ASBMB
JOURNAL OF LIPID RESEARCH

Expression and microvillar localization of scavenger receptor class B, type I (SR-BI) and selective cholesteryl ester uptake in Leydig cells from rat testis

Eve Reaven,[1] Lichun Zhan, Ann Nomoto, Susan Leers-Sucheta, and Salman Azhar

Geriatric Research, Education and Clinical Center, VA Palo Alto Health Care System, Palo Alto, CA 94304

Abstract This study investigates the relationship between the high density lipoprotein (HDL) receptor (scavenger receptors, SR-BI and SR-BII), selective lipoprotein-cholesteryl ester uptake, and testosterone production in Leydig cells of control, hypocholesterolemic and gonadotrophic hormone (hCG) treated rats. Leydig cells from mature control rats show poor efficiency in incorporation of labeled HDL-cholesteryl esters into testosterone, poor selective uptake of lipoprotein lipids overall, and a dramatic reduction of circulating levels of lipoproteins has no apparent effect on testosterone production or expression of intracellular enzymes synthesizing cholesterol. Leydig cells from control rats show minimal levels of SR-BI and SR-BII. However, similarly aged rats treated with hCG for several days undergo changes consistent with hormone-desensitization. Despite the resulting low levels of testosterone production, SR-BI levels are dramatically increased, Leydig cells now efficiently internalize HDL-supplied cholesteryl esters by the selective cholesterol uptake process, and various other cholesterol-sensitive genes of the cells are up-regulated. Only SR-BII expression remains negligible and unchanged throughout this period. It is of interest that Leydig cell SR-BI of hCG-treated rats is localized in surface microvilli, but is present also in an elaborate and complex channel system within the cytoplasm of the cells. In summary, Leydig cells differ from other rat steroidogenic cells in not depending on exogenous lipoprotein-cholesterol during periods of normal steroid hormone production. However, trophic hormone desensitization is accompanied by increased Leydig cell SR-BI expression and increased selective HDL-cholesteryl ester uptake, presumably in preparation for renewed testosterone production.—Reaven, E., L. Zhan, A. Nomoto, S. Leers-Sucheta, and S. Azhar. **Expression and microvillar localization of scavenger receptor class B, type I (SR-BI) and selective cholesteryl ester uptake in Leydig cells from rat testis.** ***J. Lipid Res.*** **2000.** 41: **343–356.**

Supplementary key words cholesterol • HDL • HDL receptor • testosterone production • testicular interstitial cells • hypocholesterolemia • gonadotropins • HMG-CoA reductase • microvilli • BODIPY-cholesteryl esters

Recent reports on the unexpectedly high expression of high density lipoprotein (HDL) scavenger receptor isoforms, SR-BI and SR-BII in rat testicular tissue (1–3) has reopened the issue of whether Leydig cells, like other steroidogenic cells of the rat (4–15), utilize exogeneous (blood-borne) lipoprotein-derived cholesteryl esters (CEs) as a source of cholesterol in hormone production, or whether Leydig cells prefer to synthesize their own cholesterol, as needed, for the production of testosterone.

A review of the literature on testicular use of lipoprotein-supplied cholesterol for testosterone production in the rat reveals findings that vary with the experimental conditions used, e.g., the use of in vivo (10, 16–18) or in vitro (18–27) techniques, dispersed interstitial cells [crude (20–22), or purified (23–27)], the dose and mode of administration of trophic hormones, LH or hCG [i.e., acute (19–21, 27), semi-chronic (23, 25), chronic (24, 26)] or even Bt_2-cAMP (21, 25), the use of agents such as 4-aminopyrazolo-[3,4 *d*]-pyrimidine (4-APP) to lower circulating levels of serum cholesterol (21, 24) or, finally, the supply of lipoproteins either in vitro or in vivo (18–27) to deliver exogenous cholesterol. Overall, the evidence indicates that under normal physiological conditions, the rat testis does not require exogenous lipoprotein-cholesterol for Leydig cell testosterone production (4, 8, 10), that a single, super-physiological injection of trophic hormone LH or hCG renders the testis desensitized for a period of time with lowered binding of trophic hormone and decreased testosterone production (28–30), but that sustained administration of trophic hormones for several days or longer can overcome the desensitization process with renewed steroidogenesis (24, 30–33) with, or without, normalized levels of trophic hormone binding (30). The question arises as to what extent the scavenger receptor proteins, SR-BI or SR-BII, are related to or mediate these changes.

In the rodent ovary (34–38), adrenal (34, 35, 39, 40), and in SR-BI transfected cells (34), SR-BI has been shown to recognize HDL and to be associated with 'selective'

Abbreviations: HDL, high density lipoprotein; CE, cholesteryl ester; hCG human chorionic gonadotropin; RT, reverse transcriptase; PCR, polymerase chain reaction.

[1] To whom correspondence should be addressed.

Downloaded from www.jlr.org by guest, on June 14, 2012

lipid uptake from HDL (34, 37), effectively stripping CEs from HDL particles at the target cell surface (37). In so doing, HDL-derived CEs are internalized by the cells (leaving the remaining HDL particle outside); these CEs may be directly used by the cell in steroidogenesis (37, 41, 42) or stored within the cell in the form of lipid droplets (37, 41, 42). In general, SR-BI protein expression in steroidogenic tissues has been shown to be regulated by physiological conditions that alter cholesterol metabolism and cell requirements for cholesterol (35, 36, 38, 39). Some evidence suggests that in addition to mediating the selective uptake of CE from HDL, SR-BI may also mediate cellular cholesterol efflux (43). A recent study has shown that the semi-chronic administration of hCG to rats induces a dramatic increase in SR-BI in Leydig cells of the testes and suggests that such cells may also utilize lipoprotein-derived CEs (1).

Recently, a variant of SR-BI (termed SR-BII) has been found to be expressed in mice and humans (2, 3). The SR-BI and SR-BII variants are identical except for the region encoding the C terminal domain. The SR-BII transcript is relatively abundant in tissues known to express SR-BI and represents ~30% of total mRNA SR-BI values in mouse adrenal, but about 80% of total SB-BI values in mouse testes (2, 3): note, conventional Northern blotting does not distinguish between SR-BI and SR-BII. SR-BII appears to mediate selective CE uptake from HDL (as well as HDL-dependent cholesterol efflux from cells), though with ~4-fold lower efficiency than SR-BI.

The present study examines these issues in some detail. We ask whether the process of Leydig cell testosterone production utilizes lipoprotein-derived cholesterol, and if so, under what physiological conditions. We ask whether the acquisition of lipoprotein CE is mediated by the selective CE uptake pathway, and whether the process is related to the expression of SR-BI or its variant, SR-BII, in Leydig cells. And we describe the localization of SR-BI and SR-BII in cells. As Leydig cells comprise only 2–3% of the normal testis, biochemical and molecular studies must be carried out on preparations of isolated Leydig cells. However, to determine the localization of HDL receptor proteins, isolated Leydig cells as well as Leydig cells from intact testes have been viewed.

The study begins with a time course outlining the consequences of acute and chronic trophic hormone administration on testosterone production under the experimental conditions of our study, and key time points have been chosen to answer all subsequent questions.

METHODS

Materials

Iodine-^{125}I radionucleotide (carrier free, ~629 GBq/mg; ~17 Ci/mg), [1β, 2β-^{3}H (N)]testosterone, and [cholesteryl-1,2,6,7-^{3}H(N)]cholesteryl oleate (2.22–3.70 TBq/mmol; 40–60 Ci/mmol) were purchased from NEN© Life Science Products, Boston, MA. [1α,2α(N)-^{3}H]cholesteryl oleolyl ether (1.78 TBq/mmol; 48.0 Ci/mmol) was obtained from Amersham, Arlington Heights, IL. The following chemicals were supplied by Sigma Chemical Co, St. Louis, MO: cholesteryl oleate, cholesterol, testosterone, bovine plasma fibronectin, egg phosphatidylcholine, bovine brain sphingomyelin, triolein, insulin, transferrin, crude human chorionic gonadotropin (hCG), poly-d-lysine, and 8-bromo-cAMP. Cholesteryl BODIPY FL C12® (BODIPY-CE) was purchased from Molecular Probes, Eugene, OR. Purified hCG (CR-121; biological potency, 13,450 IU/mg) was kindly provided by Dr. R.E. Canfield (College of Physicians and Surgeons of Columbia University, New York, NY), through the center of Population

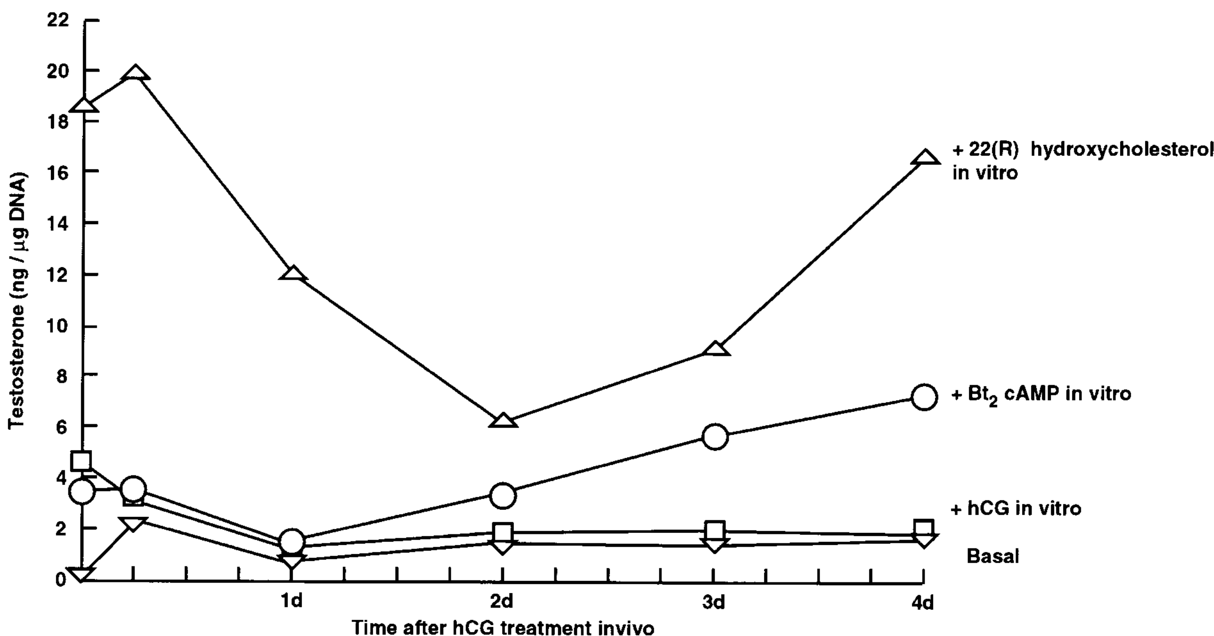


Fig. 1. Time course of testosterone production. Leydig cell-enriched interstitial cell preparations were obtained from rats given in vivo saline or hCG (50 IU) every day for 4 consecutive days. Testes were removed at 6 h or at 1, 2, 3, and 4 days of hCG treatment. Cells isolated at these time points were incubated with medium alone (basal), hCG, Bt_2cAMP, or 22(R) hydroxy-cholesterol for 4 h before samples were taken for measurement of testosterone production by RIA.

