Estrogens Induce Low-Density Lipoprotein Receptor Activity and Decrease Intracellular Cholesterol in Human Hepatoma Cell Line Hep G2†

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Received September 25, 1986; Revised Manuscript Received March 31, 1987

ABSTRACT: Administration of estrogens in pharmacologic doses to rats and rabbits induces hepatic low-density lipoprotein (LDL) receptor activity. To determine if estrogens can regulate LDL receptor activity in human cells, ^{125}I-LDL binding and ligand blotting studies were performed with the cell line Hep G2, well-differentiated cells derived from a human hepatoma, and with normal human fibroblasts. Addition of estradiol to Hep G2 cells growing in lipoprotein-deficient medium increased cell surface receptor activity by 141%, whereas fibroblast receptors were slightly reduced. Measurement of LDL internalization and degradation showed that estradiol induced the entire LDL receptor pathway and not simply surface receptors for LDL. Scatchard analysis of specific binding data in Hep G2 cells revealed that increased LDL receptor activity was due to high-affinity binding. When Hep G2 cells were incubated with LDL as well as estradiol, estradiol induction of LDL receptor activity did not occur. Estrogen treatment reduced Hep G2 free cholesterol content by 24% as determined by gas–liquid chromatography but had no significant effect on fibroblast free cholesterol, suggesting that estrogens may induce Hep G2 LDL receptor activity indirectly by lowering intracellular cholesterol. LDL receptor activity in Hep G2 cells grown in the absence of estradiol was resistant to down-regulation by LDL; incubation of cells with LDL for 48 h reduced receptor activity by only 25.8% in Hep G2 cells compared to 80.3% in fibroblasts. The Hep G2 LDL receptor was shown to be biochemically similar to the fibroblast receptor by ligand blotting and immunoblotting with IgG-C7, a monoclonal antibody to the extrahepatic LDL receptor. Hep G2 LDL receptor induction by estradiol and resistance to down-regulation by LDL were confirmed by ligand blotting. Therefore, although the same LDL receptor protein appears to be present in Hep G2 cells and fibroblasts, regulation of the LDL receptor pathway differs in these cell types.

Despite considerable recent progress in the field of hepatic lipoprotein metabolism, the exact mechanisms whereby hepatocytes interact with circulating lipoproteins remain unclear. The liver is thought to express two lipoprotein receptors that appear genetically, physiologically, and biochemically distinct, the low-density lipoprotein (LDL)[1] receptor and the apo-E or chylomicron remnant receptor (Brown & Goldstein, 1983; Hoeg et al., 1985; Hui et al., 1986). Considerable attention has been focused on the hepatic LDL receptor because of the well-defined role of the LDL receptor pathway in regulating cholesterol metabolism in extrahepatic tissues (Goldstein & Brown, 1982).

In vitro and in vivo studies of hepatic LDL receptor activity in several species have yielded results indicative of both similarities and differences between hepatic and extrahepatic LDL receptors. LDL receptors with ^{125}I-LDL binding properties resembling the classic fibroblast receptor have been well described in liver membranes from rats treated with estradiol (Kovanen et al., 1979; Windler et al., 1980) and colestipol-treated dogs or dogs subjected to a prolonged fast (Kovanen et al., 1981; Mahley et al., 1981). Human hepatic membranes assayed at 37 °C bind ^{125}I-LDL specifically, but in contrast to the fibroblast receptor, this binding is only partially EDTA sensitive (Hoeg et al., 1985, 1984). Other investigators studying human hepatic membranes found similar results in assays conducted at 4 °C (Harders-Spengel et al., 1982). Porcine hepatic tissue has been shown to possess an LDL binding site of broad specificity that does not discriminate well between LDL and HDL (Bachorik et al., 1978, 1982). Two groups have recently identified LDL receptor protein in human liver with the same apparent molecular weight as the fibroblast receptor (Hui et al., 1986; Soutar et al., 1986) while another group has detected LDL binding proteins of higher molecular weight than the fibroblast receptor in human liver (Hoeg et al., 1986).

These studies leave unanswered the question: Is the human hepatic LDL receptor subject to the same regulation as the extrahepatic receptor? The present work addresses this question by studying the human hepatoma cell line Hep G2, a well-differentiated cell line (Knowles et al., 1980) known to possess receptors for LDL binding and degradation (Havekes et al., 1983; Dashti et al., 1984).

Experimental Procedures

Cell Preparations. Hep G2 cells were kindly provided by Dr. Barbara Knowles of The Wistar Institute, Philadelphia, PA. Skin fibroblasts were obtained by biopsy of the deltoid region of a normal subject. For Western blotting studies, Hep G2 cells were plated on day 1 at 1.0 million cells per 150-mm culture dish in MEM + 10% FBS + 4 mM glutamine; fi-

† This work was supported by NIH Grant RO1-HL29229.

