Evidence for sterol-independent regulation of low-density lipoprotein receptor activity in Hep-G2 cells

Jeff L. ELLSWORTH,*† Chitra CHANDRASEKARAN* and Allen D. COOPER*†

*Research Institute, Palo Alto Medical Foundation, 860 Bryant Street, Palo Alto, CA 94301, and †Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305, U.S.A.

The relationship between the serum factor(s)-mediated induction of low-density lipoprotein (LDL) receptor activity and changes in cellular cholesterol metabolism was examined in the human hepatoma cell line Hep-G2. Relative to incubation with serum-free media [Eagle's minimal essential medium (MEM) control], short-term (< 8 h) incubation with medium containing 15 % of either calf serum (MEM + serum) or the $d > 1.25$ fraction of calf serum (MEM + $d > 1.25$) produced a time- and concentration-dependent increase in the uptake of ^{125}I -LDL. Immunoblotting with anti-(LDL receptor) antibodies demonstrated that this was correlated with a 2-fold increase in the amount of the mature 136 000 Da LDL receptor protein in detergent-solubilized Hep-G2 cell membranes. Incubation with MEM + serum, but not MEM + $d > 1.25$, increased the efflux of radiolabelled cholesterol from Hep-G2 cells. However, the induction of ^{125}I -LDL uptake by MEM + $d > 1.25$ (2.3-fold) and MEM + serum (2.2-fold) was virtually identical. Addition of the $d < 1.063$ lipoproteins of calf serum to MEM + $d > 1.25$ at their original or three times their serum concentration decreased the induction of ^{125}I -LDL uptake by MEM + $d > 1.25$ by only 20–30 %. Together, these results suggest that the stimulation of ^{125}I -LDL uptake was not due to the presence of high-density lipoprotein, the absence of LDL or the stimulation of cholesterol efflux. MEM + serum stimulated ^{125}I -LDL uptake in cells cholesterol-loaded by incubation with rat very-low-density lipoprotein with β electrophoretic mobility (β -VLDL). Compared to incubation with the MEM control, either MEM + serum or MEM + $d > 1.25$ produced time-dependent increases in the activity of 3-hydroxy-3-methylglutaryl-CoA reductase which also occurred in cholesterol-loaded cells. However, cholesterol biosynthesis, whether measured from $^3\text{H}_2\text{O}$, ^{14}C acetate or ^3H mevalonic acid, was not increased. Incubation with MEM + serum or MEM + $d > 1.25$ did not affect ^3H oleate incorporation into cellular cholesteryl esters, hydrolysis of intracellular ^3H cholesteryl esters or the cellular mass of unesterified or esterified cholesterol. Incubation with MEM + serum or MEM + $d > 1.25$ produced a transient increase in the level of LDL receptor mRNA, reaching a maximum of 5–10-fold by 2 h and decreasing to near baseline levels by 4 h. Actinomycin D blocked the serum-factor-mediated induction of LDL receptor mRNA. These data indicate that LDL receptor gene expression in liver-derived cells is rapidly and transiently increased by incubation with a factor(s) present in serum and that this induction is independent of demonstrable changes in cellular cholesterol metabolism.

INTRODUCTION

The level of plasma low-density lipoprotein (LDL) cholesterol is a major risk factor for the development of atherosclerosis [1]. The level of LDL cholesterol in the blood is maintained by a dynamic balance between the formation and catabolism of LDL. Although many organs can participate in LDL removal, the liver is the most important single site, and the majority of this uptake is mediated by the LDL receptor [2–7]. The factors that determine the number of LDL receptors expressed by the liver are not completely understood. The principal form of LDL receptor regulation demonstrated to date is by feedback, where increasing cellular cholesterol content causes a decrease in the number of cell surface LDL receptors [8]. Thus incubation of a variety of cells in the presence of LDL, cholesterol or oxygenated sterols results in a decrease in LDL receptors, an effect mediated by a decrease in transcription of the LDL receptor gene [8]. Recent studies on the mechanism of sterol-dependent regulation of LDL receptor activity have focused on a 42 bp sequence, termed the sterol regulatory element (SRE), in the 5'-flanking region of the LDL receptor gene [8–11]. This sequence contains two contiguous 16 bp imperfect repeats that differ in their mode of regulation.

Repeat number 3 is a constitutive positive transcriptional element that binds the eukaryotic transcription factor Sp1, while repeat number 2, which has the sequence CACCCCAC, designated SRE-1 [8], is thought to interact with another regulatory protein to confer strong repression upon repeat number 3 when sterols are present [11]. Although mutation of SRE-1 leads to a loss of sterol-dependent regulation, mutation of SRE-1 in the LDL receptor promoter also prevents the increase in transcription that occurs in the absence of sterols [10]. Thus, in the LDL receptor promoter, SRE-1 appears to contain the signals for positive and negative regulation by sterols. While it appears that this type of control may be important for sterol-mediated repression of the LDL receptor promoter, it is not clear whether these *cis*-acting transcriptional elements are responsible for all forms of LDL receptor regulation.

Over the last few years evidence, both direct and circumstantial, has accumulated that supports the occurrence of the regulation of LDL receptors independent of cellular sterols. In both adrenal [12–14] and ovarian granulosa [15–17] cells, LDL receptor activity is increased in response to hormone administration and occurs under conditions in which the cholesterol demand for steroidogenesis is low and LDL receptors should be down-regulated.

Abbreviations used: VLDL, very-low-density lipoprotein; β -VLDL, VLDL that migrates with β electrophoretic mobility on agarose gel electrophoresis; LDL, low-density lipoprotein; HDL, high-density lipoprotein; MEM, Eagle's minimal essential medium; EBSS, Earle's balanced salt solution; PBS, phosphate-buffered saline; HMG, 3-hydroxy-3-methylglutaryl; SRE, sterol regulatory element; PDGF, platelet-derived growth factor; ASGR, asialoglycoprotein receptor.

† To whom correspondence should be addressed.

Furthermore, regulation of LDL receptor activity in the outer (glomerulosa and fasciculata) and inner (reticularis) zones of the adrenal cortex is different. In general, in the outer zone hormonal modulation of steroid hormone production is linked to changes in LDL receptor activity, while in the inner zone adrenocorticotrophic hormone and dexamethasone regulate cholesterol metabolism, but do not significantly affect LDL receptor activity [13].

The expression of LDL receptors by cells in culture is also affected by a variety of incubation conditions. Receptor activity is increased by incubation of cells with insulin [18–20], platelet-derived growth factor (PDGF) [21–24], fibroblast-derived growth factor [21], thyroid hormone [20,25], oestrogen [26], Ca^{2+} channel blockers [27–29] and calmodulin inhibitors [30–31], and by factors present in serum [32]. It is not clear whether the effects of these growth-related factors on LDL receptor activity in the various cell types are secondary to changes in cellular cholesterol content or whether they act as primary effectors. However, conditions have been described for some of these agents in which the increase in LDL receptor activity appears to be independent of the content of or demand for cellular cholesterol.

