Regulation of low density lipoprotein receptor gene expression in HepG2 and Caco2 cells by palmitate, oleate, and 25-hydroxycholesterol[1]

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Abstract Our in vivo studies in mice have shown that LDL-receptor gene expression is regulated differently in both liver and intestine by dietary cholesterol and dietary saturated fat. While dietary cholesterol serves to regulate at transcriptional levels, dietary fatty acids do not. To study the mechanism of regulation of LDL-receptor by saturated fat and cholesterol at the cellular level, where any secondary effects of long-term feeding in vivo are minimized, we used the cultured hepatoma and colon carcinoma cells, HepG2 and Caco2. LDL-receptor activity was determined by ^{125}I-labeled LDL binding and uptake, LDL-receptor protein by Western blotting, LDL-receptor mRNA by RNase protection assay, and relative rates of LDL-receptor mRNA transcription by nuclear 'run-off' assay. Incubation of cells in lipoprotein-deficient serum (LPDS) for 48 h progressively induced LDL-receptor activity and LDL-receptor protein by 5- to 6-fold in HepG2 cells and 2- to 3-fold in Caco2 cells. Absolute levels of LDL-receptor mRNA and relative rates of LDL-receptor mRNA transcription also increased in parallel to the LDL-receptor activity and protein levels in both cell lines. These data suggest that LPDS induced the LDL-receptor gene by transcriptional mechanism. The suppressive effect of 25-hydroxycholesterol on LDL-receptor regulation was studied by incubating HepG2 and Caco2 cells grown either in 10% FCS or 10% LPDS for 24 h and then for 0–24 h with various doses of 25-hydroxycholesterol. In HepG2 cells, LDL-receptor activity and protein mass progressively decreased to 50% of zero time controls over 24 h. LDL-receptor mRNA levels and relative rates of transcription decreased in parallel. In Caco2 cells, 25-hydroxycholesterol lowered LDL-receptor activity, mRNA, and transcription by ~35%. To examine the effects of palmitate on LDL-receptor regulation, palmitate was complexed with albumin. Palmitate decreased LDL-receptor activity by 25% in HepG2 cells without altering LDL-receptor mass, mRNA levels, or rates of mRNA transcription. Similarly, in Caco2 cells, palmitate decreased LDL-receptor activity and protein mass 30% of controls, but did not change LDL-receptor mRNA levels and/or rates of transcription. The combination of palmitate (0.8 mM) and 25-hydroxycholesterol (2.5–5 μg/ml) suppressed LDL-receptor activity by 65% in HepG2 cells and by 52% in Caco2 cells. However, LDL-receptor mRNA decreased by ~50% in HepG2 cells and 30–40% in Caco2 cells. Thus, there were further decreases in LDL-receptor activity and mRNA levels when palmitate and 25-hydroxycholesterol were present together in the media as compared to 25-hydroxycholesterol alone. Oleate did not affect LDL-receptor activity. Thus, *a)* exogenously added 25-hydroxycholesterol regulates LDL-receptor gene expression by transcriptional mechanism, the amplitude of regulation being greater in HepG2 than in Caco2 cells; *b)* exogenously added palmitate regulates LDL-receptor gene expression by posttranscriptional mechanism, possibly at the posttranslational level; and *c)* a combination of cholesterol and palmitate have additive effects on the regulation of the LDL-receptor gene in part via posttranscriptional and in part via transcriptional mechanisms.–**Srivastava, R. A. K., H. Ito, M. Hess, N. Srivastava, and G. Schonfeld.** Regulation of low density lipoprotein receptor gene expression in HepG2 and Caco2 cells by palmitate, oleate, and 25-hydroxycholesterol. *J. Lipid Res.* 1995. **36:** 1434–1446.

Supplementary key words LDL-receptor · palmitate · 25-hydroxycholesterol · HepG2 · Caco2

Elevation of low density lipoproteins (LDL) levels in circulation is one of the major risk factors for coronary artery disease (1). LDL levels are determined by a balance between rates of production and rates of clearance of LDLs from circulation. The majority of plasma LDL clearance occurs via LDL-receptor-mediated uptake by

Abbreviations: LDL, low density lipoprotein; FCS, fetal calf serum; LPDS, lipoprotein-deficient serum; LDL-R, low density lipoprotein receptor; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; TCA, trichloroacetic acid; EDTA, ethylenediamine tetra acetic acid; EGTA, ethylene glycol tetra acetic acid.

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hepatocytes. The concentrations of these lipoprotein particles are influenced by nutritional and hormonal stimuli (2–5). For example, dietary cholesterol and saturated fat can separately and together elevate LDL cholesterol in humans, and alteration of hepatic LDL-receptor activities is one way that dietary lipids can change plasma LDL concentrations. Dietary lipids gain access to hepatocytes via chylomicron remnants. Dietary cholesterol is thought to decrease hepatic LDL-receptor protein and activity by transcriptional mechanisms in vivo (2, 5). Similarly, in cultured cell lines exogenously added cholesterol or oxidized cholesterol derivatives efficiently regulate LDL-receptor activity (6–8). This regulation is mediated by the interactions of a steroid receptor element in the promoter region of the LDL-receptor gene with a steroid receptor element binding protein (9). Diets high in saturated fats in the absence of dietary cholesterol also increase plasma LDL-cholesterol levels (2, 10), while concomitantly, LDL-receptor activities decline in livers. The regulation by fatty acids appears to be posttranscriptional (10). Combinations of dietary saturated fatty acids and dietary cholesterol have additive effects on plasma LDL levels (11, 12). It has been suggested that the dietary saturated fatty acid-cholesterol combination may regulate LDL-receptors by altering sizes of putative intracellular regulatory pools of cholesterol and hence presumably LDL-receptor transcription (12). Our hypothesis was that cholesterol and fatty acids, when administered separately, operated at different loci to regulate LDL-receptor gene expression. But when administered together, cholesterol and saturated fatty acids could operate through similar molecular mechanisms, e.g., regulation of mRNA levels. We used cultured cells in order to minimize any secondary effects due to adaptations that may occur in whole organisms in vivo. Both HepG2 and Caco2 cells were used because they are adequate models of human hepatocytes and enterocytes, respectively, i.e., they secrete lipoproteins and apolipoproteins, and their LDL-receptors are regulated by physiologic perturbations (8, 13, 14), and more importantly, because LDL-receptors of liver and small intestine appeared to respond dissimilarly to dietary or hormonal perturbations (2, 4, 5).

MATERIALS AND METHODS

Cell culture

HepG2 cells were obtained from the American Type Culture Collection at passage 77 (ATCC, Rockville, MD). Caco2 cells (passage 41) were a generous gift from Dr. Jeffrey Field, University of Iowa. Stocks of both cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum (FCS, J. R. Scientific, Woodland, CA), 0.1 mM nonessential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Identical media were used for both HepG2 and Caco2 cells in order to facilitate comparisons between the two cell lines. Cells were fed fresh medium every 2 days and maintained in a humidified incubator equilibrated with 5% CO_2/95% air. Cells were plated at a density of $2.5–3 \times 10^5$/10 cm^2 in 75 cm^2 tissue culture flasks (Costar, Cambridge, MA). When the cell layers reached about 80% confluence, the cells were dissociated by incubation with 0.05% trypsin/0.02% EDTA for 5 min at 37°C and passaged at a split ratio of approximately 1:10.

Cells were plated on 35-mm 6-well plastic dishes (Costar, Cambridge, MA) for ^{125}I-labeled LDL uptake and binding studies, on 100-mm tissue culture dishes (Corning Glass Works, Corning, NY) for cell membrane preparations for LDL-receptor immunoblotting, or in 75-mm Costar flasks for mRNA quantitation. When the cells were 80% confluent, the maintenance medium was removed, monolayers were washed twice with phosphate-buffered saline (PBS), and DMEM containing either 10% FCS or 10% lipoprotein-deficient serum (LPDS) was added to each dish. After incubation for 6–48 h cellular LDL-receptor protein and activities, mRNA levels, and transcription rates were determined. To test the effects of 25-hydroxycholesterol and fatty acids, cells were preincubated either in 10% FCS or 10% LPDS for 24 h, then cells were washed twice with PBS and incubated for the indicated times in serum-free DMEM supplemented with one of the following additions to give the final desired concentrations of 25-hydroxycholesterol in 0.25% ethanol with or without palmitate or oleate complexed to BSA; or 0.16 mM BSA alone. Palmitate or oleate (Sigma Chemical Co., Saint Louis, MO) were complexed with fatty acid-free BSA (Sigma Chemical Co., MO) as previously published (15). Most frequently BSA present at 0.16 mM, but concentrations were varied to obtain desired fatty acid/BSA molar ratios. Complexes were still-filtered before use. As 25-hydroxycholesterol was dissolved in ethanol, the same amount of ethanol (0.25% final concentration) was added to the control cells. Ethanol did not affect cellular protein contents per dish.

