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Plasma lipoproteins are required for both basal and stress-induced adrenal glucocorticoid synthesis and protection against endotoxicemia in mice

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Steroid hormones constitute a class of hormones that are derived from cholesterol. They regulate a vast number of physiological processes such as growth and metabolism. Steroid hormones include the sex hormones testosterone and estrogen as well as glucocorticoids and mineralocorticoids that are secreted by the adrenals. A constant supply of cholesterol is required within adrenal cells to serve as a precursor for the conversion to glucocorticoids and mineralocorticoids. The rate-limiting step for corticoid synthesis is the transport of unesterified cholesterol from the outer mitochondrial membrane to the inner membrane by steroidogenic acute regulatory protein (StAR) (6, 7, 18). However, in response to a stressful lifestyle, subjects can exhibit a sustained increase in plasma glucocorticoid (i.e., cortisol) levels, which requires relatively large amounts of cholesterol to be used for steroidogenesis within the adrenals. Theoretically, the unesterified cholesterol needed for optimal adrenal steroidogenesis under stress conditions can be derived from several different sources: 1) de novo synthesis of cholesterol from acetyl-CoA, 2) intracellular catabolism of stored cholesterol esters, and 3) uptake of extracellular cholesterol from lipoproteins [reviewed by Kraemer (13)]. The relative contribution of these three pathways to basal and stress-induced steroidogenesis has been examined extensively in adrenocortical cells in vitro. The production of glucocorticoids in cultured bovine adrenocortical cells is dependent on the de novo synthesis of cholesterol, since pharmacological inhibition of hydroxymethylglutaryl (HMG)-CoA reductase activity significantly impairs both basal and adrenocorticotropic hormone (ACTH)-stimulated cortisol secretion (24). In addition, the breakdown of cholesterol esters to free cholesterol by hormone-sensitive lipase (HSL) is also of quantitative importance for adrenal glucocorticoid synthesis since HSL deficiency is associated with a significantly lower basal and stimulated corticosterone production in mouse adrenocortical cells in vitro (14). Importantly, already in the 1950s it was shown that >95% of the adrenal cholesterol pool in rats does not originate from local sources (i.e., de novo synthesis or cellular catabolism) but rather from the plasma compartment (21). Although cholesterol is also an important building block for cell membranes, this finding does clearly suggest that lipoprotein-associated cholesterol may also make a significant contribution to adrenal steroidogenesis in vivo. To determine whether lipoproteins indeed contribute to optimal adrenal steroidogenesis in mice, in the current study we have determined the relative contribution of lipoprotein (i.e., LDL and HDL) deficiency on basal and stress-induced glucocorticoid secretion in each mouse using a Superose 6 column (3.2 x 300 mm, 300 μl). Plasma lipoproteins are required for both basal and stress-induced adrenal steroidogenesis and protection against endotoxicemia in mice.
Table 1. Plasma and adrenal lipid levels in probucol-treated and control mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Produclo</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>17.8 ± 0.5</td>
<td>17.8 ± 0.7</td>
<td>1.00</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>58 ± 2</td>
<td>6.5 ± 0.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Phospholipid, mg/dl</td>
<td>136 ± 6</td>
<td>39 ± 1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglyceride, mg/dl</td>
<td>57 ± 9</td>
<td>39 ± 3</td>
<td>0.11</td>
</tr>
<tr>
<td>Adrenals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight, mg</td>
<td>6.4 ± 1.1</td>
<td>6.2 ± 0.8</td>
<td>0.90</td>
</tr>
<tr>
<td>Weight, %BW</td>
<td>0.036 ± 0.006</td>
<td>0.035 ± 0.005</td>
<td>0.94</td>
</tr>
<tr>
<td>Total cholesterol, µg/mg</td>
<td>17 ± 1</td>
<td>8 ± 1</td>
<td>0.0015</td>
</tr>
</tbody>
</table>

Data represent means ± SE of 5 mice. %BW, percentage of body weight.