Regulation of cholesterol metabolism is more complex in liver cells than in less specialized cells such as skin fibroblasts. In the liver, newly synthesized cholesterol, or that derived from the catabolism of lipoproteins, can be used for bile acid synthesis, secreted as biliary cholesterol or used for lipoprotein production, in addition to supplying cholesterol for the formation of cell membranes [3]. Interestingly, relative to regulation of LDL receptors in fibroblasts, the LDL receptor activity of Hep-G2 cells [33–36], pig [37], rat [38] and human hepatocytes [39–41] is somewhat resistant to down-regulation by cholesterol derived from exogenous LDL. In general, an extracellular concentration of LDL that produces an 80–85% decrease in LDL receptor number in fibroblasts [33,42] fails to produce more than a 20–30% decrease in LDL receptors in hepatocytes. Maintenance of receptor-mediated transport in the presence of a high extracellular concentration of lipoprotein cholesterol may be indicative of an alternative means of control of hepatic LDL receptor activity. We have demonstrated that LDL receptor activity in the human hepatoma cell line Hep-G2 was regulated by a non-dialysable, heat-stable serum component [32]. Furthermore, serum factor stimulation of ^{125}I -LDL uptake appeared to be specific for LDL receptors in Hep-G2 cells as compared with human skin fibroblasts, and the induction of LDL receptor activity appeared to occur in the absence of changes in cellular cholesterol biosynthesis. These data suggested that a serum component may be responsible for sterol-independent regulation of hepatic LDL receptor activity. The experiments reported herein were designed to investigate in detail the serum-factor-mediated induction of LDL receptor activity and to determine whether this is mediated by a change in some other parameter of cholesterol metabolism.

EXPERIMENTAL

Materials

Male Sprague–Dawley rats (retired breeders) were obtained from Simonsen Laboratories (Gilroy, CA, U.S.A.). $\text{RS-[5-}^3\text{H(N)]-Mevalonolactone}$ (40 Ci/mmol), $^3\text{H}_2\text{O}$ (25 mCi/g) and $[4\text{-}^{14}\text{C}]\text{-cholesterol}$ (60 mCi/mmol) were purchased from Dupont–New England Nuclear (Wilmington, DE, U.S.A.). $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ (triethylammonium salt), $[1\alpha,2\alpha(\text{n})\text{-}^3\text{H}]\text{cholesterol}$ (46 mCi/mmol), $[^{14}\text{C}]\text{acetic acid}$ (sodium salt; 55 mCi/mmol), 3-hydroxy-3-methyl-[3- $^{14}\text{C}]\text{glutaryl-CoA}$ (52 mCi/mmol), $[9,10(\text{n})\text{-}^3\text{H}]\text{joleic acid}$ (5 Ci/mmol), $\text{L-[U-}^{14}\text{C}]\text{tyrosine}$ (423 mCi/mmol), Na^{125}I

(13–17 mCi/ μg) and Rainbow Protein Molecular Weight Markers were purchased from Amersham (Arlington Heights, IL, U.S.A.). Oligolabelling kits were obtained from Pharmacia–LKB (Piscataway, NJ, U.S.A.). Unesterified cholesterol and cholestane were obtained from Alltech Associates (Deerfield, IL, U.S.A.). *cis*-9-Octadecenoic acid, squalene, cholesteryl oleate, 1,3-diolein, lanosterol, DL- α -dipalmitoylphosphatidylcholine, 1-mono-oleoyl-*rac*-glycerol, 1,2-dioleoyl-*sn*-glycerol, dolichol Grade 1 $\text{C}_{80}\text{--C}_{105}$, coenzyme Q_{10} (ubiquinone 50), glucose-6-phosphate dehydrogenase, mevalonic acid lactone, sodium oleate, orosomucoid (α_1 acid glycoprotein), CHAPS and radioimmunoassay-grade BSA were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fatty-acid-poor BSA was purchased from CalBiochem (San Diego, CA, U.S.A.). SP-2250 (3%) on 80/100 Supelcoport was obtained from Supelco (Bellefonte, PA, U.S.A.). All tissue culture supplies were purchased from Gibco Laboratories (Grand Island, NY, U.S.A.), and all other chemicals were obtained from Sigma or as described in a previous publication [32].

Cell culture

Hep-G2 cells and human skin fibroblasts were cultured in medium A [Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum, 292 μg of glutamine/ml, 100 units of penicillin/ml and 100 μg of streptomycin/ml] as described previously [32]. The cells were grown at 37 °C under a humidified atmosphere of 95% air and 5% CO_2 . Cultures were routinely checked for mycoplasma contamination using the Mycoplasma TC kit (Gen-Probe, San Diego, CA, U.S.A.).

Preparation of lipoproteins and the lipoprotein-free fraction of serum

Plasma from normolipaemic fasting human subjects was isolated from blood collected into 0.15% EDTA, the erythrocytes were removed and the lipoproteins were isolated as described [32]. The LDL ($1.019 < d < 1.063$) were dialysed against phosphate-buffered saline (PBS; 0.01 M-sodium phosphate, 0.15 M-NaCl, pH 7.4) containing 0.04% EDTA at 4 °C, sterilized by passage through a 0.45 μm filter and stored at 4 °C. Methyl-LDL was prepared by reductive methylation of native LDL [43] and was dialysed, sterilized and stored as described above. LDL and methyl-LDL were radiolabelled with ^{125}I by the Iodogen procedure. The average specific radioactivities were 200 c.p.m./ng of protein and 52 c.p.m./ng of protein respectively. For each lipoprotein, greater than 95% of the ^{125}I label was precipitated with an equal volume of 20% trichloroacetic acid. Very-low-density lipoproteins with β electrophoretic mobility (β -VLDL) from hypercholesterolaemic rats were isolated as described [44]. The lipid and apoprotein composition of these particles has been reported previously [44]. To prepare the $d > 1.25$ fraction, a sample of calf serum was adjusted to a density of 1.25 g/ml with solid KBr and centrifuged for 42 h at 39000 rev./min (r_{av} , 110.2 mm) at 4 °C as described above. The lipoproteins were removed by tube slicing, and the $d > 1.25$ infranant fraction was dialysed exhaustively against PBS at 4 °C using a Spectro/Por 1 membrane (molecular mass cut-off = 6000–8000 Da). The $d > 1.25$ fraction was adjusted to the original serum volume by the addition of a small volume of PBS, sterilized by passage through a 0.45 μm filter and stored at –20 °C. The lipoprotein-containing supernatant fraction was dialysed exhaustively against PBS at 4 °C, adjusted to $d = 1.063$ with solid KBr and centrifuged for 18 h at 4 °C as described above. The VLDL + LDL ($d < 1.063$) fraction was isolated by tube slicing, dialysed, sterilized as described above and stored at 4 °C. Dialysed calf serum was prepared by extensive dialysis against PBS at 4 °C, sterilized and stored as described above.