Downloaded from www.jlr.org by guest, on June 14, 2012

Research, NICHHD, NIH, Bethesda, MD. Anti-SR-BII serum was generously supplied by Dr. Deneys R. van der Westhuyzen, University of Kentucky Medical Center, Lexington, KY. All other reagents used were of analytical grade.

Animals and hormonal (gonadotropin) and 4-APP treatment

Sprague-Dawley male rats (~3 months of age) were purchased from Charles River Laboratories, Bloomington, IN. The animals were injected (sc) with either normal saline (control rats) or 50 IU of hCG (hCG-treated rats) daily for up to 4 days. To induce hypocholesterolemia, some animals were treated (ip) with 4-aminopyrazolo-3, 4 [d] pyrimidine (4-APP; 20 mg/kg BW, every 24 h for 3 days). The animals were killed by decapitation and the testes were removed and placed in medium 199 supplemented with 1 mg/ml bovine serum albumin, plus penicillin (100 U/ml) and streptomycin (100 μg/ml).

Isolation, purification and culture of Leydig cells

Crude interstitial cells (containing 10–15% Leydig cells) were prepared by collagenase treatment of decapsulated testes as described previously (21, 44). Pure preparations of Leydig cells were obtained using a continuous iso-osmotic Percoll gradient centrifugation (44). The percentage of the Leydig cells in each preparation was examined by staining for 3β-hydroxysteroid dehydrogenase activity as described by Payne, Downing, and Wong. (45). The purified Leydig cells were maintained at 37°C for up to 24 h in a basal medium (46) [(DME: F12, 1:1) supplemented with HEPES (15 mm), bovine serum albumin (1 mg/ml), insulin (2 μg/ml), transferrin (5 μg/ml), fibronectin (2 μg/cm^2), penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (20 μg/ml)].

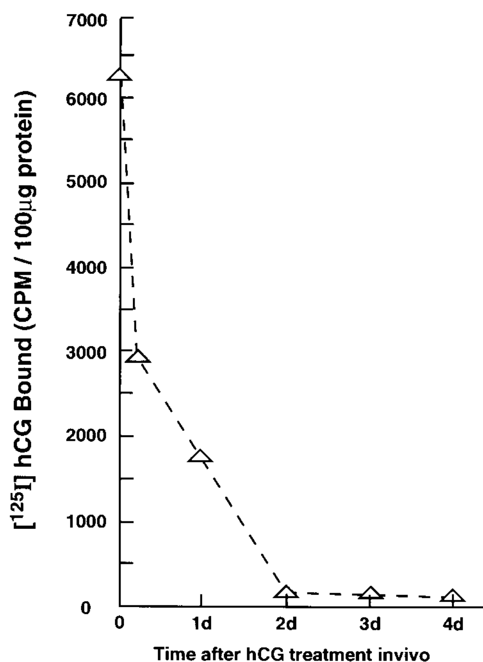
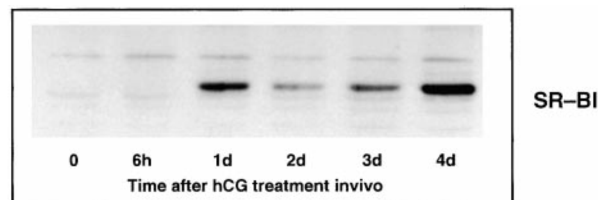


Fig. 2. Time-dependent effect of hCG administration on gonadotropin receptor (hCG) binding activity in testicular interstitial cell membranes. The crude membrane preparations from Leydig cell-enriched interstitial cell preparations of Fig. 1 were incubated with a saturating concentration (300 pm) [^{125}I]hCG in the presence or absence of excess unlabeled hCG (12.5 IU/ml) at 22°C for 3 h to monitor gonadotropin receptor binding activity. Other details were the same as described under Experimental Procedures. The results are a mean of two separate experiments.



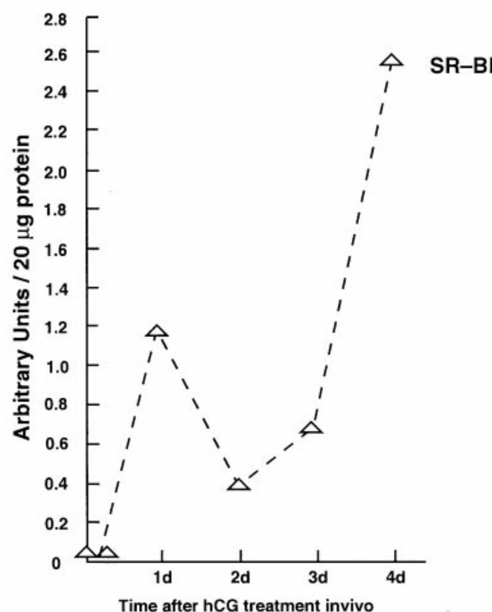


Fig. 3. Time-dependent affect of hCG administration on SR-BI expression. Leydig cell-enriched interstitial cell preparations of Fig. 1 were used for Western blot analysis of SR-BI protein expression. Immunoreactive bands are shown in top panel (arrow) and quantitative scanned data of blots are presented in graph form (lower panel).

Measurement of testosterone secretion

Freshly isolated interstitial cells (~2 × 10^6 cells), purified Leydig cells (~2 × 10^5 cells) or cultured purified Leydig cells (~2 × 10^5 cells) were incubated in incubation medium (1 ml) containing $CaCl_2$ (2.5 mm), 1 methyl-3-isobutylxanthine (IBMX, 0.1 mm) and ± purified hCG (10 ng/ml) or freely diffusible 22(R) hydroxycholesterol (10 μm). After incubation for 3 h at 37°C, the testosterone concentration in cells plus medium was determined by radioimmunoassay (44).

Gonadotropin receptors (hCG binding activity)

The gonadotropin receptor concentration was determined using a saturation concentration of ^{125}I-labeled hCG (300 pm) in the presence or absence of an excess of unlabeled hCG. Bound and free hormone was separated and specific binding was calculated as described previously (47, 48).

Lipoprotein preparation

Apo E-free high density $hHDL_3$ were isolated as described previously (11, 13, 41, 42). These human-derived lipoproteins were used exclusively because they are not recognized by the LDL-mediated 'endocytic pathway'. For uptake and internalization studies, $hHDL_3$ preparations were conjugated with residualizing labels, i.e., ^{125}I-labeled dilactitol tyramine ([^{125}I]DLT) and [^{3}H]cholesteryl oleolyl ether ([^{3}H]COE) (11, 13). For fluorescence microscopy, reconstituted (rec) cholesteryl *BODIPY*-HDL particles were prepared as recently described from this laboratory (41, 42). In addition, rec[^{3}H]cholesteryl oleate-$hHDL_3$ (40,

Downloaded from www.jlr.org by guest, on June 14, 2012

41) were used to demonstrate the conversion of lipoprotein-derived cholesteryl ester into testosterone.

Uptake and internalization of lipoprotein-derived cholesteryl esters

For these experiments, cultured Leydig cells were incubated ± hCG (10 ng/ml) and $hHDL_3$ equipped with radiolabeled, non-releaseable apolipoprotein ([^{125}I]DLT) and cholesteryl ester ([^{3}H] COE) tags that would accumulate within the cells even when degraded (11, 13, 41, 42). Incubations were carried out with [^{125}I]DLT–[^{3}H]COE–$hHDL_3$ (35 μg/ml) ± hCG (10 ng/ml) for 6–24 h at 37°C. At the end of incubation, the dishes were processed for the determination of trichloroacetic acid-insoluble and -soluble [^{125}I] and total ^{3}H radioactivities, as described previously (11, 13).

Endocytic uptake is calculated from TCA-soluble ^{125}I label only. The difference between total and TCA-soluble radioactivity is believed to be surface associated ^{125}I radioactivity. Because ^{125}I and ^{3}H labels are on the same particle, the surface bound ^{125}I is also equal to surface bound ^{3}H. Thus, total ^{3}H minus surface bound ^{3}H equals the total amount of ^{3}H internalized. To calculate 'selective' uptake of cholesteryl ester (CE), soluble ^{125}I radioactivity is subtracted from soluble ^{3}H radioactivity. Finally, to calculate mass of CE internalized, these values are divided by protein:cholesterol ratio of $hHDL_3$ (i.e., 2.73).

Utilization of lipoprotein derived CE in testosterone production

To demonstrate that rat Leydig cells can utilize lipoprotein-derived cholesteryl ester for steroid production, Leydig cells (~2 × 10^5) were incubated with [^{3}H]cholesteryl oleate (CO)-$hHDL_3$ or [^{3}H]CO-[^{14}C]free cholesterol (FC)–HDL (100 μg/ml) ± hCG (10 ng/ml) for 24 h, after which cells and media were extracted with chloroform–methanol 2:1, and the organic phase was assayed for testosterone production by thin-layer chromatography using hexane–diethyl ether–methanol 70:20:5 (v/v/v) as a mobile phase. Results are expressed as DPM of ^{3}H label or ^{3}H/^{14}C labels incorporated into testosterone (49).

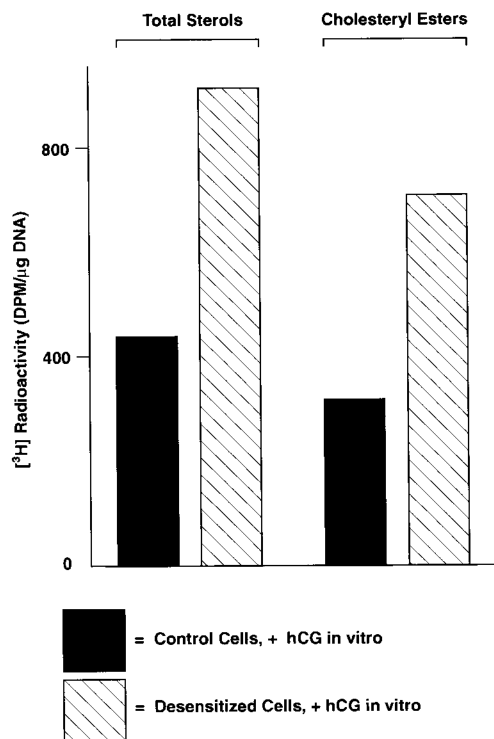


Fig. 4. Uptake of radiolabeled HDL-cholesteryl oleate by Leydig cells. Percoll-purified Leydig cells from control and 4d-hCG-treated rats were incubated with HDL labeled with [^{3}H]cholesteryl oleate and hCG in vitro for 24 h, then measured for cholesteryl ester, free cholesterol, and total sterol uptake after extraction with organic solvents followed by TLC.