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[1] Abbreviations: LDL, low-density lipoprotein; HDL, high-density lipoprotein; BSA, bovine serum albumin; LPDS, lipoprotein-deficient serum; apo, apolipoprotein; FBS, fetal bovine serum; EDTA, ethylenediaminetetraacetic acid; EDTA–saline, 0.15 M NaCl containing 0.3 mM EDTA, pH 7.4; *d*, density; MEM, minimum essential medium; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CoA, coenzyme A.

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broblasts were plated at the same density in MEM + 15% FBS. On day 4, cells were washed twice with Puck's saline G and fed MEM + 10% LPDS (Semenkovich et al., 1982) alone or supplemented with 50 μg/mL LDL in EDTA–saline or 10 μg/mL 17β-estradiol (Sigma, St. Louis, MO) in ethanol as described in the legend to Figure 5. For blotting experiments, Hep G2 cells and fibroblasts were harvested on day 6 after 48-h incubation in LPDS-containing media.

For ^{125}I-LDL binding studies, Hep G2 cells were plated on day 1 in the same medium used for blotting studies at 10 000 cells per 22-mm well; fibroblasts were plated in the same medium used for blotting studies at 25 000 cells per 22-mm well. The protein content per well of fibroblasts and Hep G2 cells was equal at cell harvest. On day 4 or 5, cells were washed twice with saline G and fed MEM + 10% LPDS ± 50 μg/mL LDL or MEM + 10% LPDS ± estradiol. If LPDS treatment took place on day 5, cells were refed initial plating media on day 4. After 48-h incubation in LPDS ± LDL media, cells were washed twice with saline G and incubated at 37 °C in a 95% air–5% CO_2 incubator in saline G for 30 min to allow internalization of previously bound LDL. Cluster dishes were then placed in a cold room at 4 °C for surface binding studies or returned to the incubator with tracer media for 37 °C binding studies.

Monoclonal Antibody Preparation. Mouse hybridoma cells producing IgG-C7, a monoclonal antibody raised against the bovine adrenal LDL receptor (Beisiegel et al., 1981), were kindly provided by Drs. Michael Brown and Joseph Goldstein. Antibody was isolated as described previously (Semenkovich & Ostlund, 1986). Monoclonal antibody recognizing apolipoprotein A1 was a gift from Dr. Gustav Schonfeld.

Western Blotting. Receptors from whole cells were solubilized in 1.6% Triton X-100, 0.3 mM leupeptin, 5.0 M urea, and 1.5 mM phenylmethanesulfonyl fluoride as reported previously (Semenkovich & Ostlund, 1986; Semenkovich et al., 1985). Ligand blotting (Daniel et al., 1983) and immunoblotting (Beisiegel et al., 1982) were performed as previously described (Semenkovich & Ostlund, 1986; Semenkovich et al., 1985) except ligand blotting buffers contained 45 mM NaCl and 25 mM Tris-HCl instead of 90 and 50 mM, respectively. Lower ionic strength buffers enhanced receptor detection. Briefly, solubilized cell extracts were electrophoresed in 0.1% SDS–6% polyacrylamide gels (Laemmli, 1970) and then transferred to unmodified nitrocellulose paper (Burnette, 1981). Nitrocellulose strips were blocked with BSA–goat LPDS buffer, incubated with apo-B-containing lipoproteins (for ligand blotting) or monoclonal antibody IgG-C7 or anti-apo-A1 (for immunoblotting), washed extensively, incubated with affinity-purified ^{125}I-labeled goat anti-LDL antibody (ligand blotting) or ^{125}I-labeled goat anti-mouse IgG antibody (immunoblotting), washed extensively, and then dried and subjected to autoradiography. In some experiments, BSA-containing buffers were adsorbed with anti-apo-B–agarose overnight at 4 °C to remove trace amounts of bovine lipoproteins in BSA.

Lipoprotein Preparation. Normal human LDL was prepared by rate-zonal ultracentrifugation (Semenkovich et al., 1985) and iodinated by the iodine monochloride technique (Semenkovich et al., 1982); acetyl-LDL was prepared as previously described (Semenkovich & Ostlund, 1986). Human postprandial d <1.0063 lipoprotein was prepared from postprandial plasma as previously described (Semenkovich & Ostlund, 1986).

^{125}I-LDL Binding Studies. Tracer media for 4 °C surface binding studies consisted of MEM buffered with 25 mM HEPES containing 10% LPDS and variable concentrations of ^{125}I-LDL ± a 25-fold excess of unlabeled LDL. Binding studies at 37 °C were performed with the same media buffered with sodium bicarbonate instead of HEPES. For surface binding experiments, incubations in the presence of ^{125}I-LDL were carried out for 2 h at 4 °C. At the end of the incubation period, the tracer media were discarded, the cells were washed extensively, and dextran sulfate releasable surface binding was determined (Semenkovich et al., 1982). For 37 °C binding experiments, dextran sulfate releasable ^{125}I-LDL, internalized ^{125}I-LDL, and degraded ^{125}I-LDL were determined (Semenkovich et al., 1982). Ligand specifically bound, internalized, or degraded was determined by subtracting ^{125}I-LDL bound, internalized, or degraded in wells containing excess unlabeled LDL from the same parameters measured in the absence of unlabeled LDL.

