The LDL receptor is not necessary for acute adrenal steroidogenesis in mouse adrenocortical cells

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The LDL receptor is not necessary for acute adrenal steroidogenesis in mouse adrenocortical cells. Am J Physiol Endocrinol Metab 292: E408–E412, 2007. First published September 19, 2006; doi:10.1152/ajpendo.00428.2006.—Steroid hormones are synthesized using cholesterol as precursor. To determine the functional importance of the low density lipoprotein (LDL) receptor and hormone-sensitive lipase (HSL) in adrenal steroidogenesis, adrenal cells were isolated from control, HSL⁻/⁻, LDLR⁻/⁻, and double LDLR/HSL⁻/⁻ mice. The endocytic and selective uptake of apolipoprotein E-free human high density lipoprotein (HDL)-derived cholesteryl esters did not differ among the mice, with selective uptake accounting for >97% of uptake. In contrast, endocytic uptake of either human LDL- or rat HDL-derived cholesteryl esters was reduced 80–85% in LDLR⁻/⁻ and double-LDLR/HSL⁻/⁻ mice. There were no differences in the selective uptake of either human LDL- or rat HDL-derived cholesteryl esters among the mice. Maximum corticosterone production induced by ACTH or dibutyryl cyclic AMP and lipoproteins was not altered in LDLR⁻/⁻ mice but was reduced 80–90% in HSL⁻/⁻ mice. Maximum corticosterone production was identical in HSL⁻/⁻ and double-LDLR/HSL⁻/⁻ mice. These findings suggest that, although the LDL receptor is responsible for endocytic delivery of cholesteryl esters from LDL and rat HDL to mouse adrenal cells, it appears to play a negligible role in the delivery of cholesterol for acute adrenal steroidogenesis in the mouse. In contrast, HSL occupies a vital role in adrenal steroidogenesis because of its link to utilization of selectively delivered cholesteryl esters from lipoproteins.

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neutral cholesteryl ester hydrolase; corticosterone; cholesterol; receptor low density lipoprotein; selective pathway; hormone-sensitive lipase

ADRENAL STEROIDOGENESIS REQUIRES a constant supply of cholesterol as a precursor for conversion to steroid hormones. This cholesterol can potentially be derived from several different sources as follows (3, 14): 1) de novo cellular cholesterol synthesis, 2) circulating lipoprotein-derived cholesteryl esters obtained by either receptor-mediated endocytic or “selective” cellular uptake, and 3) the mobilization of stored cholesteryl esters via the actions of neutral cholesteryl ester hydrolase, which we have shown to be the result of the activity of hormone-sensitive lipase (HSL; see Ref. 18). Neither endogenous cellular cholesterol synthesis nor mobilization of stored cholesteryl esters is sufficient to support maximal steroid production, since hormone-stimulated steroid production is markedly augmented in the presence of lipoproteins (31). Lipoprotein-derived cholesteryl esters can be delivered by receptor-mediated endocytic uptake in which low density lipoprotein (LDL) or other apolipoprotein B- or apolipoprotein E-containing lipoproteins are processed via the LDL receptor (5) or can be delivered by selective uptake in which a variety of cholesterol-rich lipoproteins [high density lipoprotein (HDL) or LDL, regardless of apolipoprotein composition] bind to scavenger receptor class B type I (SR-BI) and release cholesteryl esters directly in cells without internalizing the lipoprotein particle itself (10, 19, 27, 32). Selective cholesteryl ester uptake appears to quantitatively deliver more cholesterol than endocytic uptake in hormone-stimulated rodent adrenal (4, 10, 19, 24, 26, 27, 32).