^{125}I-labeled LDL uptake by cultured cells

LDL and LPDS were prepared by ultracentrifugation of serum obtained from healthy human subjects using the method of Havel, Eder, and Bragdon (16). LDL was iodinated with Na^{125}I (17). More than 95% of the radioactivity was precipitable with 10% trichloroacetic acid (TCA). Specific activities of ^{125}I-labeled LDL varied between 300 to 500 cpm per ng of protein.

^{125}I-labeled LDL binding and uptake by the cells were determined in cells grown in 35-mm dishes (18). Cell media were as indicated for individual experiments. Then, 1 ml of DMEM containing 2 mg/ml of BSA and 5 μg/ml of ^{125}I-labeled LDL in the presence or absence of 250 μg/ml of native LDL were added. Incubations at 4°C or at 37°C lasted for 3 h. The media were then removed and all subsequent operations were carried out at 4°C. Each cell monolayer was washed three times with 2 ml of buffer A containing 50 mM Tris-HCl (pH 7.4) buffer, 0.15 M NaCl, and 2 mg/ml BSA, after which a further 2 ml of buffer A was added and the monolayer was washed finally with 2 ml of buffer containing 50 mM Tris-HCl (pH 7.4) and 0.15 M NaCl. The cells were then dissolved in 1 ml of 0.1 N NaOH and collected for ^{125}I counting in a gamma counter and for the measurement of cell protein concentrations by the method of Lowry et al. (19).

Cell membrane preparation

For LDL-receptor immunoblotting, cells were grown on 100-mm culture dishes. After incubation with the test substrates for the indicated times, cells were placed on ice, washed three times with 0.15 M NaCl at 4°C, scraped from the incubation dishes with a rubber policeman, and briefly centrifuged. The cell pellet was suspended in a 10-fold (compared with pellet volume) excess of receptor solubilizing buffer containing 1.6% Triton X-100, 0.3 mM leupeptin, 5 M urea, and 1.5 mM phenylmethylsulfonyl fluoride (PMSF). After homogenization of the cells on ice in a 1.5 ml tissue homogenizer and incubation on ice for 30 min, the resulting suspension was centrifuged in a Beckman TL-100 Ultracentrifuge at 100,000 *g* for 10 min. The supernatant was removed and stored in liquid nitrogen (20).

LDL-receptor protein quantification

Six percent polyacrylamide separation gels and 3% polyacrylamide stacking gels, each containing 0.1% SDS, were prepared as described (4). Solubilized cellular membranes were mixed with a solution of 2.5% SDS, 50% glycerol, and 0.5% bromophenol blue in a ratio of 4:1. One hundred microgram of total HepG2 cell membrane or 200 μg of Caco2 cell membrane protein were electrophoresed without preheating and in the absence of dithiothreitol (DTT) for 3 h at 4°C in buffer containing 25 mM Tris-glycine (pH 8.6) and 0.1% SDS.

Electrotransfer of proteins separated on polyacrylamide gels was performed essentially as described by Towbin, Staehelin, and Gordon (21) with the exception that for nondenaturing gels methanol was omitted from the transfer buffer. Immobilin-P membrane (Millipore Corp., Bedford, MA) was presoaked in methanol, washed with water, and then soaked in transfer buffer (25 mM Tris, 192 mM glycine) for 15 min. The membrane was placed on the anode side of the gel between two sheets of porous filter paper and electrotransferred at 4°C at 4 V/cm. After the transfer, the membrane was immersed in bovine Lacto Transfer Technique Optimizer (BLOTTO) blocking buffer consisting of 5% (w/v) nonfat powdered dry milk in PBS with 0.01% antifoam A emulsion (Sigma) and 0.001% merthiolate (Sigma) for 1 h. Next, the membrane was incubated overnight at room temperature in BLOTTO containing 10 μg/ml of mouse monoclonal anti-human LDL-receptor antibody (IgG-C7), (generously provided by Drs. Y. K. Ho, Joseph L. Goldstein, and Michael S. Brown, Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas). The membrane was then washed with 250 ml of 0.1% Tween-80 and 0.1% Triton X-100 in PBS. The membrane was incubated for 4 h at room temperature in BLOTTO containing ^{125}I-labeled goat anti-mouse IgG antibody (approximately 7.5×10^5 cpm/ml), and then washed again with 250 ml of 0.1% Tween-80 and 0.1% Triton X-100 in PBS. Bands were visualized on radiographic film using autoradiography, and band densities were determined using an image analysis system (JAVA, Jandel Scientific, Corte Medera, CA).

Quantification of LDL-receptor mRNA

Cells were grown in 75-cm^2 culture flasks. Immediately after removal of the culture medium, cells were washed with serum-free DMEM, scraped off by a rubber policeman, and total RNA was isolated by a single-step RNA isolation method (22). Concentrations of LDL-receptor mRNA were determined by an RNase protection-solution hybridization assay as described (23). For the preparation of riboprobes and RNA standard, a 311 bp cDNA fragment of clone pLDLR3 (ATCC, Rockville, MD) was subcloned into *Bam*HI and *Eco*RI sites of pGEM3Zf(+) vector (Promega Corp., Madison, WI). After confirming the orientation of the insert by sequencing, ^{32}P-labeled cRNA probe and RNA standard were synthesized by in vitro transcription using T7 or SP6 RNA polymerases. The total cellular RNA and different concentrations of RNA were hybridized with ^{32}P-labeled cRNA probe (50,000 cpm). The hybridized probe was collected on glass fiber filters after TCA precipitation and counted in a scintillation counter. A standard curve was generated by hybridizing the probe with sense RNA standard and absolute levels of LDL-receptor mRNA were calculated. As an internal control, the mRNA for γ-actin was also quantified using a 267 bp γ-actin cDNA subcloned at *Hind*III-*Xba*I sites of pGEM3Zf(+) vector. To prepare riboprobe the recombinant plasmid was linearized with *Hind*III and T7 RNA polymerase was used. RNase protection assay was performed in a similar

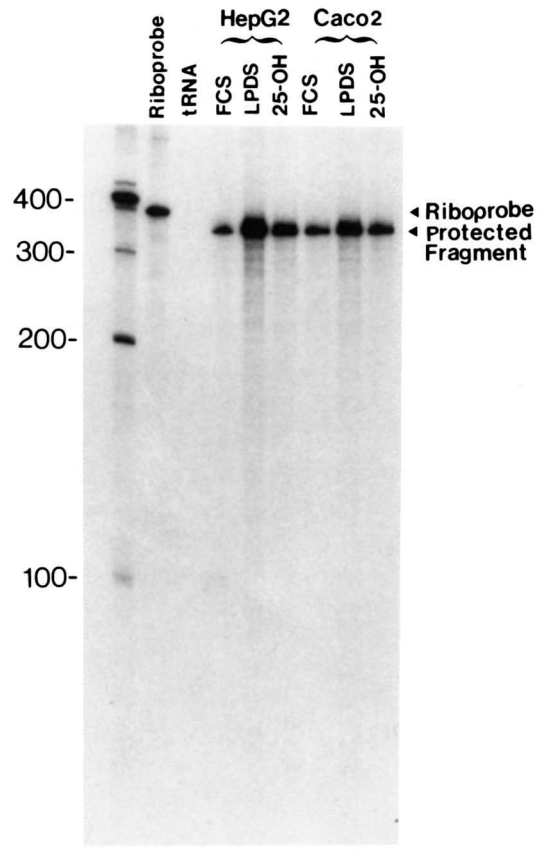

Fig. 1. Autoradiogram of RNase protection assay. Total RNA from HepG2 cells (25 μg) and from Caco2 cells (50 μg) were hybridized with LDL-receptor riboprobe (100,000 cpm), treated with RNase to digest unhybridized RNA and riboprobes, precipitated with isopropanol, dissolved in loading buffer and separated in a sequencing gel. Lane 1, RNA molecular size markers synthesized in vitro in the same way as the riboprobes using a mixture of linearized plasmids obtained from Ambion; lane 2, LDL-receptor riboprobe; lane 3, riboprobe hybridized with yeast tRNA; FCS, cells incubated in 10% fetal calf serum; LPDS, cells incubated with 10% lipoprotein-deficient serum; 25-OH, cells incubated with 5 μg/ml 25-hydroxycholesterol containing 10% FCS media. The positions of riboprobe and the protected fragments are indicated.

way as described for the LDL-receptor. **Figure 1** shows the protected fragment of the LDL-receptor mRNA in an RNase protection assay. Fluctuations in LDL-receptor mRNA by LPDS and 25-hydroxycholesterol are also evident in HepG2 and Caco2 cells.