Other Assays. Protein determinations were by the method of Lowry et al. (1951) except for monoclonal antibodies, which were assayed by the method of Bradford (1976). Free cholesterol was determined by gas–liquid chromatography of 2:1 chloroform:methanol cell extracts using 5α-cholestane as an internal standard as described (Ostlund & Yang, 1985). Total cholesterol was determined by the same procedure after saponification of cholesterol esters in 1 mL of 10% KOH in 9:1 ethanol:H_2O at 56 °C for 30 min (Sokoloff & Rothblat, 1972). Esterified cholesterol was calculated as the difference between total and free cholesterol.

RESULTS

The regulation of LDL receptor activity in Hep G2 cells and fibroblasts was first studied by assessing LDL binding activity after treatment of cultured cells with 17β-estradiol; estrogens in pharmacologic doses are known to induce LDL receptor activity in hepatic tissue from rats (Kovanen et al., 1979; Windler et al., 1980) and rabbits (Ma et al., 1986), but the effect in human hepatic tissue is unknown. When Hep G2 cells and fibroblasts were incubated for 48 h in lipoprotein-deficient media in the absence or presence of different concentrations of 17β-estradiol, LDL binding activity assayed at 4 °C was consistently induced by estradiol treatment in Hep G2 cells and consistently decreased by estradiol in fibroblasts (Figure 1, panel A). A striking increase in Hep G2 receptor activity occurred after treatment with 10 μg/mL 17β-estradiol, a dose not associated with cell toxicity. Treatment with 17α-ethinylestradiol resulted in similar increases in Hep G2 and decreases in fibroblast receptor activity (data not shown). The time course of the effect of 10 μg/mL 17β-estradiol on receptor activity in Hep G2 and fibroblasts is presented in Figure 1, panel B. Consistent differences in LDL receptor activity were detected by 18 h for both cell types.

To confirm that estradiol treatment induces the entire LDL receptor pathway and not merely surface binding of LDL, ^{125}I-LDL binding and degradation studies were performed at 37 °C in cells pretreated with estradiol (Table I). When Hep G2 cells were pretreated with 10 μg/mL 17β-estradiol and assayed at 37 °C, the amount of LDL specifically internalized increased 108% compared to untreated cells; the amount of LDL specifically degraded was increased 152% by estrogen treatment. These changes paralleled the 141% increase in specific surface binding (dextran sulfate releasable) induced by estrogen in Hep G2 cells. Estrogen treatment in fibroblasts resulted in decreases of 13%, 11%, and 24% in surface binding, internalized LDL, and degraded LDL, respectively.

Estradiol induction of LDL receptor activity was also evident in the specific binding dose–response experiment depicted in Figure 2 (panel A). When the dose–response data from panel

Table I: Effect of 17β-Estradiol on Specific LDL Binding and Degradation[a]

cell type and assay conditions	ng of LDL specifically bound or degraded/mg of cell protein: dextran sulfate releasable binding	LDL internalized	LDL degraded
Hep G2, ethanol only	6.8 ± 0.7	108.5 ± 5.8	122.0 ± 7.6
Hep G2, 10 μg/mL 17β-estradiol in ethanol	16.4 ± 0.9 ($p < 0.01$)	225.9 ± 11.2 ($p < 0.001$)	307.2 ± 17.3 ($p < 0.001$)
fibroblasts, ethanol only	57.6 ± 1.5	532.5 ± 9.1	665.6 ± 23.9
fibroblasts, 10 μg/mL 17β-estradiol in ethanol	49.9 ± 3.9	475.4 ± 15.6 ($p < 0.02$)	506.0 ± 16.1 ($p < 0.001$)

[a] After 4 days of initial cell growth, cells were washed and fed MEM + 10% LPDS + 0.05% ethanol with or without 10 μg/mL β-estradiol. Forty-eight hours later, the media were placed with MEM + 10% LPDS + 7.5 μg/mL ^{125}I-LDL ± 187.5 μg/mL unlabeled LDL and incubated for 2 h at 37 °C in a 95% air–5% CO_2 incubator. The cells and media were then processed for LDL binding and degradation.