Once “selectively” delivered to the cell, cholesteryl esters must be hydrolyzed by nonlysosomal cholesteryl ester hydrolases before the free cholesterol can be transferred to mitochondria for steroid production (19). We have recently shown that HSL is functionally linked to the selective pathway and is critically involved in the intracellular processing and availability of cholesterol for adrenal steroidogenesis (17). Using HSL⁻/⁻ mice, we showed that basal (50%) and hormone-stimulated (75–85%) corticosterone production in the absence or presence of LDL or HDL was markedly reduced in isolated adrenocortical cells. Moreover, we showed that HSL was critical for the conversion of HDL cholesteryl esters into corticosterone by demonstrating that the conversion of HDL cholesteryl esters into corticosterone was reduced 97% in adrenal cells from HSL⁻/⁻ mice. Interestingly, the severity of the defect in hormone-stimulated corticosterone production observed in vitro in isolated adrenocortical cells was attenuated in vivo, although a defect was observed (17, 18). This attenuation was probably because of compensatory changes in the multiple pathways that can supply cholesterol requirements to the cell under physiological conditions. In particular, a threefold increase in the adrenal expression of LDL receptors was observed (17), suggesting a potential increase in the supply of cholesterol via receptor-mediated endocytic uptake.

The current studies were undertaken to test whether the absence of LDL receptors would exacerbate the defects observed in adrenal steroid production in HSL⁻/⁻ mice by eliminating this potential compensatory pathway. In addition, because we are not aware of any prior studies examining acute adrenal steroidogenesis in LDLR⁻/⁻ mice, we also examined the role of LDL receptors on lipoprotein-derived cholesteryl esters for corticosterone production in the mouse.

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MATERIALS AND METHODS

Chemicals. Reagents were obtained from the following sources: BSA (fraction V; Intergen, Purchase, NY); cholesterol oleate (Sigma Chemical, St. Louis, MO); [3H]cholesterol oleate, Na125I, and [3H]cholesterol oleoyl ether (Perkin-Elmer Life Sciences, Boston, MA); and chloroform, methanol, and heptane (J. T. Baker, Phillipsburg, NJ). All other chemicals were obtained from standard commercial sources.

Animals and isolation of culture of mouse adrenocortical cells. HSL+/− mice were generated as described previously and were backcrossed for at least six generations in a C57BL/6J genetic background (21). LDLR−/− mice in a C57BL/6J genetic background were purchased from Jackson Laboratories (JAX mice, Bar Harbor, ME). HSL−/− and LDLR−/− mice were interbred to yield HSL+/−/LDLR+/− mice, which were then mated to yield HSL+/−+/LDLR+/− and HSL−/−/LDLR−/− mice for studies. All animal experimentation was conducted in accord with accepted standards of humane animal care and was approved by the VA Palo Alto institutional committee on animal care.

Mouse adrenocortical cells were isolated from control (HSL+/−+/LDLR+/+), HSL−/−, LDL−/−, and double-HSLLDLR−/− mice by a procedure described previously (17). In brief, mice were killed by cervical dislocation, and the adrenal glands were aseptically removed and dissected free of fat. A group of 20 adrenals (from 10 mice) was finely minced with scissors, and tissue fragments were suspended in sterilized medium 199 containing 40 mg/ml of BSA, 3.7 mg/ml of insulin, 5 µg/ml transferrin, and 2 µg/cm2 fibronectin) plus other test substances, as specified under Figs. 1–5.

Preparation of lipoproteins. Human LDL, human apolipoprotein E-free HDL (HDL3), and rat HDL were isolated and characterized as previously described (25). For uptake and internalization studies, lipoproteins were modified with two nonreleasable labels, i.e., 125I-labeled dilactitol tyramine to mark lipoprotein proteins and [3H]cholesterol oleoyl ether to mark lipoprotein cholesteryl esters, as described previously (4).

Measurement of corticosterone secretion. To assay steroidogenesis, triplicate samples of freshly isolated cells were incubated without (basal) or with ACTH (1–24) (10 ng/ml) or Bt2cAMP (2.5 mM) for 3 h, and subsequently samples of incubation medium were frozen and stored until assayed for corticosterone. To examine lipoprotein-supported corticosterone production, isolated adrenocortical cells were cultured for 24 h ± Bt2cAMP (2.5 mM), ± human HDL3 (500 µg protein/ml), ± human LDL (100 µg protein/ml), or ± rat HDL (130 µg protein/ml), and after incubation the incubation media were collected, frozen, and stored frozen until analysis. Results are expressed as nanograms corticosterone produced per milligram cell protein and represent the means ± SE of duplicate determination of three different samples.