Smart-System; Pharmacia). Total cholesterol content of the effluent was determined using enzymatic colorimetric assays (Roche Diagnostics). Seven-micrometer cryosections of snap-frozen adrenals were prepared on a Leica CM3050-S cryostat. Cryosections were routinely stained with hematoxylin (Sigma) for nuclei and Oil Red O (Sigma) for lipid visualization. Images were obtained with a Leica image analysis system consisting of a Leica DMRE microscope coupled to a camera and Leica Qwin Imaging software (Leica, Cambridge, UK).

Analysis of gene expression by real-time quantitative PCR. Quantitative gene expression was performed using real-time SYBR Green technology (Eurogentec). Primers were validated for identical efficiencies [slope = −3.3 for a plot of threshold cycle (Ct) vs. log ng cDNA]. Primer sequences are available on request. Hypoxanthine guanine phosphoribosyl transferase (HPRT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, and acidic ribosomal phosphoprotein P0 (36B4) were used as the standard housekeeping genes. Relative gene expression numbers were calculated by subtracting the Ct number of the target gene from the average Ct of HPRT, GAPDH, β-actin, and 36B4 (Ct housekeeping) and raising 2 to the power of this difference. The average Ct of four housekeeping genes was used to exclude changes in the relative expression caused by variations in the expression of the separate housekeeping genes.

Corticosterone and TNFα response upon a lipopolysaccharide challenge. Blood was drawn between 0800 and 0900 via tail cut using a mouse restrainer for basal “nonstressed” plasma values of corticosterone and TNFα levels. Subsequently, mice were intravenously injected at 0900 with a sublethal dose of 50 µg/kg lipopolysaccharide (LPS) from Salmonella Minnesota R595 (List Biological Laboratories, Hornby, ON, Canada) into the tail vein. At the indicated times following LPS injection, tail cut blood samples were collected and plasma corticosterone and TNFα levels determined by [125I]radioimmunoassay (RIA) from MP Biomedicals (Irvine, CA) and ELISA (OptEIA kit; BD Biosciences Pharmingen, San Diego, CA), respectively.

Tissue uptake of 3Hcholesteryl ether HDL. Human HDL was isolated from blood of healthy subjects by differential ultracentrifugation and dialyzed against PBS with 1 mM EDTA. HDL (1.063 < d < 1.21) was labeled with 3Hcholesteryl ether (CET) via exchange from donor particles. A dose of 200 µg of apolipoprotein (±1.2 × 106 dpm) of 3HHDLCET (total volume 100 µl) was injected into the tail vein. For analysis of tissue cholesteryl ether uptake, 2 h after tracer injection tissues were excised, weighed, solubilized, and counted for 3Hradioactivity in a Packard liquid scintillation unit. A correction was made for the radioactivity in the blood present in the tissues at the time of sampling. Values were expressed as percent of the injected dose per microgram of tissue. To determine the effect of LPS on the tissue uptake of 3HHDLCET, mice were preinjected with 50 µg/kg LPS 1 h before injection of the 3HHDLCET.

Data analysis. Statistical analyses were performed using two-tailed unpaired t-test or two-way ANOVA, using Graphpad Prism Software (http://www.graphpad.com; Graphpad Software, San Diego, CA). Normality testing of the experimental groups was performed using the method of Kolmogorov and Smirnov (Graphpad Instat Software). P < 0.05 was considered significant.

RESULTS

To induce an acute and short-term depletion of plasma lipoproteins, C57BL/6 mice were fed a regular chow control diet or a chow diet supplemented with the lipid-lowering drug probucol for 2 wk. As anticipated, treatment with probucol induced a marked decrease in plasma total cholesterol (~89%, P < 0.001) and phospholipid (~71%, P < 0.001) levels in ad libitum-fed mice (Table 1). Lipoprotein distribution analysis revealed that the decrease in the plasma total cholesterol level upon probucol treatment could be attributed to an 87% decrease in HDL cholesterol levels and a 77% decrease in LDL cholesterol levels (Fig. 1). VLDL/chylomicron cholesterol levels were unchanged after probucol treatment (Fig. 1). In parallel, no significant difference was detected in plasma triglyceride levels between probucol-fed and control-fed mice (Table 1).