Table II: Effect of LDL and 17β-Estradiol on Specific LDL Surface Binding[a]

cell type	ng of LDL specifically bound/mg of cell protein for preincubation conditions: LPDS	50 μg/mL LDL	10 μg/mL 17β-estradiol	50 μg/mL LDL + 10 μg/mL 17β-estradiol
Hep G2	11.7 ± 2.5	6.5 ± 0.7	29.1 ± 4.4 ($p < 0.01$ vs. LPDS)	8.9 ± 1.3
fibroblasts	71.7 ± 3.1	5.1 ± 1.0	55.0 ± 5.1	5.5 ± 0.6

[a] After 4 days of initial cell growth, cells were washed and fed MEM + 10% LPDS containing EDTA–saline + 0.05% ethanol (LPDS condition), 50 μg/mL LDL, 10 μg/mL 17β-estradiol, or 50 μg/mL LDL + 10 μg/mL 17β-estradiol. Thirty-six hours later, the cells were washed with saline G and prechilled MEM + 25 mM HEPES + 10% LPDS + 7.5 μg/mL ^{125}I-LDL ± 187.5 μg/mL unlabeled LDL was added to each well. The cells were incubated at 4 °C for 2 h and then assayed for LDL surface binding.

Table III: Effect of 17β-Estradiol on Cellular Cholesterol and Cholesterol Ester Content[a]

cell type	treatment	cholesterol content (μg of cholesterol/mg of protein)	cholesterol ester content (μg of cholesterol ester/mg of protein)
Hep G2	no estradiol	34.5 ± 2.6 ($p < 0.02$)	7.5 ± 1.9 (NS)
	10 μg/mL 17β-estradiol	26.2 ± 1.7	7.3 ± 1.8
fibroblasts	no estradiol	28.5 ± 3.5 (NS)	1.8 ± 0.7 (NS)
	10 μg/mL 17β-estradiol	26.9 ± 3.4	3.0 ± 1.0

[a] Hep G2 cells were plated at 100 000 cells/60-mm dish and fibroblasts at 450 000 cells/100-mm dish in order to measure similar amounts of sterol in each cell type. After 2 days of growth in initial plating medium, cells were refed. One day later, cells were washed and then fed MEM + 10% LPDS containing 0.05% ethanol only (no estradiol condition) or 0.05% ethanol containing 17β-estradiol to yield a final concentration of 10 μg/mL estradiol. After 48 h, cells were washed, extensively and cholesterol and cholesterol ester content was determined by gas–liquid chromatography. Results (mean ± SEM) represent measurements performed on 11 dishes for Hep G2/no estradiol, 12 dishes for Hep G2/+estradiol, 8 dishes for fibroblasts/no estradiol, and 8 dishes for fibroblasts/+estradiol. Estrogen addition did not affect protein concentration in either cell type.

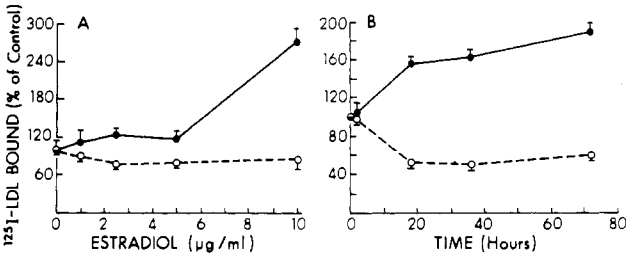

FIGURE 1: Dose–response (panel A) and time course (panel B) effects of 17β-estradiol on specific ^{125}I-LDL surface binding in Hep G2 cells (closed circles) and fibroblasts (open circles). For panel A, after 2–3 days of initial cell growth, Hep G2 cells were refed initial plating media. Twenty-four hours later, cells were washed and fed MEM + 10% LPDS + 0.05% ethanol with or without variable concentrations of 17β-estradiol. Forty-eight hours later, incubation media were removed and MEM + 10% LPDS + 25 μg/mL ^{125}I-LDL ± 625 μg/mL unlabeled LDL added to each well. Cells were incubated at 4 °C for 2 h, and specific binding was determined. For panel B, after 3 days of initial cell growth, cells were washed and fed MEM + 10% LPDS + 0.01% dimethyl sulfoxide with or without 10 μg/mL 17β-estradiol. At varying times over the following 3 days the media of certain dishes treated initially with only dimethyl sulfoxide were aspirated and replaced with fresh LPDS medium containing dimethyl sulfoxide + 10 μg/mL estradiol. On the last day, all cells were assayed for ^{125}I-LDL surface binding with tracer media containing 25 μg/mL ^{125}I-LDL ± 625 μg/mL unlabeled LDL. Similar results were obtained in experiments using 0.05% ethanol as solvent instead of 0.01% dimethyl sulfoxide. For both panel A and panel B results are expressed as percentage of specific binding as compared to controls (panel A, 9.5 ± 1.4 ng/mg for Hep G2, 74.7 ± 2.2 ng/mg for fibroblasts; panel B, 23.3 ± 2.1 ng/mg for Hep G2, 164.5 ± 6.0 ng/mg for fibroblasts).