Preparation of nuclei from cultured cells

Cells were grown in 75-cm^2 culture flasks, and were washed and scraped off by a rubber policeman as above. Nuclei were prepared as previously described (2, 4) with some modifications for the cultured cells. Cells were washed with 2.5 ml of ice-cold wash solution (20 mM Tris-HCl, pH 7.5, 15 mM NaCl, 1.1 mM sucrose) and pelleted by centrifugation at 300 *g* at 4°C for 10 min. The cells were resuspended in 2.5 ml of ice-cold hypotonic solution (20 mM Tris-HCl, pH 8.0, 4 mM $MgCl_2$, 6 mM $CaCl_2$, 0.5 mM DTT) and allowed to remain on ice for 5 min. After the addition of 2.5 ml of lysis buffer (0.6 M sucrose, 0.2% Nonidet P-40, 0.5 mM DTT), cells were homogenized with the tight-fitting pestle of a homogenizer. The homogenate was centrifuged at 1,500 *g* at 4°C for 10 min and the pellet was resuspended in 0.5 ml of 0.3 M sucrose solution in buffer B (60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 M spermidine, 14 mM β-mercaptoethanol, 0.5 mM EGTA, 2 mM EDTA, 15 mM HEPES buffer, pH 7.5). The crude nuclear suspension was layered over a 2.5 ml cushion of 1.5 M sucrose in buffer C (same as buffer B but EGTA and EDTA concentrations reduced to 0.1 mM each) and centrifuged for 1 h at 4°C at 45,000 rpm in a Beckman TL-100 Ultracentrifuge. The clean nuclei were transferred to a microfuge tube and suspended in cold nuclei storage buffer (20 mM Tris-HCl, pH 7.9, 75 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT, 0.125 mM PMSF, 50% glycerol). Prepared nuclei were either used immediately for in vitro nuclear run-off assay or stored at −70°C for up to 4 weeks without loss in activity.

Measurements of relative rates of LDL-receptor mRNA transcription

Details of nuclear 'run-off' assay have been described earlier (2, 4). The transcription assay was performed in 200 μl of buffer containing 100 mM Tris-HCl, pH 7.9, 50 mM NaCl, 0.4 mM EDTA, 0.1 mM PMSF, 1.2 mM DTT, 1 mg/ml heparin sulfate, 2 mM $MnCl_2$, 4 mM $MgCl_2$, 10 mM creatine phosphate, 1 mM each of ATP, CTP, and GTP, 40 U human placental ribonuclease inhibitor, 30% glycerol, 10 million nuclei and 100 μCi [α-^{32}P]UTP. Transcription was allowed to occur at 26°C for 20 min in the presence or absence of 2 μg/ml α-amanitin and the reaction was terminated by the addition of 30 U of DNase I (RNase-free). Nuclear RNA was then isolated using RNAzol™ B. The isolated RNA was washed with 70% ethanol and dissolved in hybridization buffer (20 mM PIPES, pH 6.7, 50% deionized formamide, 2 mM EDTA, 0.8 M NaCl, 0.2% SDS, 0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA, 500 μg/ml denatured salmon sperm DNA). An aliquot was counted to determine the incorporation of [^{32}P]UTP in RNA. The extracted nuclear RNA was hybridized to membrane bound LDL-receptor cDNA (1.9 kb) at 42°C for 50 h. A 1.9 kb LDL-receptor cDNA of clone pLDLR3 was subcloned into *Bam*HI site of pGEM3Zf(+) vector. As an internal control 267 bp human γ-actin cDNA fragment subcloned into *Hind*III and *Xba*I site was used. This clone was kindly provided by Dr. Clay Semenkovich, Department of Internal Medicine, Washington University, Saint Louis. For background counts nonrecombinant pGEM vector was linearized and hybridized the

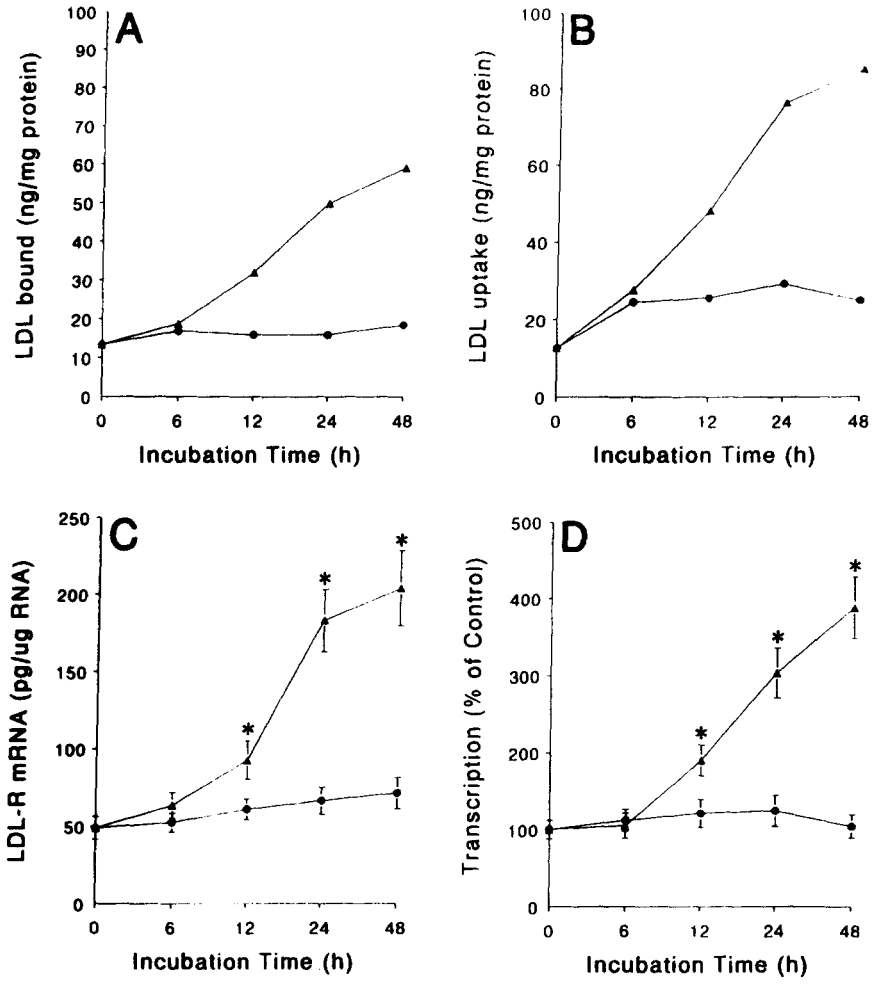

Fig. 2. Effects of LPDS on LDL-receptor gene expression in HepG2 cells. HepG2 cells were grown to 80% confluency in 20% FCS, washed, and then incubated with 10% LPDS or FCS for the times indicated. A: Shows binding of iodinated LDL isolated from human blood. This assay was performed at 4°C as described in the text. Each assay point indicates mean values of duplicate dishes. Circles represent values obtained with 10% FCS and triangles indicate values obtained with 10% LPDS media. B: Shows binding and uptake of iodinated LDL performed at 37°C. This assay was also performed in duplicate and the mean values are plotted. C: Shows absolute levels of LDL-receptor mRNA quantified by RNase protection assay. Each assay was performed in triplicate using total RNA from three dishes, and 25 μg total RNA was taken for each assay. Details of LDL-receptor mRNA quantification are described in the text; *, significantly different compared to initial values and values obtained with FCS. Each value represents mean ± SEM. As an internal control absolute levels of γ-actin were also determined. These did not change in the LPDS media. D: Shows results of relative rates of LDL-receptor mRNA transcription. Nuclear 'run-off' assay was performed as described in the text. Each assay was performed in triplicate and the values represent mean ± SEM. As an internal control the relative rates of γ-actin mRNA transcription were also performed. The maximum change was a 12% increase in the rates of γ-actin mRNA transcription at the 24-h time point. Experiments shown here were repeated and similar results were obtained.

same way. After the hybridizations, the membrane was washed as described (2), and counted. Transcription rates were expressed as percent in relation to a control group that was assigned a value of 100%.

