Attenuated corticosterone response to chronic ACTH stimulation in hepatic lipase-deficient mice: evidence for a role for hepatic lipase in adrenal physiology

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Dichek, Helén L., Nalini Agrawal, Nazim El Andaloussi, and Kun Qian. Attenuated corticosterone response to chronic ACTH stimulation in hepatic lipase-deficient mice: evidence for a role for hepatic lipase in adrenal physiology. Am J Physiol Endocrinol Metab 290: E908–E915, 2006. First published December 20, 2005; doi:10.1152/ajpendo.00442.2005.—Hepatic lipase (HL), a liver-expressed lipolytic enzyme, hydrolyzes triglycerides and phospholipids in lipoproteins and promotes cholesterol delivery through receptor-mediated whole particle and selective cholesterol uptake. HL activity also occurs in the adrenal glands, which utilize lipoprotein cholesterol to synthesize glucocorticoids in response to pituitary ACTH. It is likely that the role of adrenal HL is to facilitate delivery of exogenous cholesterol for glucocorticoid synthesis. On this basis, we hypothesized that HL deficiency would blunt the glucocorticoid response to ACTH. Furthermore, because exogenous cholesterol also is derived from the LDL receptor (LDLR) pathway, we hypothesized that LDLR deficiency would blunt the response to ACTH. To test these hypotheses, we compared the corticosterone response to eight daily ACTH injections in HL-deficient (hl+/−), LDLR-deficient (Ldlr−/−), and HL− and LDLR doubly deficient (Ldlr−/− hl+/−) mice with that in wild-type (WT) mice. Plasma corticosterone levels were measured on days 2, 5, and 8. Differences in plasma corticosterone levels between genotypes were analyzed by Kruskal-Wallis one-way ANOVA on ranks and pairwise multiple comparisons by Dunn’s test. Our results demonstrate a trend toward reductions in plasma corticosterone levels on day 2 and significant reductions on day 5 and day 8 in the knockout models. Thus, on day 5, plasma corticosterone levels were reduced by 57, 70, and 73% (all P < 0.05) and on day 8 by 76, 59, and 63% (all P < 0.05) in hl+/−, Ldlr−/−, and Ldlr−/− hl+/− mice, respectively. These results demonstrate that HL deficiency, like LDLR deficiency, blunts the adrenal response to chronic ACTH stimulation and suggest a novel role for HL in adrenal physiology.

adrenal cholesterol; endothelial lipase; hydroxymethylglutaryl coenzyme A reductase; low-density lipoprotein receptor; steroidogenic acute regulatory protein

HEPATIC LIPASE (HL) IS A LIPOLYTIC ENZYME, produced by the liver, that hydrolyzes triglycerides and phospholipids in remnants, low-density lipoproteins (LDLs), and high-density lipoproteins (HDLs) (5, 20) to yield smaller particles. In humans, rats, and hamsters, the majority of HL is anchored by heparan sulfate proteoglycans to hepatocytes and endothelial cells of the liver sinusoids (24, 34) and is thereby positioned to facilitate cellular lipoprotein cholesterol delivery (25–27, 29). Also, in humans, rats, and hamsters, HL is produced exclusively in the liver; however, HL activity and protein also exist in the adrenal glands, which depend on cholesterol for steroid hormone synthesis (9, 21–23). Interestingly, HL is not expressed in the adrenal glands in these species (12, 36); however, HL is transported there on plasma lipoproteins (8). The presence of HL in the adrenal, taken together with its known lipoprotein processing function, suggests that HL may play a role in adrenal cholesterol delivery by facilitating the steroidogenic response to stress (7).

Unlike in humans, in whom the majority of HL binds to cell surfaces, in mice the majority of HL circulates (32). Immunohistochemical staining for mouse HL (using anti-rat HL antibodies that cross-react with mouse HL) demonstrated binding of HL to mouse adrenocortical cell surfaces (35). Additional evidence that HL exists in mouse adrenals is the presence of HL activity in mouse adrenal extracts that is suppressible by HL antibodies (35). We postulate that adrenal HL facilitates cholesterol delivery for steroid synthesis.