Asialo-orosomucoid was prepared and iodinated by the chloramine-T procedure as described previously [32]. The specific radioactivity of the ^{125}I -asialo-orosomucoid was 2600 c.p.m./ng of protein, and more than 95% of the radioactivity was precipitable by an equal volume of 20% trichloroacetic acid. The specific uptake of ^{125}I -asialo-orosomucoid, determined in the presence and absence of a 100-fold excess of unlabelled asialo-orosomucoid, was always greater than 90% of the total.

Lipoprotein binding studies

Hep-G2 cells were seeded into 6- or 12-well tissue culture plates at a density of $(1-2) \times 10^5$ cells/cm² (day 0) and were grown in medium A as described above. By day 3 or 4 the cells had formed dense monolayers of 4.5×10^5 cells/cm² and 0.1 mg of cell protein/cm². The cells were placed on ice, the medium was removed and the monolayers were washed once with 2-4 ml of ice-cold Earle's balanced salt solution (EBSS) containing 0.02 M-Hepes, pH 7.4. The washes were discarded and each dish received fresh MEM containing 292 μg of glutamine/ml with either no additions (MEM control), 15% (v/v) dialysed calf serum (MEM + serum) or 15% (v/v) $d > 1.25$ fraction of calf serum (MEM + $d > 1.25$). The cells were returned to the incubator, and after the indicated time (generally 5-6 h) the incubation media were removed and the cells were placed on ice and washed. The cells were then incubated with the indicated concentration of ^{125}I -LDL in 0.4 ml (for 12-well plates) of medium B (MEM containing 0.02 M-Hepes, pH 7.4, and 40 mg of BSA/ml). Incubations at 37 °C were started by the addition of the incubation mixture to the cells and terminated by placing the cells on ice. The binding media were removed and the cells were washed as described [32]. The cells were solubilized with 1.0 ml of 1.0 M-NaOH and the cell-associated radioactivity was determined. The uptake of ^{125}I -LDL was determined in the presence (non-specific uptake) and absence (total uptake) of a 100-fold excess of unlabelled LDL. The specific uptake was calculated as the difference between the total and non-specific values, and represented about 80% of the total. Unless specified otherwise, the specific uptake of ^{125}I -LDL is presented in the Tables and Figures. The measured radioactivity was normalized per mg of cell protein, which was determined in an aliquot of the cell suspension. Human skin fibroblasts were cultured and used in lipoprotein binding studies as described previously [32].

Cholesterol loading of Hep-G2 cells

Hep-G2 cells were cholesterol-loaded by incubation with β -VLDL isolated from hypercholesterolaemic rats as follows. On day 3 or 4 of culture, the medium was removed from each dish of cells, the cell media were combined and any cellular debris was removed by centrifugation at 1000 g for 10 min at 4 °C. An aliquot of the conditioned media pool was added to each dish of cells, β -VLDL was added in the indicated amounts to the appropriate dishes and the cells were returned to the incubator. After 18-24 h at 37 °C the cells were removed from the incubator and placed on ice. The cell media were removed and the cells were washed with ice-cold EBSS + 0.02 M-Hepes, pH 7.4. Cells were incubated with MEM, MEM + serum or MEM + $d > 1.25$ for the indicated time at 37 °C and then taken for determination of cellular cholesterol mass, uptake of ^{125}I -LDL or assay of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase (EC 1.1.1.34) activity.

Determination of HMG-CoA reductase activity and incorporation of [^{14}C]acetate, [^3H]mevalonate, $^3\text{H}_2\text{O}$ and [^{14}C]tyrosine into cellular lipids

To measure HMG-CoA reductase activity Hep-G2 cells were cultured in medium A and incubated with MEM, MEM + serum

or MEM + $d > 1.25$ as described above. At the appropriate time the cells were removed from the incubator and placed on ice, and the monolayers were washed with ice-cold PBS. The cells were scraped from the dishes, centrifuged at 2000 g for 5 min at 4 °C in a Beckman J6 centrifuge and taken immediately for determination of HMG-CoA reductase activity [44] or stored at -86 °C until use.

To measure the incorporation of radiolabelled precursors into cellular lipids, Hep-G2 cells were cultured in 6-well plates as described above, and on day 3 or 4 the cell media were removed and the cells were washed with 2 ml of serum-free MEM. The wash was discarded and to each dish was added 40 μCi of [^{14}C]acetate (in sterile water) or 25 μCi of [^3H]mevalonic acid lactone in 0.8 ml of fresh serum-free MEM, and the cells were returned to the incubator for 1 h at 37 °C. For labelling with $^3\text{H}_2\text{O}$ the cells were incubated with 8 mCi/ml for 2 h at 37 °C. A 0.15 ml sample of dialysed calf serum, the $d > 1.25$ fraction of calf serum or an equivalent volume of sterile PBS was added to the appropriate dishes of cells, and the cells were incubated for 5 h at 37 °C. The cell media were removed, centrifuged to remove any cell debris, quick-frozen in liquid N_2 and stored at -20 °C, or the total lipids in the cell medium and the cells were extracted immediately with chloroform/methanol (2:1, v/v) as described [32]. In some instances the lipids from the cellular extracts were dried under N_2 , resuspended in 2 ml of 90% methanol containing 15% KOH and 100 μg of butylated hydroxytoluene/ml, and saponified by incubation at 80 °C for 1 h. The lipids were extracted with 2 vol. of hexane, the aqueous phases were re-extracted with hexane, and the hexane phases were combined and backwashed with $\frac{1}{3}$ vol. of 50% saturated NaHCO_3 and then with $\frac{1}{3}$ vol. of 70% ethanol. For [^{14}C]acetate incorporation a sample of [^3H]cholesterol (12 500 d.p.m.), and for [^3H]mevalonic acid and $^3\text{H}_2\text{O}$ incorporation a sample of [^{14}C]cholesterol (5000 d.p.m.), was added prior to lipid extraction to quantify recoveries. The lipid extracts were dried under N_2 and for [^3H]mevalonic acid and $^3\text{H}_2\text{O}$ incorporation into cellular cholesterol, and radiolabel incorporation into cell media lipids, the plates were developed in a single dimension using hexane/diethyl ether/acetic acid (86:16:1.5, by vol.) as solvent. A sample of unesterified cholesterol was applied to each plate as standard. The plates were dried, the lipid-containing areas were visualized with I_2 vapour and were scraped into scintillation vials for quantification of radioactivity by scintillation counting.

To quantify [^{14}C]acetate incorporation into cellular isoprenoids, before spotting the samples on to the plates a sample of a mixture of unlabelled unesterified cholesterol (60 μg), mono-olein (50 μg), 1,2-diolein (12 μg), 1,3-diolein (12 μg), oleic acid (80 μg), lanosterol (50 μg), dolichol (15 μg), ubiquinone (30 μg), squalene (10 μg) and cholesteryl oleate (75 μg) was added as standard. The lipids were dried under N_2 and separated by t.l.c. on silica gel G plates using the two-dimensional solvent system as described [45]. The lipids were separated in the first dimension by developing the plate in hexane/diethyl ether/glacial acetic acid (47:20:1, by vol.). The plates were briefly dried and the lipids were separated in the second dimension by developing the plates in benzene. The plates were dried and radiolabel incorporation into cellular lipids was determined as described above.