RESULTS

Effects of 10% LPDS or 10% FCS on LDL-receptor gene expression

Media of 80% confluent cells were changed from 20% FCS to 10% LPDS (or 10% FCS as indicated) for varying lengths of time to up-regulate LDL-receptors. As expected, LDL-receptor mass and activity increased progressively with increasing incubation time and reached a maximum of 5- to 6-fold increase after 24 h of incubation in 10% LPDS (**Fig. 2A and 2B**). Incubation in 10% FCS produced much lower degrees of LDL-receptor up-regulation. Parallel increases are noted in LDL-receptor mRNA levels and relative rates of transcription (Figs. 2C and 2D). The mRNA for γ-actin was also quantified. No significant changes were seen. LDL-receptor activity and mass also increased in parallel with LDL-receptor mRNA levels and rates of transcription in Caco2 cells

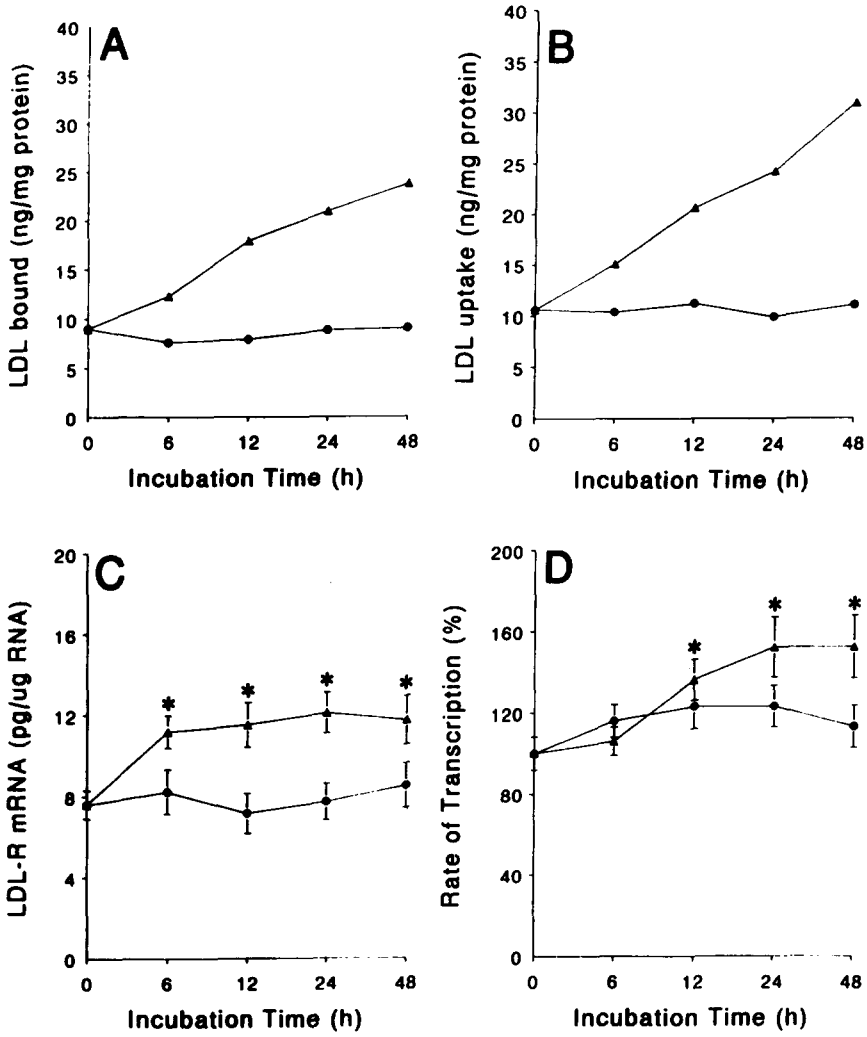

Fig. 3. Effects of LPDS media on LDL-receptor gene expression in Caco2 cells. The experimental details are provided in the legend to Fig. 2. A: Iodinated LDL binding at 4°C; B: iodinated LDL uptake and degradation at 37°C; C: LDL-receptor mRNA; D: LDL-receptor mRNA transcription. Filled circle represents values obtained with 10% FCS media and triangle represents values obtained with 10% LPDS media.

(**Fig. 3**). However, LDL-receptors were induced less in Caco2 cells than in HepG2 cells.

Effects of 25-hydroxycholesterol on LDL-receptor expression

In HepG2 cells grown in 10% FCS or 10% LPDS for 48 h (down from the 20% FCS used for routine maintenance) showed 25-hydroxycholesterol dose-dependent (**Fig. 4**) and time-dependent (**Fig. 5**) decreases in the LDL-receptor activity. Thus, 0.1 and 5 μg of 25-hydroxycholesterol decreased LDL-receptor activity by 20% and 50%, respectively (Fig. 4). In the presence of 2.5 μg/ml of 25-hydroxycholesterol, LDL-receptor activity decreased to ~50% of basal over 24 h (Figs. 5A and 5C). LDL-receptor binding was higher in cells whose receptors had been up-regulated in LPDS, and the extent of 25-hydroxycholesterol-induced decrease was also greater. Nevertheless, the 25-hydroxycholesterol-induced fractional decreases in 10% FCS incubated cells suggested that 10% FCS alone did not produce complete down-regulation of receptors. LDL-receptor protein mass decreased in parallel with LDL-receptor activity (Figs. 5A and 5C). LDL-receptor mRNA levels and relative rates of transcription also decreased after as little as 6 h of incubation of HepG2 cells in 10% FCS (Fig. 5B) or with 10% LPDS (Fig. 5D). Results in Caco2 cells resembled those in HepG2 cells, i.e., 25-hydroxycholesterol (5 μg/ml) decreased LDL-receptor activity and protein mass to 65% and 60% of initial values, respectively (**Fig. 6A**), while concomitantly, LDL-receptor mRNA concentrations and relative rates of transcription both decreased to about 60% (Fig. 6B).

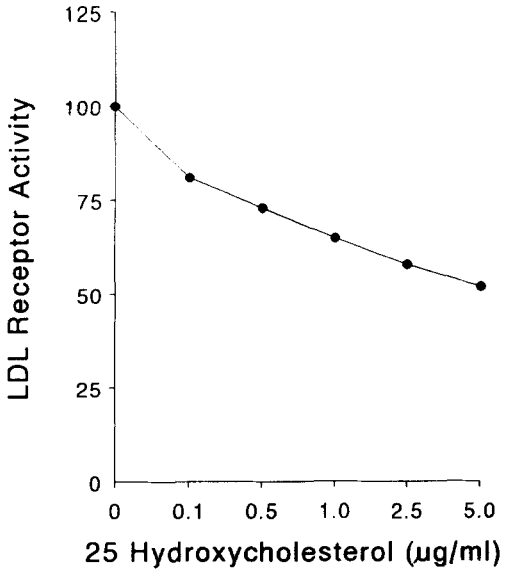

Fig. 4. Effects of 25-hydroxycholesterol concentrations on LDL-receptor activity in HepG2 cells. HepG2 cells were grown to 80% confluency, washed with PBS, incubated in 10% LPDS for 24 h, and then further incubated with DMEM containing varying amounts of 25-hydroxycholesterol for another 24 h. The values represent means of duplicate dishes.

Effects of oleate, palmitate, and 25-hydroxycholesterol combinations on LDL-receptor expression

Three concentrations of oleate at three different oleate/BSA molar ratios did not affect LDL-receptor activity (**Fig. 7A**). By contrast, a maximum ~30% decrease in LDL-receptor activity was noted after 24 h incubation in 0.5 mM palmitate complexed with BSA in a ratio of 9:1 (Fig. 7B). Lesser degrees of lowering were obtained at lower concentrations and molar ratios. In a different experiment performed with 0.8 mM palmitate/0.16 mM BSA, LDL-receptor activities fell by 20–30%, but LDL-receptor protein, mRNA levels and rates of transcription were not altered (**Table 1**). Addition of 1 μg/ml 25-hydroxycholesterol to palmitate produced lowering of LDL-receptor activities at every concentrations of palmitate and molar ratio of palmitate/BSA (Fig. 7B). Conversely, adding a fixed amount of palmitate (0.8 mM) to varying doses of 25-hydroxycholesterol also produced additive effects on LDL-receptor activities in both HepG2 and Caco2 cells (**Fig. 8**). Compatible results were obtained whether preincubations were in 10% LPDS or 10% FCS.

In confirmation of the experiment reported in Table 1, 0.5 mM palmitate in the absence of 25-hydroxycholesterol did not alter LDL-receptor mRNA levels, while addition of varying concentrations of 25-hydroxycholesterol to media containing the 0.5 mM palmitate decreased LDL-receptor mRNA in a 25-hydroxycholesterol dose-dependent manner while the mRNA for γ-actin remained unchanged (**Fig. 9**). Incubation of HepG2 cells in 5 μg/ml 25-hydroxycholesterol and 0.8 mM palmitate (palmitate/BSA ratio of 5) lowered LDL-receptor mRNA to a greater extent than did palmitate or 25-hydroxycholesterol alone (**Fig. 10**). Control incubation of cells in the same amounts of BSA (0.16–0.64 mM) used to produce the various fatty acid/BSA ratios, in the absence of either fatty acids or 25-hydroxycholesterol, had no effect on any of the parameters measured (not shown).