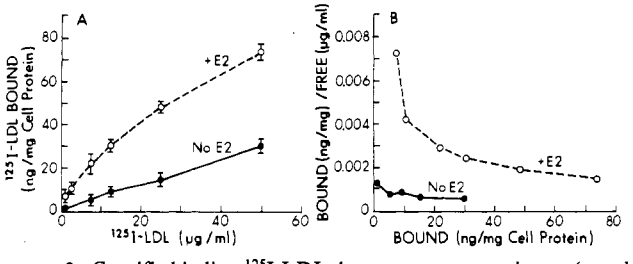

FIGURE 2: Specific binding ^{125}I-LDL dose–response experiment (panel A) and Scatchard analysis (panel B) in Hep G2 cells in the presence and absence of 17β-estradiol (E2). Panel A: Hep G2 cells were grown and refed on day 4 as described in the legend to Figure 1. Twenty-four hours later, cells were washed and fed MEM + 10% LPDS + 0.05% ethanol in the presence (open circles) or absence (closed circles) of 10 μg/mL 17β-estradiol. Forty-eight hours later, cells were chilled to 4 °C and specific surface binding was determined. Panel B: Scatchard analysis of the same experiment depicted in panel A.

A were subjected to Scatchard analysis (panel B), increased apparent affinity of binding as well as increased capacity for LDL was detected in estrogen-treated cells.

Since intracellular cholesterol is thought to regulate the LDL receptor pathway in fibroblasts and since the current data indicate that estrogens induce LDL receptor activity in Hep G2 cells but not fibroblasts, experiments were performed to test the hypothesis that estrogens regulate Hep G2 receptor activity independently of changes in cell cholesterol (Table II). When preincubated with 17β-estradiol, Hep G2 receptor activity increased 149%, but this induction was abolished when LDL was included with estradiol in the preincubation medium, suggesting that 17β-estradiol does not independently regulate LDL receptor activity in Hep G2 cells.

Because of the importance of intracellular cholesterol in regulating the fibroblast LDL receptor pathway and since

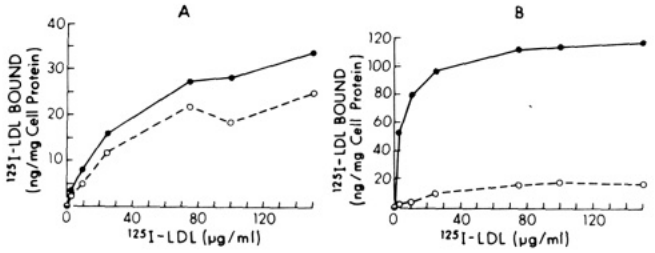

FIGURE 3: Suppression of specific LDL binding by LDL preincubation in Hep G2 cells (panel A) and fibroblasts (panel B). Cells grown for 48 h in LPDS-containing medium (closed circles) or the same medium supplemented with 50 μg/mL human LDL (open circles) were washed with saline G, incubated for 30 min in saline G at 37 °C, and then cooled to 4 °C. Saline G was replaced with prechilled media containing various concentrations of ^{125}I-LDL, then cells were incubated at 4 °C for 2 h and washed extensively, and specific dextran sulfate releasable surface binding was determined.

17β-estradiol does not increase Hep G2 LDL receptor activity in the presence of LDL, measurements of intracellular free cholesterol and cholesterol esters in Hep G2 cells and human fibroblasts with and without estradiol treatment were performed by gas–liquid chromatography (GLC) (Table III). Incubation of Hep G2 cells in LPDS with 10 μg/mL 17β-estradiol for 48 h resulted in a 24% reduction in intracellular free cholesterol compared to cells incubated in LPDS only (p <0.02, Student's t test). Estrogen treatment had no significant effect on intracellular free cholesterol levels in fibroblasts. Significant changes in cholesterol ester levels were not seen after estrogen treatment in either cell type.

Although LDL was capable of preventing estradiol induction of Hep G2 LDL receptors (Table II), specific LDL receptor activity in Hep G2 cells was resistant to suppression by LDL (Figure 3, panel A) compared to fibroblasts (Figure 3, panel B). In 11 experiments in Hep G2 cells, preincubation for 48 h in LPDS media containing 50 μg/mL LDL resulted in suppression of ^{125}I-LDL binding by 25.8 ± 3.8% (mean ± SEM) compared to cells exposed only to LPDS media. In 19 experiments, fibroblast receptor activity was suppressed 80.3 ± 3.8% by exposure to LDL (p <0.001). Specific ^{125}I-LDL binding in the same experiments was 50.8 ± 21.1 ng/mg of cell protein for Hep G2 vs. 90.4 ± 7.0 ng/mg for fibroblasts. The poor down-regulation of Hep G2 cells was not due to the inability to internalize and degrade LDL since the ratio of ^{125}I-LDL that was surface bound and degraded at 37 °C was similar in fibroblasts and Hep G2 cells (see Table I). Figure 3A shows that specific ^{125}I-LDL receptor binding did not easily saturate in Hep G2 cells, suggesting lower affinity of the Hep G2 receptor for LDL.

