Overexpression of hormone-sensitive lipase in Chinese hamster ovary cells leads to abnormalities in cholesterol homeostasis

Fredric B. Kraemer,[1,*] Loren Fong,[†] Shailja Patel,[*] Vanita Natu,[*] and Michael C. Komaromy[2,†]

Department of Veterans Affairs Medical Center,* Palo Alto, CA 94304; Division of Endocrinology, Gerontology, and Metabolism,* Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305; and Research Institute Palo Alto Medical Foundation,[†] Palo Alto, CA 94301

Abstract Hormone-sensitive lipase (HSL) is an intracellular enzyme that functions as both a neutral triglyceride and cholesteryl ester hydrolase. In order to explore the effects of HSL on cholesterol homeostasis, Chinese hamster ovary (CHO) cells were transfected with rat HSL and several different stable cell lines that overexpress HSL mRNA, HSL protein, and HSL activity approximately 600-fold were isolated. Cells transfected with HSL contained less cholesteryl esters and unesterified cholesterol than control cells. HSL transfectants expressed 20–60% fewer LDL receptors than control cells when grown in lipid-depleted media or in the presence of mevinolin, as assessed by binding and degradation of LDL and immunoblotting of LDL receptors. In contrast, the rate of cholesterol synthesis and the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase were increased 3- to 14-fold in HSL transfectants grown in sterol replete media. The rate of cholesterol synthesis and the activity of HMG-CoA reductase increased when cells were grown in lipid-depleted media, and remained markedly elevated compared to control cells. These results show that the regulation of LDL receptor expression and cholesterol synthesis can be dissociated through the actions of HSL and suggest multiple control mechanisms for sterol-responsive genes.—**Kraemer, F. B., L. Fong, S. Patel, V. Natu, and M. C. Komaromy.** Overexpression of hormone-sensitive lipase in Chinese hamster ovary cells leads to abnormalities in cholesterol homeostasis. *J. Lipid Res.* 1997. **38:** 1553–1561.

Supplementary key words hormone-sensitive lipase • LDL receptors • 3-hydroxy-3-methylglutaryl coenzyme A reductase • cellular cholesterol • transfection

Hormone-sensitive lipase (HSL) is an intracellular neutral lipase that is primarily found in adipose tissue, adrenal, ovary, testis, and, to a lesser extent, in skeletal muscle and macrophages (1, 2). In adipose tissue HSL functions presumably as the rate-limiting enzyme in lipolysis, mediating the hydrolysis of triacylglycerol and diacylglycerol after the phosphorylation of the enzyme by protein kinase A activated via a variety of hormonal stimuli (3–5). In addition to functioning as a triglyceride lipase, HSL also has neutral cholesteryl ester hydrolase activity, mediating the hydrolysis of cholesteryl esters to unesterified cholesterol and free fatty acid (3, 5). Recently, we showed that transfection of adipogenic cells with HSL prevented the accumulation of triglyceride in the adipocytes, demonstrating that high expression of HSL could alter cellular triacylglycerol metabolism through its actions as a triglyceride lipase (6). However, the consequences on cellular metabolism of HSL functioning as a neutral cholesteryl ester hydrolase are not clear.

Cellular cholesterol homeostasis is a tightly regulated system in which the amount of unesterified cholesterol within the cell is controlled by the rate of endogenous cholesterol synthesis, the rate of lipoprotein cholesterol uptake, the rate of esterification of cholesterol, and the rate of hydrolysis of stored cholesteryl esters. Depleting the regulatory pool of unesterified cholesterol by depriving cells of cholesterol or, conversely, expanding the regulatory pool of unesterified cholesterol by overloading cells with cholesterol results in the up- or down-regulation, respectively, of the rate of transcription of a variety of sterol responsive genes, such as hydroxymethylglutaryl (HMG) CoA reductase, HMG-CoA synthase, farnesyl diphosphate synthase, squalene synthase, and the low density lipoprotein (LDL) receptor, that

Abbreviations: HSL, hormone-sensitive lipase; CHO, Chinese hamster ovary; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; SREBP, sterol regulatory element binding protein; ACAT, fatty acyl:cholesterol acyltransferase.

[1]To whom correspondence should be addressed.

[2]Deceased.

are mediated via changes in sterol regulatory element binding proteins (SREBPs) (7–11). Likewise, expanding the regulatory pool of intracellular unesterified cholesterol by preventing the formation of cholesteryl esters via inhibition of fatty acyl:cholesterol acyltransferase (ACAT) activity down-regulates LDL receptors and HMG-CoA reductase (12). The current studies were conducted to explore whether the overexpression of HSL would expand the sterol regulatory pool by increasing the hydrolysis of cholesteryl esters and, thus, cause a down-regulation of the expression of LDL receptors and cholesterol synthesis.

MATERIALS AND METHODS

Chemicals

Reagents were obtained from the following sources: bovine serum albumin (fraction V) (Intergen Co., Purchase, NY); sodium deoxycholate, Triton X-100, L-α phosphatidylcholine, cholesterol oleate, leupeptin, aprotinin, anti-rabbit IgG FITC conjugate, hygromycin B (Sigma Chemical Co., St. Louis, MO); carrier-free ^{125}I, cholesteryl[1-^{14}C]oleate, [2-^{14}C]acetate, 3-hydroxy-3-methyl[3-^{14}C]glutaryl coenzyme A, DL-[2-^{3}H]mevalonic lactone, [1α,2α(n)-^{3}H]cholesterol (E.I. DuPont de Nemours and Co., Boston, MA); fetal bovine serum (Gemini Bio-Products, Inc., Calabasas, CA); Coon's F12/Dulbecco's Modified Eagle's medium, lipofectin reagent (GIBCO BRL, Grand Island, NY); ECL Western blotting detection reagents, horseradish peroxidase-linked whole antibody anti-rabbit IgG, [α-^{32}P]-dCTP (Amersham Life Sciences Products, Arlington Heights, IL); nitrocellulose paper (Schleicher and Schuell, Keene, NH); oligolabeling kit (Pharmacia LKB Biotechnology, Piscataway, NJ). All other chemicals were obtained from standard commercial sources.