Analytic procedures. Protein was measured with a bichinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL). The procedure of Markwell et al. (20) was used to quantify protein content of human HDL3, human LDL, rat HDL, and doubly-labeled lipoprotein preparations. Cholesterol content of human HDL3, human LDL, rat HDL, and doubly-labeled lipoproteins was determined according to the procedure of Tercyak (30).

Statistics. Data are expressed as means ± SE. Statistical analyses were performed by ANOVA and comparisons among groups by Fisher’s Protected t-tests using SPSS software (SPSS, Chicago, IL) on a Power Macintosh computer.

RESULTS

To characterize the phenotype of the mice on the basis of the uptake of lipoprotein-derived cholesteryl esters, we measured total, LDL receptor-mediated (endocytic), and selective uptake using double-labeled lipoproteins ([125I]-labeled dilactitol-[3H]cholesterol oleoyl ether) in isolated adrenal cells. As shown in Fig. 1A, endocytic uptake of human HDL3-derived cholesteryl esters was extremely low (<3%) compared with selective uptake, consistent with the vast majority of HDL3 cholesteryl ester delivery occurring via SR-BI; however, there were no differences in either total, receptor-mediated “endo- cyt,” or selective uptake of HDL cholesteryl esters among control, LDLR−/−, HSL−/−, or double-LDLR/HSL−/− mice. For comparison (Fig. 1B), we measured total, receptor-mediated, and selective uptake of double-labeled human LDL ([125I]-labeled dilactitol-[3H]cholesterol oleoyl ether-human LDL). As opposed to HDL3, substantial amounts of LDL cholesteryl esters were derived from endocytic uptake (42% of total uptake in controls); yet selective uptake still accounted for the majority of cholesteryl ester delivery. There were no detectable differences in either total, receptor-mediated endocytic, or selective uptake of LDL cholesteryl esters between control and HSL−/− mice. In contrast, as would be expected from the absence of LDL receptors, total uptake of LDL cholesteryl esters was diminished equivalently in both LDLR−/− and double-LDLR/HSL−/− mice (34%). The decrease in total uptake of LDL cholesteryl esters reflected a 77–82% reduction (P < 0.01) in receptor-mediated endocytic uptake in both LDLR−/− and double-LDLR/HSL−/− mice, without any changes in selective uptake. Because the above studies of uptake of lipoprotein-derived cholesteryl esters were performed using human lipoproteins, for further comparison we measured total, receptor-mediated, and selective uptake of double-labeled rat HDL ([125I]-labeled dilactitol-[3H]cholesterol oleoyl ether HDL), which contains substantial amounts of apolipoprotein E. As shown in Fig. 1C, endocytic uptake of rat HDL-derived cholesteryl esters was greater than observed with human HDL3 (24% of total); however, selective uptake still accounted for the vast majority of HDL cholesteryl ester delivery. There were no detectable differences in either total, receptor-mediated endocytic, or selective uptake of LDL cholesteryl esters between control and HSL−/− mice. However, similar to the observations with LDL, receptor-mediated endocytic uptake was decreased 82–87% (P < 0.01) in both LDLR−/− and double-LDLR/HSL−/− mice, most likely reflecting apolipoprotein E-mediated uptake. Thus the genotypes of the mice resulted in the expected phenotypes for the mechanisms of lipoprotein-derived cholesteryl ester delivery.

Although LDL receptor-mediated endocytic uptake of cholesterol is known to support adrenal steroidogenesis (9), we are not aware of any prior studies examining adrenal steroidogenesis in LDL receptor knockout mice. Therefore, we compared steroid hormone production from adrenal cells isolated from LDLR−/− mice with adrenal cells from control and HSL−/− mice (Fig. 2). Basal (absence of ACTH) corticosterone production from adrenal cells was similar in control and LDLR−/− mice but, as seen previously, was reduced ~50% in HSL−/− mice (P < 0.02). Exposure of adrenal cells to ACTH (10 ng/ml) or Bt2cAMP (2.5 mM) increased corticosterone production ~10-fold in both control and LDLR−/− mice, but only ~3-
to 4-fold in HSL−/− mice. Therefore, corticosterone production induced by ACTH or Bt2cAMP was ~85–90% lower in adrenal cells from HSL−/− mice compared with either control or LDLR−/− mice (P < 0.02). Exposure of adrenal cells to HDL or LDL had no effect on corticosterone production in either control, LDLR−/−, or HSL−/− mice, but the combination of either ACTH or Bt2cAMP and HDL or LDL increased corticosterone production 18- to 25-fold in both control and LDLR−/− mice, but only ~7- to 10-fold in HSL−/− mice. Therefore, maximum corticosterone production induced by ACTH or Bt2cAMP and HDL or LDL was similar in control and LDLR−/− mice, but was ~80% lower in adrenal cells from HSL−/− mice (P < 0.005). Thus the absence of LDL receptors does not appear to impair lipoprotein-cholesteryl ester-supported murine adrenal steroidogenesis, whereas the absence of HSL severely interferes with steroid hormone production.