Strikingly, the gross appearance of the adrenals was markedly different between probucol-treated mice and control mice. Although we did not observe a difference in weight of the adrenals (Table 1), adrenals of probucol-treated mice appeared darker (more red) compared with those of controls (Fig. 2A). In parallel, a significant 54% decrease (P = 0.0015) in total adrenal cholesterol stores was observed upon probucol treatment (Table 1). Oil Red O staining revealed that the neutral lipid (i.e., cholesteryl ester) content of the adrenal cortex was clearly decreased in probucol-fed mice compared with control-fed mice. In particular, a decrease in Oil Red O-positive neutral lipid stores was detected in the zona fasciculata, the area of the adrenal cortex that contains the glucocorticoid-producing cells, whereas the lipid content of mineralocorticoid-producing zona glomerulosa seemed to be essentially unaffected (Fig. 2B).

Importantly, a significant decrease in the plasma corticosterone levels was noted in probucol-treated mice under basal nonstressed conditions (P = 0.016; Fig. 3A). In the adrenals, probucol did not affect the relative gene expression level of...
StAR (Fig. 3B), the rate-limiting factor for acute adrenal steroidogenesis. In addition, no significant change was observed in the mRNA expression level of cytochrome P450scc (CYP11A1; Fig. 3B) that catalyzes the first and rate-limiting step in the biosynthesis of hormones in all steroidogenic tissues, namely the conversion of cholesterol to pregnenolone. Furthermore, no significant change in the relative mRNA expression level of the HDL receptor scavenger receptor BI (SR-BI), the LDL receptor, HSL, or acyl-coenzyme A:cholesterol acyltransferase-1 (ACAT1) was observed (Fig. 3B), suggesting that the activity of lipoprotein uptake as well as intracellular cholesterol ester synthesis and catabolism systems was unaltered by probucol treatment. In contrast, probucol treatment significantly increased the adrenal relative expression level of HMG-CoA reductase (+47%; P = 0.036; Fig. 3B). This suggests a compensatory stimulation of the rate of cholesterol synthesis from acetyl-CoA in response to probucol treatment. Thus it seems that the decrease in the basal adrenal glucocorticoid synthesis rate after probucol feeding was not due to a change in the expression level of essential steroidogenic enzymes but more likely due to impaired substrate (unesterified cholesterol) availability.

The liver is a key target organ of glucocorticoids, since via the action of the nuclear glucocorticoid receptor they are able to modulate the hepatic expression of genes involved in glucose, cholesterol, and bile acid metabolism (5, 27). Probucol did not change the expression of the glucocorticoid-responsive genes apolipoprotein A4 and cholesterol 7α-hydroxylase under basal feeding (nonstressed) conditions (Fig. 3C). This suggests that the decrease in plasma corticosterone levels upon probucol treatment was not associated with a concomitant change in hepatic glucocorticoid receptor activity under basal conditions.

As a first-line defense protective “stress” response to overcome inflammation-associated morbidity (i.e., sepsis) and mortality, adrenals readily secrete high levels of anti-inflammatory glucocorticoids upon exposure to LPS. To investigate the effect of probucol on the adrenal response to stress, probucol-fed and chow-fed mice were therefore exposed to a sublethal dose of LPS. As anticipated, exposure to LPS induced a rapid time-dependent increase in plasma corticosterone levels in control-treated mice (Fig. 4A). In probucol-fed mice, an acute rise in plasma corticosterone levels also occurred within the first hour after the injection with LPS. Subsequently, however, the plasma corticosterone concentration did not increase further and actually declined gradually in the probucol-treated mice, resulting in a fourfold lower (P < 0.001) plasma corticosterone level 3 h after the LPS injection and a significantly decreased overall corticosterone response compared with control-treated mice (P < 0.001; Fig. 4A). Thus it seems that probucol has no major effect on the “acute” (<1 h) steroidogenesis but does impair the “sustained” adrenal corticosterone response to stress.

The initial response of adrenocortical cells to stress (i.e., ACTH stimulation) is an increase in cholesterol esterase activ-

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**Fig. 2.** The effect of probucol treatment on the gross morphology of the adrenals (A) and adrenal cortex lipid levels (B). Note the clear darkening of the adrenals in probucol-fed mice. Cryosections of adrenals were stained with Oil Red O for neutral lipids and counterstained with hematoxylin for nuclei. ZG, zona glomerulosa; ZF, zona fasciculata.