The Hep G2 LDL receptor was further characterized by the blotting experiments shown in Figure 4. When Hep G2 cells were grown in lipoprotein-deficient medium for 48 h, a single receptor species of M_r 140 000 was detected by ligand blotting (lane 1). With this technique the cell extracts were electrophoresed in SDS gels, transferred to nitrocellulose paper, and incubated with d <1.0063 human postprandial lipoprotein followed by ^{125}I-labeled anti-LDL antibody. The Hep G2 receptor consistently migrated to the same position as the human fibroblast receptor (lane 3); when Hep G2 and fibroblast extracts were mixed and electrophoresed in the same lane (lane 2), a single band appeared. When cell extracts of lanes 1–3 were electrophoresed and transferred to nitrocellulose paper but lipoproteins omitted in the workup, no bands were detected (lane 4, fibroblasts; lane 5, Hep G2).

Evidence that the Hep G2 receptor is expressed on the cell surface is presented in lanes 6 and 7 of Figure 4. Hep G2 LDL receptor synthesis was induced by growth in lipoprotein-deficient medium, and then cells were incubated at 37 °C for 20 min in saline G without (lane 6) or with 6 μg/mL Pronase (lane 7). The majority of Hep G2 LDL receptor protein was destroyed by Pronase treatment. The Hep G2 receptor bound LDL as well as postprandial d <1.0063 lipoprotein on ligand blots (data not shown); incubation of nitrocellulose-immobilized receptor protein with acetyl-LDL (a modification of LDL known to abolish lipoprotein recognition by the receptor) followed by ^{125}I-labeled anti-LDL antibody (known to recognize acetyl-LDL as well as native LDL) yielded no receptor band on ligand blots (lane 8).

Lanes 9–12 of Figure 4 represent immunoblots; in these experiments receptors immobilized on nitrocellulose paper were treated with IgG-C7 (lanes 9 and 10), a monoclonal antibody directed to the extrahepatic LDL receptor, or a monoclonal antibody directed to apo-A1 (lanes 11 and 12). Bands were imaged by incubating blots with ^{125}I-labeled goat anti-mouse IgG antibody. One hundred forty kilodalton bands were de-

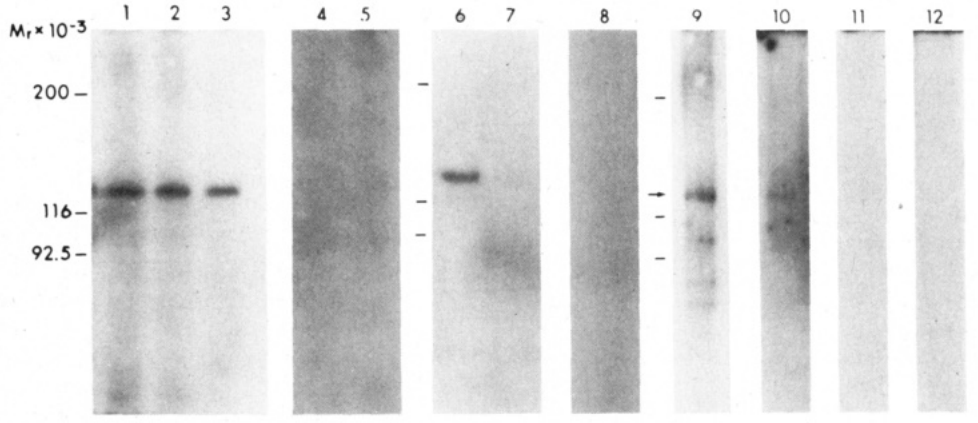

FIGURE 4: Ligand blots (lanes 1–8) and immunoblots (lanes 9–12) of Hep G2 and fibroblast LDL receptors. Extracts derived from cells incubated in LPDS-containing media were electrophoresed in SDS–polyacrylamide gels and electrophoretically transferred to nitrocellulose paper. Ligand blots were incubated in buffer containing 30 μg/mL human postprandial d <1.0063 lipoprotein (lanes 1–3, 6, 7) or 20 μg/mL acetyl-LDL (lane 8) or in buffer containing no lipoproteins (lanes 4, 5) and then treated with ^{125}I-labeled goat anti-apo-B antibody (lanes 1–8). Immunoblots were incubated in buffer containing 12 μg/mL IgG-C7, a monoclonal antibody to the extrahepatic LDL receptor (lanes 9, 10), or 12 μg/mL anti-apo-A1 monoclonal antibody (lanes 11, 12). Lane 1: Hep G2 (50 μg of protein). Lane 2: Hep G2 (25 μg) + normal fibroblasts (5 μg). Lane 3: Fibroblasts (10 μg). Lane 4: Fibroblasts (25 μg), no ligand. Lane 5: Hep G2 (75 μg), no ligand. Lane 6: Hep G2 (50 μg), incubated in saline G at 37 °C for 20 min before preparation of extracts. Lane 7: Hep G2 (50 μg), incubated in saline G with 6 μg/mL Pronase before preparation of extracts. Lane 8: Hep G2 (50 μg), acetyl-LDL as ligand. Lane 9: Hep G2 (75 μg), IgG-C7. Lane 10: Fibroblasts (25 μg), IgG-C7. Lane 11: Hep G2 (75 μg), anti-apo-A1. Lane 12: Fibroblasts (25 μg), anti-apo-A1.