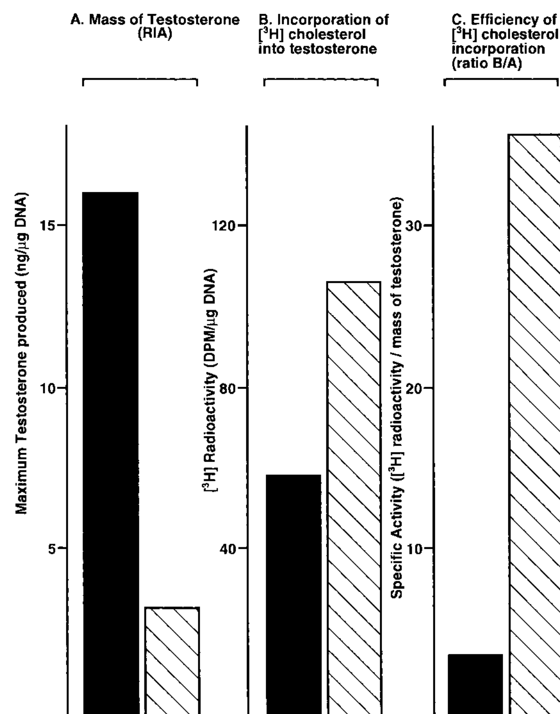


Fig. 5. Utilization of HDL-derived cholesteryl oleate in testosterone production by Leydig cells. Purified Leydig cells as in Fig. 4 were incubated with [^{3}H]cholesteryl oleate and hCG in vitro for 24 h, after which the cells and media were extracted with chloroform–methanol and the organic phase was assayed for mass of testosterone produced by RIA (panel A), incorporation of [^{3}H]cholesterol into testosterone by TLC (panel B), and efficiency of [^{3}H]cholesterol incorporation ([^{3}H]radioactivity/mass of testosterone produced) (panel C). □, control + hCG; (▨), 4d-hCG + hCG.

Fluorescence microscopy of HDL-derived CEs

For fluorescence microscopy of internalized BODIPY-CE, Leydig cells were grown on 25-mm-diameter glass coverslips coated

TABLE 1. Effect of 4-APP treatment on testosterone secretion by purified Leydig cells

	Testosterone Secretion	
Addition	Untreated Control	4-APP
	ng/μg DNA	
Basal	0.61 ± 0.12	0.45 ± 0.09
+hCG	22.00 ± 3.10	26.00 ± 5.60

Aliquots of Leydig cells (~2 × 10^5) from control and 4-APP treated rats (20 mg/kg BW, every 24 h for 3 days, i.p.) were incubated with or without hCG (10 ng/ml) plus 0.1 mm 1-methyl-3-isobutylxanthine (IBMX). After incubation for 3 h at 37°C, the testosterone concentration in the medium plus cells was determined by radioimmunoassay. Results are means ± SE of four separate experiments.

Downloaded from www.jlr.org by guest, on June 14, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR

TABLE 2. Rates of incorporation of endogenously synthesized cholesterol [^{14}C]acetate-labeled sterols and HDL derived [^{3}H]-cholesteryl oleate into secreted testosterone by Leydig cells from control and 4d-hCG-treated rats

	[^{3}H]Cholesteryl Oleate		[^{14}C]Acetate	
	Total Cholesterol	Testosterone	Total Cholesterol	Testosterone
	dpm/μg DNA		*dpm/μg DNA*	
Control	358.5 ± 72.5	28.0 ± 6.7	11783 ± 1577	825 ± 236
4d-hCG	1343.0 ± 179	195.0 ± 30	29367 ± 6055	74 ± 8

Leydig cells from control and 4d-hCG-treated rats were incubated with [^{14}C]acetate + HDL labeled with [^{3}H]cholesteryl oleate + hCG in vitro for 24 h. The cells and medium were extracted with organic solvents and, after TLC, the incorporation of ^{3}H/^{14}C, into testosterone was quantified by liquid scintillation counting. The results are expressed as a mean (dpm/μg DNA) ± SE of four separate experiments.

with fibronectin. After washing, cells were incubated ± hCG and/or rec HDL-BODIPY-CE (50 μg/ml) at 37°C for 24 h and processed for confocal fluorescence microscopy (42).

SR-BI and SR-BII antibodies

Polyclonal antibodies raised against peptides to the carboxy terminus of mouse SR-BI (amino acids 489–509: AYSESLMSP AAKGTVLEQEAKL (36, 38) and SR-BII (amino acids 491–506: PLLEDSLSGQPTSAMA (2) were prepared and characterized as described previously (3, 36, 38).

Polyacrylamide gel electrophoresis and immunoblot analysis

Aliquots of interstitial or Leydig cell lysates (25–50 μg protein) containing equal protein mass were resolved by SDS-polyacrylamide gel and the proteins were electrophoretically transferred onto immobilon PVDF transfer membranes. The membranes were subsequently blocked, probed with polyclonal anti-SR-BI or SR-BII followed by reaction with peroxidase-conjugated secondary antibody (36, 38). After extensive washing, chemiluminescent substrate was added (Amersham, Arlington Heights, IL), the radiographic chemiluminescence was detected at various times (1–10 min), and appropriate films were subjected to densitometric scanning.

Identification of SR-BI and SR-BII by immunofluoresence

For immunofluorescent confocal microscopy of SR-BI and SR-BII, Leydig cells were grown on fibronectin-coated glass coverslips under basal conditions. After the appropriate treatment, cells were washed in phosphate-buffered saline (PBS), fixed 10 min in 4% paraformaldehyde, washed in PBS, treated with ethanolamine, permeabilized with 0.2% Triton X-100, placed in normal goat serum and 5% non-fat dry milk in PBS, for 1 h at 37°C, and incubated with anti-SR-BI or SR-BII (1:1000–1500) at 4°C overnight. Subsequently, the cells were washed free of antibody and incubated at room temperature in a buffer containing biotinylated goat anti-rabbit IgG (1 h), followed by FITC-avidin for 1 h. After washing, coverslips were mounted on slides using Fluoromount G (Fisher Scientific, Pittsburgh, PA) and viewed by confocal fluorescent microscopy (Cell Science Imaging Facility, Stanford University, Stanford).

TABLE 3. Incorporation of HDL-derived [^{3}H]cholesteryl oleate ([^{3}H]CO) and [^{14}C]free cholesterol ([^{14}C]FC) into testosterone by Leydig cells from control and 4d-hCG-treated rats

Conditions	Incorporation into Testosterone: [^{3}H]CO	Incorporation into Testosterone: [^{14}C]FC	^{3}H/^{14}C Ratio
	dpm/μg DNA		
Control	41.25 ± 8.8	4.75 ± 1.19	8.68
4d-hCG	169.50 ± 26.0	15.80 ± 4.8	10.73

Leydig cells from control and 4d-hCG-treated rats were incubated with doubly labeled ([^{3}H]CO and [^{14}C]FC HDL and hCG in vitro for 24 h and incorporation of ^{3}H/^{14}C, labels into testosterone was measured by TLC. The result are mean ± SE of four separate experiments.

Immunoelectron microscopy

For immunoelectron microscopic localization of SR-BI, intact testes of control and 4d-hCG-treated rats were perfused for 30 min with 4% paraformaldehyde-0.5% glutaraldehyde; the tissue was diced into tiny cubes and fixed for 3–5 h longer in the original fixative before being placed in 4% paraformaldehyde overnight. Tissue blocks were processed and embedded in LR gold resin (Ted Pella Co., Redding, CA) using techniques described by Berryman and Rodewald (50). Ultrathin sections on grids were blocked with 5% normal goat serum (1 h, room temperature), incubated with primary antibody (SR-BI, 300 μg protein/ml) or pre-immune IgG 1–2 h at room temperature, and labeled with goat anti-rabbit IgG–10 nm gold (EM Sciences, Fort Washington, PA) for 1 h at room temperature. Finally, the sections were stained with osmium vapor and lead citrate and viewed with the electron microscope (38).

Isolation of total RNA and reverse transcription (RT) polymerase chain reaction (PCR) analysis

A sensitive semi-quantitative assay for multiple mRNAs by RT-PCR (36,51) was adopted to assess the expression of two cholesterol-sensitive genes within Leydig cells [LDL (B/E) receptor (52, 53), and the key enzyme involved in cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reduc-

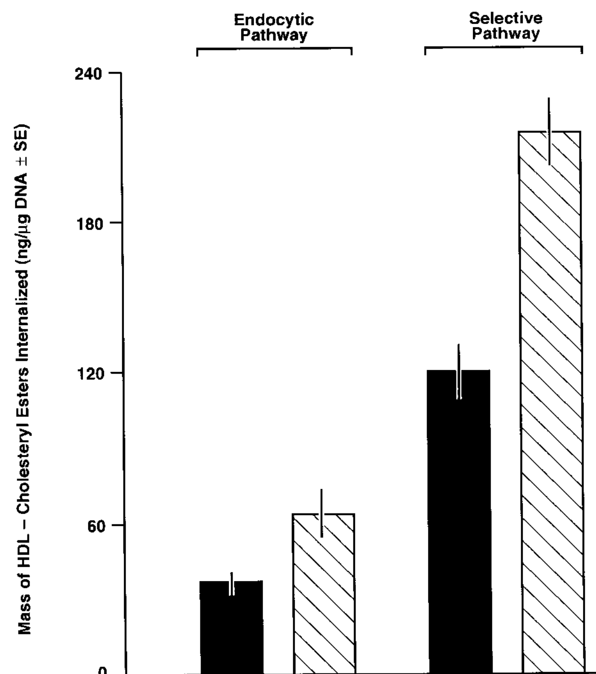


Fig. 6. Uptake of radiolabeled HDL-CEs by selective versus endocytic mechanisms. Purified Leydig cells from control (■) and 4d-hCG-treated (▨) rats were provided with hCG + HDL with radiolabeled, non-releasable apolipoprotein ([^{125}I]DLT) and cholesteryl ester ([^{3}H]COE) tags that would accumulate within cells. Uptake by the endocytic or selective pathways was determined as described under Experimental Procedures.

Downloaded from www.jlr.org by guest, on June 14, 2012

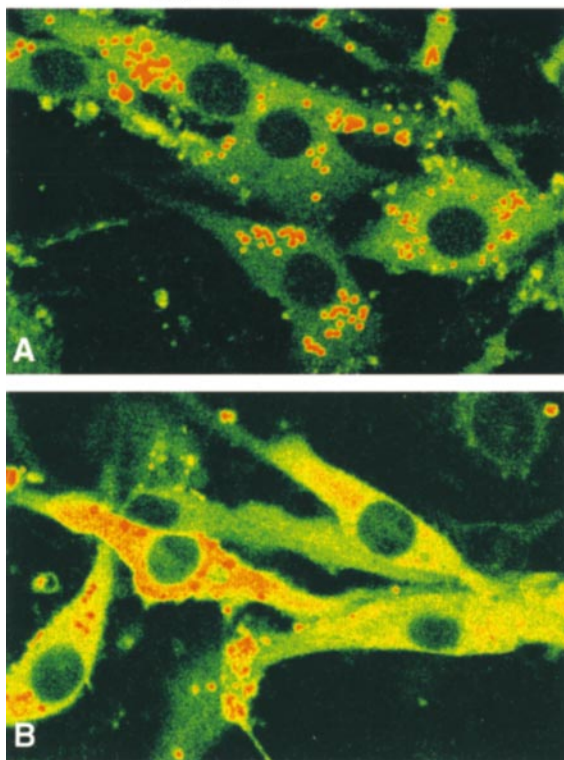


Fig. 7. HDL-fluorescent-CE uptake by Leydig cells. Leydig cells from control (panel A) and 4d-hCG rats (panel B) were provided with HDL-BODIPY-CEs for 24 h, then viewed by confocal microscopy. Some cells of the control animals were able to take in CEs and store them in lipid droplets (red fluorescence, panel A). Cells of the desensitized animals took in far more CE (yellow and red fluorescence, panel B) than control cells, but this CE appeared to be stored in membranes rather than discrete fat droplets.

tase (54)]. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a stable internal control (56) as preliminary experiments indicated that L19 expression increased in Leydig cells of 4d-hCG-treated rats (36, 51). Total RNA was isolated from Leydig cell preparations by the guanidine–isothiocyanate–phenol–chloroform extraction procedure (55). For mRNA analysis by RT-PCR, the following oligonucleotide primers were designed based on known cDNA sequences of the various target genes.