To measure [^{14}C]acetate incorporation into total cellular protein, Hep-G2 cells were labelled with 25 μCi of [^{14}C]acetate/ml and incubated with MEM, MEM + serum or MEM + $d > 1.25$ as described above. After 5 h at 37 °C the cells were placed on ice, washed and the proteins were precipitated by the addition of an equal volume of 20% trichloroacetic acid. After incubation on ice for 20 min the protein precipitates were collected by centrifugation (1000 g , 5 min), and the lipids were removed by

sequential extraction of the protein pellet with 2.0 ml each of hexane (twice), ethanol and acetone. The cell protein was dissolved in 0.5 ml of 1 M-NaOH and a sample was removed for protein determination and quantification of radioactivity.

To measure [^{14}C]tyrosine incorporation into cellular ubiquinone Hep-G2 cells were subcultured in T-75 flasks as described above. On day 3 each dish of cells (~ 1 mg of cell protein) was washed with 2 ml of MEM and received 10 μCi of [^{14}C]tyrosine in 1.7 ml of MEM. After 2 h at 37 °C a 0.3 ml sample of MEM or $d > 1.25$ (serum containing $d > 1.25$ lipoproteins) was added to the appropriate dishes. After 5 h at 37 °C the media were removed and the cells were harvested and lipids extracted as described above. A 30 μg sample of unlabelled ubiquinone was added to each sample before application of the lipid extracts to the plates. The lipids were separated by two-dimensional t.l.c., visualized with I_2 and radioactivity measured as described above.

Assay of cholesterol efflux

The cell media of Hep-G2 cells (day 3 or 4 of culture) were removed and combined. A 1.0 ml sample of the conditioned media containing 7.0 μCi of [^3H]mevalonic acid was then added to each dish and the cells were returned to the incubator. After 18–22 h the cell media were removed and the cells were washed with EBSS containing 0.02 M-Hepes, pH 7.4. A 1.0 ml sample of MEM, MEM+serum or MEM+ $d > 1.25$ was added, and the cells were incubated for 5 h at 37 °C. The cell media were removed and lipids were extracted as described above. A sample of [^{14}C]cholesterol (5000 d.p.m.) was added before lipid extraction for estimation of recoveries. The lipids were spotted on to silica gel G plates and were separated by development in hexane/diethyl ether/acetic acid (86:16:1.5, by vol.). The areas corresponding to unesterified and esterified cholesterol were identified by comparison with unesterified and esterified cholesterol which were co-chromatographed on each plate. The lipid-containing areas were scraped from the plate and radioactivity was quantified by scintillation counting.

Incorporation of [^3H]oleate and [^3H]cholesterol into cellular cholesteryl esters

Complexes of [^3H]oleic acid/albumin were prepared as described previously [44] and were sterile-filtered (0.45 μm filter) before use. Dishes of Hep-G2 cells were cultured as described above, and on day 3 or 4 the cell media were removed and the cells were washed. A 30 μl aliquot (5.0 μCi) of the [^3H]oleate/albumin complexes in 1 ml of serum-free MEM was added to each dish and the cells were returned to the incubator. In preliminary studies this amount of [^3H]oleate/albumin was found to be saturating with respect to cholesteryl [^3H]oleate formation. After 1 h at 37 °C, a 150 μl sample of MEM, whole serum or the $d > 1.25$ fraction of serum was added to the appropriate dishes and the cells were returned to the incubator. To label endogenously synthesized cholesteryl esters, on day 3 or 4 of culture the medium from each dish of cells was removed and combined together. A 1.0 ml sample of the conditioned cell media containing 3.5 μCi of [^3H]cholesterol in ethanol (final ethanol concentration = 1%) was added to each dish. After 20 h at 37 °C the dishes were removed from the incubator and washed with 2 ml of EBSS. The cells were then incubated with MEM, MEM+serum or MEM+ $d > 1.25$ as described above. After 5 h at 37 °C the cells from each experiment were placed on ice and the media were removed and centrifuged for 2 min in a Microfuge and were extracted immediately or stored at -20 °C before extraction for total lipids as described above. Each dish of cells was washed with 2 ml of ice-cold PBS and the cells were scraped

from the dish into 1 ml of ice-cold PBS. The cells were centrifuged at 1000 g for 5 min and were resuspended in a small volume of ice-cold PBS. A sample was removed for protein determination and the remainder was extracted for total lipids as described above. The lipid extracts were dried under N_2 and the radioactivity in unesterified and esterified cholesterol was determined by t.l.c. on silica gel G using the single dimension system as described above.

Measurement of cell cholesterol mass

Hep-G2 cells were subcultured in T-25 flasks at a density of 2×10^5 cells/cm 2 . On day 3 the cell media were removed and the cells were washed. Each flask of cells received 3 ml of MEM containing no additions, 15% serum or the $d > 1.25$ fraction of serum. After incubation for 5 h at 37 °C the media were removed, the cells were washed with 2 ml of ice-cold PBS and the cells were scraped from the dishes into 1 ml of ice-cold PBS. The cells were then centrifuged at 1000 g for 5 min and either extracted immediately for total lipids as described above or quick-frozen in liquid N_2 and stored at -20 °C until used. The cellular mass of cholesterol was measured by gas chromatography on 3% SP-2250 on 80/100 Supelcoport as described previously [32].

Isolation and quantification of mRNA

Total cellular RNA was isolated from Hep-G2 cells by the single-step guanidinium thiocyanate procedure [46], and poly(A) $^+$ RNA was selected by chromatography on oligo(dT)-cellulose according to standard procedures [47]. RNA samples were further assessed by Northern blot and slot-blot hybridization (see below).

cDNA probes

A 1.8 kb *Eco*R1–*Xho*I fragment of pDLR-3 [48] was used to detect LDL receptor mRNA, a 404 bp *Eco*R1 fragment of the human cytoplasmic β -actin clone pHF β A-3' UT-HF [49] was employed to detect β -actin mRNA and a 1.2 kb *Hind*III–*Eco*R1 fragment of the human asialoglycoprotein receptor (ASGR) H1 clone pSA1 [50] was used to detect ASGR mRNA. Inserts were excised from the plasmids with the appropriate restriction enzymes and were extracted from 1% low-melting agarose gels using the hexadecyltrimethylammonium bromide procedure [51] and were labelled with [^{32}P]dCTP to a specific radioactivity of $(0.5\text{--}1.0) \times 10^9$ c.p.m./ μg by random priming with Klenow DNA polymerase.