The cholesterol for adrenal steroid synthesis is derived from exogenous (~80%) and endogenous (~20%) sources (2). Exogenous cholesterol is derived from circulating lipoproteins, including LDL and HDL (31, 41). This was originally demonstrated in DBA/2J mice that were made lipoprotein deficient by treatment with 4-amino-pyrazolopyrimidine. In these mice, infusion of either labeled LDL or HDL restored the depleted adrenal cholesteryl ester levels (28). Also, adrenal uptake of labeled LDL and HDL increased in mice upon stimulation with pituitary adrenocorticotropic hormone (ACTH, which regulates corticosterone production) and decreased with suppression of ACTH secretion by dexamethasone (28). These studies demonstrate that both LDL and HDL serve as exogenous cholesterol sources for adrenal steroidogenesis in mice.

Exogenous cholesterol delivery requires intact lipoprotein uptake mediated by the LDL receptor (LDLR) and the HDL receptor-scavenger receptor (SR)-B1 pathways (40). The contribution of the LDLR pathway to adrenal steroidogenesis was examined in patients with abetalipoproteinemia, in whom LDL (a ligand for the LDLR) is absent due to absent apolipoprotein (apo)B. When subjected to continuous infusion of ACTH lasting 24–36 h, their excretion of urinary free cortisol (reflecting adrenal glucocorticoid synthesis) was substantially reduced (18). Similar studies in patients with homozygous familial hypercholesterolemia who lack functional LDLR and as a result have high plasma LDL levels (17) also demonstrated reduced excretion of urinary free cortisol. These two human
studies demonstrated the important roles of LDL and the LDLR pathway in providing the adrenal with cholesterol for steroid hormone synthesis.

The contribution of the HDL-SR-B1 pathway was demonstrated in apoA-1 null mice and in rats. In apoA-1 null mice, the lack of apoA-1, the major structural protein of HDL, results in HDL deficiency. These mice had reduced adrenal cholesterol and increased SR-B1 mRNA compared with control C57BL/6 mice (40). Also, in another study in rats, inhibition of HL activity by anti-HL antibodies increased SR-B1 mRNA (39). ACTH stimulation further increased SR-B1 mRNA during HL inhibition (39). These two studies in mice and rats demonstrated the important roles of HL and the SR-B1 pathway in providing the adrenal with cholesterol for steroid hormone synthesis (39, 40). In addition, these studies suggest that HL activity contributes to the SR-B1-mediated cholesterol uptake. Taken together, these in vivo data demonstrate that both LDL and HDL provide exogenous cholesterol for adrenal steroidogenesis.

In addition to exogenous cholesterol sources, the adrenal utilizes endogenous cholesterol sources for steroidogenesis. The endogenous cholesterol sources include cholesterol synthesized de novo from acetate in a pathway dependent on the rate-limiting enzyme hydroxymethylglutaryl (HMG)-CoA reductase (31). HMG-CoA reductase is upregulated by ACTH. The role that HL plays in the adrenal gland is not clear. Because HL mediates the transfer of cholesterol from lipoproteins into cells, and because cholesterol is the substrate for steroid synthesis, we reasoned that HL may facilitate the adrenal corticosterone response to ACTH. On this basis, we hypothesized that the absence of HL would lead to an attenuated adrenal corticosterone response to ACTH. Also, on the basis of studies demonstrating a role of the LDLR pathway (17, 18) in adrenal steroidogenesis, we hypothesized that the absence of LDL would also result in an attenuated response to ACTH in the mouse model. Finally, because the dual absence of HL and the LDLR would render both the SR-B1 and the LDLR pathways of cholesterol delivery less efficient or inactive, we predicted that the dual absence of HL and the LDLR would further attenuate the adrenal corticosterone response to ACTH. To test our hypotheses, we compared the adrenal response to chronic ACTH stimulation in gene-targeted HL-deficient (hl−/−), LDLR-deficient (Ldlr−/−), and doubly deficient (Ldlr−/− hl−/−) mice with that in wild-type (WT) mice.

MATERIALS AND METHODS

Experimental Animals

WT, hl−/− (16), and Ldlr−/− (19) mice were originally from Jackson labs and have been in our breeding pool for at least five generations. Ldlr−/− mice were bred with hl−/− mice, and the resulting offspring were bred with each other to yield mice that were homozygous for the gene-targeted LDLR and mouse HL genes (Ldlr−/− hl−/−) (10, 11). Homozygosity for the gene-targeted LDLR gene and mouse HL genes was determined by polymerase chain reaction (PCR) (11, 14). All mice were female. Mice were housed in a full-barrier facility with a 12:12-h light-dark cycle and fed regular mouse chow containing 4.5% fat (Picolab Rodent Diet 20, no. 5053; PMI Feeds, St. Louis, MO). All studies were approved by the Institutional Animal Care and Use Committees of the University of Washington and Children’s Hospital Oakland Research Institute, and all animal experimentation was conducted in accordance with accepted guidelines published in the NIH Guide for the Care and Use of Laboratory Animals.

