>20±2</td>
<td>N.S.</td>
</tr>
<tr>
<td>Cholesteryl esters (CE; mg/dl)</td>
<td>50±2</td>
<td>7±1</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Total cholesterol (TC; mg/dl)</td>
<td>71±3</td>
<td>27±2</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>FC/ CE ratio</td>
<td>0.41±0.01</td>
<td>3.31±0.57</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>93±4</td>
<td>134±18</td>
<td>$&lt;0.05$</td>
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Activation of the hypothalamus-pituitary-adrenal axis results in the secretion of glucocorticoids by the adrenals at levels that effectively activate downstream glucocorticoid receptor signalling, an essential part of the body’s response to physiological stressors. As anticipated, adrenocortical cell activation upon the administration of a synthetic mimetic of the pituitary-derived hormone ACTH (tetracosactide; 200 µg i.p.) – a potent activator adrenal steroidogenesis – was associated with an acute rise in plasma corticosterone levels in C57BL/6 wild-type mice. Plasma corticosterone reached a plateau concentration at 1 hour after the tetracosactide injection, which remained maximal until 3 hours after the administration (figure 2A). Tetracosactide exposure also increased plasma corticosterone levels in Lcat knockout mice, however, the peak concentration of corticosterone in plasma of Lcat knockout mice was 37% lower than the level detected in C57BL/6 mice after 1 hour of tetracosactide exposure (365±31 ng/ml vs. 584±34 ng/ml; P<0.001; figure 2A). In contrast to wild-type mice, the concentration of corticosterone rapidly declined in Lcat knockout mice after 1 hour and returned to basal levels at the 3 hours (figure 2A). This suggests that the adrenals of Lcat knockout mice are only capable of realizing a short attenuated glucocorticoid response upon activation of the hypothalamus-pituitary-adrenal axis.

Endogenous glucocorticoids protect against sepsis and other inflammation-associated pathologies. In line with an essential role for glucocorticoids in the response to infection,
exposure to a sub-lethal dose of endotoxin (lipopolysaccharide [LPS]; 50 µg/kg i.p.) also induced a rapid increase in plasma corticosterone levels in C57BL/6 mice, which reached a plateau of 586±24 ng/ml at 2 hours after endotoxin exposure (figure 2B). Plasma corticosterone levels did rise in Lcat knockout mice upon endotoxin exposure (figure 2B). However, Lcat knockout mice displayed a more gradual increase up to 3 hours after LPS exposure (341±24 ng/ml), which was markedly lower as compared to the maximal level observed in C57BL/6 mice (593±31 ng/ml; P<0.001; figure 2B). Of note, the observed maximum plasma corticosterone levels (~600 ng/ml for C57BL/6 mice vs ~300 ng/ml for Lcat knockout mice) as well as the area-under-the-curve (AUC; ~1400 ng/ml.h for C57BL/6 mice vs ~800 ng/ml.h for Lcat knockout mice) were similar for either genotype after tetracosactide and endotoxin exposure. It is therefore suggested that both treatments induced a maximal acute adrenal steroid output, which is apparently 40-50% lower in Lcat knockout mice.

Since glucocorticoids through activation of the nuclear glucocorticoid receptor are important regulators of gluconeogenesis and glucose utilization, overnight fasting is associated with an obligatory stimulation of adrenal glucocorticoid secretion to overcome hypoglycemia.10,18,19 In line, after ~18 hours of fasting we observed 51% lower (P<0.001) corticosterone levels in Lcat knockout mice as compared to C57BL/6 controls (figure 2C). Combined, these findings suggest that HDL deficiency in Lcat knockout mice is associated with a diminished adrenal corticosterone output in response to stress.

Adrenals of Lcat knockout mice are deprived of neutral lipids despite compensatory upregulation of genes associated with cholesterol acquisition

No significant difference in the weight of the adrenals between Lcat knockout mice and C57BL/6 mice during endotoxemia, i.e. 4 hours after LPS exposure, or under fasting stress conditions was noted (figure 3A). Ng et al. have previously described that adrenals from Lcat knockout mice are under normal conditions deprived of cholesteryl esters.14 In our

![Figure 2](image_url)

**Figure 2.** Plasma corticosterone levels in wild-type C57BL/6 (n=10) and LCAT knockout (LCAT KO; n=8) mice. Concentrations in plasma were measured after (A) injection with the human ACTH analogue tetracosactide, (B) injection with a sub-lethal dose of lipopolysaccharide (50 µg/kg LPS), or (C) ~18 hours of overnight fasting. ** P<0.01, *** P<0.001 vs C57BL/6 mice.
group of *Lcat* knockout mice, this deficiency of neutral lipids in adrenocortical cells could be verified using Oil red O neutral lipid staining. As evident from *figure 3B*, specifically the glucocorticoid-producing adrenocortical cells within the zona fasciculata in *Lcat* knockout mice lack the intense Oil red O staining as seen in the C57Bl/6 mice.