DISCUSSION

Many investigators have used HepG2 cells as a model system to study various aspects of the regulation of cholesterol biosynthesis and metabolism and LDL-receptor regulation (7, 8, 24–29). The aim of the present investigation was to ascertain how saturated fatty acids in the absence or presence of 25-hydroxycholesterol decreased LDL-receptor activity. It had been reported previously that cholesterol or its derivatives, particularly 25-hydroxycholesterol, efficiently reduced LDL-receptor activity, protein and mRNA levels in vivo (2, 4), and in cultured cells (6–8, 24). Our data confirm and extend these findings in Caco2 and HepG2 cells. We show that removal of extracellular sources of cholesterol (i.e., culturing in 10% LPDS) caused increases in LDL-receptor activity, protein, mRNA, and rates of transcription, while supplying external sources of 25-hydroxycholesterol produced time- and dose-dependent decreases that were in the same direction and similar in magnitude. The findings were alike for both cell types, but the amplitudes of the variations were greater for HepG2 cells. Switching either HepG2 or Caco2 cells from 20% to 10% FCS produced only small degrees of up-regulation of receptors, while addition of 25-hydroxycholesterol to 10% FCS cells produced receptor inhibition, suggesting that 10% FCS did not completely suppress receptor activity. In human fibroblasts, stimulation of LDL-receptor activity and mRNA levels by LPDS was not similar in magnitude. Therefore, additional regulation of LDL-receptor gene at the translational level was suggested (24). However, unlike in fibroblasts, complete down-regulation of LDL-receptor gene was not observed in HepG2 cells when they were incubated with 25-hydroxycholesterol. We found about 40–50% inhibition in receptor activity and mRNA levels of LDL-receptor. Similar results have been obtained in other studies where LDL-receptor mRNA was quantified by slot-blot technique (7, 26, 29). More effective suppression of LDL-receptors by 25-hydroxycholesterol than by LDL has been reported (7, 8). However, β-VLDL and 25-hydroxycholesterol down-regulated LDL-receptor activity to almost the same extent (8). Based on our

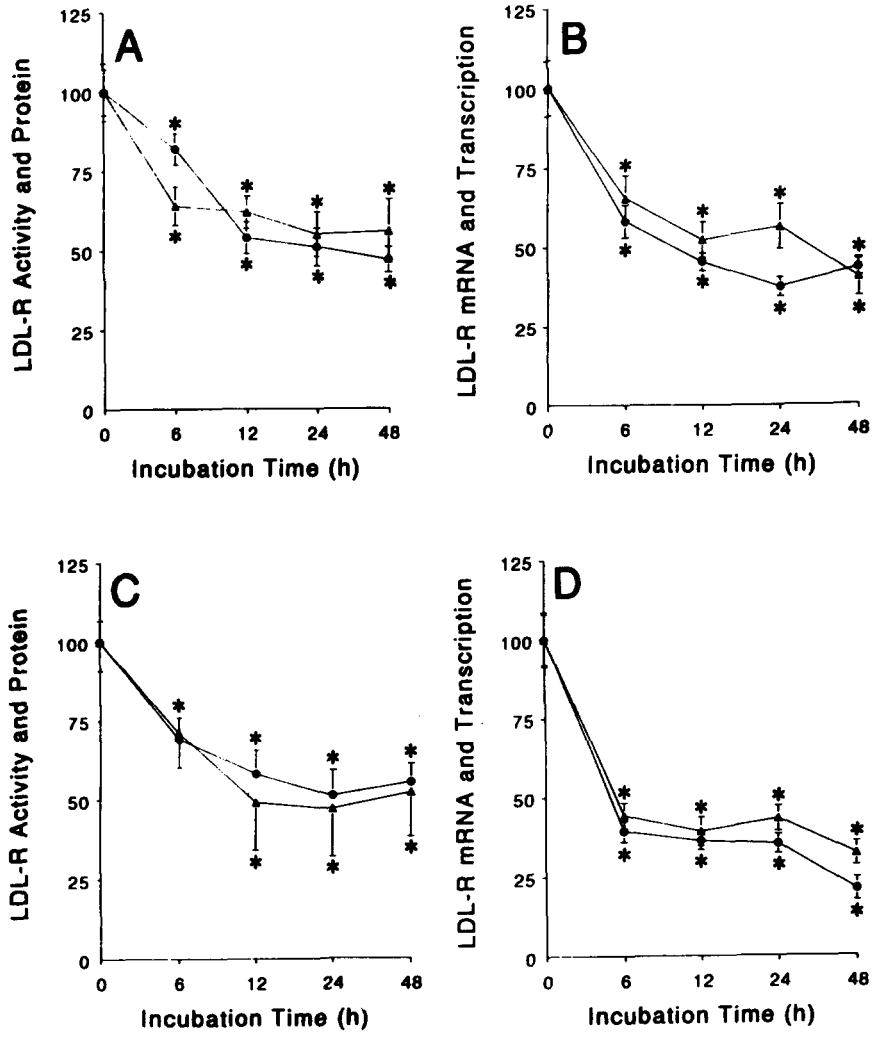

Fig. 5. Effects of 25-hydroxycholesterol on LDL-receptor gene expression in HepG2 cells. HepG2 cells were grown to 80% confluency in 20% FCS, washed with cold PBS, and incubated in media containing 10% FCS or 10% LPDS. After 24 h incubation, the cells were washed with PBS and incubated with DMEM, 0.25% (vol/vol) ethanol, and 2.5 μg/ml of 25-hydroxycholesterol for the indicated times. Panels A and B show results obtained with cells preincubated with 10% FCS, and panels C and D show values obtained with cells preincubated in 10% LPDS. Cells were also incubated with equivalent amounts (0.25% vol/vol) of ethanol but without 25-hydroxycholesterol. After each incubation period, LDL-receptor activity, protein mass, mRNA, and relative rates of transcription were measured. Panels A and C show LDL-receptor activity (circles) and protein (triangles). Panels B and D show LDL-receptor mRNA (circles) and transcription (triangles). Results are means of triplicate dishes ± SEM. *Significantly different compared to zero time values. The absolute values at zero time point for HepG2 cells preincubated with FCS were: LDL-receptor activity, 22 ± 2 ng ^{125}I-labeled LDL/mg cell protein, LDL-receptor mRNA, 43.1 pg/μg RNA. The densitometric scanning of LDL-receptor protein band and counts of LDL-receptor mRNA transcription were assigned a value of 100. The absolute values at zero time point for HepG2 cells preincubated in LPDS were: LDL-receptor activity, 59.6 ± 5.5 ng ^{125}I-labeled LDL/mg cell protein, LDL-receptor mRNA, 197.6 ± 6 pg/μg RNA.

data, we suggest that β-VLDL may also down-regulate LDL-receptor gene by transcriptional mechanism as seen with 25-hydroxycholesterol. The differences in the extent of down-regulation of LDL-receptor gene by LDL and β-VLDL (8) may reflect the abilities of these particles to deliver cholesterol to intracellular pools and regulatory sites for the LDL-receptor (9).

In order to understand how saturated fatty acids regulate the LDL-receptor gene, HepG2 and Caco2 cells were incubated with varying oleate and palmitate concentrations and palmitate/BSA molar ratios. Oleate produced no effects, while palmitate produced modest decreases in LDL-receptor activity in both cell lines. In contrast, with 25-hydroxycholesterol, palmitate evoked no responses in LDL-receptor protein or mRNA levels, or in rates of mRNA transcription. These findings are in agreement with the in vivo studies where saturated fat did not alter LDL-receptor mRNA (2, 5), yet recep-