Overexpression of HSL

CHO cells were grown in Coon's F12/Dulbecco's Modified Eagle's media containing 1 g/l glucose (50/50) and supplemented with 10% fetal bovine serum, penicillin (10,000 units/ml) and streptomycin (10,000 μg/ml). CHO cells were transfected with pCEP4-HSL as described previously (6). pCEP4-HSL contains nucleotides 581–2923 of rat HSL and encompasses the entire HSL coding region. The full length cDNA of rat HSL (13) used to produce pCEP4-HSL was kindly obtained from Drs. C. Holm, P. Belfrage, and M. Schotz (University of Lund, Lund, Sweden, and UCLA). Transfected cells were selected by hygromycin resistance and cells overexpressing HSL were subcloned as described previously (6).

Immunoblotting

For immunoblotting HSL, cells were scraped and briefly sonicated (3 s) in 1 ml of ice-cold lysis buffer containing 0.15 M NaCl, 3% Triton X-100, 0.1% lauryl sarcosyl, and 1 unit/ml leupeptin. Homogenates were centrifuged at 10,000 *g* for 15 min and the supernatants were taken for electrophoresis. For immunoblotting LDL receptors, cells were homogenized at 18°C in 20 mM Tris-HCl (pH 7.4), 1.0 mM EDTA, 255 mM sucrose, and 100 μM leupeptin, and total membranes were prepared by centrifugation at 100,000 *g* for 60 min in a Beckman 70 Ti rotor. Samples were electrophoresed on 10% polyacrylamide gels under reducing conditions, transferred to nitrocellulose, incubated with anti-rat HSL/fusion protein IgG (2) or with anti-rat LDL receptor/fusion protein IgG (14), and visualized by chemiluminescence as described previously. The relative amounts of immuno-detectable protein contained in each lane were determined by scanning with an LKB Ultra scan XL enhancer laser densitometer and Gel scan XL software (Pharmacia LKB Biotechnology, Piscataway, NJ) on a NEC computer.

RNA isolation and measurement

Total cellular RNA was extracted by $CHCl_3$–phenol extraction as described (15). RNA pellets were dissolved in sterile water and quantified by standard UV absorbency. After denaturation with 1 M glyoxal, 50% dimethyl sulfoxide, RNA was analyzed by Northern blot hybridization after electrophoresis on 1% agarose gels. Probes used were: rat HSL cDNA and rat 18S ribosomal RNA cDNA. Probes were labeled with [^{32}P]-dCTP to a specific activity of 1–2 × 10^9 dpm/μg with an oligolabeling kit. Prehybridization and hybridization procedures were performed as previously described (15). Autoradiographs were visualized in a PhosphorImager® (Molecular Dynamics, Sunnyvale, CA).

Lipoprotein metabolism

Human LDL (d 1.019–1.063 g/ml) were isolated from EDTA-treated plasma by density gradient ultracentrifugation and dialyzed against phosphate-buffered saline containing 0.01% EDTA as described previously (16). The lipoproteins were radiolabeled with Na^{125}I using Iodogen beads (Pierce Chemical Co.). Lipoprotein metabolism studies (Figs. 2, 3, and 5) were performed with the 3F9 clone of HSL transfectants. Cells were subcultured in 24-well tissue culture plates and incubated in Coon's F12:Dulbecco's Modified Eagle's media (1:1) containing 10% fetal bovine serum for 2 days. The medium was removed and the cells were incubated

overnight in fresh medium containing 10% lipoprotein-deficient fetal bovine serum (LPDS), unless otherwise stated. The cells were washed three times and the metabolism of lipoproteins was measured as described previously (17). Briefly, the binding of lipoproteins was measured at 4°C in medium containing 10% LPDS. The amount of specific binding was calculated by subtracting the amount bound in the presence of an excess (≥20-fold) of unlabeled lipoprotein (non-specific binding) from the amount bound in the absence of unlabeled lipoprotein (total binding). The K_d and B_o values were calculated by Scatchard analysis. The cell association (bound and intracellular) and degradation of lipoproteins were measured at 37°C after a 5-h incubation. The amount of specific cell association and degradation were calculated as described for lipoprotein binding. All values were normalized for differences in the amount of cell protein per each well.

Enzyme activities and cholesterol synthesis

Measurement of HSL activity was performed using a cholesteryl[^{14}C]oleate emulsion as described previously (2). Cells were scraped into 1 ml of 50 mM Tris-HCl and 1 mM EDTA containing 1 unit/ml leupeptin and homogenized. After centrifuging the homogenates at 14,000 *g* for 15 min, the supernatants were removed, and aliquots (50–100 μl) were assayed in duplicate for neutral cholesterol esterase activity (2). The activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of cholesterol biosynthesis, was assayed as described previously (18). Cells were washed, homogenized in 0.1 M KPO_4, 0.25 μM sucrose, 10 mM EDTA, 30 mM KCl, 5 mM dithiothreitol, pH 7.4, and disrupted by sonication. After centrifugation (1,000 *g*, 15 min, 4°C), the post nuclear homogenate supernatants were assayed in duplicate for HMG-CoA reductase activity. Cholesterol synthesis was measured as the incorporation of [^{14}C]acetate into cholesterol (19). Cells were incubated with 1 μCi/ml [U-^{14}C]acetate for 1 h at 37°C. After scraping, the cells were saponified in ethanolic KOH, the nonsaponifiable sterols were extracted with hexane, and resolved by thin-layer chromatography with toluene–ethyl acetate 4:1. The location corresponding to the migration of purified cholesterol was scraped and the radioactivity was determined in a liquid scintillation spectrophotometer.