Fig. 1. Total, endocytic, and selective uptake of lipoprotein-derived cholesteryl esters by mouse adrenal cells from human apolipoprotein E-free high density lipoprotein (HDL; A), human low density lipoprotein (LDL; B), and rat high density lipoprotein (HDL; C). Adrenocortical cells were isolated from control, wild-type (WT), LDL receptor (LDLR) knockout (KO), hormone-sensitive lipase (HSL) KO, and HSL/LDLR KO mice as described in MATERIALS AND METHODS. Cells were incubated with 125I-labeled dilactitol-[3H]cholesteryl oleoyl ether-human HDL3 (A), 125I-labeled dilactitol-[3H]cholesteryl oleoyl ether-human LDL (B), or 125I-labeled dilactitol-[3H]cholesteryl oleoyl ether-rat HDL (C) for 24 h at 37°C. At the end of incubation, the cell samples were washed and then solubilized in 2 ml of 0.1 N NaOH. Aliquots (1 ml) were precipitated with an equal volume of 20% TCA to determine insoluble and soluble 125I radioactivity or extracted with organic solvents to determine 3H radioactivity. Endocytic uptake is calculated from TCA-soluble 125I label only. The difference between total and TCA-soluble activity is taken as the surface-bound 125I radioactivity. Because both 125I and 3H labels are on the same particle, the surface-bound 125I equals the total amount of 3H internalized. To calculate “selective” uptake of cholesteryl ester, soluble 125I radioactivity is subtracted from soluble 3H radioactivity. Results are means ± SE of 4 in each group. A P < 0.01 compared with control mice.

Fig. 2. Comparison of corticosterone secretion by cultured mouse adrenocortical cells. Adrenocortical cells were isolated from control, wild-type (WT), LDLR KO, and HSL KO mice as described in MATERIALS AND METHODS. Cells were incubated in the absence or presence of ACTH (10 ng/ml), Bt2cAMP (2.5 mM), HDL3 (500 µg protein/ml), LDL (100 µg protein/ml), or both ACTH (10 ng/ml) or Bt2cAMP (2.5 mM) and HDL3 (500 µg protein/ml) or LDL (100 µg protein/ml) for 24 h, and the amount of corticosterone secreted in the media was measured. Results are means ± SE of 3 independent experiments. A P < 0.02 compared with control mice. B P < 0.005 compared with control mice.
Because our previous studies with HSL 
−/−
mice showed a threefold increase in adrenal LDL receptor expression, which could provide a potential compensatory mechanism for maintaining corticosterone production in adrenal cells from HSL 
−/−
mice, we generated double-LDLR/HSL 
−/−
 mice for study. Corticosterone production following Bt2cAMP in the presence of or absence of lipoproteins was then examined in adrenal cells isolated from control, LDLR 
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, HSL 
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, or double-LDLR/HSL 
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 mice. As shown in Fig. 3, corticosterone production was identical between LDLR 
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 and double-LDLR/HSL 
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 mice, whether stimulated with Bt2cAMP in the absence or presence of human HDL3, human LDL, or rat HDL, and was ~80% lower than control. Moreover, there were no differences in corticosterone production between LDLR 
−/−
 and control mice under any conditions. Thus LDL receptor deficiency alone had no effect on steroid production, and the addition of LDL receptor deficiency to HSL deficiency did not impact the marked defect in steroid production observed with HSL deficiency alone.