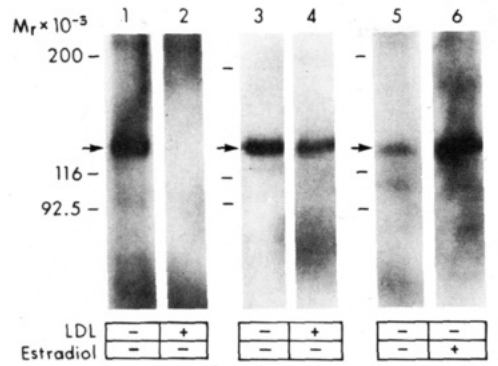

FIGURE 5: Effect of preincubation with LDL or 17β-estradiol on LDL receptor expression in fibroblasts and Hep G2 cells by ligand blotting. Fibroblasts (lanes 1, 2) and Hep G2 cells (lanes 3–6) were grown as described under Experimental Procedures. Twenty-four hours after refeeding plating medium, cells were washed and fed MEM + 10% LPDS with or without 50 μg/mL LDL in EDTA–saline and with or without 10 μg/mL 17β-estradiol in ethanol (0.05%). After forty-eight hours, receptors were solubilized, electrophoresed, transferred to nitrocellulose paper, and detected by ligand blotting by use of d <1.0063 lipoprotein and ^{125}I-labeled anti-apo-B antibody. Lanes 1 and 2 contained 25 μg of cell protein per lane, and lanes 3–6 contained 50 μg of cell protein per lane. Addition of carrier only (EDTA–saline or ethanol) in repeated experiments had no effect on control bands. Lane 1: Fibroblasts without LDL. Lane 2: Fibroblasts + LDL. Lane 3: Hep G2 without LDL. Lane 4: Hep G2 + LDL. Lane 5: Hep G2 without estradiol. Lane 6: Hep G2 + estradiol.

tected by IgG-C7 in Hep G2 cells (lane 9) and fibroblasts (lane 10), but no bands were detected by the control antibody (lanes 11 and 12). Thus, the Hep G2 receptor is biochemically similar to the fibroblast LDL receptor.

The differential regulation of LDL receptor activity in Hep G2 cells and fibroblasts by 17β-estradiol and LDL was also studied by ligand blotting (Figure 5). When fibroblasts were preincubated with LDL in EDTA–saline or EDTA–saline alone and receptors solubilized and then detected by blotting, a dark LDL receptor band was seen in cells preincubated without LDL (lane 1) but no band was detected in cells preincubated with LDL (lane 2). In contrast, Hep G2 cells preincubated with LDL continued to express LDL receptor protein detectable by blotting (lane 4) although receptor bands were decreased in comparison to Hep G2 receptors grown in the absence of LDL (lane 3). Hep G2 cells induced in the presence of 17β-estradiol clearly expressed more LDL receptor protein as detected by blotting (lane 6) when compared to cells induced in the absence of estradiol (lane 5). No difference in band intensity was detected when the same experiment was performed in fibroblasts (blots not shown).

Discussion

The current studies show that the LDL receptor pathway is significantly induced in Hep G2 cells and slightly suppressed in fibroblasts after incubation with pharmacologic doses of 17β-estradiol (Table I, Figures 1, 2, and 5). However, the estrogen-induced increase in Hep G2 receptor activity is prevented by concomitant incubation with LDL (Table II), and estrogen treatment results in decreased intracellular free cholesterol in Hep G2 but no significant change in fibroblasts (Table III). Estrogens are known to induce hepatic LDL receptors in rats (Kovanen et al., 1979; Windler et al., 1980) and rabbits (Ma et al., 1986), but we are unaware of prior reports of estrogen induction of LDL receptors in human hepatic tissues or in cultured cells. Further, although estrogen treatment is associated with increased hepatic mRNA for the LDL receptor in rabbits, the exact mechanism of LDL receptor induction by estrogens is unclear. The current results suggest that 17β-estradiol may induce hepatic LDL receptor activty indirectly by lowering intracellular free cholesterol. This concept is supported by findings in the rat hepatic hydroxymethylglutaryl-CoA reductase (HMG-CoA reductase) system, in which estrogens stimulate HMG-CoA reductase activity but this stimulation is abolished by cholesterol feeding (Abul-Hajj, 1981).