Other assays

Protein was measured with a bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL). Cellular cholesteryl ester, unesterified cholesterol, and triglyceride mass were measured as described previously (20).

Statistical analysis

Data are expressed as mean ± SEM. Statistical analyses were performed by analysis of variance and comparisons among groups by Bonferroni/Dunn using StatView™ software (ABACUS Concepts, Berkeley, CA) on a Power Macintosh computer.

RESULTS

After transfection of CHO cells with pCEP4-HSL and selection with hygromycin B, several cells were isolated that expressed HSL. These cells were subcloned by serial dilution to yield several different clonal lines that stably expressed HSL. **Figure 1** displays the expression of HSL in four different clones stably transfected with pCEP4-HSL. A Northern blot of HSL mRNA showed that HSL mRNA was undetectable in control CHO cells, but large amounts of HSL mRNA were observed in the cells transfected with pCEP4-HSL (Fig. 1A). To document that the high levels of HSL mRNA expressed in the cells transfected with pCEP4-HSL were translated, extracts of the CHO cells were immunoblotted with anti-rat HSL/fusion protein antibodies (Fig. 1B). As observed for HSL mRNA, immunoreactive HSL protein was not detected in control CHO cells, but large quantities of 84 kD immunoreactive HSL protein were detected in the cells transfected with pCEP4-HSL. HSL activity paralleled the amount of HSL mRNA and HSL protein (Fig. 1C). A very low level of HSL activity was detected in control CHO cells (1.2 ± 0.6 nmol/mg/h), while high, but slightly variable, HSL activities (ranging from 658 ± 102 to 866 ± 79 nmol/mg/h) were seen in the cells transfected with pCEP4-HSL. Thus, several CHO cell lines have been isolated that have been stably transfected with HSL and that overexpress HSL mRNA, HSL protein and HSL activity approximately 600-fold.

The levels of cellular cholesterol (both esterified and unesterified) and triglyceride were measured in order to document potential alterations in cellular lipid content resulting from the overexpression of HSL. As shown in **Table 1,** small amounts of cholesteryl esters were found in control CHO cells cultured in cholesterol replete conditions in fetal calf serum that were reduced to barely detectable levels when the cells were cultured in cholesterol deplete conditions in lipoprotein-deficient serum ($P < 0.001$). Consistent with the actions of HSL, cells overexpressing HSL contained barely detectable levels of cholesteryl esters when cultured in either cholesterol replete or deplete conditions ($P < 0.001$). Cellular unesterified cholesterol was greatest in control CHO cells cultured in cholesterol replete media and

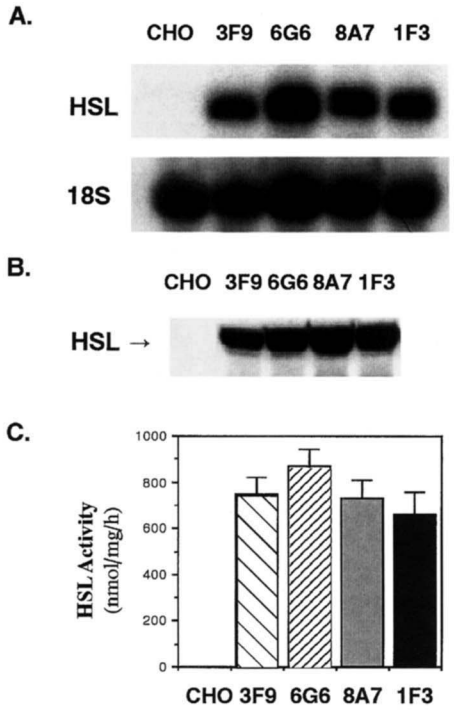

Fig. 1. HSL expression in transfected CHO cells. CHO cells were transfected with pCEP4-HSL, selected by hygromycin resistance, and stably transfected cell lines were cloned as described in Materials and Methods. Panel A: Northern blot analysis of HSL mRNA and 18S ribosomal RNA mRNA in control CHO cells and four different clonal lines stably transfected with pCEP4-HSL. Total RNA (20 μg) was electrophoresed on 1% agarose gels, transferred to nylon, and hybridized with an HSL cDNA and 18S ribosomal RNA cDNA. Panel B: Immunoblot of HSL protein in control CHO cells and four different clonal lines stably transfected with pCEP4-HSL. Cell extracts (25 μg) were electrophoresed on SDS-PAGE, transferred to nitrocellulose filters, immunoblotted with anti-HSL/fusion protein IgG, and visualized by enhanced chemiluminescence. The film was developed after a 10 s exposure. Panel C: HSL activity in control CHO cells and four different clonal lines stably transfected with pCEP4-HSL. Neutral cholesteryl ester hydrolase activity was measured in postnuclear extracts of cells as described in Materials and Methods. Results are the mean ± SEM of triplicate wells.