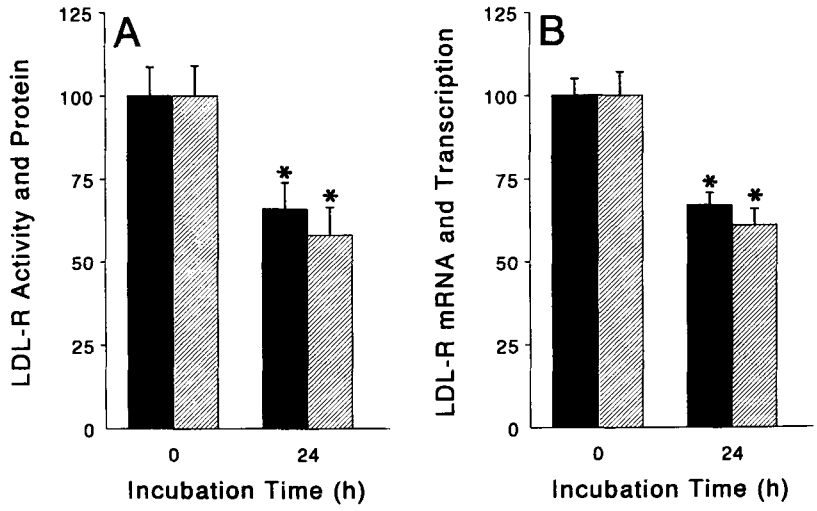

Fig. 6. Effects of 25-hydroxycholesterol on LDL-receptor in Caco2 cells. Experimental design was same as shown in Fig. 5, but the results obtained with cells preincubated further with 10% FCS and incubated for another 24 h with DMEM and 5 μg/ml 25-hydroxycholesterol in 0.25% ethanol are only shown. LDL-receptor activity, mass, mRNA, and transcription were determined. A: Shows LDL-receptor activity (filled bar) and LDL-receptor protein mass (diagonal bar); B: Shows LDL-receptor mRNA (filled bar) and mRNA transcription (diagonal bar). *, Significantly different when compared to control and zero hour values. Similar results were obtained when Caco2 cells were preincubated with 10% LPDS for 24 h, washed, and incubated with DMEM containing equivalent amounts of 25-hydroxycholesterol. The absolute values at zero time point preincubated in FCS were: LDL-receptor activity, 3.2 ± 0.3 ng ^{125}I-labeled LDL/mg protein, LDL-receptor mRNA, 4.94 ± 1.2 pg/μg RNA. The zero time point values preincubated with LPDS were: LDL-receptor activity 14.4 ng ^{125}I-labeled LDL/mg protein, LDL-receptor mRNA, 8.86 pg/μg RNA.

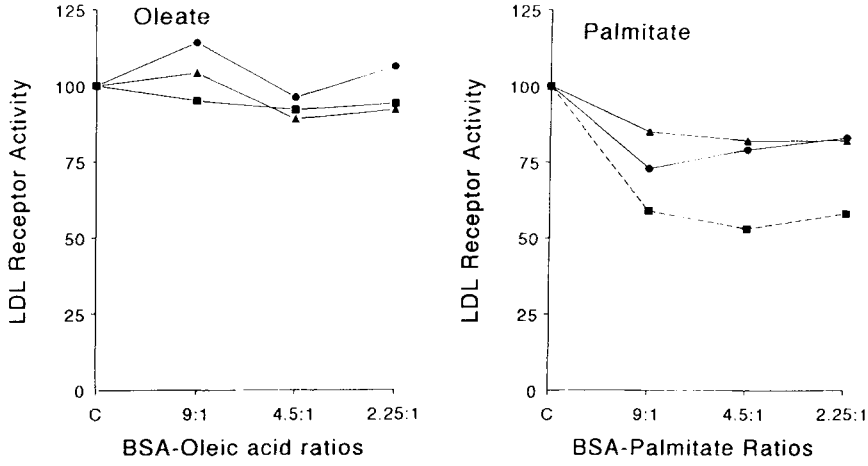

Fig. 7. Effects of oleate and palmitate on LDL-receptor activity in HepG2 cells. Three different concentrations of oleate (0.25 mM, 0.5 mM, 1.0 mM) were complexed with BSA in varying oleate/BSA ratios as indicated. Two concentrations (0.25 mM and 0.5 mM) of palmitate at three different molar ratios were used. HepG2 cells were grown to 80% confluency in 20% FCS, washed with cold PBS, and incubated for 24 h in 10% FCS, washed again, and then incubated with the indicated fatty acid-BSA complex in DMEM. At the end of 24 h incubation, LDL-receptor activity was measured. Left panel, triangles, 0.25 mM; squares, 0.5 mM; circles, 1 mM oleate. Right panel, triangles, 0.25 mM; circles, 0.5 mM; and dotted lines with squares, 0.5 mM palmitate plus 1 μg/ml 25-hydroxycholesterol. The values presented are means of duplicate dishes and given as percents of zero hour values. Similar results were obtained when LDL-receptors were up-regulated by preincubation with 10% LPDS and then transferred to DMEM containing indicated amounts of fatty acid-BSA complex (not shown).

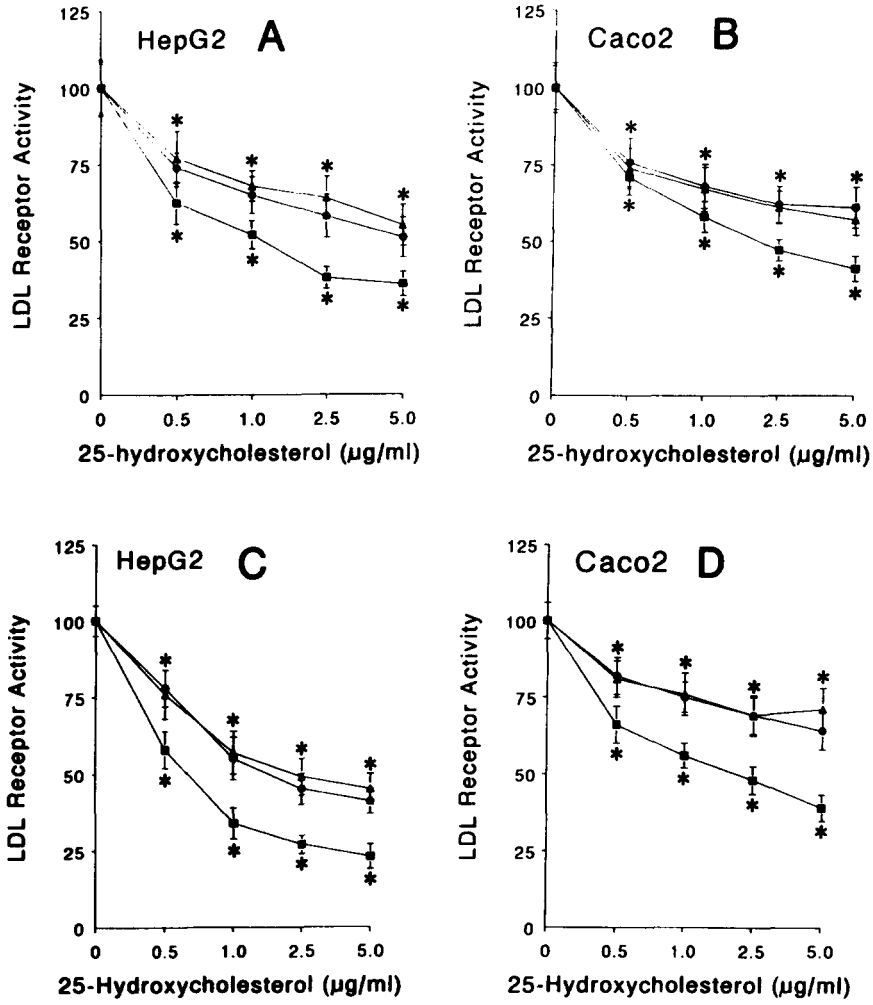

Fig. 8. Effects of combination of 25-hydroxycholesterol and palmitate (0.8 mm) on LDL-receptor activity in HepG2 and Caco2 cells. Palmitate was complexed with bovine serum albumin. Cells were grown to 80% confluency in 20% FCS, washed with PBS incubated for 24 h in 10% FCS (panels A and B) and then incubated in DMEM containing 0.8 mm palmitate, 0.16 mm BSA, and the indicated amounts of 25-hydroxycholesterol. Initial (zero point) values represent values obtained in the absence of palmitate and 25-hydroxycholesterol, and rest of the values represent percents of the initial values. Circles represent values obtained in the presence of 25-hydroxycholesterol alone; triangles represent values obtained in the presence of 0.16 mm bovine serum albumin and varying concentrations of 25-hydroxycholesterol; squares represent values obtained in the presence of 0.8 mM palmitate plus varying concentrations of 25-hydroxycholesterol. Each assay was performed in triplicate and the experiment was repeated. *, Significantly different compared to initial values. The absolute values of LDL-receptor activity at zero time point without 25-hydroxycholesterol for HepG2 cells were: DMEM, 37.3 ± 3.6, DMEM + BSA (0.16 mm), 33 ± 3, DMEM + palmitate/BSA (0.8 mm and 0.16 mm, respectively), 36.3 ng ^{125}I-labeled LDL/mg proteins. For Caco2 cells, the values were: DMEM + BSA, 12 ± 1.15, DMEM + palmitate/BSA, 10.9 ± 0.9. C and D show effects of combination of palmitate (0.8 mM) and 25-hydroxycholesterol on LDL-receptor activity in HepG2 and Caco2 cells preincubated with 10% LPDS for 24 h, washed with cold PBS, and incubated with DMEM and indicated amounts of palmitate/BSA alone or in combination with 25-hydroxycholesterol. The zero time point values of LDL-receptor activity for HepG2 cells were: DMEM + BSA, 77.6 ± 7, DMEM + palmitate/BSA, 76.1, Caco2 cells, DMEM + BSA 25.1 ± 2.16, DMEM + palmitate/BSA, 23.9 ± 1.9 ng ^{125}I-labeled LDL/mg protein.

tor-mediated clearance of LDL from plasma decreased (30, 31), and plasma LDL levels increased as a consequence. Thus, it is clear that the suppressive effect of fatty acids is most likely to be posttranslational. The decreased LDL-receptor activity is not due to the presence of fatty acids in the media during the binding assay. Bihain and associates (32) have reported that fatty acids directly affect LDL binding to fibroblast LDL-receptors. However, in our experiments the fatty acid-BSA complexes were removed, cells were washed with DMEM, and the assay was then performed in the absence of added fatty acids. Palmitic acid produced the effect on LDL-receptor binding and oleate did not, demonstrating the specificity of the saturated fatty acid effect and confirming the absence of any effects of media fatty acids on the binding assays. The action of palmitate is not unique. Posttranscriptional regulation of the LDL-receptor has been reported for growth hormones (33).