**Fig. 3.** The effect of probucol treatment on the plasma corticosterone level (A), the adrenal relative mRNA expression levels of steroidogenic acute regulatory protein (StAR), cytochrome P450scc (CYP11A1), scavenger receptor BI (SR-BI), the LDL receptor (LDLR), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), hormone-sensitive lipase (HSL), and acyl-coenzyme A:cholesterol acyltransferase-1 (ACAT1) (B), and the hepatic relative mRNA expression levels of glucocorticoid-responsive genes cholesterol 7α-hydroxylase (CYP7A1) and apolipoprotein A4 (apoA4) under basal “nonstressed” conditions (C). Data represent means ± SE of 5–10 mice. *P < 0.05 compared with controls.
ity, presumably reflecting the physiological purpose of having a readily accessible store of substrate for steroidogenesis (25).

Also, upon LPS exposure, no change in the mRNA expression level of cholesteryl ester synthesis (ACAT1) or catabolism (HSL) genes was observed between probucol-fed and control mice (Fig. 4B). However, we detected a 2.4- and 5.3-fold increase (P < 0.001 for both) in the adrenal relative mRNA expression of the LDL receptor and HMG-CoA reductase from probucol-fed mice compared with control-fed mice under high steroidogenic pressure conditions (Fig. 4B). This suggests a compensatory upregulation of a cholesterol delivery route by the LDL receptor and an increased cholesterol synthesis by HMG-CoA reductase in mice that exhibit relative lipoprotein deficiency in response to probucol treatment.

To determine whether the impaired response in glucocorticoid secretion to LPS due to probucol feeding was physiologically relevant in terms of the downstream glucocorticoid effects, the relative expression level of glucocorticoid receptor-responsive genes involved in inflammation was examined. Four hours after LPS exposure, proinflammatory cytokine IL-6 and TNFα mRNA expression was significantly higher in livers of probucol-treated mice compared with control mice (P < 0.05 for both), whereas that of the activated macrophage marker MARCO (macrophage receptor with collagenous domain) did not differ between the two groups of mice (Fig. 5A). In parallel with the observed higher hepatic TNFα mRNA expression level, the LPS-induced TNFα level in plasma was significantly increased in probucol-treated mice (area under the curve: 498 ± 68 vs. 263 ± 28, P = 0.012; Fig. 5B). Combined, these findings suggest that a probucol-mediated reduction in plasma lipoprotein levels is associated with adrenal glucocorticoid insufficiency due to a lack of substrate availability, which is paralleled by a lower hepatic glucocorticoid receptor activity and an enhanced susceptibility to endotoxemia.