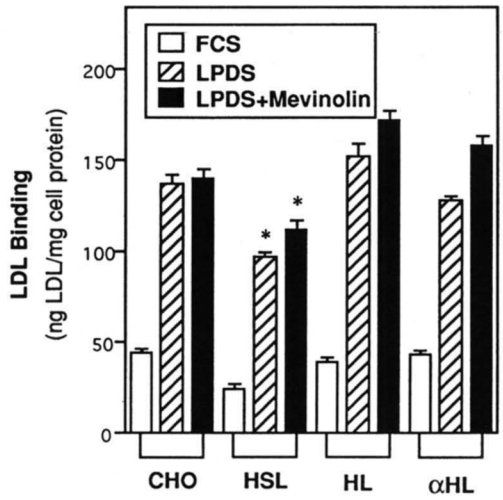

Fig. 2. LDL binding in transfected CHO cells. After reaching 70% confluence, the media were changed and control CHO cells, CHO cells stably transfected with hormone-sensitive lipase (HSL), CHO cells stably transfected with hepatic lipase (HL), and CHO cells stably transfected with hepatic lipase in the wrong orientation (αHL) were cultured for 24 h in 10% fetal calf serum (FCS), 10% lipoprotein-deficient fetal calf serum (LPDS), or 10% lipoprotein-deficient fetal calf serum plus 20 μM mevinolin (LPDS + Mevinolin) prior to measuring the specific binding of 10 μg/ml ^{125}I-labeled LDL at 4°C as described in Materials and Methods. The results are the mean ± SD of triplicate wells and are representative of three separate experiments. *, $P < 0.05$ compared to respective control CHO cells.

was reduced by the overexpression of HSL and by culturing cells in cholesterol deplete media ($P < 0.001$). Cellular triglycerides were extremely low in both control and HSL transfected CHO cells.

The availability of these HSL transfectants allowed the effects of overexpression of HSL on LDL receptor expression to be examined by comparing the surface binding of ^{125}I-labeled LDL to control and HSL-transfected cells (**Fig. 2**). When cells were maintained in fetal calf serum (conditions in which cellular cholesterol pools should be expanded and LDL receptors down-regulated), no differences in LDL binding were

TABLE 1. Cellular lipid mass in transfected CHO cells

	Cholesteryl Ester		Unesterified Cholesterol		Triglyceride	
Groups	FCS	LPDS	FCS	LPDS	FCS	LPDS
	μg/mg cell protein					
Control	1.64 ± 0.51	0.15 ± 0.03[a]	60.2 ± 4.8	26.3 ± 2.6[a]	0.06 ± 0.01	0.09 ± 0.01[a]
HSL	0.15 ± 0.06[a]	0.09 ± 0.04	34.7 ± 2.1[a]	14.4 ± 1.6[b]	0.06 ± 0.01	0.08 ± 0.01[b]

After reaching 70% confluence, the media were changed and control CHO cells and one clone (3F9) of CHO cells stably transfected with hormone-sensitive lipase (HSL) were cultured for 24 h in either 10% fetal calf serum (FCS) or 10% lipoprotein-deficient fetal calf serum (LPDS) prior to measuring cellular lipid mass as described in the Materials and Methods. The results are the mean ± SEM of sextuplicate wells.

[a] $P < 0.001$ compared to control CHO cells grown in FCS.

[b] $P < 0.001$ compared to HSL transfectants grown in FCS.

observed between control and HSL-transfected cells. However, when cells were cultured in lipoprotein-deficient serum for 24 h to induce LDL receptors, there was a marked increase in specific LDL binding in both control and HSL-transfected cells, but cells transfected with HSL bound ~25–40% less LDL than control cells ($P < 0.01$). The addition of mevinolin to inhibit endogenous cholesterol synthesis increased LDL binding further in both control and HSL-transfected cells, but the HSL transfectants still bound ~25–40% less LDL. No differences in nonspecific LDL binding were observed among the control or HSL-transfected cells in any of the culture conditions. In order to document that the decrease in LDL receptor binding observed in the HSL transfectants was not due to the process of transfection with pCEP4 and subcloning the cells to isolate stable transfectants, the binding of LDL was examined in a cloned CHO cell line that was stably transfected with a secreted form of rat hepatic lipase (HL) or with rat hepatic lipase transfected in the wrong orientation (αHL) (21). In contrast to cells transfected with HSL, where LDL binding was reduced ~25% under culture conditions that induce LDL receptors, cells transfected with HL, in either the sense or reverse orientation, bound LDL to equivalent degrees as control cells whether cultured in fetal calf serum, lipoprotein-deficient serum, or in the presence of lipoprotein-deficient serum and mevinolin (Fig. 2). Thus, neither the transfection, selection in hygromycin, nor the cloning of the CHO cells contributed to the decrease in LDL binding observed in the HSL transfectants.

To explore whether the reduction in LDL binding was due to a change in the number of LDL receptors, binding isotherms of ^{125}I-labeled LDL were performed (**Fig. 3**). There was a decrease in LDL binding in the HSL transfectants at each concentration of ^{125}I-labeled LDL. Scatchard analysis (inset) of these binding data documented a 20% decline in the number of LDL receptors without any change in their affinity. To confirm that the observed changes in LDL binding were due to changes in the expression of LDL receptors, LDL receptors were quantitated by immunoblotting total membranes from control and HSL-transfected cells that had been cultured in fetal calf serum or lipoprotein deficient serum (**Fig. 4**). LDL receptor expression was low and no differences were observed when cells were cultured in fetal calf serum. However, when cells were cultured in lipoprotein-deficient serum, LDL receptors increased in control and all HSL-transfected cells, but HSL transfectants expressed 35–60% fewer LDL receptors than control ($P < 0.05$). Therefore, overexpression of HSL in CHO cells did not affect LDL receptor expression when cells were grown under cholesterol replete conditions; however, HSL overexpression resulted

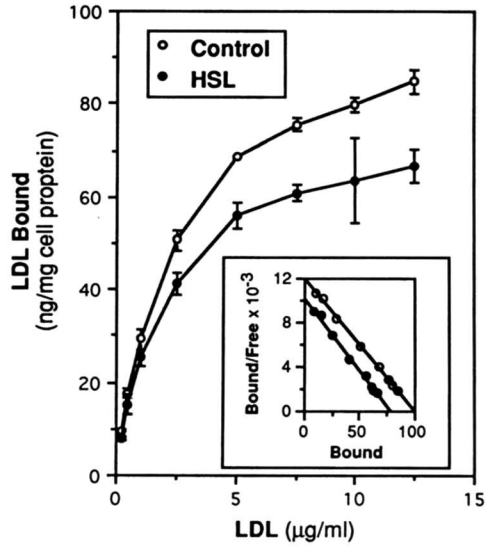

Fig. 3. LDL binding in transfected CHO cells. After reaching 70% confluence, the media were changed and control CHO cells (○) and CHO cells stably transfected with hormone-sensitive lipase (HSL) (●) were cultured for 24 h in 10% lipoprotein-deficient fetal calf serum prior to measuring the specific binding of the indicated concentrations of ^{125}I-labeled LDL at 4°C as described in Materials and Methods. The results are the mean ± SD of triplicate wells and are representative of three separate experiments. Inset: Scatchard analysis of the binding data.