	Rat LDL (B/E) receptor (LDL-R)
(*upper primer*)	5′-GAAGTCGACACTGTACTGACCACC-3′
(*lower primer*)	5′-CTCCTCATTCCCTCTGCCAGCCAT-3′
	Rat HMG-CoA reductase
(*upper primer*)	5′-CCAAGGGTACGGAGAAAGCAC-3′
(*lower primer*)	5′-CAATGTAGATGGCAGTGACGA-3′
	Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
(*upper primer*)	5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′
(*lower primer*)	5′-CATGTAGGCCATGAGGTCCACCAC-3′

Suitable aliquots of total RNA (250–5,000 ng) were reverse transcribed for 60 min at 37°C using 500 ng oligo $(dT)_{12-18}$ and 200 U of Moloney murine leukemia virus reverse transcriptase (SUPERSCRIPT II RT) in a 20 μl reaction mixture (1.0 mm dNTPs, 1.5 mm $MgCl_2$, 50 mm Tris-HCl, pH 8.4, 75 mm KCl, 20 mm DTT, and 40 U RNAse inhibitor, RNasin). Two microliters of the reverse transcription reaction mixture was then used for PCR in a final solution of 100 μl containing 1 × PCR buffer, 0.8 mm dNTPs, 1.0 μm of appropriate oligonucleotide primers (**Table 1**), 2 μCi [α-^{32}P]dCTP, and 2.5 U of *Taq* DNA polymerase, as well as 1.0 μM oligonucleotide primers for GAPDH (56) used as the stable internal control.

Unless otherwise stated, 30 cycles were performed consisting of denaturation (94°C, 1 min), annealing (GAPDH and HMG-CoA reductase, 56°C, and LDL receptor, 62°C; 3 min), and extension (72°C, 2 min). PCR products were subjected to electrophoresis on 5% polyacrylamide gels in 0.5 × Tris-borate-EDA buffer. The gels were dried under vacuum and heat and exposed to X-ray films for 1–4 h at room temperature. Appropriate films were subjected to densitometric scanning, and the radioactivity in each of the PCR bands was normalized to the radioactivity of the GAPDH band as an internal control.

Miscellaneous techniques

The DNA content of the cells was quantified colorimetrically (57). The procedure of Markwell et al. (58) was used to quantify protein content of $hHDL_3$ and reconstituted HDL preparations. Protein in the cellular lysates was determined as described by

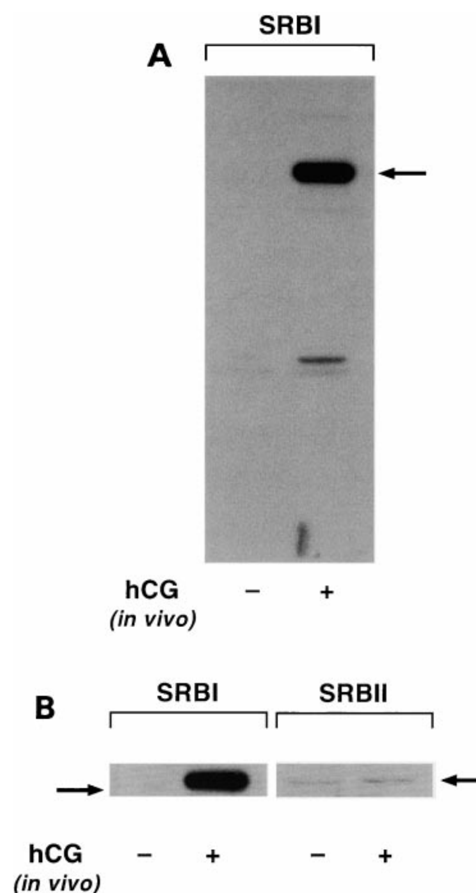


Fig. 8. Western blot analysis of SR-BI and SR-BII protein expression. Purified Leydig cells from either control or 4d-hCG-treated rats were used. Total cell lysates were prepared as described in Experimental Procedures and 25 μg of the lysate from each group was subjected to immunoblot analysis using either rabbit anti-SR-BI or anti-SR-BII. The SR-BI and SR-BII proteins were visualized by chemiluminescence. In 8A, SR-BI protein (arrow) is compared in purified Leydig cell preparations from control and 4d-hCG: in 8B, SR-BI and SR-BII expression is compared in cells from control and 4d-hCG-treated rats.

Downloaded from www.jlr.org by guest, on June 14, 2012

Peterson (59). Cholesterol content of the $hHDL_3$ and reconstituted HDL was determined colorimetrically according to the procedure of Tercyak (60).

RESULTS

Throughout these studies, animals that were treated with vehicle alone are referred to as controls; animals treated in vivo with gonadotropins are referred to as hCG rats. For experiments in which testosterone is measured, cell preparations from both groups of animals are also given hCG in vitro.

Time course of Leydig cell function

Testosterone production. Leydig cell testosterone production after acute and chronic stimulation with the trophic hormone, hCG, is shown in **Fig. 1**. Rats were injected for 4 days with hCG (or vehicle) and testicular samples were obtained at 6 h, or 1, 2, 3, and 4 days. Interstitial cell preparations from these samples were treated with buffer (basal) or hCG, Bt_2cAMP or 22[R] hydroxy-cholesterol in vitro for 3 h prior to measurement of testosterone production. Depending on the in vitro treatment, testosterone production varied but, in general, the capacity of Leydig cells to produce hormone fell after 6 h of hCG treatment and reached a low point after 1–2 days of in vivo hCG. Some recovery was seen at 3 days when the cells were provided with freely diffusible cholesterol analog or Bt_2cAMP in vitro, but no recovery was seen in cells provided with hCG in vitro.

hCG binding. **Figure 2** indicates that Leydig cell hCG binding in interstitial cell preparations drops precipitously after commencement of hCG treatment in vivo and remains low for the duration of the test period.

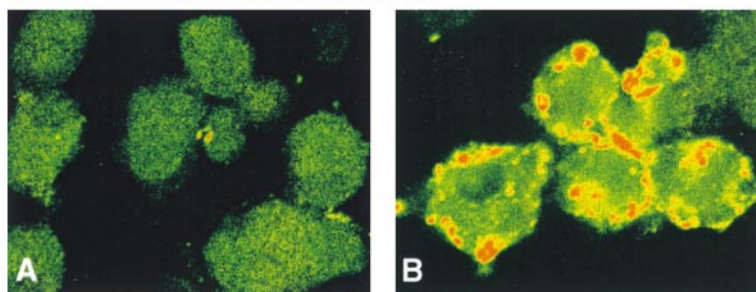
SR-BI expression. Western blots of the above interstitial cell preparations show that expression of the HDL-scavenger receptor (SR-BI) is not seen at zero time or 6 h, but is highly expressed by 24 h after hCG treatment, and continues to be highly expressed for the remainder of the experimental period (**Fig. 3**).

The large differences in the response of cells from control and 4d-hCG-treated animals provided the basis for choosing these two animal models to further clarify the interaction of SR-BI/SR-BII and selective CE uptake in rat Leydig cells. In the following studies purified Leydig cell preparations were used throughout.

Uptake and utilization of metabolizable cholestrol oleate

Uptake of HDL-derived cholesteryl oleate. Purified Leydig cells from control and 4d-hCG-treated animals incubated with [^{3}H]cholesteryl oleate for 24 h were measured for uptake of radiolabeled cholesteryl esters (CEs) and total sterols. **Figure 4** indicates that cells from 4d-hCG rats took up ~2 times the amount of lipid taken in by control cells.

Utilization of HDL-derived CE for testosterone production. In a similar protocol, purified Leydig cells from control and 4d-hCG-treated animals were incubated with radiolabeled [^{3}H]cholesteryl oleate hHDL + hCG in vitro for 24 h to determine the efficiency of incorporation of HDL-cholesterol into testosterone. As shown in **Fig. 5** (panel A), the mass of testosterone produced in control cells is 4 to 5-fold greater than the mass of testosterone produced in 4d-hCG rats. However, the incorporation of ^{3}H-labeled HDL-cholesterol into testosterone (panel B) in control cells is relatively low and, therefore, the efficiency of [^{3}H]cholesterol utilization for testosterone production (ratio B/A) in control cells is also low (panel C). This suggests that under normal circumstances, control cells do not utilize an exogenous source of cholesterol for testosterone production. A comparable study done with cells from hCG-treated rats



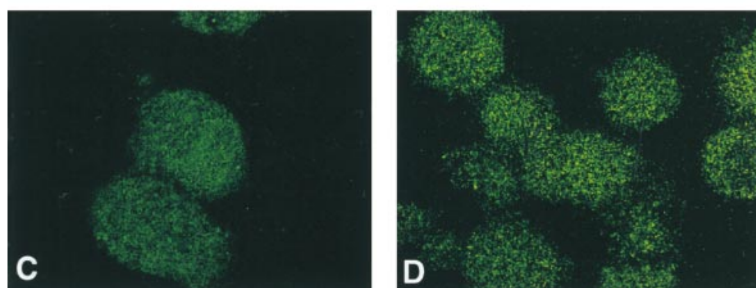


Fig. 9. SR-BI/SR-BII immunofluorescent staining of Leydig cells. Cells from control and 4d-hCG-treated rats were immunostained for SR-BI (9A, 9B) or SR-BII (9C, 9D) and examined by confocal microscopy. Cells from 4d-hCG rats (9B) showed patchy SR-BI expression (red color) near the surface of most cells; cells from control animals showed borderline labeling (9A, light green), but neither preparation of Leydig cells stained for SR-BII showed specific labeling (9C, 9D dark green color).

Downloaded from www.jlr.org by guest, on June 14, 2012
ASBMB JOURNAL OF LIPID RESEARCH

shows that the mass of testosterone produced by hCG cells is far lower than seen in control cells, but the incorporation of ^{3}H-labeled cholesterol into this testosterone is high. Thus, the efficiency of the process (ratio B/A) in cells from hCG rats is far greater than that seen in cells of control rats (panel C). These data suggest that while hCG cells do not make much hormone they do have an efficient process by which to obtain precursor cholesterol from exogenous sources when needed.