Quantification of RNA by Northern blotting and slot-blot hybridization

For Northern blots RNA denaturation and electrophoresis was according to standard procedures [47]. Immediately after electrophoresis the RNA was transferred to Nitroplus 2000 as described [47]. For slot-blot hybridization a range of RNA concentrations was applied to Nytran membranes using a slot-blot apparatus (Schleicher & Schuell) to yield a linear response of the hybridization signal to the amount of RNA applied to the membrane. The filters were prehybridized in a solution that consisted of 50% formamide, 10% dextran sulphate, $5 \times \text{SSC}$ ($1 \times \text{SSC} = 0.15 \text{ M-NaCl}/0.015 \text{ M-sodium citrate}$), 4 $\times$ Denhardt's solution, 0.1% SDS and 0.1 mg of salmon sperm DNA/ml for 18 h at 42 °C. Filters were hybridized at 42 °C for 18–48 h in a solution that consisted of 50% formamide, 10% dextran sulphate, $2 \times \text{SSC}$, 0.1% SDS, 1.0 mM-EDTA and 0.1 mg of salmon sperm DNA/ml. The filters were then washed at room temperature for 20 min in $2 \times \text{SSC}$ and then successively for 10 min at 68 °C in $2 \times$, $1 \times$, $0.5 \times$, $0.2 \times$ and finally $0.1 \times \text{SSC}$. The filters from the Northern blot and slot-blot hybridizations were air-dried prior to baking for 2 h at 80 °C. Autoradiographs of the slot-blots were quantified by scanning densitometry.

Preparation of cell membranes and their solubilization

A 500–105 000 *g* cell membrane fraction was prepared from Hep-G2 cells and solubilized with CHAPS exactly as described previously [52]. For each incubation condition, seven T-75 flasks containing approx. 3×10^8 cells were used.

Immunoblotting with anti-(rat liver LDL receptor) antibodies

Samples of detergent-solubilized membranes (25–100 μ g of protein) were adjusted to final concentrations of 0.002 % (w/v) Bromophenol Blue, 0.5 % (w/v) SDS and 13 % (w/v) glycerol and were applied to 6 % polyacrylamide gels containing 0.1 % (w/v) SDS under non-reducing conditions as described previously [52]. Gels were calibrated with a 15 μ l sample of the Rainbow Protein Molecular Weight Markers containing myosin (200 kDa), phosphorylase *b* (92.5 kDa), BSA (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.3 kDa). Proteins were electrophoretically transferred to nitrocellulose paper as described previously [52]. Immunoblotting was carried out as described [52], except that incubations with the anti-(rat liver LDL receptor) antiserum and the horseradish peroxidase-conjugated goat anti-rabbit IgG were extended to 3 h and 2 h respectively. Immunoblots were developed with 4-chloro-1-naphthol as described [52] and the amount of LDL receptor protein was quantified by scanning densitometry of the dried membrane.

Other methods

Cellular and lipoprotein protein was measured with the Bio-Rad (Richmond, CA, U.S.A.) protein assay kit using BSA as standard. The total cholesterol content of the lipoproteins was measured enzymically with the Cholesterol 20 kit (Sigma). Statistical analyses were performed using a paired or unpaired two-tailed Student's *t*-test or by analysis of variance.

RESULTS

Kinetics and specificity of the serum-mediated induction of LDL receptor activity in Hep-G2 cells

In a previous study [32] we noted that the number of cell surface LDL receptors in Hep-G2 cells increased during 24–48 h of incubation with serum or a partially purified fraction of serum. Further, in incubations of 8 h or less, the serum-mediated increase in 125 I-LDL uptake occurred in the absence of changes in cholesterol biosynthesis. This suggested that the change in LDL receptors occurred independently of changes in cell sterol metabolism. To further explore this, the kinetics of induction of LDL receptor activity were examined in greater detail. When Hep-G2 cells were incubated in the absence of serum (MEM control) there was little change in 125 I-LDL uptake over this time course (Fig. 1a, ○). In contrast, after a lag of approx. 2 h, incubation with MEM + 15 % calf serum (MEM + serum) produced a time-dependent increase in cell-associated 125 I-LDL (Fig. 1a, ●). The serum-mediated increase in 125 I-LDL uptake was also dependent on the concentration of serum used in the incubation. The uptake of 125 I-LDL increased in a dose-dependent manner to a maximum of 2.3-fold at a serum concentration of 20 % (Fig. 1b). Addition of higher concentrations of calf serum, up to 80 %, did not increase the uptake of 125 I-LDL beyond this level. Similar to our previous results [32], incubation with MEM + serum had no significant effect on the amount of cell protein per dish (results not shown).

To evaluate whether the induction of 125 I-LDL uptake was specific for transport through the LDL receptor pathway, the ability of anti-(rat liver LDL receptor) antibodies [53] to compete for uptake of 125 I-LDL was determined. The anti-(LDL receptor)

antibodies were as effective as unlabelled LDL in competition for 125 I-LDL uptake in serum-stimulated cells (Fig. 2a). Furthermore, the uptake of 125 I-methyl-LDL was not increased by incubation in the presence of serum-containing medium (Table 1), indicating that incubation with MEM + serum had no effect on the LDL-receptor-independent uptake of 125 I-LDL. Immunoblotting with the anti-(LDL receptor) antibodies revealed that incubation of Hep-G2 cells with MEM + serum or MEM + *d* > 1.25 increased the amount of the mature 136 000 Da LDL receptor protein in detergent-solubilized Hep-G2 cell membranes by 2-fold (Fig. 2b). The increase in the amount of LDL receptor was apparent over a large range (25–250 μ g) of solubilized Hep-G2 cell membrane protein applied to the gel (results not shown). Thus LDL receptor expression in Hep-G2 cells can be increased in a rapid, specific and dose-dependent, manner by factors present in serum.

To evaluate whether the induction of 125 I-LDL uptake was specific for Hep-G2 cells or could be generalized to other cell types, the effect of incubation with MEM + serum or MEM + *d* > 1.25 on the uptake of 125 I-LDL by human skin fibroblasts was examined. Similar to our previous report, in which a 24–48 h incubation with MEM + serum was used [32], incubation with either MEM + serum or MEM + *d* > 1.25 for 5 h at 37 °C had no effect on the amount of cell-associated 125 I-LDL in the fibroblast cultures. Relative to incubation with MEM, incubation of Hep-G2 cells with MEM + serum or MEM + *d* > 1.25 increased the specific uptake of 125 I-LDL from 124 ± 13 ng/mg of cell protein to 320 ± 14 ($P < 0.001$) and 299 ± 23 ($P < 0.001$) ng/mg of cell protein (means $\pm$ s.d. of measurements on triplicate dishes) respectively. With skin fibroblasts incubated with MEM, MEM + serum and MEM + *d* > 1.25 the values were 431 ± 9 , 387 ± 22 ($P = 0.48$) and 338 ± 61 ($P = 0.22$) ng/ml of cell protein respectively ($n = 3$). Moreover, the serum-factor-mediated induction of 125 I-LDL uptake in Hep-G2 cells was relatively specific for LDL receptors and was not due to a generalized increase in cell surface proteins, since incubation of Hep-G2 cells with MEM + *d* > 1.25 for 5 h at 37 °C had no significant effect on the uptake of 125 I-asialo-orosomucoid (results not shown). Taken together, these data indicate that liver-derived cells respond rapidly to stimulation by a serum factor(s) by induction of LDL receptor activity.