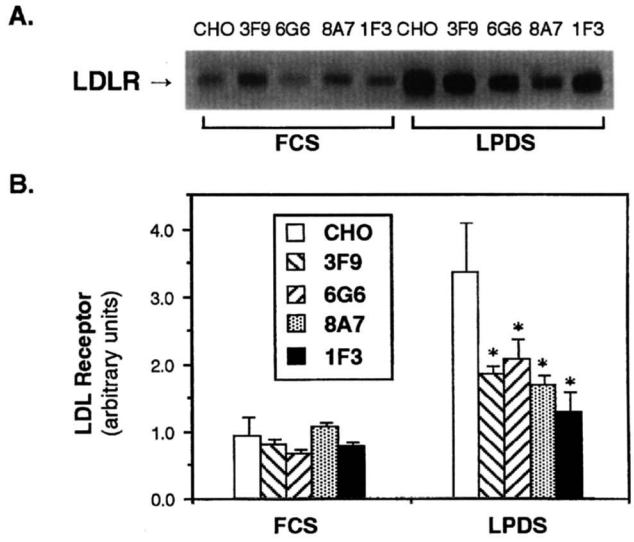

Fig. 4. Immunoblot of LDL receptors in transfected CHO cells. Panel A: Extracts (50 μg) of cell membranes prepared from control CHO cells and four different clonal lines stably transfected with pCEP4-HSL that were cultured for 24 h in either 10% fetal calf serum (FCS) or 10% lipoprotein-deficient fetal calf serum (LPDS) were electrophoresed on SDS-PAGE, transferred to nitrocellulose filters, immunoblotted with anti-LDLR/fusion protein IgG, and visualized by enhanced chemiluminescence. The film was developed after a 30 s exposure. Panel B: Densitometric analysis of immunoreactive LDL receptors in transfected CHO cells. The results are the mean ± SEM of triplicate wells. *, $P < 0.05$ compared to respective control CHO cells.

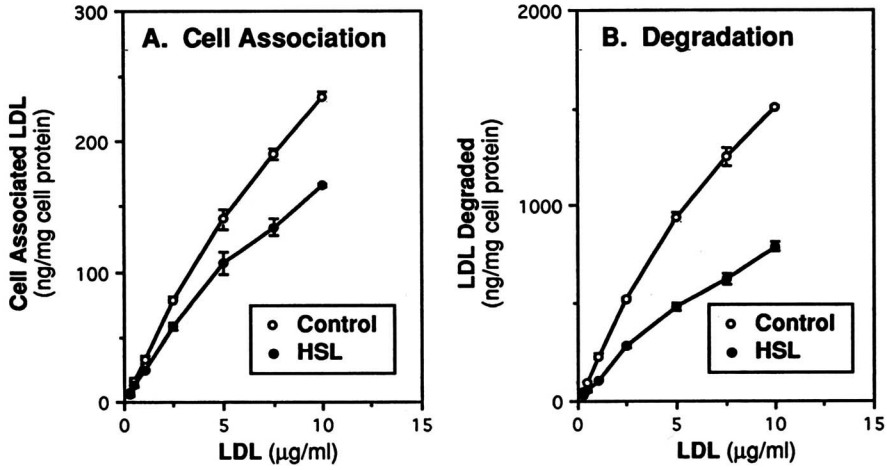

Fig. 5. Cell association (A) and degradation (B) of LDL in transfected CHO cells. After reaching 70% confluence, the media were changed and control CHO cells (○) and CHO cells stably transfected with hormone-sensitive lipase (HSL) (●) were cultured for 24 h in 10% lipoprotein-deficient fetal calf serum prior to measuring the specific cell association and degradation of the indicated concentrations of ^{125}I-labeled LDL at 37°C as described in Materials and Methods. The results are the mean ± SD of triplicate wells and are representative of three separate experiments.

in an impaired ability of cells to increase LDL receptor expression in response to conditions where exogenous cholesterol is deficient. As an additional means of substantiating the decline in LDL receptors observed in HSL-transfected cells, the cell association and degradation of ^{125}I-labeled LDL by control and HSL transfectants was investigated (**Fig. 5**). Similar to the decline in binding observed at 4°C, ^{125}I-labeled LDL cell association was 30% ($P < 0.01$) and degradation 50% ($P < 0.01$) lower in HSL transfectants than controls.