To prove that cholesterol is actually acquired from plasma lipoproteins by the adrenals under conditions of high steroidogenic pressure, the uptake of [3H]-labeled HDL cholesteryl ether ([3H]HDL-CEt) was determined under “basal” and LPS-induced “stress” conditions. As shown in Fig. 6, a relatively high uptake of [3H]HDL-CEt per microgram of tissue was detected in the adrenals and liver of wild-type mice under basal conditions. This was to be expected since both the liver and adrenals express high mRNA levels of the HDL receptor SR-BI (1). As
not able to compensate for a loss of lipoprotein-derived cholesterol. Thus it can be suggested that plasma lipoproteins are required to maintain optimal steroidogenesis in mice and probably are the major contributor to glucocorticoid synthesis under basal conditions. Apolipoprotein A1 (apoA1)-knockout mice that lack the main apolipoprotein constituent of HDL, apoA1, exhibit a similarly lowered plasma HDL level, as detected in the current study. In contrast, basal plasma corticosterone levels are not different in apoA1-knockout mice compared with their wild-type littermates (23). In addition, mice that are deficient in the HDL receptor SR-BI and have an impaired adrenal uptake of HDL-associated cholesterol esters also do not suffer from adrenal glucocorticoid insufficiency in the basal state (9, 10). Thus it seems that a combination of increased endogenous cholesterol synthesis and receptor-mediated uptake of lipoproteins other than HDL (i.e., LDL) can overcome glucocorticoid insufficiency in these animals. Importantly, a defect in uptake of LDL via the LDL receptor alone also does not affect adrenal steroidogenesis (15). In vitro studies have indicated that both HDL and LDL can be utilized as substrate for steroid synthesis in adrenocortical cells (2, 7, 8, 12). Combined, these findings suggest that both HDL and LDL may be substrates actually used for basal steroidogenesis in mice in vivo. However, the relative contribution of the two different lipoproteins for steroid hormone synthesis under basal conditions in wild-type mice remains to be established. Our current studies also show that probucol-induced relative lipoprotein deficiency is associated with a hampered protective adrenal corticosterone response to an inflammatory stimulus and an enhanced susceptibility to LPS-induced endotoxemia. From our previous findings in SR-BI-knockout mice with and without expression of the cholesteryl ester transfer protein (10), it can be expected that the diminished glucocorticoid stress response to LPS in probucol-treated mice can contribute to the lowered plasma HDL but not LDL level. We now also show that the uptake of HDL cholesterol esters is increased specifically in the adrenals but not other SR-BI-expressing organs such as liver, indicating that cholesterol associated with HDL is indeed used efficiently for adrenal steroidogenesis in mice in vivo. However, the relative contribution of the two different lipoproteins for steroid hormone synthesis under basal conditions in wild-type mice remains to be established. Our current studies also show that probucol-induced relative lipoprotein deficiency is associated with a hampered protective adrenal corticosterone response to an inflammatory stimulus and an enhanced susceptibility to LPS-induced endotoxemia. From our previous findings in SR-BI-knockout mice with and without expression of the cholesteryl ester transfer protein (10), it can be expected that the diminished glucocorticoid stress response to LPS in probucol-treated mice can contribute to the lowered plasma HDL but not LDL level. We now also show that the uptake of HDL cholesterol esters is increased specifically in the adrenals but not other SR-BI-expressing organs such as liver, indicating that cholesterol associated with HDL is indeed used efficiently for adrenal steroidogenesis under high steroidogenic pressure conditions (i.e., upon LPS exposure) in mice. Probucol has potent antioxidant and anti-inflammatory properties (16). However, probucol-treated mice do still exhibit a higher TNFα response upon exposure to a sublethal dose of LPS. Glucocorticoids, via the action of the nuclear glucocorticoid receptor, inhibit the expression of proinflammatory cytokines in immune cells such as dendritic cells and macrophages (17, 22). Glucocorticoid insufficiency as a result of adrenal removal (adrenalectomy) is associated with an enhanced susceptibility to endotoxemia in mice (22). Therefore, it is anticipated that the anti-inflammatory effect of probucol is overruled by the proinflammatory effect of adrenal glucocorticoid insufficiency induced by probucol in mice.

In a multivariate analysis in human patients with liver failure and patients post-liver transplantation, low HDL cholesterol levels were the only variable predictor of adrenal insufficiency (19). Furthermore, in critically ill patients, in whom the need for anti-inflammatory steroid hormone production is high, a low plasma HDL cholesterol level was associated with an impaired glucocorticoid (cortisol) response to synacthen stimulation (26). This suggests that HDL is also required for stress-induced adrenal glucocorticoid secretion in the human...
situation, similar to what was observed for mice in the current study. In humans, partial HDL deficiency or hypo-α-lipoproteinemia, defined as an age- and sex-adjusted plasma HDL cholesterol concentration below the 10th percentile, can be caused by a defect in several genes essentially involved in HDL metabolism, including ATP-binding cassette transporter A1, apoA1, lecithin-cholesterol acyltransferase, and phospholipid transfer protein (11, 20). To further establish the importance of HDL for stress-induced adrenal glucocorticoid synthesis in the human situation, it will be of interest to measure the cortisol response to synacthen/ACTH stimulation in carriers of functional mutations in these gene products.

In conclusion, we have shown that relative lipoprotein deficiency in mice as a result of protocol feeding is associated with decreased adrenal cortex cholesterol levels, a lower basal and stress-induced plasma glucocorticoid level, and an increased susceptibility to LPS-induced inflammation. Therefore, it is suggested that plasma lipoproteins are required for optimal adrenal steroidogenesis and protection against endotoxia in mice.

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DISCLOSURES
All authors have nothing to disclose.

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