Role of high-density-lipoprotein (HDL)-mediated cholesterol efflux in serum stimulation of LDL receptor activity

A number of studies have shown that incubation of a variety of cells in culture with HDL₃ or VLDL can promote the efflux of cholesterol from cells and thus stimulate LDL receptor activity [54,55]. To study this, Hep-G2 cells were incubated with [3 H]mevalonate in order to radiolabel cellular cholesterol, and the appearance of [3 H]cholesterol in the medium was monitored. When cells were labelled with the radiolabelled precursors for 1 h prior to incubation with MEM containing the various serum fractions, little radiolabelled cholesterol was found in the cell medium (results not shown). In order to ensure labelling of slowly equilibrating cholesterol pools the cells were prelabelled with [3 H]mevalonate for 22 h. The cells were then incubated with MEM containing the various serum fractions in the absence of radiolabelled mevalonate for 5 h at 37 °C, and the appearance of radiolabel in the extracellular medium was determined. In the absence of serum there was little radiolabelled cholesterol found in the cell media. Incubation of cells with MEM + serum increased the radiolabelled cholesterol in the cell media by 3.3 ± 0.6 -fold (mean $\pm$ s.e.m. of three separate experiments of triplicate dishes of cells; $P < 0.02$). Analysis of the cell medium [3 H]cholesterol by density gradient ultracentrifugation revealed that more than 95 % of the radiolabel was found in the lipoprotein-containing

Fig. 1. Time course of the serum-mediated induction of ¹²⁵I-LDL uptake in Hep-G2 cells

Hep-G2 cells were subcultured in 12-well plates as described in the Experimental section. (a) On day 3 the culture media were removed, the cells were washed and a 0.5 ml sample of MEM or MEM + 15% calf serum, prewarmed to 37 °C, was added to the appropriate dishes of cells. After incubation for the indicated period of time at 37 °C, the cells were placed on ice, the media were removed, the cells were washed and each dish was incubated with ¹²⁵I-LDL (5 µg of protein/ml) in the presence or the absence of a 100-fold excess of unlabelled LDL. After incubation for 2 h at 37 °C, the specific cell-associated ¹²⁵I-LDL was determined. Values are means ± S.E.M. of quintuplicate dishes from two separate experiments. There was a significant difference ($P < 0.003$) between MEM + serum (●) and MEM (○) at 8 h. The zero time value of cell-associated ¹²⁵I-LDL was 85 ± 3 ng/mg of cell protein. (b) On day 3 each dish of cells received the indicated concentration of serum in a final volume of 1 ml of MEM. After incubation for 6 h at 37 °C the cells were placed on ice, washed and the specific cell-associated ¹²⁵I-LDL was determined as described above. Values are means ± S.E.M. of quadruplicate dishes from two separate experiments. There was a significant difference ($P < 0.02$) from control. The control value (0% serum) was 421 ± 146 ng/mg of cell protein. Where not shown, the error bars are contained within the symbols.

$d < 1.25$ supernatant fraction, indicating that the radiolabelled cholesterol was lipoprotein-associated. In contrast, incubation with MEM containing the $d > 1.25$ fraction of serum had no significant effect on the efflux of [³H]cholesterol (induction of 1.3 ± 0.2 -fold; mean ± S.E.M. of three separate experiments of triplicate dishes, $P = 0.24$). For cells incubated with MEM, MEM + serum or MEM + $d > 1.25$ the cell medium radiolabelled cholesterol represented $13 \pm 7\%$, $37 \pm 14\%$ and $16 \pm 4\%$ respectively of that found in the cells. These data indicated that the lipoprotein-containing fraction of whole serum, most likely HDL, does promote the efflux of a small amount of cholesterol from Hep-G2 cells.

Fig. 2. The serum-stimulated uptake of ¹²⁵I-LDL is mediated by LDL receptors in Hep-G2 cells

Hep-G2 cells were cultured as described in the legend to Fig. 1. (a) Cells were incubated with MEM + 15% serum for 5 h, placed on ice and each dish of cells was incubated with the indicated concentration of anti-(LDL receptor) or non-immune IgG in a final volume of 0.4 ml of medium B. After 1 h at 4 °C, ¹²⁵I-LDL was added to each dish to a final concentration of 1 µg of protein/ml and unlabelled LDL was added at the indicated concentration to the appropriate dishes of cells. After incubation for 2.5 h at 37 °C the cells were placed on ice and washed, and the specific cell-associated ¹²⁵I-LDL was determined as described in the legend to Fig. 1. Values are means of duplicate dishes. A representative of two separate experiments is shown. Differences were significant between non-immune IgG (○) and either LDL (▲, $P < 0.001$) or anti-(LDL receptor) IgG (●, $P < 0.003$). (b) Hep-G2 cells were cultured in T-75 flasks as described in the Experimental section. On day 4 the flasks were divided into three groups of approx. 3×10^8 cells each, the media were removed, the cells were washed and 2 ml of serum-free MEM, MEM + 15% calf serum or MEM + 15% $d > 1.25$ was added to the appropriate dishes of cells. After 5 h at 37 °C the cells were washed and cell membranes were prepared and solubilized as described in the Experimental section. A 50 µg protein sample of the solubilized membranes from cells incubated with MEM (lane 1), MEM + serum (lane 2) and MEM + $d > 1.25$ (lane 3) was applied to the polyacrylamide gel. After electrophoresis the proteins were transferred electrophoretically to nitrocellulose membranes and the blots were probed successively with 1.4 µl of rabbit anti-(rat liver LDL receptor) antibodies/ml and 1.0 µl of horseradish peroxidase-conjugated goat anti-rabbit IgG/ml. A representative experiment is shown.

To evaluate whether the observed efflux of cellular cholesterol is responsible for the induction of ¹²⁵I-LDL uptake by the serum factor, Hep-G2 cells were incubated with MEM, MEM + serum or MEM + $d > 1.25$ and the uptake of ¹²⁵I-LDL was determined.

Table 1. Effect of serum on the uptake of 125 I-LDL and 125 I-methyl-LDL in Hep-G2 cells

Hep-G2 cells were incubated with MEM or MEM + serum for 5 h at 37 °C as described in the legend to Fig. 1. Each dish of cells was then incubated with either 10 μ g of 125 I-LDL protein/ml in the presence and absence of 500 μ g of unlabelled LDL protein/ml, or 10.0 μ g of 125 I-methyl-LDL protein/ml in a final volume of 0.4 ml of medium B. After incubation for 2.5 h at 37 °C the specific cell-associated 125 I-LDL and cell-associated 125 I-methyl-LDL was determined as described in the Experimental section. The specific cell-associated 125 I-LDL represented 72 % and 83 % of the total for cells incubated with MEM and MEM + serum respectively. Values are means $\pm$ S.D. of measurements in triplicate dishes: * $P < 0.005$, ** $P < 0.006$ compared with the MEM control.