In order to examine whether other aspects of cholesterol homeostasis were impaired by the overexpression of HSL in CHO cells, the rates of cholesterol synthesis were measured in control and HSL-transfected cells grown in fetal calf serum or lipoprotein-deficient serum (**Fig. 6**). Surprisingly, when cultured in cholesterol-replete conditions in fetal calf serum, the incorporation of [^{14}C]acetate into cholesterol was elevated 3- to 10-fold among the HSL transfectants compared to control cells ($P < 0.001$). When cultured in cholesterol-deplete conditions in lipoprotein-deficient serum, cholesterol synthesis increased 9-fold in control and 7- to 11-fold in the HSL transfectants, but cholesterol synthesis was still elevated 3- to 14-fold in HSL transfectants compared to control ($P < 0.001$). Although the degree of overexpression of HSL was relatively similar among the HSL transfectants, the degree to which cholesterol synthesis was increased among the HSL transfectants varied several fold. Because the incorporation of [^{14}C]acetate into cholesterol can be an inaccurate estimation of the rate of cholesterol synthesis under conditions where acetate pool size is altered and the overexpression of HSL might be expected to alter the acetate pool size, the activity of HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, was determined as another measure of the effects of HSL overexpression on cholesterol synthesis (**Fig. 7**). When cultured in cholesterol replete conditions in fetal calf serum, HMG-CoA reductase activity was elevated 3- to 14-fold among the HSL transfectants compared to control cells ($P < 0.05$). When cultured in cholesterol deplete conditions in lipoprotein-deficient serum, HMG-CoA reductase activity increased 4-fold in control and 2- to 9-fold in the HSL transfectants, but HMG-CoA reductase activity was still

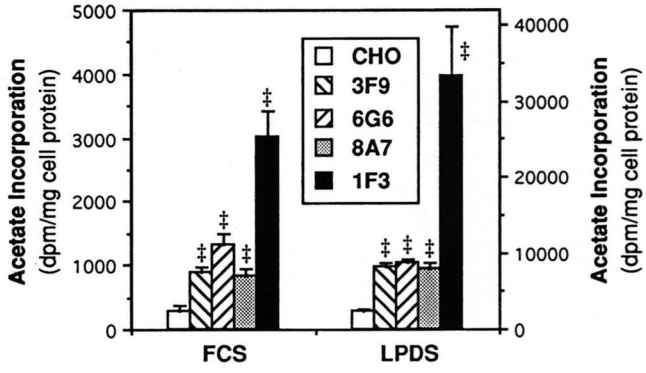

Fig. 6. Cholesterol synthesis in transfected CHO cells. Control CHO cells and four different clonal lines stably transfected with pCEP4-HSL were cultured for 24 h in either 10% fetal calf serum (FCS) or 10% lipoprotein-deficient fetal calf serum (LPDS) and the incorporation of [^{14}C]acetate into cholesterol was measured as described in the Materials and Methods. The results are the mean ± SEM of triplicate wells and are representative of two separate experiments. ‡, $P < 0.001$ compared to respective control CHO cells.

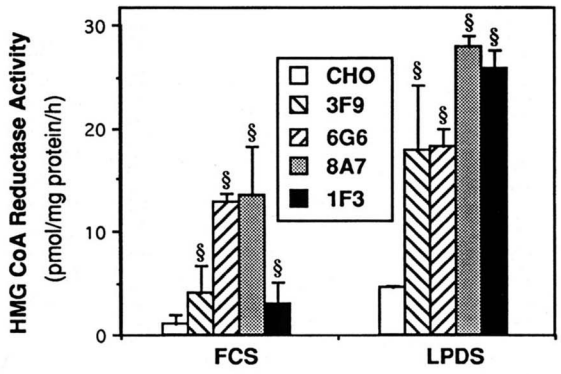

Fig. 7. Activity of HMG-CoA reductase in transfected CHO cells. Control CHO cells and four different clonal lines stably transfected with pCEP4-HSL were cultured for 24 h in either 10% fetal calf serum (FCS) or 10% lipoprotein-deficient fetal calf serum (LPDS) and HMG-CoA reductase activity was measured as described in the Materials and Methods. The results are the mean ± SEM of triplicate wells and are representative of two separate experiments. §, $P < 0.02$ compared to respective control CHO cells.

elevated 4- to 6-fold in HSL transfectants compared to control ($P < 0.02$). Thus, overexpression of HSL appears to dissociate the regulation of LDL receptor expression from cholesterol synthesis, causing an increase in cholesterol synthesis while decreasing LDL receptor expression.

DISCUSSION

A portion of cellular unesterified cholesterol functions as a putative, regulatory pool that is responsible for modulating the release from the endoplasmic reticulum of SREBPs, transcription factors that activate the transcription of a variety of sterol responsive genes, such as the LDL receptor and HMG-CoA reductase (7–11). Under most in vivo and in vitro conditions, depletion of cellular sterols is associated with a decline in both unesterified and esterified cholesterol that is accompanied by an increased expression of LDL receptors and cholesterol synthesis due to the activation of transcription of LDL receptors and enzymes involved in cholesterol biosynthesis. Conversely, overaccumulation of cellular sterols is associated with an increase primarily in cholesteryl esters that is accompanied by a decrease in expression of LDL receptors and cholesterol synthesis due to a decline in transcription of these genes caused by a fall in the release of SREBPs from the endoplasmic reticulum. In each of these conditions, the changes in SREBP occur in response to alterations in the size of the putative regulatory pool of cholesterol. Cellular cholesterol and, presumably, the putative regulatory pool of cholesterol are in equilibrium with stored cholesteryl esters through the actions of ACAT, which esterifies cholesterol to cholesteryl esters, and of neutral cholesterol esterase, which hydrolyzes cholesteryl esters to unesterified cholesterol.