Characterization of Plasma Lipids

Plasma was collected by orbital vein bleeding after a 4-h fast. For each genotype, pooled plasma from three mice was fractionated by fast-protein liquid chromatography (FPLC) to obtain lipoprotein profiles, (9, 11). Cholesterol and triglyceride concentrations in plasma and in FPLC fractions were measured by standard enzymatic assays (cholesterol, Abbott Clinical Chemistry, Abbott Park, IL; triglycerides, Roche/Hitachi Diagnostics, Indianapolis, IN). Lipoproteins were also separated by agarose gel electrophoresis (Titan gels; Helena Laboratories, Beaumont, TX). For each genotype, 2-μl of fast plasma from 4–5 mice were applied to two separate gels. One gel was stained with Fat Red 7B to visualize lipoproteins, and the stained gel was scanned and image analysis performed to facilitate comparison between genotypes. For image analysis, NIH Image version 1.63 (National Institutes of Health, Bethesda, MD) was used. Another agarose gel was subjected to Western blot analysis to visualize apoA-1 (11).

Study Protocol

All mouse experiments were performed between 10 AM and 12 noon to minimize variability in corticosterone levels due to diurnal secretory pattern. In preparation for the study, mice were habituated to handling for 5–10 min daily for 3–4 days. Each mouse served as its own control. The study consisted of a control (saline) and an experimental (ACTH) period (30). The saline period served as control for the corticosterone response to manipulation and handling. The experimental period examined the corticosterone response to chronic ACTH stimulation. Each period lasted 8 days. This length was chosen to deplete adrenal cholesteryl ester stores by the end of the ACTH period.

During the control period, mice received daily intraperitoneal injections of 150 μl of saline, and during the experimental period the same mice received daily intraperitoneal injections of 1 U of α1–24 ACTH (Cortrosyn, Organon) in 150 μl of saline. Plasma corticosterone was measured on days 2, 5, and 8 of each time period.

In separate experiments, in different mice, adrenals were harvested for RNA analysis and protein and lipid measurements on day 8 of the ACTH period.

Biochemical Measurements

Plasma corticosterone. Corticosterone concentrations were measured on plasma collected 45 min postinjection using the Coat-a-Count rat corticosterone RIA kit [Diagnostic Products, Thousand Oaks, CA (33)]. Intra- and interassay coefficients of variation were 8.3 and 14.9%, respectively. Samples from the same time point were measured in the same assay. For each time point, plasma from all four genotypes was assayed. Corticosterone values from the saline period represent the response to endogenous ACTH release in response to handling and manipulation, whereas the values from the experimental (ACTH) period represent the response to pharmacological levels of ACTH.

Adrenal protein. Four to eight adrenals were pooled and homogenized in a buffer containing 0.10 M sucrose, 0.05 M KCl, 0.04 M KH2PO4, and 0.03 M EDTA, pH 7.4, for protein extraction. Protein concentration was determined by the Lowry method using Protein Assay Kit no. P 5656 (Sigma Diagnostics, St. Louis, MO). The protein was used for Western blot analyses.

Adrenal lipid. Adrenal glands were harvested, and peripheral fat tissue was removed and discarded to ensure that only adrenal lipid was measured. For each genotype, three adrenal glands from three individual mice were pooled. Two sets of adrenal cholesterol mea-