The unesterified cholesterol pool used for steroidogenesis can be supplied by (1) endogenous synthesis of cholesterol in which HMG-CoA reductase catalyzes the rate-limiting step, (2) hydrolysis of stored cholesteryl esters, or (3) uptake of exogenous lipoprotein-associated cholesterol. To identify potential compensatory gene regulation, using quantitative real-time PCR, we measured gene expression levels in adrenals harvested from 18 hour fasted *Lcat* knockout mice and C57BL/6 wild-type controls (*figure 3C*). A 6-fold stimulation of HMG-CoA reductase (HMGCR; P<0.001) mRNA expression levels was detected in the adrenals of *Lcat* knockout mice. The relative expression levels of enzymes crucially involved in synthesis (acetyl-CoA acetyltransferase 1; ACAT-1) and hydrolysis

**Figure 3.** (A) Adrenal weights in wild-type C57BL/6 (n=10) and LCAT knockout (LCAT KO; n=8) mice that either suffered from sub-lethal endotoxemia or were fasted overnight for ~18 hours. (B) Representative Oil red O neutral lipid staining of cortical zones in adrenals from C57BL/6 and LCAT KO mice. ZG, zona glomerulosa; ZF, zona fasciculata. (C) Relative mRNA expression levels of cholesterol metabolism-associated genes in adrenals of LCAT KO mice (n=4) as fold compared to those found in C57BL/6 controls (n=11). *** P<0.001 vs C57BL/6 mice.
(hormone-sensitive lipase; HSL) of cholesteryl esters within the adrenals were unaffected. Adrenal scavenger receptor BI (SR-BI) expression was unaltered, while the LDL receptor (LDLR) expression level was significantly increased (+384%; P<0.001) in Lcat knockout mice. It thus seems that adrenals of Lcat knockout mice as compared to those of wild-type controls, probably as a compensatory response, attempt to synthesize more cholesterol and acquire increased amounts of LDL-cholesterol through receptor-mediated uptake.

**Lcat knockout mice display hepatocyte but not leukocyte glucocorticoid insufficiency**

The glucocorticoid receptor is highly expressed in leukocytes where it modulates inflammatory responses as well as in hepatocytes where it regulates glucose metabolism. Since leukocyte glucocorticoid insufficiency is associated with an enhanced endotoxemia-associated cytokine profile, we determined the effect of Lcat deficiency on the LPS-induced tumor necrosis factor-α (TNF-α) response. Plasma levels of the early response cytokine TNF-α were not significantly different at any time point measured after LPS (50 µg/kg i.p.) exposure (0-3 hours; figure 4A). It thus seems that Lcat knockout and C57BL/6 wild-type mice reach a similar (optimal) anti-inflammatory glucocorticoid receptor signalling level in leukocytes after LPS exposure.

As anticipated, Lcat mRNA expression was completely absent in livers of Lcat knockout mice (P<0.001; figure 4B). The relative expression level of the hepatocyte-specific glucocorticoid-induced gene APOA4 encoding apolipoprotein A4, however, was also significantly reduced in livers of overnight fasted Lcat knockout mice (-66%; P<0.01; figure 4B). Furthermore, Lcat deficiency was associated with a marked 3.7-fold increase in the expression level of the corticosteroid binding globulin (CBG).

**Figure 4.** (A) Plasma tumor necrosis factor-α (TNF-α) levels in wild-type C57BL/6 (n=10) and LCAT knockout (LCAT KO; n=10) mice. Concentrations in plasma were measured before and after injection with a sub-lethal dose of lipopolysaccharide (50 µg/kg LPS). (B) Relative mRNA expression levels of LCAT, apolipoprotein A4 (APOA4) and corticosteroid binding globulin (CBG) in livers of LCAT KO mice (n=4) as fold compared to those found in C57BL/6 controls (n=11). ** P<0.01, *** P<0.001 vs C57BL/6 mice.
in the relative hepatic mRNA expression level of the glucocorticoid carrier protein corticosteroid binding globulin (CBG), whose gene expression level in hepatocytes is subject to negative feedback control by glucocorticoids. Thus, downstream glucocorticoid receptor signalling pathways appear to be relatively de-activated in hepatocytes within the liver of Lcat knockout mice. Combined, these findings suggest that Lcat knockout mice do not suffer from total body glucocorticoid insufficiency, but rather display hepatocyte-specific glucocorticoid insufficiency.