In the current studies in CHO cells the overexpression of HSL, an active neutral cholesterol esterase, resulted in a decline in LDL receptors, documented by LDL binding and degradation and by immunoblot analysis, when LDL receptors were induced by lipid deprivation. The decrease in LDL receptor expression in HSL transfectants was not due to an artifact of transfection and cloning as cells transfected with a secreted form of hepatic lipase or with hepatic lipase in the wrong orientation bound LDL normally, as described previously (22). Although CHO cells accumulate very little cholesteryl esters under normal conditions (see Table 1), this observation of a decrease in LDL receptor expression is consistent with the overexpression of HSL hydrolyzing whatever cellular cholesteryl esters that had been formed through the actions of ACAT, thereby causing an expansion of the putative regulatory pool of cholesterol with the resultant decline in LDL receptors. It is interesting that the overexpression of HSL decreased the accumulation not only of cholesteryl esters, but also of total unesterified cholesterol. Thus, any expansion of a putative regulatory pool of cholesterol would appear to have occurred simultaneously with a decline in total cellular cholesterol. A similar decrease in LDL receptor expression has been observed previously when ACAT inhibitors were utilized to expand the regulatory pool of cholesterol by preventing the conversion of cholesterol to cholesteryl esters (12). However, when ACAT inhibitors were used to expand the regulatory pool of cholesterol, there was not only a decline in LDL receptors observed, but also a decrease in HMG-CoA reductase activity under conditions where LDL receptor expression and HMG-CoA reductase activity were induced by lipid depletion. Surprisingly, expansion of the regulatory pool of cholesterol by overexpression of HSL in the current studies did not suppress, but caused a large increase in HMG-CoA reductase activity and cholesterol synthesis in both sterol replete and sterol depleted conditions. The reasons for the dissociation between the regulation of LDL receptor expression and cholesterol synthesis with overexpression of HSL are unclear; however, LDL receptor expression, cholesterol synthesis, and HMG-CoA reductase activity apparently remained responsive to some aspects of sterol regulation as sterol depletion resulted in substantial increases in LDL receptor expression, cholesterol synthesis, and HMG-CoA reductase activity compared to sterol replete conditions. Although not measured in the current studies, it is possible that transcriptional regulation of LDL recep-

tors and HMG-CoA reductase remained coordinated, while post-translational events associated with the overexpression of HSL might have contributed to their apparent dissociation.

In addition to hydrolyzing any cholesteryl esters that might form, overexpression of HSL could disturb other processes through its hydrolysis of triacylglycerol, diacylglycerol, and monoacylglycerol substrates (3). Thus, it is possible that overexpression of HSL could have inhibited acetoacetyl CoA thiolase or HMG-CoA synthase through the release of products of tri-, di-, and monoacylgylcerol hydrolysis. Inhibition of acetoacetyl CoA thiolase or HMG-CoA synthase would result in an increase in HMG-CoA reductase activity (23, 24), while LDL receptor expression would be expected to be suppressed due to an expansion of the regulatory pool of cholesterol. However, this scenario is unlikely as acetate incorporation into cholesterol was increased by overexpression of HSL, suggesting that all the enzymes involved in the synthesis of cholesterol from acetate were normal or increased.

A dissociation between LDL receptor expression and cellular cholesterol synthesis or cellular cholesterol and cholesteryl ester accumulation has been observed under certain conditions. For instance, fasting or insulin deficiency produces a decrease in LDL receptor expression in adipose cells that occurs while cellular total and esterified cholesterol levels also fall (25, 26). This observation was thought to be due to the unusual feature of adipose cells where the triglyceride lipid droplet solubilizes significant amounts of unesterified cholesterol. Thus, as the adipose cells shrink, there is an expansion of the regulatory pool of cholesterol by the cholesterol released from the depletion of the lipid droplet. In addition, certain fatty acids have been shown to alter LDL receptor expression, presumably through their ability to induce changes in the regulatory pool of cholesterol, without altering cellular cholesterol synthesis (27). It is possible that release of certain fatty acids during HSL-mediated hydrolysis of various lipid substrates might have contributed to the decrease in LDL receptor expression and increase in cholesterol synthesis.

It has recently been shown that cellular sterol content, acting through SREBPs, can regulate not only the expression of LDL receptors and enzymes in the cholesterol biosynthetic pathway, but also enzymes involved in fatty acid synthesis, such as fatty acid synthase and acetyl CoA carboxylase (28, 29), suggesting a coordinated control of cholesterol and triglyceride metabolism. Data from the current studies suggest that HSL, an enzyme involved in both triglyceride and cholesteryl ester hydrolysis, can dissociate the regulation of some aspects of cholesterol homeostasis.

This work was supported in part by Research Grants from the Research Service of the Department of Veterans Affairs and by grants DK 46942 and 49705 (FBK) from the NIH.

Manuscript received 29 October 1996 and in revised form 17 April 1997.

REFERENCES

1. Holm, C., T. G. Kirchgessner, K. L. Svenson, G. Fredrikson, S. Nilsson, C. G. Miller, J. E. Shively, C. Heinzmann, R. S. Sparkes, T. Mohandas, A. J. Lusis, P. Belfrage, and M. C. Schotz. 1988. Hormone-sensitive lipase: sequence, expression, and chromosomal localization to 19 cent-q13.3. *Science.* **241:** 1503–1506.
2. Kraemer, F. B., S. Patel, M. S. Saedi, and C. Sztalryd. 1993. Detection of hormone-sensitive lipase in various tissues. 1. Expression of an HSL/bacterial fusion protein and generation of anti-HSL antibodies. *J. Lipid Res.* **34:** 663–671.
3. Fredrikson, G., P. Stralfors, N. O. Nilsson, and P. Belfrage. 1981. Hormone-sensitive lipase of rat adipose tissue: purification and some properties. *J. Biol. Chem.* **256:** 6311–6320.
4. Stralfors, P., H. Olsson, and P. Belfrage. 1987. Hormone-sensitive lipase. *In* The Enzymes. P. D. Boyer and E. G. Krebs, editors. Academic Press, New York. 147–177.
5. Yeaman, S. J. 1990. Hormone-sensitive lipase—a multipurpose enzyme in lipid metabolism. *Biochim. Biophys. Acta.* **1052:** 128–132.
6. Sztalryd, C., M. C. Komaromy, and F. B. Kraemer. 1995. Overexpression of hormone-sensitive lipase prevents triglyceride accumulation in adipocytes. *J. Clin. Invest.* **95:** 2652–2661.
7. Goldstein, J. L., and M. S. Brown. 1990. Regulation of the mevalonate pathway. *Nature.* **343:** 425–430.
8. Wiang, X., R. Sato, M. S. Brown, X. Hua, and J. L. Goldstein. 1994. SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis. *Cell.* **77:** 53–62.
9. Ericsson, J., S. M. Jackson, and P. A. Edwards. 1996. Sterol regulatory element binding protein binds to a *cis* element in the promoter of the farnesyl diphosphate synthase gene. *Proc. Natl. Acad. Sci. USA.* **93:** 945–950.
10. Lloyd, D. B., and J. F. Thompson. 1996. Transcriptional modulators affect in vivo protein binding to the low density lipoprotein receptor and 3-hydroxy-3-methylglutaryl coenzyme A reductase promoters. *J. Biol. Chem.* **270:** 25812–25818.
11. Guan, G., G. Jiang, R. L. Koch, and I. Shechter. 1995. Molecular cloning and functional analysis of the promoter of the human squalene synthase gene. *J. Biol. Chem.* **270:** 21958–21965.
12. Tabas, I., D. A. Weiland, and A. R. Tall. 1986. Inhibition of acyl coenzyme A:cholesterol acyl transferase in J774 macrophages enhances down-regulation of the low density lipoprotein receptor and 3-hydroxy-3-methylglutaryl-coenzyme A reductase and prevents low density lipoprotein-induced cholesterol accumulation. *J. Biol. Chem.* **261:** 3147–3154.
13. Holm, C., T. G. Kirchgessner, K. L. Svenson, A. J. Lusis, P. Belfrage, and M. C. Schotz. 1988. Nucleotide sequence of rat adipose hormone sensitive lipase cDNA. *Nucleic Acids Res.* **16:** 9879.