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sures were done. Adrenal cholesterol was measured according to Folch (13) and cholesterol recovery determined as described by Brown (4), with minor modifications. Tissues were homogenized in 1 ml of chloroform-methanol (2:1, vol/vol) containing as an internal standard for recovery tracer amounts of [1-14C]cholesterol olate (2.2 × 10^8 dpm) in a 1.5-ml tissue grinder (Kontes-Dual) attached to a Fisher Dyna-mix homogenizer (Fisher Scientific). Aliquots of homogenates were taken for scintillation counting and for protein determination by Lowry’s method. Phase separation was accomplished by adding 0.5 ml of distilled water followed by 30 s of vigorous vortexing and centrifugation at 2,000 rpm (900 g) for 5 min. The lower, organic phase was washed with 0.75 ml of chloroform-methanol-water (15:240:235) and centrifuged at 2,000 rpm for 5 min. The washed lower phase was evaporated to dryness under nitrogen. The lipids were resuspended in 0.5 ml of PBS containing 0.5% vol/vol Triton X-100. Total cholesterol was measured in quadruplicate by standard enzymic assays (6) (total cholesterol, Abbott Spectrum). Cholesterol recoveries were calculated by dividing the radioactivity in the final sample by the radioactivity in the homogenate. Cholesterol concentrations were divided by the recovery to correct for loss during the extraction procedure. Free cholesterol was measured in duplicate using the Free cholesterol kit, (Wako Chemicals, Neuss, Germany). Cholesterol ester value was calculated by subtracting the free cholesterol value from total cholesterol values. All lipid values were corrected for protein concentration of the homogenate.

Adrenal Histology

In a separate experiment to determine whether deficiency of HL, LDLR, or both reduced adrenal cholesterol ester, adrenal sections were stained for Oil Red O (ORO) to visualize neutral lipids. Adrenals were flash-frozen in liquid nitrogen and embedded in optimal cutting temperature compound, and 10-μm sections were cut on a cryostat (Leica). Tissue sections were stained with ORO, and nuclei were counterstained with hematoxylin. Sections were photographed using a Nikon Coolpix camera and an inverted microscope (Leica DMIL). Red staining was quantitated using the NIH Image Program version 1.63f.

Western Blot Analyses of Adrenal Proteins

To determine the extent of ACTH-induced compensatory upregulation of proteins associated with lipoprotein processing, uptake, and steroidogenesis, we examined endothelial lipase (EL) [an extracellular triglyceride and phospholipase expressed in the adrenal (15)], SR-B1, LDLR, and steriodogenic acute regulatory protein (StAR) (37). Aliquots of adrenal protein extract were analyzed by Western blot. The same amount of protein was loaded into each well of a single gel. To ensure reproducibility, at least three Western blots were performed for each protein on the same protein extract. The same amount of protein (15–20 μg) was loaded onto each blot. For the LDLR Western, three gels were loaded with 20 μg and one gel with 30 μg. Equal loading was verified by Ponceau S staining of the Western blot membrane. Immunoblots were analyzed by densitometry on a GelDoc 2000 (Bio-Rad, Hercules, CA) using the Quantity One software package (Bio-Rad).

EL. Samples were electrophoresed through 12% SDS-PAGE and transferred to nitrocellulose followed by incubation with a 1:500 dilution of a rabbit polyclonal anti-mouse EL antibody (C18PEP4 no. 3, gift from Thomas Quertermous, Stanford University).

SR-B1. Samples were electrophoresed through 12% SDS-PAGE, followed by electrotransfer to nitrocellulose and incubation with a 1:1,000 polyclonal rabbit anti-SR-B1 antiserum (Novus Biologicals, Littleton, CO).

LDLR. Samples were electrophoresed through 7.5% SDS-PAGE and transferred to nitrocellulose followed by incubation with a 1:10,000 dilution of a rabbit polyclonal anti-human LDLR antibody (gift from Sandra Erickson and Janet Boyles, Univ. of California, San Francisco).

StAR. Samples were electrophoresed through 12% SDS-PAGE. After electrotransfer to nitrocellulose membrane, blots were incubated with a 1:1,000 dilution of rabbit polyclonal antiserum against StAR (gift from Dr. Douglas Stocco, Texas Tech University, Lubbock, TX).

Western blots were incubated with goat anti-rabbit biotinylated second antibody, and the respective proteins were visualized by ECL (Amersham Pharmacia Biotech).