TABLE 1. Effects of palmitate/BSA (0.8 mM/0.16 mM) on LDL-receptor gene expression in HepG2 and Caco2 cells

Cell Types	Media	Incubation Time	LDL-Receptor Activity[a]	LDL-Receptor Protein[b]	LDL-Receptor mRNA[c]	LDL-Receptor mRNA Transcription[d]
		h				
HepG2	BSA	0	64.5 ± 5.7	100 ± 7.7	163.4 ± 16.2	100 ± 9.5
		24	61.0 ± 5.2	93.8 ± 4.6	147.3 ± 13.5	91.2 ± 8.4
HepG2	BSA/palmitate	0	65.2 ± 4.7	100 ± 6.4	146.6 ± 16.8	100 ± 9.3
		24	47.3 ± 3.2	92.1 ± 5.4	139.5 ± 12.3	87.8 ± 8.3
Caco2	BSA	0	16.0 ± 1.2	100 ± 8.7	10.37 ± 0.9	100 ± 10.1
		24	15.2 ± 0.7	89.1 ± 6.1	10.82 ± 1.1	81.3 ± 8.3
Caco2	BSA/palmitate	0	15.5 ± 1.1	100 ± 11.2	10.54 ± 1.1	100 ± 10.8
		24	11.7 ± 0.8	78 ± 8.4	11.75 ± 0.9	77 ± 7.8

[a]LDL-receptor activity shown as ng of ^{125}I-labeled LDL per mg protein.
[b]LDL-receptor protein was determined by Western blotting and values represent densitometric scanning of LDL-receptor protein band. Values at zero time point have been assigned 100.
[c]LDL-receptor mRNA was determined by RNase protection assay using 25 μg total RNA for each assay. Results are in pg mRNA/μg total RNA.
[d]LDL-receptor mRNA transcriptions were measured by "nuclear run-off" assay on isolated nuclei. Values obtained at zero time point have been assigned 100. Cells were grown to 80% confluency in 20% FCS, washed with cold PBS and incubated in 10% LPDS for 24 h to up-regulate LDL-receptor, again washed with cold PBS, and then incubated for indicated time in DMEM containing either BSA alone or with palmitate.

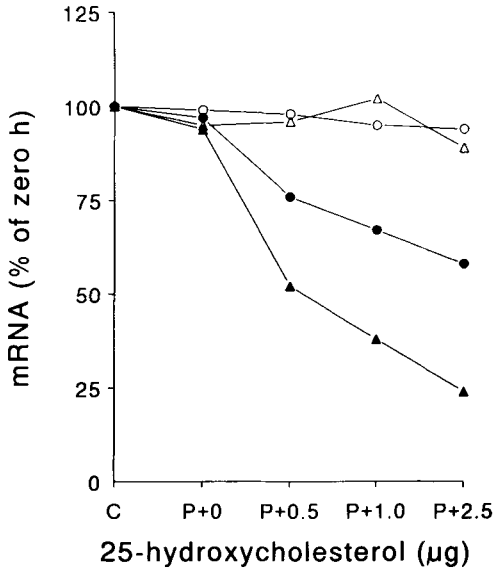

Fig. 9. Effects of palmitate on LDL-receptor and γ-actin mRNA in HepG2 cells. Cells were grown to 80% confluency in 20% FCS, washed, incubated either in 10% FCS (circles) or in 10% LPDS (triangles) for 24 h, and then further incubated in DMEM containing various concentrations and molar ratios of palmitate/BSA. At the end of 24 h, total RNA were prepared and LDL-receptor (filled circles and triangles) and γ-actin (empty circles and triangles) mRNA were quantified by RNase protection assay shown in Fig. 1. C indicates control levels after 24 h in 10% LPDS or 10% FCS. P indicates levels after 24 h in palmitate (0.5 mM) but in the absence of 25-hydroxycholesterol. P + 0.5, P + 1.0, and P + 2.5 indicate mRNA levels after 24 h in 0.5 mM palmitate to which the indicated increasing concentrations of 25-hydroxycholesterol (in μg/ml) were added. Values represent means of duplicate dishes. Clearly, palmitate alone did not affect LDL-receptor mRNA levels (compare C vs. P + 0); adding 25-hydroxycholesterol produced lower LDL-receptor mRNA levels (P + 0 vs. P + 0.5, etc.).

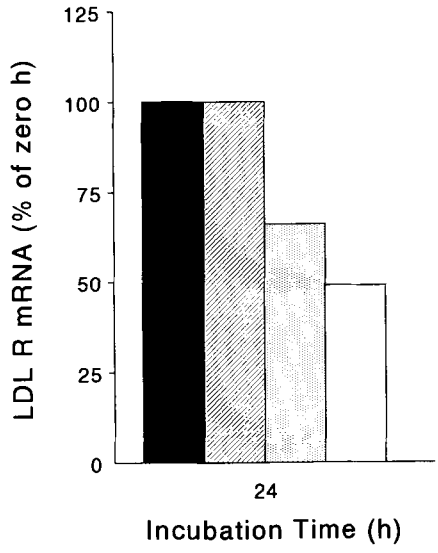

Fig. 10. Effects of combination of 25-hydroxycholesterol and palmitate on LDL-receptor mRNA in HepG2 cells. Cells were grown to 80% confluency in 20% FCS, washed with PBS, incubated for 24 h in 10% FCS, and then further incubated for 24 h with DMEM and palmitate-BSA (0.8 mm and 0.16 mm, respectively) or 5 μg/ml of 25-hydroxycholesterol. Filled bar, DMEM plus ethanol; diagonal bar, DMEM plus ethanol plus BSA; dotted bar, DMEM and ethanol plus 25-hydroxycholesterol plus BSA; empty bar, DMEM plus ethanol, plus 25-hydroxycholesterol plus BSA plus palmitate. Values of LDL-receptor mRNA are in % of zero hour values and represent means of duplicate dishes.

The posttranslational mechanism(s) by means of which saturated fatty acids reduce LDL-receptor activity is(are) still not clear, but some possibilities are suggested by a recent study on the effects of palmitic and linoleic acids on triglyceride synthesis and lipoprotein secretion in Caco2 cells (34). Palmitic acid increased intracellular saturated phospholipid cell membrane contents of cells by much more than did linoleic acid, while linoleic acid increased the triglyceride contents of cells and the secretion of triglycerides from cells more than did palmitic acid. The accumulation of the newly synthesized saturated phospholipid in cells incubated with palmitic acid may alter the cycling of LDL-receptors between the inside and the outside of cells or it may alter the physical characteristics of membranes and affect the activity of LDL-receptors by this mechanism.

Combining palmitate with 25-hydroxycholesterol produced additive effects on LDL binding in both cell lines at each concentration of palmitate and palmitate/BSA molar ratio tested. Adding increasing amounts of 25-hydroxycholesterol to a fixed amounts of palmitate also produced additive effects. In hamsters, dietary saturated fat augmented the effects of dietary cholesterol in suppressing hepatic LDL-receptor activity (10). With respect to mRNA levels, while 25-hydroxycholesterol alone down-regulated LDL-receptor mRNA levels to a maximum of ~50% of control values and palmitate alone did not significantly reduce LDL-receptor mRNA levels at all, combinations of 25-hydroxycholesterol and palmitate had additive effects on LDL-receptor mRNA levels (Fig. 10). These results support the postulate by Daumerie, Woollett, and Dietschy (12) that the combination of dietary saturated fatty acids and cholesterol may be regulating LDL-receptor activity by altering the sizes of "regulatory pools" of cholesterol. The implication of their postulate is that the combination may be enhancing transcriptional regulation. Thus, saturated fatty acids may be suppressing LDL-receptors by two different mechanisms, one affecting membrane fluidity and/or receptor recycling and the other in conjunction with cholesterol, affecting rates of receptor mRNA transcription.