14. Kraemer, F. B., V. Natu, A. Singh-Bist, S. Patel, M. C. Komaromy, S. Medicherla, S. Azhar, and C. Sztalryd. 1996. Isoproterenol decreases LDL receptor expression in rat adipose cells: activation of cyclic AMP-dependent proteolysis. *J. Lipid Res.* **37:** 237–249.
15. Kraemer, F. B., K. Tavangar, and A. R. Hoffman. 1991. Developmental regulation of hormone-sensitive lipase mRNA in the rat: changes in steroidogenic tissues. *J. Lipid Res.* **32:** 1303–1310.
16. Ellsworth, J., F. B. Kraemer, and A. D. Copper. 1987. Transport of β-VLDL and chylomicron remnants by macrophages is mediated by the low density lipoprotein receptor. *J. Biol. Chem.* **262:** 2316–2325.
17. Ellsworth, J. L., L. G. Fong, F. B. Kraemer, and A. D. Cooper. 1990. Differences in the processing of chylomicron remnants and β-VLDL by macrophages. *J. Lipid Res.* **31:** 1399–1411.
18. Ellsworth, J., A. D. Cooper, and F. B. Kraemer. 1986. Evidence that chylomicron remnants and β-VLDL are transported by the same receptor pathway in J774 murine macrophage-derived cells. *J. Lipid Res.* **27:** 1062–1072.
19. Kraemer, F. B. 1986. Insulin deficiency alters cellular cholesterol metabolism in murine macrophages. *Diabetes.* **35:** 764–770.
20. Kraemer, F. B., Y-D. I. Chen, R. D. Lopez, and G. M. Reaven. 1983. Characterization of binding sites on thioglycolate-stimulated mouse peritoneal macrophages that mediate the uptake of very low density lipoproteins. *J. Biol. Chem.* **258:** 12190–12197.
21. Komaromy, M., and M. Reed. 1991. Expression of rat hepatic lipase in heterologous systems: evidence for different sites for interface binding and catalysis. *J. Lipid Res.* **32:** 963–975.
22. Choi, S. Y., M. Komaromy, J. Chen, L. G. Fong, and A. D. Cooper. 1994. Acceleration of uptake of LDL but not chylomicrons or chylomicron remnants by cells that secrete apoE and hepatic lipase. *J. Lipid Res.* **35:** 848–859.
23. Clinkenbeard, K. D., W. D. Reed, R. A. Mooney, and M. D. Lane. 1975. Intracellular localization of the 3-hydroxy-3-methylglutaryl coenzyme A cycle enzymes in liver. *J. Biol. Chem.* **250:** 3108–3116.
24. Schnitzer-Polokoff, R., C. von Gunten, J. Logel, R. Torget, and M. Sinensky. 1982. Isolation and characterization of a mammalian cell mutant defective in 3-hydroxy-3-methylglutaryl coenzyme A synthase. *J. Biol. Chem.* **257:** 472–476.
25. Kraemer, F. B., C. Laane, B. Park, and C. Sztalryd. 1994. Low-density lipoprotein receptors in rat adipocytes: regulation with fasting. *Am. J. Physiol.* **266:** E26–E32.
26. Swami, S., C. Sztalryd, and F. B. Kraemer. 1996. Effects of streptozotocin-induced diabetes on low density lipoprotein receptor expression in rat adipose tissue. *J. Lipid Res.* **37:** 229–236.
27. Daumerie, C. M., L. A. Woollett, and J. M. Dietschy. 1992. Fatty acids regulate hepatic low density lipoprotein receptor activity through redistribution of intracellular cholesterol pools. *Proc. Natl. Acad. Sci. USA.* **89:** 10797–10801.
28. Bennett, M. K., J. M. Lopez, H. B. Sanchez, and T. F. Osborne. 1995. Sterol regulation of fatty acid synthase promoter: coordinate feedback regulation of two major lipid pathways. *J. Biol. Chem.* **270:** 25578–25583.
29. Lopez, J. M., M. K. Bennett, and T. F. Osborne. 1996. Sterol regulation of acetyl coenzyme A carboxylase: a mechanism for coordinate control of cellular lipid. *Proc. Natl. Acad. Sci. USA.* **93:** 1049–1053.