To more carefully examine the source of cholesterol for control Leydig cells, three additional studies were carried out. In the first study, hypocholesterolemic (3d-4APP-treated) rats were examined for testosterone production. **Table 1** indicates that the Leydig cell testosterone response from control and 4APP rats was similar despite a 90% fall in total cholesterol levels of the 4APP-treated rats, again suggesting that the rat Leydig cell does not depend on exogenous lipoproteins for cholesterol. In a more direct study, Leydig cells from control and 4d-hCG rats were incubated with [^{14}C]acetate or [^{3}H]cholesteryl oleate-tagged HDL. **Table 2** indicates that control cells used ~10 times more of the de novo synthesized cholesterol (from [^{14}C]acetate) and 4 times less cholesteryl oleate than did hCG cells in the production of testosterone. Finally, to determine whether Leydig cells depend on free cholesterol as a precursor source for testosterone production, Leydig cells of control and 4d-hCG rats were incubated with HDL doubly radiolabeled with [^{3}H]cholesterol oleate and [^{14}C]free cholesterol. Although total incorporation of the two sterols into testosterone (and the amount of testosterone produced) differed significantly, Leydig cells from both groups of rats utilized similar ratios of cho-

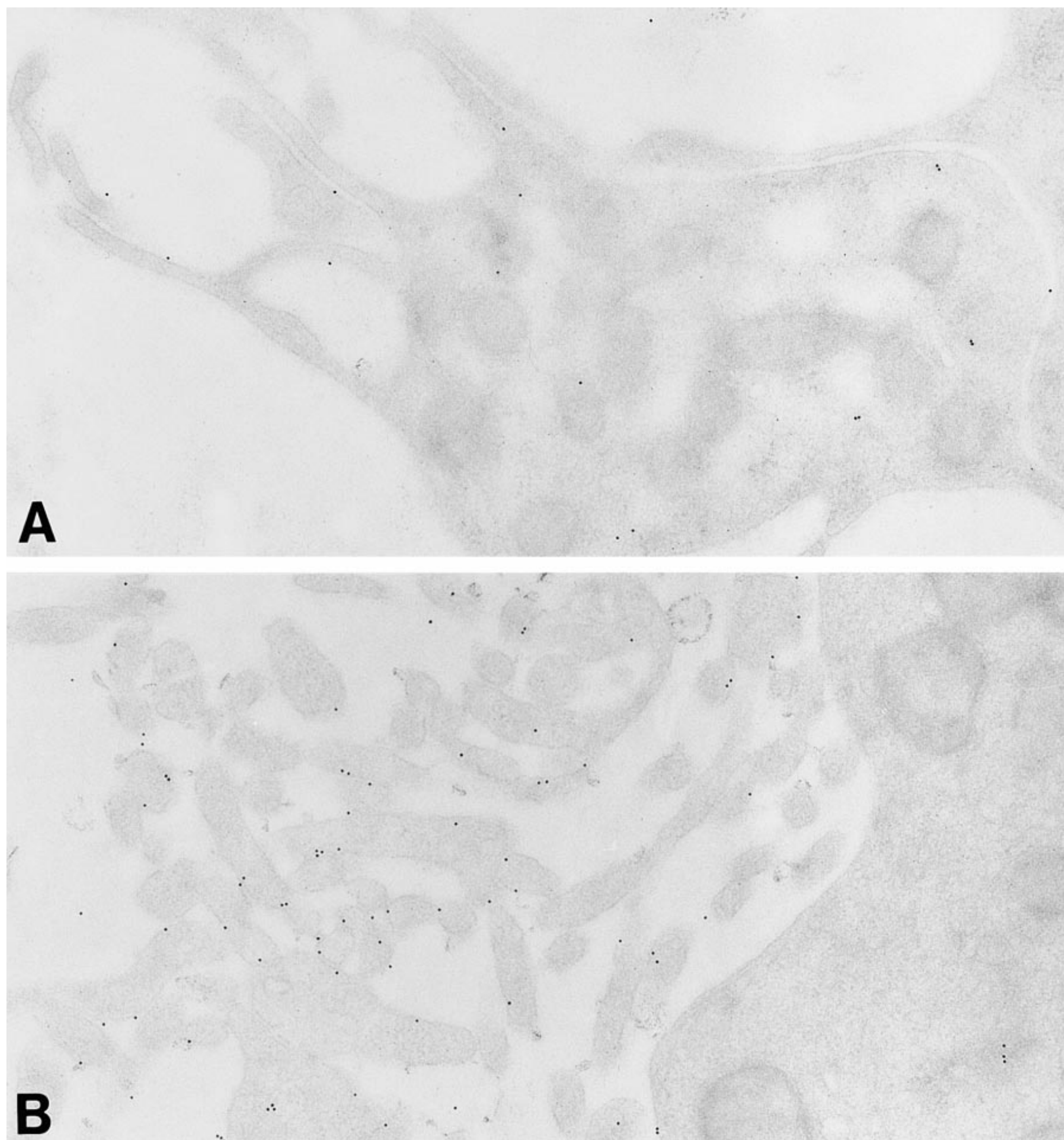


Fig. 10.

Downloaded from www.jlr.org by guest, on June 14, 2012

lesteryl oleate to free cholesterol for hormone production (**Table 3**). Thus, free cholesterol does not constitute a major source of cholesterol for control Leydig cells.

Leydig cell selective HDL-CE uptake

Mass of HDL-CEs internalized by Leydig cells. Leydig cells from control and 4d-hCG-treated rats were provided with ^{125}I-labeled dilactitol (DLT)–[^{3}H]cholesteryl oleoyl ether (COE)–hHDL to measure the mass of CEs internalized (**Fig. 6**). DLT and COE are non-releasable, non-metabolizable apolipoprotein and cholesterol tags that accumulate within cells even when degraded and distinguish between endocytic and selective pathway-mediated cholesterol uptake from HDL (11, 13, 41, 42). Figure 6 shows that while both the endocytic and selective pathways are operative in Leydig cells, the major proportion of HDL-CE uptake takes place via the selective pathway. In any case, cells from 4d-hCG animals take up substantially more (~2-fold) HDL-derived CEs compared to cells from control animals. These results are similar whether (or not) Leydig cells from control and hCG animals are treated with hCG in vitro.

HDL-BODIPY-CE uptake. Using BODIPY-CE as a fluorescent morphological marker for the uptake of HDL-CEs, we can confirm the fact that Leydig cells from control rats do take up HDL-CEs (**Fig. 7A**), but the amount of CE uptake is low; i.e., even after the long 24 h incubation period with BODIPY-labeled HDL, only 50% of Leydig cells from control animals show any lipid stored in droplets, and even in such active cells, the lipid droplets are relatively sparse. Cells from 4d-hCG-treated rats (Fig. 7B) show generally heightened fluoresence compared to the control rats (i.e., more yellow and red colors representing medium and high levels of fluorescence). However, in these cells, the newly internalized (fluorescent) lipid appears confined to endomembranes and lipid storage droplets are only rarely seen. These results are similar whether or not Leydig cells are treated with hCG in vitro.

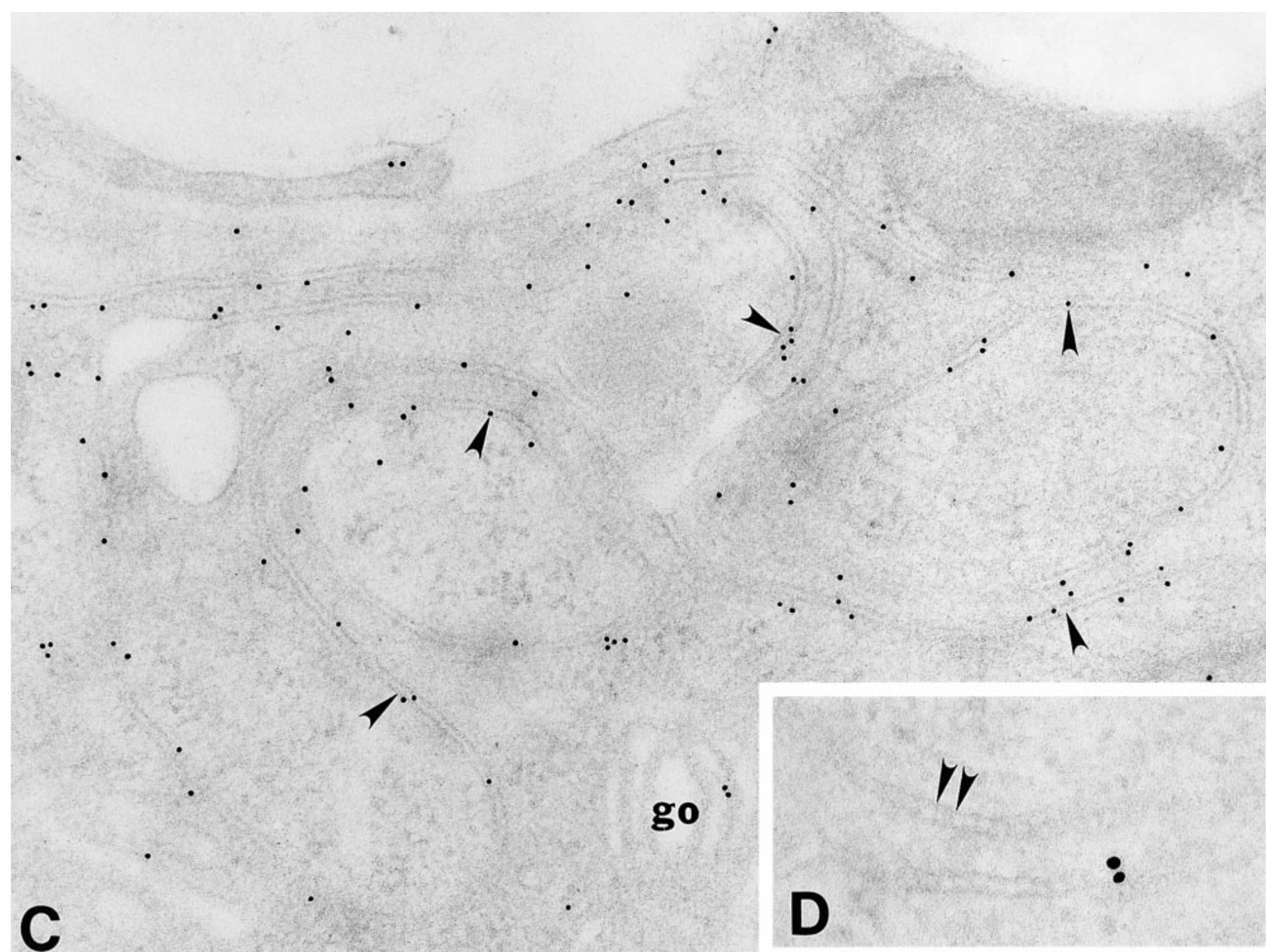


Fig. 10. SR-BI immunocytochemical staining of Leydig cells at the electron microscope level. A low magnification view shows Leydig cells from control animals (10A) with occasional SR-BI -associated gold particles, and cells from 4d-hCG animals (10B) with pronounced immunogold labeling of microvilli when these structures are found to protrude from the surface of the cells. Many Leydig cells from hCG rats also show immunogold labeling of SR-BI in the cell interior. At higher magnification (10C), this gold labeling is found associated with membranes of a complex series of channels (10C, arrowheads) spiraling through the peripheral cytoplasm of the cells. The channels are believed to be inverted portions of the plasma membrane (or microvilli) and in fortuitous high magnification images (10D), one sees striations within the double membranes of the channels (arrowheads) which may be remnants of native HDL.

Downloaded from www.jlr.org by guest, on June 14, 2012

Leydig cell expression of SR-BI and SR-BII

SR-BI/SR-BII protein. Whereas the expression of SR-BI is barely detectable in Leydig cells from control animals, it is highly expressed in Western blots of cells from 4d-hCG-treated animals (**Fig. 8**, arrow). In Fig. 8B, the expression of SR-BI and SR-BII are compared in Western blots. Again, SR-BI is prominent in cells of hCG rats; in contrast, SR-BII expression is low in cells of both control and hCG treated animals.