**DISCUSSION**

Faust et al. (9) originally reported that LDL cholesterol was used by mouse Y-1 adrenal cells for steroid hormone production via a receptor-mediated pathway and that HDL did not provide cholesterol for steroid synthesis in these cells, thus first documenting the involvement of the LDL receptor in adrenal steroidogenesis. In contrast, other investigators reported that cholesterol was transferred into and converted into steroids in rat adrenals from HDL at a higher rate than from LDL (11–13). Thus it was apparent that LDL and HDL cholesterol uptake in the rat (and the mouse) adrenal occurred by separate mechanisms but that HDL cholesterol appeared to be the major substrate based on quantitative data of the kinetics (2). The importance of the LDL receptor for delivery of cholesterol in adrenal steroid production has been supported by studies in rabbits and humans. First, studies in Watanabe heritable hyperlipidemic rabbits, which lack LDL receptors, showed that ACTH-stimulated corticosterone production was reduced compared with normal rabbits, although basal corticosterone concentrations were normal (15). Studies of human fetal adrenals documented the importance of LDL receptors in mediating cholesterol delivery to the adrenal for steroidogenesis; in fact, the impact of adrenal LDL receptors is such that adrenal LDL receptor-mediated uptake of LDL for steroid production is a major determinant of circulating LDL concentrations in the fetus (6). Nonetheless, subjects with homozygous familial hypercholesterolemia who lack LDL receptors were found to have a normal cortisol response to an acute injection of ACTH (1). In addition, plasma cortisol concentrations were also normal during prolonged infusions of ACTH in homozygous familial hypercholesterolemics with typical LDL deficiency (23). Moreover, SR-BI-mediated cholesteryl ester uptake from HDL (22, 29) and LDL (28) into adrenal cells. The absence of SR-BI results in an 85–90% reduction in cholesteryl ester uptake from HDL (22). This is similar to the effect observed with apolipoprotein AI deficiency, where adrenal cholesteryl esters are ~95% depleted along with a reduction in basal and ACTH-stimulated corticosteroids (20). Moreover, SR-B1-mediated selective cholesteryl ester uptake appears to quantitatively deliver more cholesterol than endocytic uptake in hormone-stimulated rodent adrenals (4, 10, 19, 24, 26, 27, 32). The cholesteryl esters that are selectively delivered to adrenocortical cells are hydrolyzed by HSL to unesterified cholesterol before transfer to mitochondria for steroid production (7, 18). Using...
isolated adrenocortical cells from HSL−/− mice, we previously showed that HSL is critically involved in the intracellular processing and availability of cholesterol derived from intracellular stores of cholesteryl esters and from lipoprotein-derived cholesteryl esters for adrenal steroidogenesis by demonstrating both a marked defect in corticosterone production under basal and hormone-stimulated conditions and a marked defect in the conversion of HDL cholesteryl esters into corticosterone (17). The severity of this defect in steroid production in vitro was attenuated in vivo and appeared to be due, at least in part, to a threefold upregulation of LDL receptor expression in the adrenals of HSL−/− mice (17). Thus we reasoned that the addition of LDL receptor deficiency to HSL deficiency would create a scenario whereby adrenal steroid production would be severely impaired, because of disruption of the supply of free cholesterol through loss of the following three sources of cholesterol: 1) receptor-mediated endocytic uptake, 2) utilization from selective uptake, and 3) mobilization of stored cholesteryl esters. Surprisingly, the addition of LDL receptor deficiency to HSL deficiency did not impact the marked defect in steroid production observed with HSL deficiency alone.

In conclusion, although the LDL receptor is responsible for receptor-mediated endocytic delivery of cholesteryl esters from apolipoprotein B (LDL)- and apolipoprotein E-containing lipoproteins (rat HDL) to mouse adrenal cells, it appears to play a negligible role in the delivery of cholesteryl esters for acute adrenal steroid hormone production in the mouse. In contrast, HSL occupies a vital role in adrenal steroidogenesis because of the requirement for its action on cholesteryl esters selectively delivered from lipoproteins via SR-BI to be efficiently utilized. (11–13)

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