In conclusion, our data demonstrate that the LDL-receptor gene in a human hepatoma cell line and a human colon carcinoma cell line is subject to up-regulation by LPDS, and down-regulation by 25-hydroxycholesterol at the transcriptional level. Saturated fat regulated LDL-receptor in both cell lines by posttranslational mechanism. We also showed that the effect of combination of 25-hydroxycholesterol and saturated fat on LDL-receptor activity and mRNA levels was additive. Thus, cholesterol and saturated fat when administered separately affect the LDL-receptor gene at different loci of regulation. When administered together, saturated fatty acids may enhance the regulatory effects of cholesterol at the transcriptional level, perhaps by affecting regulatory pools of cholesterol (10, 12, 30, 31).

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REFERENCES

1. Gofman, J. W., W. Young, and R. Tandy. 1966. Ischemic heart disease, atherosclerosis and longevity. *Circulation.* **34:** 679–697.
2. Srivastava, R. A. K., S. Jiao, J. Tang, B. Pfleger, R. T. Kitchens, and G. Schonfeld. 1991. In vivo regulation of low density lipoprotein receptor and apolipoprotein B gene expressions by dietary fat and cholesterol in inbred strains of mice. *Biochim. Biophys. Acta.* **1086:** 29–43.
3. Srivastava, R. A. K., J. Tang, E. S. Krul, B. Pfleger, R. T. Kitchens, and G. Schonfeld. 1992. Dietary fatty acids and dietary cholesterol differ in their effect on the in vivo regulation of apolipoprotein A-I and apolipoprotein A-II gene expression in inbred strains of mice. *Biochim. Biophys. Acta.* **1125:** 251–261.
4. Srivastava, R. A. K., D. Baumann, and G. Schonfeld. 1993. In vivo regulation of low density lipoprotein receptors by estrogen differs at the post-transcriptional level in rat and mouse. *Eur. J. Biochem.* **216:** 527–538.
5. Sorci-Thomas, M., M. D. Wilson, F. L. Johnson, D. L. Williams, and L. L. Rudel. 1989. Studies on the expression of genes encoding apolipoproteins B-100 and B-48 and the low density lipoprotein receptor in nonhuman primates. *J. Biol. Chem.* **264:** 9039–9045.
6. Südhof, T. C., D. R. van der Westhuyzen, J. L. Goldstein, M. S. Brown, and D. W. Russell. 1987. Three direct repeats and a TATA-like sequence are required for regulated expression of the human low density lipoprotein receptor gene. *J. Biol. Chem.* **262:** 10773–10779.
7. Molowa, D. T., and G. M. Cimis. 1989. Co-ordinate regulation of low-density-lipoprotein receptor and 3-hydroxy-3-methylglutaryl-CoA reductase and synthase gene expression in HepG2 cells. *Biochem. J.* **260:** 731–736.
8. Kamps, J. A. A. A. M., and T. J. C. Van Berkel. 1992. Complete down-regulation of low density lipoprotein receptor activity in the human hepatoma cell line HepG2 by β-migrating very low density lipoprotein and non-lipoprotein cholesterol: different cellular regulatory pools of cholesterol. *Eur. J. Biochem.* **206:** 913–918.
9. Wang, 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.
10. Spady, D. K., and J. M. Dietschy. 1988. Interaction of dietary cholesterol and triglycerides in the regulation of hepatic low density lipoprotein transport in the hamster. *J. Clin. Invest.* **81:** 300–309.
11. Schonfeld, G., W. Patsch, L. L. Rudel, C. Nelson, M. Epstein, and R. E. Olson. 1982. The effects of dietary cholesterol and fatty acids on plasma lipoproteins. *J. Clin. Invest.* **69:** 1072–1080.
12. Daumerie, C. M., L. A. Woollett, and J. M. Dietschy. 1992. Fatty acids regulate hepatic low density lipoprotein recep-

tor activity through redistribution of intracellular cholesterol pools. *Proc. Natl. Acad. Sci. USA.* **89:** 10797–10801.
13. Javitt, N. B. 1990. HepG2 cells as a resource for metabolic studies: lipoprotein, cholesterol and bile acids. *FASEB J.* **4:** 161–168.
14. Dashti, N. 1992. The effect of low density lipoproteins, cholesterol, and 25-hydroxycholesterol on apolipoprotein B gene expression in HepG2 cells. *J. Biol. Chem.* **267:** 7160–7169.
15. Van Harken, D. R., C. W. Dixon, and M. Heimberg. 1969. Hepatic lipid metabolism in experimental diabetes. *J. Biol. Chem.* **244:** 2278–2285.
16. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
17. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoproteins. I. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta.* **260:** 212–221.
18. Goldstein, J. L., and M. S. Brown. 1974. Binding and degradation of low density lipoproteins by cultured human fibroblasts. Comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *J. Biol. Chem.* **249:** 5153–5162.
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
20. Kovanen, P. T., M. S. Brown, and J. L. Goldstein. 1979. Increased binding of low density lipoprotein to liver membranes from rats treated with 17-alpha-ethinyl estradiol. *J. Biol. Chem.* **254:** 11367–11373.
21. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* **76:** 4350–4354.
22. Srivastava, R. A. K., N. Srivastava, and G. Schonfeld. 1992. Expression of low density lipoprotein receptor, apolipoprotein A-I, apolipoprotein A-II and apolipoprotein A-IV in various rat organs utilizing an efficient and rapid method for RNA isolation. *Biochem. Int.* **27:** 85–95.
23. Srivastava, R. A. K., B. Pfleger, and G. Schonfeld. 1991. Expression of LDL-receptor, apolipoprotein B, apolipoprotein A-I and apolipoprotein A-IV messenger RNA in various mouse organs as determined by a novel RNA-excess solution hybridization assay. *Biochim. Biophys. Acta.* **1090:** 95–101.
24. Tam, S-P., L. Brissette, R. Ramharack, and R. Deeley. 1991. Differences between the regulation of 3-hydroxy-3-methylglutaryl-coenzyme-A reductase and low density lipoprotein receptor in human hepatoma cells and fibroblasts reside primarily at the translational and post-translational levels. *J. Biol. Chem.* **266:** 16764–16773.
25. Cohen, L. H., M. Griffioen, L. Havekes, D. Schouten, V. Van Hinsbergh, and H. J. Kempen. 1984. Effects of compactin, melvalonate and low density lipoprotein on 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity and low density lipoprotein receptor activity in the human hepatoma cell line HepG2. *Biochem. J.* **222:** 35–39.
26. Boogaard, A., M. Griffioen, and L. H. Cohen. 1987. Regulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase in human hepatoma cell line HepG2. *Biochem. J.* **241:** 345–351.
27. Wade, D. P., B. L. Knight, and A. K. Soutar. 1988. Hormonal regulation of low density lipoprotein (LDL) receptor activity in human hepatoma HepG2 cells. Insulin increases LDL-receptor activity and diminishes its suppression by exogenous LDL. *Eur. J. Biochem.* **174:** 213–218.
28. Ellsworth, J. L., C. Brown, and A. D. Cooper. 1988. Stimulation of LDL-receptor activity in HepG2 cells by a serum factor(s). *J. Cell. Physiol.* **135:** 213–223.
29. Kraft, H. G., S. J. Demosky, Jr., K. Schumacher, H. B. Brewer, Jr., and J. M. Hoeg. 1992. Regulation of LDL-receptor, apoB, and apoE protein and mRNA in HepG2 cells. *DNA Cell Biol.* **11:** 291–300.
30. Woollett, L. A., D. K. Spady, and J. M. Dietschy. 1992. Saturated and unsaturated fatty acids independently regulate low density lipoprotein receptor activity and production rate. *J. Lipid Res.* **33:** 77–88.
31. Woollett, L. A., D. K. Spady, and J. M. Dietschy. 1992. Regulatory effects of the saturated fatty acids 6–0 through 18–0 on hepatic low density lipoprotein receptor activity in the hamster. *J. Clin. Invest.* **89:** 1133–1141.
32. Bihain, B. E., R. J. Deckelbaum, F. T. Yen, A. M. Gleeson, Y. A. Carpentier, and L. D. Witte. 1989. Unesterified fatty acids inhibit the binding of low density lipoproteins to the human fibroblast low density lipoprotein receptor. *J. Biol. Chem.* **264:** 17316–17321.
33. Rudling, M., and B. Angelin. 1993. Stimulation of rat hepatic low density lipoprotein receptors by glucagon. Evidence of a novel regulatory mechanism in vivo. *J. Clin. Invest.* **91:** 2796–2805.
34. van Greevenbroek, M. M. J., W. M. Voorhout, D. W. Erkelens, G. van Meer, and T. W. A. de Bruin. 1995. Palmitic acid and linoleic acid metabolism in Caco2 cells: different triglyceride synthesis and lipoprotein secretion. *J. Lipid Res.* **36:** 13–24.