The effects of estrogen on cellular sterol content have not been extensively studied. Merola and colleagues detected increased cholesterol in whole rat liver after treatment with estrone (Merola et al., 1968), an effect in retrospect perhaps due to induction of hepatic LDL receptor activity and uptake of plasma lipoproteins. Hough and Zilversmit recently reported that estrogen treatment of hypercholesterolemic rabbits significantly decreased the free cholesterol and cholesterol ester content of arterial tissue without significantly affecting serum cholesterol levels (Hough and Zilversmit, 1986). In the same study, no significant change in the free cholesterol content of liver was noted with estrogen treatment.

The mechanism by which 17β-estradiol decreases cellular cholesterol in Hep G2 cells is unknown. Estrogens can affect several different steps in the cholesterol pathway, and the overall effect of estrogens on hepatic cholesterol metabolism is probably complex. Estrogens stimulate HMG-CoA reductase in rat liver (Abul-Hajj, 1981), an effect opposite to that seen in human fibroblasts (Brown & Goldstein, 1974). Cholesterol biosynthesis in cultured human fetal hepatocytes is also stimulated by 17β-estradiol (Carr & Simpson, 1984). In the hyperestrogenic state of pregnancy, human hepatic cholesterol secretion increases in relation to bile acid and phospholipid secretion (Kern et al., 1981). In rats, serum cholesterol is increased by treatment with low-dose estrogens and decreased by high-dose estrogens (Ferreri & Naito, 1978). Finally, the rate-limiting enzyme for the conversion of cholesterol to bile acids, 7α-hydroxylase, is stimulated by estrogen administration in rats (Ferreri & Naito, 1977).

The present studies also show that LDL receptor expression is resistant to down-regulation by LDL in Hep G2 cells (Figures 3 and 5). This finding confirms the work of Havekes and colleagues in Hep G2 cells (Havekes et al., 1983) and extends their work by demonstrating that LDL-suppressed Hep G2 cells continue to express LDL receptor protein detectable by blotting in contrast to LDL-suppressed fibroblasts (Figure 5). This demonstration is important since liver has been shown to possess in addition to the LDL receptor a specific low-affinity binding site for LDL of uncertain physiologic significance (Kita et al., 1981).

Resistance to down-regulation of the LDL receptor by incubation with LDL may be characteristic of hepatic tissue. Such resistance has been seen in cultured porcine hepatocytes (Pangburn et al., 1981) and cultured human hepatocytes (Edge et al., 1986) in addition to Hep G2 cells, and Spady et al. have shown that LDL receptor expression in rat liver is not suppressed despite increases in liver cholesterol (Spady et al., 1985b). The hepatic LDL receptor's resistance to suppression by LDL may represent an adaptive mechanism to allow continued LDL receptor expression in the lipoprotein-rich environment of the liver. This hypothesis is supported by recent studies suggesting that in some species, including humans, the majority of LDL clearance takes place in the liver by receptor-dependent mechanisms (Spady et al., 1983, 1985a; Bilheimer et al., 1984).

Finally, the current work incudes characterization of the Hep G2 LDL receptor by blotting techniques (Figure 4). Cultured Hep G2 cells express an LDL receptor with the same

molecular weight in unreduced polyacrylamide gels as the fibroblast receptor and the same binding properties as the fibroblast receptor. Like the fibroblast receptor, the Hep G2 receptor was Pronase-sensitive and recognized by a monoclonal antibody (IgG-C7) to the extrahepatic LDL receptor. Therefore, apparently the same LDL receptor protein is expressed in Hep G2 cells and fibroblasts. We did not detect high molecular weight LDL binding proteins like those detected by Hoeg and colleagues (Hoeg et al., 1986). However, the Hep G2 receptor in the current work appears biochemically similar to the LDL receptor recently characterized by Hui et al. and Soutar et al. in human liver biopsies (Hui et al., 1986; Soutar et al., 1986).

Thus, Hep G2 cells and fibroblasts appear to express the same LDL receptor protein, but LDL receptor regulation differs in these cell types. The similarities between Hep G2 cells and hepatic tissue from other sources suggest that cultured Hep G2 cells may be a model system for future studies of the LDL receptor pathway and its regulation in human liver.

ACKNOWLEDGMENTS

We are indebted to Dr. Barbara Knowles for providing Hep G2 cells, Drs. Michael Brown and Joseph Goldstein for providing the IgG-C7 hybridoma, Dr. Gustav Schonfeld for providing anti-apo-A1 antibody, Kay Zorn for secretarial expertise, and Stephen Block for technical assistance. We thank Dr. Philip Cryer for his critical reading of the manuscript.

Registry No. Estradiol, 50-28-2; cholesterol, 57-88-5.

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