Semiquantitative PCR Analysis of Adrenal HMG-CoA Reductase Expression

To establish the extent of compensatory upregulation of endogenous cholesterol synthesis in response to ACTH, HMG-CoA reductase expression was determined by semiquantitative PCR. Total RNA was isolated using the RNeasy Lipid Tissue minikit (Qiagen) with minor modifications. Tissue (3–4 mg) was homogenized in 0.5 ml of Qiazol reagent in a 1.5-ml microcentrifuge tube with a Bellco homogenization pestle and BioVortexer motor (Bellco Glass, Vineland, NJ). One microgram of total RNA was reverse transcribed (RT) at 42°C for 50 min using Sensiscript Reverse Transcriptase and oligo(dT) to yield the cDNA. For the HMG-CoA reductase PCR the following primers were used: HMG-1, 5′-TTC-GGC-TGC-ATG-TCA-ATG-TTG-G-3′ (nt 641–662, mouse HMG-CoA reductase gene, acc. no. BC019782); and HMG-2, 5′-TCA-TTA-GGT-CGT-GGC-TGC-ATG-G-3′ (nt 1338–1359, mouse HMG-CoA reductase gene). HMG-CoA reductase expression was standardized using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH was amplified using forward primer (GAPDH-F) 5′-CTG CCA TTT GCA GTG GCA AAG TGG-3′ and reverse primer (GAPDH-R) 5′-TTG TCA TGG ATG ACC TTC GCC AGG-3′. The HMG-CoA reductase and GAPDH cDNAs were amplified in a program consisting of 4 min at 94°C denaturation followed by 32 cycles of 20 s at 94°C denaturation, 30 s at 60°C annealing, 90 s at 72°C elongation, and ending with 5 min at 72°C elongation. The amplified product was separated on a 2% agarose gel containing ethidium bromide and visualized with ultraviolet transillumination. Gel bands were photographed on a GelDoc 2000 and relative densities assessed using the Quantity One software package.

Statistical Analyses

Results are presented as means ± SE. Plasma cholesterol and triglyceride concentrations were analyzed by unpaired t-test. For each time point, differences in corticosterone concentrations among genotypes were assessed by Kruskal-Wallis one-way analysis of variance on ranks. Pairwise multiple comparisons were by Dunn’s method. Paired t-tests were used to compare quantitation of Western blots for adrenal protein expression.

RESULTS

Plasma Lipid and Lipoprotein Concentrations

Compared with WT mice, cholesterol concentrations increased modestly in hl−/− mice (1.4-fold, P < 0.02), and markedly in Ldlr−/− and Ldlr−/−hl−/− mice (3- and 6-fold, respectively, both P < 0.0003; Table 1). Plasma triglyceride concentrations increased modestly in hl−/− and Ldlr−/− mice and markedly in Ldlr−/−hl−/− mice (Table 1).

Lipoprotein cholesterol. In WT mice, cholesterol distributed in a small LDL peak and a moderate HDL peak, whereas in hl−/− mice cholesterol mainly distributed in a large HDL peak (Fig. 1). In Ldlr−/− mice, cholesterol distributed in a minimal VLDL peak and moderate IDL/LDL and HDL peaks. In Ldlr−/−hl−/− mice, cholesterol distributed in a small VLDL peak and large IDL/LDL and HDL peaks.
Table 1. Plasma cholesterol and triglyceride concentrations

<table>
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<th>Genotype</th>
<th>Cholesterol</th>
<th>Triglyceride</th>
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<tr>
<td>WT</td>
<td>52±8</td>
<td>31±10</td>
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<tr>
<td>ht&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>73±10&lt;sup&gt;±&lt;/sup&gt;</td>
<td>47±12&lt;sup&gt;‡&lt;/sup&gt;</td>
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<tr>
<td>Ldlr&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>158±18&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>54±14&lt;sup&gt;‡&lt;/sup&gt;</td>
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<tr>
<td>Ldlr&lt;sup&gt;−/−&lt;/sup&gt;ht&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>299±32&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>113±18&lt;sup&gt;‡&lt;/sup&gt;</td>
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Values are means ± SE, concentrations in mg/dl. WT, wild type; ht<sup>−/−</sup>, hepatic lipase deficient; Ldlr<sup>−/−</sup>, LDL receptor deficient. *P < 0.02 vs. WT cholesterol conc.; †P < 0.0003 vs. WT cholesterol conc.; ‡P < 0.03 vs. WT triglyceride conc. §P < 0.001 vs. WT triglyceride conc.

Lipoprotein triglyceride. In WT mice, lipoprotein triglyceride occurred in a small VLDL peak and a minimal LDL peak, and in ht<sup>−/−</sup> mice triglycerides occurred in a moderate VLDL peak. In Ldlr<sup>−/−</sup> mice triglycerides occurred in small VLDL and LDL peaks, whereas in Ldlr<sup>−/−</sup>ht<sup>−/−</sup> mice triglycerides occurred in a large VLDL peak and a broad IDL/LDL peak. (Fig. 1).