Incubation	Cell-associated 125 I-lipoprotein (ng/mg of cell protein)	
	125 I-LDL	125 I-Methyl-LDL
MEM	87 $\pm$ 8	17 $\pm$ 1
MEM + serum	183 $\pm$ 27*	12 $\pm$ 3**

Table 2. Removal of serum HDL has no effect on the serum-mediated induction of 125 I-LDL uptake in Hep-G2 cells

On day 3 each dish of cells received MEM or MEM containing 15 % serum, 15 % $d > 1.25$ or 15 % $d > 1.25$ containing the indicated cholesterol concentration of the $d < 1.063$ lipoprotein fraction of calf serum. After 5 h at 37 °C the medium was removed, the cells were washed and the specific cell-associated 125 I-LDL was determined as described in the legend to Fig. 1. The values are means $\pm$ S.E.M. of measurements on six dishes from two separate experiments. The data were normalized to the amount of cell-associated 125 I-LDL measured in cells incubated with MEM (212 $\pm$ 22 ng/mg of cell protein). All values are significantly different from the MEM control ($P < 0.001$). *Significant difference ($P < 0.03$) from incubation with MEM + $d > 1.25$.

Preincubation	$d < 1.063$ lipoproteins (μ g of cholesterol/ml)	Cell-associated 125 I-LDL (MEM + addition/ MEM)
MEM + 15 % serum	None	2.2 $\pm$ 0.2
MEM + 15 % $d > 1.25$	None	2.3 $\pm$ 0.1
MEM + 15 % $d > 1.25$	8.0	1.9 $\pm$ 0.1*
MEM + 15 % $d > 1.25$	24.0	1.8 $\pm$ 0.2*

Incubation with MEM + $d > 1.25$, which does not promote cholesterol efflux, was as effective in stimulating the uptake of 125 I-LDL as incubation with MEM + whole serum (Table 2). In fibroblast cultures, incubation of cells for 24 h with lipoprotein-deficient serum results in an increase in LDL receptor activity thought to be due to the removal of apo-B-containing lipoproteins [42]. However, the short-term 5 h incubation of fibroblasts with MEM + serum or MEM + $d > 1.25$ had no effect on the uptake of 125 I-LDL (see above). Further, when the $d < 1.063$ lipoproteins of calf serum were added back to the $d > 1.25$ fraction, either at the original (8 μ g of cholesterol/ml) or at 3 times (24 μ g of cholesterol/ml) the concentration of $d < 1.063$ lipoproteins (VLDL + LDL) found in calf serum, the induction of 125 I-LDL uptake was decreased by only 20 % relative to that on incubation with MEM + serum or MEM + $d > 1.25$ (Table 2). These data suggest that the rapid stimulation of LDL receptor activity induced by serum or the $d > 1.25$ fraction of serum is due

Fig. 3. Effect of incubation with MEM and MEM + serum on HMG-CoA reductase activity in Hep-G2 cells

On day 3 of culture the cells were washed and to each dish was added 2 ml of either MEM (○) or MEM containing 15 % serum (●). After incubation for the indicated time at 37 °C the dishes were placed on ice and the cells were harvested for measurement of HMG-CoA reductase activity as described in the Experimental section. Values are means $\pm$ S.E.M. of measurements on six dishes from two separate experiments. There was a significant difference ($P < 0.002$) between MEM and MEM + serum at 4 and 6 h. Where not shown, the error bars are contained within the symbols.

neither to the removal of the $d < 1.063$ lipoproteins nor to the presence of HDL.

Effects of incubation with MEM + serum on cellular cholesterol mass

In general, cholesterol homeostasis is maintained by a feedback loop whereby the number of cell surface LDL receptors is regulated by a change in the cellular content of cholesterol [1]. To directly evaluate whether the serum-mediated induction of LDL receptor activity is due to changes in cellular cholesterol mass, Hep-G2 cells were incubated with MEM or MEM + serum for 5 h at 37 °C and the cellular content of unesterified and esterified cholesterol was measured. For cells incubated with MEM and MEM + serum, the cellular mass of unesterified cholesterol was 32.98 $\pm$ 2.38 and 30.85 $\pm$ 1.42 μ g/mg of cell protein respectively (means $\pm$ S.E.M. of measurements on 6 dishes; difference between MEM and MEM + serum not significant; $P = 0.48$). The corresponding values for cellular cholesteryl ester mass were 15.18 $\pm$ 2.16 and 11.96 $\pm$ 1.76 μ g/mg of cell protein respectively ($n = 6$, $P = 0.29$). Similarly there were no significant differences in the cellular content of unesterified or esterified cholesterol between cells incubated with MEM and MEM + $d > 1.25$ (results not shown). Thus the serum-mediated induction of LDL receptor activity in Hep-G2 cells occurred in the absence of measurable changes in cellular cholesterol mass.

Effects of incubation with MEM + serum and MEM + $d > 1.25$ on HMG-CoA reductase activity and cholesterol biosynthesis

Measurement of the total mass of cellular cholesterol may not accurately reflect changes in a regulatory cholesterol pool, the size of which is small in relation to the total cell cholesterol mass. To examine the effects of the serum factor on cholesterol metabolism in Hep-G2 cells in greater detail, several approaches were taken. The effect of incubation of Hep-G2 cells with MEM, MEM + serum or MEM + $d > 1.25$ on cellular HMG-CoA reductase activity was determined. Incubation with MEM alone produced little change in HMG-CoA reductase activity over this time period (Fig. 3). In contrast, after a lag of approx. 2–3 h, incubation with MEM + serum produced a time-dependent in-

Table 3. Incorporation of labelled precursors into cellular cholesterol in Hep-G2 cells incubated with various serum fractions

On day 3 or 4 Hep-G2 cells were incubated with the various radiolabelled precursors for 1 h at 37 °C as described in the Experimental section. For experiments 3 and 4 the concentration of [¹⁴C]acetate used was 40 and 10 µCi/ml respectively. Each dish of cells then received a 0.15 ml sample of either MEM, serum or *d* > 1.25 and the cells were returned to the incubator. After 5 h at 37 °C, the cells were harvested, the cell lipids were extracted and saponified, and radiolabel incorporation into cellular cholesterol was determined as described in the Experimental section. For expts. 1 and 4 the lipids were not saponified before extraction. In expts. 1 and 2, each value is the mean ± S.E.M. of measurements in triplicate dishes. In expt. 3, each value is the mean ± S.E.M. of two separate experiments in triplicate dishes (*n* = 6). In expt. 4, each value is the mean ± S.E.M. of three separate experiments in triplicate dishes (*n* = 9). n.d., not determined. *Significant difference (*P* < 0.05) from MEM control.

Incubation (5 h, 37 °C)	10 ⁻⁴ × Precursor radioactivity incorporated into cholesterol (d.p.m./mg of protein)			
	³ H]Mevalonate (expt. 1)	³ H ₂ O (expt. 2)	¹⁴ C]Acetate	
			Expt. 3	Expt. 4
MEM control	27.7 ± 3.2	0.34 ± 0.05	89.3 ± 9.2	20.4 ± 5.0
MEM + serum	28.5 ± 1.9	0.42 ± 0.04	n.d.	14.6 ± 1.7
MEM + <i>d</i> > 1.25	n.d.	0.34 ± 0.03	64.6 ± 6.3*	n.d.