SRBI/II immunofluorescence. Leydig cells from control and hCG animals were maintained on coverslips, immunostained for SR-BI or SR-BII, and viewed by confocal microscopy. The color photos in **Fig. 9** indicate that control cells show very weak SR-BI staining (Fig. 9A, green coloration) and essentially no expression of SR-BII (9C, dark green color). Leydig cells from 4d-hCG rats show increased immunostaining for SR-BI (9B, red color prominent near the cell surface), but essentially no expression of SR-BII (9D, dark green).

SR-BI immunocytochemistry. At the electron microscope level, Leydig cells from control rats showed no immunogold labeling for SR-BI (**Fig. 10A**), while surface-associated microvilli were consistently labeled for SR-BI in Leydig cells of 4d-hCG rats (10B). Many Leydig cells of the hCG rats, however, did not exhibit a profusion of surface microvilli, but instead revealed deeply invaginated cell surface membranes. These invaginated membranes often formed a remarkably complex network of circular or spiral channels, which coursed through the peripheral cytoplasm of the cells. Most of the cytoplasmic channels were outlined with immunogold (Fig. 10C) representing sites of SR-BI localization and, at higher magnifications, it was often possible to recognize striations between the double membranes of the channels which in previous publications from this laboratory (38, 61, 62) have been shown to be intact HDL (Fig. 10D). As such, these channels which appear to be inside the cells, are probably connected to the outside and represent an inverted microvillar compartment with trapped HDL.

Leydig cell expression of SR-BI and LDL-R in hypocholesterolemic rats

HDL(SR-BI) receptor protein. The Western blot/graph of **Fig. 11A** indicates that SRBI expression is very low in Leydig cells of control animals. Cells from animals treated with 4APP for 3 days show similar levels of SR-BI (despite a 90% reduction in circulating cholesterol levels), but once again, cells from animals treated with hCG for 4 days show dramatically increased SR-BI expression.

LDL (B/E) receptor protein. The response pattern for LDL-R and SR-BI expression is similar; i.e., cells from con-

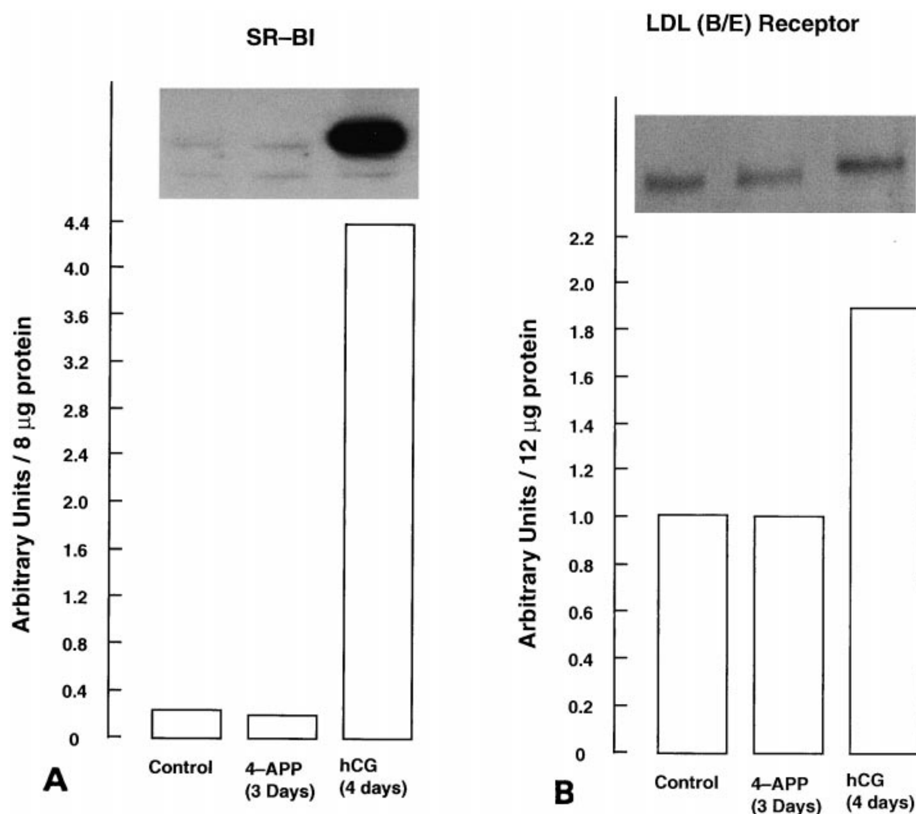


Fig. 11. Western blot analysis of SR-BI (panel A) and LDL-R (panel B) proteins in Leydig cells of control, hypocholesterolemic, and 4d-hCG-treated rats. Suitable aliquots of pure Leydig cell lysates (40–50 μg protein) were separated by SDS-PAGE on 7% (LDL-R) or 10% (SR-BI) polyacrylamide gel. SR-BI and LDL-R proteins were detected by Western blot using specific antibodies. Hypocholesterolemia had no effect on Leydig cell SR-BI or LDL-R expression in control cells, but both receptor proteins were greatly increased after chronic hormone stimulation.

Downloaded from www.jlr.org by guest, on June 14, 2012

trol and 4APP-treated rats show equal expression of LDL-R but a 2-fold increase in expression of LDL-R was seen in cells from hCG treated rats (Fig. 11B).

Leydig cell expression of cholesterol-sensitive genes

RT-PCR analyses (radioautographs and scanned images) of Leydig cell HMG-CoA reductase and LDL-R mRNAs from control and 4d-hCG-treated rats are shown in **Fig. 12**. Panels A and B show variation in expression of the proteins in the two separate RT-PCR experiments carried out, but a similar result overall, i.e., increased expression of HMG-CoA reductase and LDL-R mRNA in the cells of 4d-hCG rats as compared to control rats.

DISCUSSION

Fundamental to understanding the findings of this study is the question of whether Leydig cells, like other steroidogenic cells of the rat, normally utilize lipoprotein-provided CEs for steroid hormone production. In the current report, a variety of experiments were used to address this question. The results from these studies were compatible with the view that Leydig cells from control rats do not depend on exogenous lipoproteins, but instead tend to utilize endogenous sources of cholesterol for testosterone production. These experiments have shown that control Leydig cells can bring in some lipids from the outside if provided with radiolabeled cholesterol oleate or fluorescently labeled (BODIPY) HDL-CEs, but the amount of lipid introduced into the cells is minor. Although Leydig cells from control animals produce large amounts of testosterone, they show poor efficiency in the incorporation of labeled HDL-CEs into testosterone, they show little uptake of radiolabeled cholesterol oleate, and they show little HDL-CE uptake by either the endocytic or selective cholesterol uptake pathways. Indeed, such control cells show no increase in the expression of cholesterol-sensitive genes such as LDL receptor protein (LDL-R, mRNA or protein content), or in HMG-CoA reductase mRNA even after animals were treated for 3 days with 4APP (to reduce circulating cholesterol levels), strongly suggesting that Leydig cells do not use HDL or LDL-supplied CE for the purpose of testosterone production. Indeed, testosterone production is not altered by this treatment. Also, when control and hCG cells were directly incubated with [^{14}C]acetate or [^{3}H]cholesteryl oleate-HDL, control cells utilized ~10 times more acetate-derived cholesterol and ~5 times less HDL-CO for testosterone production than did hCG cells. Finally, the two groups of Leydig cells did

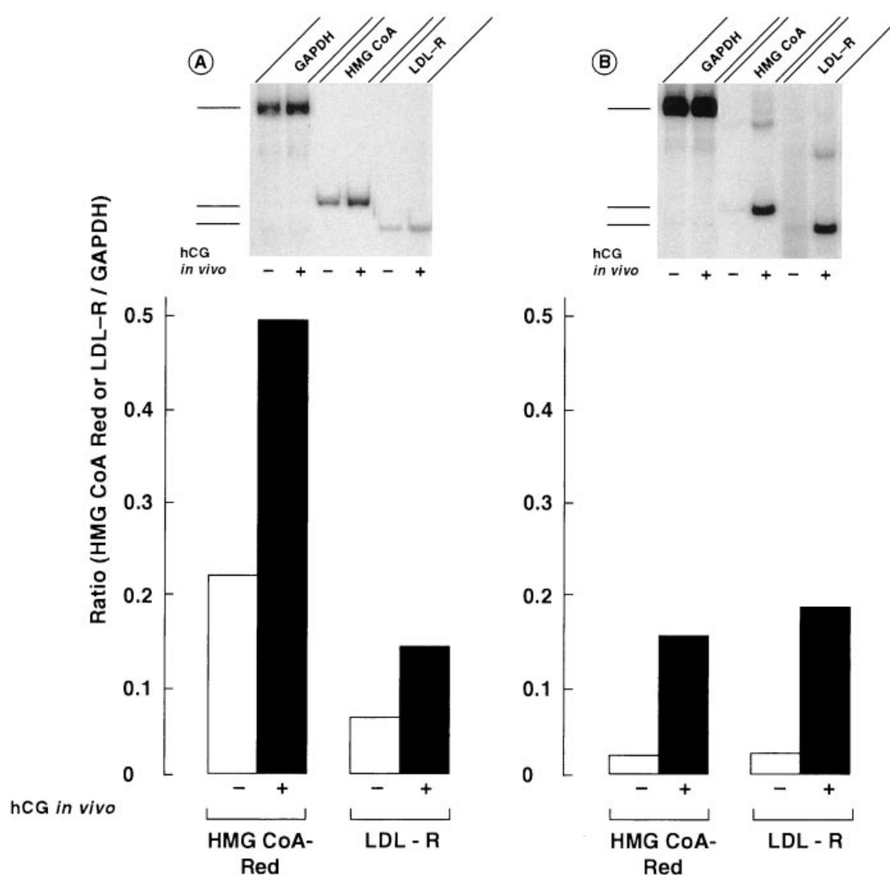


Fig. 12. The expression of LDL-R and HMG-CoA reductase mRNAs in pure Leydig cell preparations from rats treated with or without hCG for 4 days. LDL-R, HMG-CoA reductase, and GAPDH mRNAs were amplified using a semi-quantitative RT-PCR protocol as described under Experimental Procedures. The relative levels of expression in Leydig cells from control (−) and hCG (+)-treated animals are shown.

Downloaded from www.jlr.org by guest, on June 14, 2012

JOURNAL OF LIPID RESEARCH
ASBMB

not differ in their use of HDL-derived free cholesterol for testosterone production. Given these data supporting the idea that control Leydig cells utilize endogenous sources of cholesterol for testosterone production, it is no surprise that Leydig cells express only minimal levels of SR-BI and little or no SR-BII. Control Leydig cells produce large amounts of hormone, but given the above results + high expression of HMG-CoA reductase mRNA in the cells, one must conclude that ample levels of cholesterol are produced endogenously to support testosterone production in these animals.