HDL characterization. Pooled plasma HDL concentrations were 30 mg/dl in WT mice, 49 mg/dl in ht<sup>−/−</sup> mice, 34 mg/dl in Ldlr<sup>−/−</sup> mice, and 83 mg/dl in Ldlr<sup>−/−</sup>ht<sup>−/−</sup> mice. HDL triglyceride concentration ranged between 2 and 6 mg/dl in all genotypes. The increased HDL cholesterol in the ht<sup>−/−</sup> and Ldlr<sup>−/−</sup>ht<sup>−/−</sup> mice results from the absence of HL hydrolysis of HDL triglyceride and phospholipid. Agarose gel electrophoresis demonstrates increased Fat Red 7B staining in large HDL in ht<sup>−/−</sup> and Ldlr<sup>−/−</sup>ht<sup>−/−</sup> mice, also reflecting the absence of HDL hydrolysis (Fig. 2A). ApoA-1 Western blot analysis demonstrates apoA-1 in large HDL in ht<sup>−/−</sup> and Ldlr<sup>−/−</sup>ht<sup>−/−</sup> mice (Fig. 2B). Scanning of the Fat Red 7B-stained agarose gel illustrates the earlier onset of the HDL peak in ht<sup>−/−</sup> and Ldlr<sup>−/−</sup>ht<sup>−/−</sup> plasma, reflecting the lack of hydrolysis by endogenous HL (Fig. 2C).

Plasma Corticosterone Concentrations

Saline period. As expected, there were no consistent differences in plasma corticosterone responses to saline among the genotypes (Fig. 3, top).

ACTH period. On day 2, plasma corticosterone levels were moderately reduced in ht<sup>−/−</sup> and Ldlr<sup>−/−</sup> mice (by 30 and 37%, respectively) and markedly reduced in Ldlr<sup>−/−</sup>ht<sup>−/−</sup> mice (by 65%, P < 0.05; Fig. 3, bottom). The reduction in Ldlr<sup>−/−</sup>ht<sup>−/−</sup> mice was significantly different from that in WT mice (P < 0.05). On day 5, corticosterone levels were reduced by 57, 70, and 73% (all P < 0.05) in ht<sup>−/−</sup>, Ldlr<sup>−/−</sup>, and Ldlr<sup>−/−</sup>ht<sup>−/−</sup> mice, respectively. On day 8, corticosterone levels were reduced by 76, 59, and 63% (P < 0.05) in ht<sup>−/−</sup>, Ldlr<sup>−/−</sup>, and Ldlr<sup>−/−</sup>ht<sup>−/−</sup> mice, respectively. These findings demonstrate that loss of either or both HL or LDLR blunts the corticosterone production in response to chronic ACTH stimulation in mice.

Adrenal Protein Expression

EL protein expression. Because EL, like HL, hydrolyzes HDL phospholipids and triglycerides, it is possible that EL compensates for the lack of HL in ht<sup>−/−</sup> mice. Therefore, we analyzed adrenal EL expression by Western blot (Figs. 4 and 5). For each genotype, three separate Western blots were analyzed. In ht<sup>−/−</sup> mice, EL expression increased 1.7-fold (1.7 ± 0.2, P = 0.021). There were also trends toward an increase in EL in Ldlr<sup>−/−</sup> and Ldlr<sup>−/−</sup>ht<sup>−/−</sup> mice. The increase in EL in ht<sup>−/−</sup> mice and the trend toward an increase in Ldlr<sup>−/−</sup> and Ldlr<sup>−/−</sup>ht<sup>−/−</sup> mice may indicate an attempt at compensatory upregulation of EL in situations of reduced adrenal cholesterol uptake (including that seen in LDL receptor deficiency; Figs. 4 and 5).

SR-B1 expression. Because SR-B1 mediates adrenal cholesterol uptake and because SR-B1 expression is regulated by adrenal cholesterol content, we predicted a compensatory up-regulation of SR-B1 in ht<sup>−/−</sup>, Ldlr<sup>−/−</sup>, and Ldlr<sup>−/−</sup>ht<sup>−/−</sup> mice subjected to chronic ACTH stimulation. However, there were no significant differences in SR-B1 expression after ACTH stimulation (Figs. 4 and 5).