**Discussion**

Although it is widely acknowledged that both HDL and non-HDL apoB-containing lipoproteins can be used as source of cholesterol for the production of glucocorticoids by adrenocortical cells, the relative contribution of the separate lipoprotein classes to this process in vivo is not exactly known. In the current study, we assessed glucocorticoid function in Lcat knockout mice to investigate the contribution of specifically HDL-associated cholesteryl esters to adrenal steroidogenesis. Despite relatively high circulating non-HDL cholesterol levels and a compensatory upregulation of genes associated with adrenal cholesterol acquisition, Lcat knockout mice that lack HDL-cholesteryl esters are unable to execute a full glucocorticoid response (40-50% lower as compared to C57BL/6 wild-type controls) to three types of stress being tetracosactide administration, LPS exposure, and overnight fasting. The VLDL cholesteryl ester composition (saturation state) is significantly changed in Lcat KO mice, which can theoretically also affect the adrenal glucocorticoid output. However, isolation of VLDL fractions from pooled plasma of C57BL/6 and Lcat KO mice (n=13 each) using ultracentrifugation indicated that both types of mice do not have sufficiently high levels of VLDL to carry out in vitro steroidogenesis studies using H295R adrenocortical cells (data not shown). This suggests that any differences in the VLDL cholesteryl ester composition are unlikely to have a major impact on the glucocorticoid insufficiency phenotype observed in Lcat KO mice. We thus consider our current findings clear proof that HDL-associated cholesteryl esters make an essential contribution to in vivo adrenal glucocorticoid production. Accordingly, data from studies in human LCAT deficient subjects further support this concept, since LCAT deficiency in humans is associated with a lower adrenal steroid output as measured by a decrease in the urinary excretion of 17-ketogenic steroids and 17-hydroxycorticoids.

Our previous data from Scarb1 knockout mice that lack a functional HDL receptor indicated that disrupted adrenal uptake of cholesteryl esters from HDL is associated with glucocorticoid insufficiency as these mice display 1) a lowered hepatic glucocorticoid signalling and 2) an enhanced susceptibility to endotoxemia. In the current study we observed that Lcat knockout mice that completely lack HDL-cholesteryl esters also display diminished glucocorticoid signalling in hepatocytes as they exhibit a lowered hepatic APOA4 mRNA expression level and a compensatory upregulation of liver CBG expression. In line with a generally lower hepatic glucocorticoid signalling in Lcat knockout mice, Ng et al. have previously detected a lower relative expression level of the glucocorticoid-responsive gene PEPCK and a parallel decrease...
in plasma glucose levels in response to Lcat deficiency. In contrast to the change in hepatic glucocorticoid action in Lcat knockout mice, leukocyte-specific glucocorticoid signalling is apparently normal in these mice, as the susceptibility to endotoxemia is unaffected. Such a difference in cell-specific glucocorticoid sensitivity, i.e. high glucocorticoid receptor sensitivity in leukocytes and low glucocorticoid receptor sensitivity in hepatocytes has also been detected in our previous adrenalectomy and adrenal transplantation studies.

Total Scarb1 knockout mice thus display a more severe glucocorticoid insufficiency phenotype than that observed in Lcat knockout mice. SR-BI is the sole molecule involved in the selective uptake of HDL-associated cholesteryl esters. As both types of mice display hepatocyte-specific glucocorticoid insufficiency, we anticipate that the HDL / SR-BI interaction is essential to generate the bulk of corticosterone needed to effectively activate the glucocorticoid receptor in hepatocytes. However, the difference in leukocyte glucocorticoid insufficiency and endotoxemia susceptibility between the Lcat and Scarb1 knockout mice seems to rely on a HDL-cholesteryl ester independent corticosterone response. SR-BI knockout mice fail to increase their plasma corticosterone levels in response to LPS exposure, whereas the present study shows that Lcat knockout mice are able to increase – albeit to a lower extent – their plasma glucocorticoid levels in response to endotoxemia. Both our Lcat knockout mice and Scarb1 knockout mice display an increase in the adrenal relative expression level of the LDL receptor, the primary protein involved in the clearance of apoB-containing lipoproteins such as VLDL and LDL. Since previous findings by Kraemer et al. have suggested that the LDL receptor does not supply cholesterol the steroidogenic pathway, a qualitative role for the LDL receptor / apoB-lipoprotein interaction in the synthesis of this distinct corticosterone pool in Lcat knockout mice can be excluded. Importantly, SR-BI is also able to clear apoB-containing lipoproteins. Lcat knockout adrenals - in contrast to Scarb1 knockout adrenals - may thus actually acquire cholesteryl esters from VDL/LDL particles through receptor-mediated uptake by SR-BI, which is sufficient to maintain plasma glucocorticoid levels that effectively activate the glucocorticoid receptor in leukocytes (but not hepatocytes). Future studies with specific SR-BI mutants that are selective for either only HDL or non-HDL binding and the associated selective uptake, as identified by for example the group of Dr. Monty Krieger, will unequivocally show the possible contribution of the non-HDL / SR-BI interaction to total adrenal steroid production.

In conclusion, our studies show that HDL-cholesteryl ester deficiency in Lcat knockout mice is associated with a 40-50% lower adrenal glucocorticoid stress response and, as a result, a hepatocyte-specific glucocorticoid insufficiency phenotype. These findings further highlight the important novel role for HDL as cholesterol donor for the synthesis of glucocorticoids by the adrenals.

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