When such rats are treated with hCG for several days, their Leydig cells undergo changes consistent with hormone-desensitization. That is, hCG binding and testosterone production are reduced reaching a low point 24 h after the initiation of daily hormone treatment, and recovery of trophic hormone binding and steroid hormone production does not fully occur for the 4-day duration of the study. However, HDL (SR-BI) receptor expression, not seen in cells of control animals or for some hours after the initiation of the hCG treatment, is found in cells sampled at 24 h, and remains prominent for 4 days of hCG treatment. Experiments comparing control cells and 4d-hCG cells indicate that the hormone-treated cells now efficiently take up HDL-derived cholesterol esters (cholesteryl oleate) and efficiently use this cholesterol in testosterone synthesis (though relatively less testosterone is actually made throughout the test period), that HDL-provided cholesteryl esters are taken in by both the selective and endocytic pathways, that expression of SR-BI and LDL-R protein is prominent, and that mRNA for HMG-CoA reductase, LDL-R and SR-BI are all highly expressed. Indeed, in this scenario, there is a dramatic reversal in the expression of both endocytic (LDL-R) and selective (SR-BI) receptor-related uptake of lipoprotein-derived CEs from outside of cells, but also continued good expression of HMG-CoA reductase, suggesting that cholesterol synthesis inside the cell continues at a high level. Only SR-BII expression remains negligible and unchanged during the course of the 4-day period of hCG treatment, and it is likely that this variant of SR-BI plays no role in maintaining cholesterol levels in rat Leydig cells. Thus, it appears that rat Leydig cells do not utilize circulating (exogenous) lipoprotein cholesterol for the purposes of testosterone production under normal circumstances, but they are capable of doing so under emergency situations induced by chronic trophic hormone administration.

Why control Leydig cells of the rat differ so dramatically from steroidogenic cells of the ovary (4, 5, 7–10, 13, 41, 42) or adrenal (6, 11, 12, 15) in obtaining cholesterol for steroid hormone production is not yet clear. One wonders whether the variable ambient temperature of the testis makes it an inhospitable environment for the direct (selective) uptake of a neutral lipid such as cholesteryl ester, and whether Leydig cells, which rely on a steady supply of cholesterol for testosterone production, may have circumvented this problem by synthesizing their own unesterified cholesterol as needed. Whatever the reason, Leydig cells from normal mature rats have very few lipid droplets [the normal storage depot for cholesteryl esters in other steroidogenic tissues (63)], and their lipid droplet number is reduced even further after in vivo treatment with trophic hormones such as hCG (24). This situation may explain why Leydig cells from 4d-hCG animals store newly incorporated (fluorescent-labeled) HDL-derived CE in membranes of the cell instead of in lipid droplets [as is the case with human and rodent granulosa-luteal cells under similar circumstances of SR-BI up-regulation (41, 42, 51, 64)]. It may be that adult rat Leydig cells have lost the machinery necessary for efficient lipid droplet formation and storage, and that a large influx of non-metabolizable CEs, such as the HDL BODIPY-labeled CE, has no place to go after being incorporated into membrane compartments of the cell.

The relationship of the membrane-stored fluorescent lipids to sites of SR-BI expression in Leydig cells is also of interest. The immunofluorescent images of Fig. 9B indicate that SR-BI is located in irregular patches near, but not necessarily on, the exterior surface of these cells. In ultrastructural studies of similar Leydig cells from the intact testis, we see that the localization of SRBI is, in fact, associated with microvilli, and that occasionally one sees a Leydig cell with a profusion of microvilli as in Fig. 10B. More often, however, one sees Leydig cells with complex infoldings of the plasma membrane which form numerous double-membraned channels within the cortical cytoplasm of the cells. As it appears that HDL are trapped within the channels (61, 62), it is likely that the channels are (or were at some point) open to the exterior of the cells. The channel membranes stain prominently with antibodies to the HDL receptor protein, SR-BI, and we speculate that these immunolabeled channel membranes represent the patchy SR-BI immunofluorescence seen near the surface of the cells in the confocal images of Fig. 9B. Whether the incoming CE is also trapped in the plasma membrane lining of the channels, or has moved into the endomembranes of the cell, remains to be seen.

Thus, Leydig cells from control rats do not efficiently utilize exogenous lipoprotein to supply cholesterol for testosterone production, but attempt to do so when chronically stimulated by trophic hormone. Under the latter circumstances, the cells up-regulate HMG-CoA reductase, increase their de novo synthesis of cholesterol, and double the expression of LDL-R which regulates the endocytic uptake of low density lipoproteins. In addition, the cells are induced to express SR-BI and they show increased selective uptake of HDL-derived CEs. The process by which the latter occurs is similar to that seen in hormone-desensitized rat ovarian models, in that the increased expression of SR-BI is localized to a plasma membrane (microvillar) compartment of the target cells (38) and, as a result, exogenous lipoprotein-derived CEs are brought into the Leydig cells (though little steroid hormone is produced) during the trophic hormone treatment period. However, two aspects of selective uptake process show unusual expression in Leydig cells; first, most of the SRBI-labeled plasma membranes in Leydig cells appear to be in the form of convoluted membrane channels embedded within the cytoplasm of the Leydig cell rather than in typical surface

Downloaded from www.jlr.org by guest, on June 14, 2012

microvilli, and second, despite increased uptake of lipoprotein-derived CEs, little, if any, of the internalized lipid is stored in lipid droplets as is typical for steroidogenic cells of the adrenal and ovary (7, 41, 42, 61). We speculate that as mature rat Leydig cells do not normally rely on exogenous cholesterol for steroidogenesis, structural and metabolic machinery that normally increase the efficiency of the selective cholesterol uptake process in other steroidogenic cells of the rat may be absent in Leydig cells.

This work was supported by the Office of Research and Development, Medical Research Service, Department of Veterans Affairs, and grants HL-33881 and DK-49705 from the National Institutes of Health.

Manuscript received 28 May 1999 and in revised form 30 September 1999.

REFERENCES

1. Landschulz, K. T., R. K. Pathak, A. Rigotti, M. Krieger, and H. H. Hobbs. 1996. Regulation of scavenger receptor, class B, Type I, a high density lipoprotein receptor, in liver and steroidogenic tissues of the rat. *J. Clin. Invest.* **98:** 984–995.
2. Webb, N. R., W. J. S. de Villers, P. M. Connell, F. C. de Beer, and D. R. van der Westhuyzen. 1997. Alternative forms of the scavenger receptor BI (SR-BI). *J. Lipid Res.* **38:** 1490–1495.
3. Webb, N. R., P. M. Connell, G. A. Graff, E. J. Smart, W. J. S. de Villiers, F. C. de Beer, and D. R. van der Westhuyzen. 1998. SR-BII, an isoform of the scavenger receptor BI containing an alternate cytoplasmic tail, mediates lipid transfer between high density lipoprotein and cells. *J. Biol. Chem.* **273:** 15241–15248.
4. Gwynne, J. T., and J. F. Strauss III. 1982. The role of lipoproteins in steroidogenesis and cholesterol metabolism in steroidogenic glands. *Endocr. Rev.* **3:** 299–329.
5. Azhar, S., and K. M. J. Menon. 1981. Receptor-mediated gonadotropin action in the ovary: rat luteal cells preferentially utilize and are acutely dependent upon the plasma lipoprotein-supplied sterols in gonadotropin-stimulated steroid production. *J. Biol. Chem.* **256:** 6548–6555.
6. Vereschoor-Klootwyk, A. H., L. Verschoor, S. Azhar, and G. M. Reaven. 1982. Role of exogenous cholesterol in regulation of adrenal steroidogenesis in the rat. *J. Biol. Chem.* **257:** 7666–7671.
7. Reaven, E., Y-D. I. Chen, M. Spicher, and S. Azhar. 1984. Morphological evidence that high density lipoproteins are not internalized by steroid-producing cells during in situ organ perfusion. *J. Clin. Invest.* **74:** 1384–1397.
8. Nestler, J. E., G. K. Chacko, and J. F. Strauss, III. 1985. Stimulation of rat ovarian cell steroidogenesis by high density lipoproteins modified with tetranitromethane. *J. Biol. Chem.* **260:** 7316–7321.
9. Reaven, E., Y-D. I. Chen, M. Spicher, S.-F. Hwang, C. E. Mondon, and S. Azhar. 1986. Uptake of low density lipoproteins by rat tissues: special emphasis on the luteinized ovary. *J. Clin. Invest.* **77:** 1971–1984.
10. Pedersen, R. C. 1988. Cholesterol biosynthesis, storage, and mobilization in steroidogenic organs. *In* Biology of Cholesterol. P. L. Yeagle, editor. CRC Press, Boca Raton, FL. 39–69.
11. Azhar, S., D. Stewart, and E. Reaven. 1989. Utilization of cholesterol-rich lipoproteins by perfused rat adrenals. *J. Lipid Res.* **30:** 1799–1810.
12. Gwynne, J. T., and D. D. Mahaffee. 1989. Rat adrenal uptake and metabolism of high density lipoprotein cholesteryl ester. *J. Biol. Chem.* **264:** 8141–8150.
13. Azhar, S., L. Tsai, and E. Reaven. 1990. Uptake and utilization of lipoprotein cholesteryl esters by rat granulosa cells. *Biochim. Biophys. Acta.* **1047:** 148–160.
14. Hammami, M., S. Meunier, G. Maume, P. Gambert, and B. F. Maume. 1991. Effect of rat plasma high density lipoprotein with or without apoliporotein E on the cholesterol uptake and on the induction of the corticosteroid biosynthetic pathway in newborn rat adrenocortical cell cultures. *Biochim. Biophys. Acta.* **1094:** 153–160.
15. Azhar, S., J. A. Frazier, L. Tsai, and E. Reaven. 1994. Effect of okadaic acid on utilization of lipoprotein-derived cholesteryl esters by rat steroidogenic cells. *J. Lipid Res.* **35:** 1161–1176.
16. Morris, M. D., and I. L. Chaikoff. 1959. The origin of cholesterol in liver, small intestine, adrenal gland, and testis of the rat: dietary versus endogenous contributions. *J. Biol. Chem.* **234:** 1095–1097.
17. Andersen, J. M., and J. M. Dietschy. 1978. Relative importance of high and low density lipoproteins in the regulation of cholesterol synthesis in the adrenal gland, ovary, and testis of the rat. *J. Biol. Chem.* **253:** 9024–9032.
18. Freeman, D. A. and F. F. G. Rommerts. 1996. Regulation of Leydig cell cholesterol transport. *In* The Leydig Cell. A. H. Payne, M. P. Hardy, and L. D. Russell, editors. Cache River Press, Vienna, IL. 231–240.
19. Charreau, E. H., J. C. Calvo, K. Nozu, O. Pignataro, K. J. Catt, and M. L. Dufau. 1981. Hormonal modulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in gonadotropin-stimulated and -desensitized testicular Leydig cells. *J. Biol. Chem.* **256:** 12719–12724.
20. Quinn, P. G., L. J. Dombrausky, Y-D. I. Chen, and A. H. Payne. 1981. Serum lipoproteins increase testosterone production in hCG-desensitized Leydig cells. *Endocrinology.* **109:** 1790–1792.
21. Azhar, S., and K. M. J. Menon. 1982. Receptor mediated gonadotropin action in gonadal tissues: relationship between blood cholesterol levels and gonadotropin stimulated steroidogenesis in isolated Leydig and luteal cells. *J. Steroid Biochem.* **16:** 175–184.
22. Schreiber, J. R., D. B. Weinstein, and A. J. Hsueh. 1982. Lipoproteins stimulate androgen production by cultured rat testis cells. *J. Steroid Biochem.* **16:** 39–43.
23. Klinefelter, G. R., and L. L. Ewing. 1988. Optimizing testosterone production by purified adult rat Leydig cells in vitro. *In Vitro Cell Dev. Biol.* **24**: 545–549.
24. Andreis, P. G., L. Cavallini, G. Mazzocchi, V. Meneghelli, and G. G. Nussdorfer. 1990. Lipid droplets in the secretory response of Leydig cells of normal and hCG-treated rats. *J. Submicrosc. Cytol. Pathol.* **22:** 361–366.
25. Hedger, M. P., and G. P. Risbridger. 1992. Effect of serum and serum lipoproteins on testosterone production by adult rat Leydig cells in vitro. *J. Steroid Biochem. Mol. Biol.* **43**: 581–589.
26. Risbridger, G. P., and M. P. Hedger. 1992. Adult rat Leydig cell cultures: Minimum requirements for maintenance of luteinizing hormone responsiveness and testosterone production. *Mol. Cell. Endocrinol.* **83:** 125–132.
27. Frayne, J., and H. D. Nicholson. 1994. Regulation of oxytocin production by purified adult rat Leydig cells in vitro: effects of LH, testosterone and lipoproteins. *J. Endocrinol.* **143**: 325–332.
28. Tsuruhara, T., M. L. Dufau, S. Cigorraga, and K. J. Catt. 1977. Hormonal regulation of testicular luteinizing hormone receptors: effects on cyclic AMP and testosterone responses in isolated Leydig cells. *J. Biol. Chem.* **252:** 9002–9009.
29. Saez, J. M., F. Haour, and A. M. Cathiard. 1978. Human chorionic gonadotropin-induced Leydig cell refractoriness to gonadotropin stimulation. *Mol. Pharmacol.* **14:** 1054–1062.
30. Payne, A. H., K-L. Wong, and M. M. Vega. 1980. Differential effects of single and repeated administrations of gonadotropins on luteinizing hormone receptors and testosterone synthesis in two populations of Leydig cells. *J. Biol. Chem.* **255:** 7118–7122.
31. Risbridger, G. P., D. M. Robertson, and D. M. de Kretser. 1982. The effects of chronic human chorionic gonadotropin treatment on Leydig cell function. *Endocrinology.* **110:** 138–145.
32. Ewing, L. L., T-Y. Wing, R. C. Cochran, N. Kromann, and B. R. Zirkin. 1983. Effect of luteinizing hormone on Leydig cell structure and testosterone secretion. *Endocrinology.* **112:** 1763–1769.
33. Andreis, P. G., L. Cavallini, L. K. Malendowicz, A. S. Belloni, P. Rebuffat, G. Mazzocchi, and G. G. Nussdorfer. 1989. Morphological and functional responses of rat Leydig cells to a prolonged treatment with human chorionic gonadotropins. *J. Submicrosc. Cytol. Pathol.* **21:** 703–711.
34. Acton, S., A. Rigotti, K. T. Landschulz, S. Xu, H. H. Hobbs, and M. Krieger. 1996. Identification of scavenger receptor SR-Bi as a high density lipoprotein receptor. *Science.* **271**: 518–520.
35. Wang, N., W. Weng, J. L. Breslow, and A. R. Tall. 1996. Scavenger receptor BI (SR-BI) is up-regulated in adrenal gland in apolipoprotein A-1 and hepatic lipase knock-out mice as a response to depletion of cholesterol stores: in vivo evidence that SR-Bi is a functional high density lipoprotein receptor under feedback control. *J. Biol. Chem.* **271:** 21001–21004.
36. Azhar, S., A. Nomoto, S. Leers-Sucheta, and E. Reaven. 1998. Si-