LDLR expression. Because LDL and the LDLR pathway also contribute to adrenal cholesterol delivery, we predicted that the LDLR would increase in the absence of HL to compensate for the lack of HL-facilitated cholesterol delivery. Instead, we uncovered an ~20% reduction (P < 0.03) in LDLR expression in ht<sup>−/−</sup> mice after chronic ACTH compared with control, WT mice (Fig. 4). As expected, there was no LDLR protein expression in Ldlr<sup>−/−</sup> or Ldlr<sup>−/−</sup>ht<sup>−/−</sup> mice.
StAR protein expression. Because StAR protein facilitates cholesterol delivery across the outer to the inner mitochondrial membrane, the rate-limiting step in adrenal steroidogenesis, we determined adrenal expression of StAR by Western blot analysis. The StAR-mediated process is upregulated by ACTH and is expected to be intact in all four genotypes. Accordingly, there were no significant differences in StAR protein expression, indicating that all four genotypes had intact mitochondrial membrane transport mechanisms (Figs. 4 and 5).

Adrenal HMG-CoA Reductase RNA Expression

Compared with WT mice, all knockout genotypes (hl^{-/-}, Ldlr^{-/-}, and Ldlr^{-/-} hl^{-/-}) demonstrated similar HMG-CoA reductase/GAPDH expression ratios (data not shown).

Adrenal Cholesterol Content

To assess the relationship between HL-facilitated exogenous cholesterol uptake and ACTH-stimulated corticosterone secretion, we measured the adrenal cholesterol content in two pools of three adrenals per genotype. All cholesterol determinations were corrected for recovery, which ranged between 30 and 60%. The average cholesteryl ester concentrations in lipid extracts from pooled adrenals were WT 39 μg/mg, hl^{-/-} 34 μg/mg, Ldlr^{-/-} 26 μg/mg, and Ldlr^{-/-} hl^{-/-} 24 μg/mg protein. The cholesteryl ester content was measured in two pooled samples, precluding statistical data analysis. In a separate experiment, adrenal cholesteryl ester content was visualized by ORO staining of frozen sections. Quantitative analysis of ORO-stained frozen sections revealed no differences in staining intensity among the four genotypes (Fig. 6). The adrenal free cholesterol content was 15–20% of total cholesterol content in all genotypes.

DISCUSSION

This study demonstrates that HL deficiency attenuates the adrenal corticosterone response to chronic ACTH stimulation,

Fig. 2. Agarose gel electrophoresis of pooled plasma from wild-type control (WT), hepatic lipase-deficient (hl^{-/-}), LDL receptor-deficient (Ldlr^{-/-}), and doubly deficient (Ldlr^{-/-} hl^{-/-}) mice. A: agarose gel stained with Fat Red 7B. Note reduced mobility and increased staining of HDL in hl^{-/-} and Ldlr^{-/-} hl^{-/-} plasma. B: apolipoprotein (apo)A-I Western blot analysis of agarose gel. Immunostaining was with a rabbit anti-mouse apoA-I antibody. Note that the absence of HL is associated with larger HDL particles in hl^{-/-} and Ldlr^{-/-} hl^{-/-} mice. C: scanned image of Fat Red 7B-stained agarose gel. Note the earlier onset of HDL peaks in hl^{-/-} and Ldlr^{-/-} hl^{-/-} plasma.

Fig. 3. Plasma corticosterone concentrations in response to saline (top) and ACTH (bottom) injected ip in WT, hl^{-/-}, Ldlr^{-/-}, and Ldlr^{-/-} hl^{-/-} mice. Each mouse first received 8 days of 150 μl of saline followed by 8 days of 1 U of α1-24 ACTH. Plasma was collected for corticosterone measurement 45 min postinjection on days 2, 5, and 8 of each period. For each time point, no. of mice measured is indicated in the bar. Results are presented as average ± SE. Statistical analysis of differences in corticosterone values between genotypes is by Kruskal-Wallis 1-way ANOVA on ranks. Pairwise multiple comparisons were done by Dunn’s method.
consistent with a role for HL in adrenal physiology. In addition, this study demonstrates that LDLR deficiency also attenuates the adrenal corticosterone response to chronic ACTH stimulation, consistent with a role for the LDLR pathway in adrenal physiology. There was no additional effect of double deficiency of both HL and the LDLR, consistent with an interdependence of HL and the LDLR pathway in adrenal cholesterol delivery. Taken together, these results support a role for HL and the LDLR to facilitate the adrenal corticosterone response to chronic ACTH stimulation (7).

HL may contribute to the adrenal corticosterone response through several different mechanisms that facilitate adrenal cholesterol delivery. First, HL-mediated hydrolysis of HDL phospholipids and triglycerides modifies particle composition and facilitates selective cholesterol uptake through the SR-B1 pathway (29). Second, hydrolysis of IDL and remnants modifies their composition and increases their affinity for SR-B1 for selective cholesterol uptake (1). Third, hydrolysis of remnants increases the number of exposed apoE molecules on the lipoprotein surface and increases the affinity for receptors, including the LDLR, for uptake (3). Fourth, hydrolysis of IDL forms LDL and provides LDL cholesterol for the LDLR pathway (5).

To determine whether the attenuated corticosterone response in the knockout models results from defective cholesterol supply (plasma lipoproteins) (2, 31, 41), cholesterol delivery (EL, SR-B1, and LDLR), or steroidogenesis (StAR), we examined plasma lipoproteins and protein markers of cholesterol delivery and steroidogenesis.

Analysis of plasma lipids and lipoproteins revealed mild to marked increases in plasma cholesterol that distributed in either or both LDL and HDL in all knockout genotypes. These results are compatible with an adequate supply of lipoprotein cholesterol for adrenal delivery and eliminates defective cholesterol supply as a cause for the attenuated adrenal corticosterone response to chronic ACTH stimulation.

Because HL activity may contribute to both SR-B1- and LDLR-mediated cholesterol delivery, we reasoned that lack of HL activity in HL deficiency would increase SR-B1 and LDLR protein expression. However, in HL-deficient mice, neither SR-B1 nor LDLR expression increased above that in WT mice, indicating a lack of compensatory upregulation of these pathways in response to chronic ACTH.

A similar lack of compensatory upregulation of SR-B1 and LDLR was observed in apoE-deficient mice, another model of defective adrenal cholesterol delivery (38). Thus, in apoE-deficient mice, long-term ACTH stimulation reduced adrenal cholesterol content (by ~30%) without compensatory upregulation of the LDLR or SR-B1 protein expression (38). Interestingly, in the apoE-deficient mice, plasma corticosterone response was actually increased compared with WT control mice. We think that the discrepancy in corticosterone results may be related to the different frequency of ACTH stimulation between the two studies. Alternatively, the discrepancy may relate to differences in genetic background in the two studies.

Because HL-mediated hydrolysis of lipoproteins optimizes them for receptor-mediated whole particle and selective cholesterol uptake, we reasoned that the related enzyme EL might
provide the necessary hydrolysis in the absence of HL. This reasoning is supported by a nearly twofold increase in adrenal EL protein. However, this increase did not raise corticosterone levels and suggests that EL plays only a minor role in adrenal cholesterol delivery. This is compatible with the previously reported low adrenal expression of EL compared with other tissues (15).

We also evaluated the mechanism for delivering cholesterol to the inner mitochondrial membrane for initiation of steroidogenesis by determining the adrenal expression of Sf-Ar protein. As expected, there were no differences in expression of Sf-Ar protein between the genotypes, consistent with an intact mechanism for initiation of steroidogenesis.

Because reduced adrenal cholesterol content may upregulate endogenous de novo cholesterol synthesis, thereby providing the substrate for increased corticosterone production, we determined HMG-CoA reductase expression levels. Interestingly, there was no evidence of compensatory upregulation of HMG-CoA reductase in any of our mouse models compared with control WT mice. This result again is compatible with the findings in apoE-deficient mice, in which long-term ACTH stimulation reduced adrenal cholesterol content without compensatory upregulation of HMG-CoA reductase activity (38). Although we measured HMG-CoA reductase by different methods, our results were similar. We believe that the failure of HMG-CoA reductase to upregulate in the face of increased demands for endogenous cholesterol production contributes to the attenuated corticosterone response to chronic ACTH stimulation.

Finally, we considered the possibility that the reduced corticosterone response to chronic ACTH stimulation might reflect increased clearance of corticosterone in the absence of HL. However, this would not account for the reduced corticosterone response in Ldlr<sup>−/−</sup> mice in whom the endogenous mouse HL is intact and is therefore a less likely explanation for the reduced corticosterone response to chronic ACTH in these models.

In summary, our results support a role for HL in the process of adrenal cholesterol delivery. Our results indicate that the role of HL is distinct from the roles of other adrenal proteins involved in cholesterol delivery (EL, SR-B1, and LDLR) and steroidogenesis (Sf-Ar) and demonstrate a novel role for HL as a physiological component of the adrenal response to chronic ACTH stimulation.

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