Downloaded from www.jlr.org by guest, on June 14, 2012

multaneous induction of an HDL receptor protein (SR-BI) and the selective uptake of HDL-cholesteryl esters in a physiologically relevant steroidogenic cell model. *J. Lipid Res.* **39:** 1616–1628.
37. Johnson, M. S. C., P-A. Svensson, K. Helou, H. Billig, G. Levan, L. M. S. Carlsson, and B. Carlsson. 1998. Characterization and chromosomal localization of rat scavenger receptor class B type I, a high density lipoprotein receptor with a putative leucine zipper domain and peroxisomal targeting sequence. *Endocrinology.* **139:** 72–80.
38. Reaven, E., A. Nomoto, S. Leers-Sucheta, R. Temel, D. L. Williams, and S. Azhar. 1998. Expression and microvillar localization of scavenger receptor, class B, type I (a high density lipoprotein receptor) in luteinized and hormone-desensitized rat ovarian models. *Endocrinology.* **139:** 2847–2856.
39. Rigotti, A., E. R. Edelman, P. Seifert, S. N. Iqbal, R. B. DeMattos, R. E. Temel, M. Krieger, and D. L. Williams. 1996. Regulation by adrenocorticotropic hormone of the in vivo expression of scavenger receptor class B type I (SR-BI), a high density lipoprotein receptor, in steroidogenic cells of the murine adrenal gland. *J. Biol. Chem.* **271:** 33545–33549.
40. Temel, R. E., B. Trigatti, R. B. DeMattos, S. Azhar, M. Krieger, and D. L. Williams. 1997. Scavenger receptor class B, type I (SR-BI) is the major route for the delivery of high density lipoprotein cholesterol to the steroidogenic pathway in cultured mouse adrenocortical cells. *Proc. Natl. Acad Sci. USA.* **94**: 13600–13605.
41. Reaven, E., L. Tsai, and S. Azhar. 1995. Cholesterol uptake by the 'selective' pathway of ovarian granulosa cells: early intracellular events. *J. Lipid Res.* **36:** 1602–1617.
42. Reaven, E., L. Tsai, and S. Azhar. 1996. Intracellular events in the "selective" transport of lipoprotein-derived cholesteryl esters. *J. Biol. Chem.* **271:** 16208–16217.
43. Ji, Y., B. Jian, N. Wang, Y. Sun, M. de la Llera Moya, M. C. Phillips, G. H. Rothblat, J. B. Swaney, and A. R. Tall. 1997. Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux. *J. Biol. Chem.* **272:** 20982–20985.
44. Liao, C., E. Reaven, and S. Azhar. 1993. Age-related decline in the steroidogenic capacity of isolated rat Leydig cells: a defect in cholesterol mobilization and processing. *J. Steroid Biochem. Mol. Biol.* **46:** 39–47.
45. Payne, A. H., J. R. Downing, and K-L. Wong. 1980. Luteinizing hormone receptors and testosterone synthesis in two distinct populations of Leydig cells. *Endocrinology.* **106:** 1424–1429.
46. Mauduit, C., F. Gasnier, C. Rey, M-A. Chauvin, D. M. Stocco, P. Louisot, and M. Benahmed. 1998. Tumor necrosis factor-α inhibits Leydig cell steroidogenesis through a decrease in steroidogenic acute regulatory protein expression. *Endocrinology.* **139:** 2863–2868.
47. Chen, Y-D. I., F. B. Kraemer, and G. M. Reaven. 1980. Identification of specific high density lipoprotein-bindung sites in rat testis and regulation of binding by human chorionic gonadotropin. *J. Biol. Chem.* **255:** 9162–9167.
48. Azhar, S., Y-D. I. Chen, and G. M. Reaven. 1983. Stimulation of lipoprotein receptors and role of lipoprotein and cellular cholesterol during gonadotropin-induced desensitization of steroidogenic response in luteinized rat ovary. *J. Biol. Chem.* **258:** 3735–3740.
49. Hou, J. W., D. C. Collins, and R. L. Schleicher. 1990. Source of cholesterol for testosterone biosynhesis in murine Leydig cells. *Endocrinology.* **127:** 2047–2055.
50. Berryman, M. A., and R. D. Rodewald. 1990. An enhanced method for the post-embeding immunocytochemical staining which preserves cell membranes. *J. Histochem. Cytochem.* **38:** 159–170.
51. Azhar, S., Y. Luo, S. Medicherla, and E. Reaven. 1999. Up-regulation of selective cholesteryl ester uptake pathway in mice with deletion of low-density lipoprotein receptor function. *J. Cell. Physiol.* **180:** 190–202.
52. Lee, L. Y., W. A. Mohler, B. L. Schafer, J. S. Freudenberger, N. Byrne-Connoly, K. B. Eager, S. T. Mosley, J. K. Leighton, R. N. Thrift, R. A. Davis, and R. D. Tanaka. 1989. Nucleotide sequence of the rat low density lipoprotein receptor cDNA. *Nucleic Acids Res.* **17:** 1259-1260.
53. Shimomura, I., H. Shimano, J. D. Horton, J. L. Goldstein, and M. S. Brown. 1997. Differential expression of exons 1a and 1c in mRNAs for sterol regulatory element binding protein-1 in human and mouse organs and cultured cells. *J. Clin. Invest.* **99:** 838–845.
54. Medicherla, S., S. Azhar, A. Cooper, and E. Reaven. 1996. Regulation of cholesterol responsive genes in ovary cells: Impact of cholesterol delivery system. *Biochemistry.* **35:** 6242–6250.
55. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **161:** 156–159.
56. Fort, P., L. Marty, M. Piechaczyk, S. El Sabrouty, C. Dani, P. Jeanteur, and J. M. Blanchard. 1985. Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate dehydrogenase multigenic family. *Nucleic Acids Res.* **13:** 1431–1442.
57. Natarajan, N., G. E. Shambaugh III, K. M. Elseth, G. K. Haines, and J. A. Radosevich. 1994. Adaptation of the diphenylamine (DPA) assay to a 96-well plate tissue culture format and comparison with the MTT assay. *BioTechniques.* **17:** 166–171.
58. Markwell, M. A. K., S. M. Hass, N. E. Tolbert, and L. L. Bieber. 1981. Protein determination in membrane and lipoprotein samples: Manual and automated procedures. *Methods Enzymol.* **72:** 296–303.
59. Peterson, G. L. 1977. A simplification of the protein assay method of Lowry et al. Which is more generally applicable. *Anal. Biochem.* **83:** 346–356.
60. Tercyak, A. M. 1991. Determination of cholesterol and cholesterol esters. *J. Nutr. Biochem.* **2:** 281–292.
61. Reaven, E., M. Spicher, and S. Azhar. 1989. Microvillar channels: a unique plasma membrane compartment for concentrating lipoproteins on the surface of rat adrenal cortical cells. *J. Lipid Res.* **30:** 1551–1560.
62. Reaven, E., X-Y. Shi, and S. Azhar. 1990. Interaction of lipoproteins with isolated ovary plasma membranes. *J. Biol. Chem.* **265:** 19100–19111.
63. Benton, L., L-X. Shan, and M. P. Hardy. 1995. Differentiation of adult Leydig cells. *J. Steroid Biochem. Mol. Biol.* **53:** 61–68.
64. Azhar, S., L. Tsai, S. Medicherla, Y. Chandrasekher, L. Giudice and E. Reaven. 1998. Human granulosa cells use high density lipoprotein cholesterol for steroidogenesis. *J. Clin. Endocrinol. Metab.* **83:** 983–991.

ASBMB JOURNAL OF LIPID RESEARCH JLR

Downloaded from www.jlr.org by guest, on June 14, 2012