Table 4. Effect of incubation of Hep-G2 cells with MEM + serum or MEM + *d* > 1.25 on the synthesis and hydrolysis of cellular cholesteryl esters

To measure [³H]oleate incorporation into cholesteryl esters, on day 3 of culture each dish of cells (110 µg of cell protein/dish) was incubated with [³H]oleic acid/albumin complexes (5.0 µCi, 1.5 mg of albumin) in 0.85 ml of MEM. After 1 h at 37 °C a 0.15 ml sample of either MEM or *d* > 1.25 was added to the appropriate dishes. After 5 h at 37 °C the cell media were removed, the cells were harvested, total lipids were extracted and radiolabel incorporation into cellular cholesteryl ester was determined as described in the Experimental section. To measure cholesteryl ester hydrolysis, dishes of cells were incubated with 3.3 µCi of [³H]cholesterol dissolved in ethanol (final ethanol concentration = 1.2%) in 1 ml of MEM for 18 h at 37 °C. The cell media were removed and the cells were washed and incubated with 1.0 ml of either MEM or MEM + 15% *d* > 1.25. After 5 h at 37 °C the cells were harvested, the lipids were extracted and the content of [³H]cholesteryl esters was determined. For conversion of oleate, values are means ± S.E.M. of three separate experiments, two of triplicate and one of duplicate dishes of cells. For cholesteryl ester content values are means ± S.E.M. of four separate experiments of triplicate dishes of cells. The difference between MEM and MEM + *d* > 1.25 were not significant.

Incubation (5 h, 37 °C)	10 ⁻⁵ × [³ H]Oleate → cholesteryl [³ H]oleate	10 ⁻⁶ × [³ H]Cholesteryl ester content
	(d.p.m./mg of cell protein)	(d.p.m./mg of cell protein)
MEM control	1.72 ± 0.69	3.54 ± 0.85
MEM + 15% <i>d</i> > 1.25	1.41 ± 0.54	3.08 ± 0.69

crease in the activity of HMG-CoA reductase (Fig. 3). A similar time-dependent induction of HMG-CoA reductase activity was observed for cells incubated with MEM + *d* > 1.25 (results not shown).

To determine if the serum-factor-mediated increase in HMG-CoA reductase activity is associated with enhanced cholesterol

synthesis, the effects of incubation of Hep-G2 cells with MEM + serum or MEM + *d* > 1.25 on the incorporation of various cholesterol precursors was determined. In these experiments various precursors were added to the media, followed 1 h later by MEM, serum or the *d* > 1.25 fraction of serum. After an additional 5 h at 37 °C, radiolabel incorporation into cell cholesterol was determined. Relative to incubation with MEM alone, incubation of Hep-G2 cells with MEM + serum or MEM + *d* > 1.25 did not increase incorporation of [³H]mevalonate, [¹⁴C]acetate or ³H₂O into cholesterol (Table 3). In fact, [¹⁴C]acetate incorporation into cholesterol was decreased by 30% in cells incubated with MEM + *d* > 1.25. The absence of an increase in cholesterol biosynthesis was not due to an efflux of radiolabelled cholesterol, since short-term incubation with MEM + serum or MEM + *d* > 1.25 as described did not change the amount of radiolabelled cholesterol found in the cell medium (results not shown and see above), and the radiolabelled cholesterol found in the cell medium was less than 10% of that found in the cells. Furthermore, the absence of an increase in [¹⁴C]acetate incorporation into cholesterol did not appear to be due to a serum-factor-mediated decrease in the specific radioactivity of the cellular acetate pool. In this experiment [¹⁴C]acetate incorporation into cellular triacylglycerol [(268 ± 16) × 10⁴ versus (219 ± 28) × 10⁴ d.p.m./mg of cell protein, *P* = 0.06], diacylglycerol [(36 ± 6) × 10⁴ versus (38 ± 9) × 10⁴ d.p.m./mg of cell protein, *P* = 0.81] and non-esterified fatty acid [(0.28 ± 0.09) × 10⁴ versus (0.25 ± 0.10) × 10⁴ d.p.m./mg of cell protein, *P* = 0.75] in cells incubated with MEM and MEM + serum respectively was relatively constant. Thus incubation of Hep-G2 cells with MEM plus serum or the *d* > 1.25 fraction of serum did not increase cellular cholesterol biosynthesis over this time period. These data indicate that the serum-factor-mediated increase in HMG-CoA reductase activity may be due to a requirement for another mevalonate-derived product.

To explore this, the effect of the serum factor on [¹⁴C]acetate flow through the isoprenoid pathway was determined. Hep-G2 cells were incubated with [¹⁴C]acetate for 1 h at 37 °C and MEM, MEM + serum or MEM + *d* > 1.25 were added for an additional 5 h at 37 °C, and radiolabel incorporation into total cell protein and two other products of mevalonate metabolism, dolichol and ubiquinone, was determined. In addition, radiolabel incorporation into the two major intermediates in the sterol pathway, squalene and lanosterol, was quantified. Relative to incubation with MEM alone, incubation with MEM + serum or MEM + *d* > 1.25 had no effect on radiolabel incorporation into total cell protein or into any of the isoprenoid compounds examined (results not shown). Of the total non-saponifiable lipids, 80–90% of the radioactivity on average was recovered in cholesterol, with the balance recovered in squalene and lanosterol. Similar results were obtained when the incorporation of [¹⁴C]tyrosine, a precursor of the quinone ring of ubiquinone, was measured: 436 d.p.m./mg of cell protein and 433 d.p.m./mg of cell protein for cells incubated with MEM and MEM + *d* > 1.25 respectively. Thus dolichol and ubiquinone synthesis and the bulk of [¹⁴C]acetate flow through the sterol pathway were relatively unaffected by incubation with MEM + serum or MEM + *d* > 1.25, despite the induction of LDL receptor and HMG-CoA reductase activities.

To determine if serum factor stimulation of Hep-G2 cells were associated with changes in cellular cholesteryl ester metabolism, Hep-G2 cells were prelabelled with [³H]oleate or [³H]cholesterol and incubated with MEM or MEM + *d* > 1.25, and [³H]oleate incorporation into cholesteryl [³H]oleate and the hydrolysis of intracellular [³H]cholesteryl esters were determined. As compared with the MEM control, incubation with MEM + *d* > 1.25 had no significant effect on [³H]oleate incorporation into cellular chol-

Fig. 4. Effect of cholesterol loading of Hep-G2 cells on the serum stimulation of ¹²⁵I-LDL uptake

(a) Hep-G2 cells were cholesterol-loaded by incubation with the indicated concentration of rat β-VLDL as described in the Experimental section. After 18–22 h the media were removed, the cells were washed and each dish was incubated with either MEM or MEM + serum for 5 h at 37 °C. The uptake of ¹²⁵I-LDL was then measured by incubating each dish with 40 μg of ¹²⁵I-LDL protein/ml in the presence and absence of a 100-fold excess of unlabelled LDL. After 2 h at 37 °C the specific cell-associated ¹²⁵I-LDL was determined as described in the Experimental section. The values represent means ± s.d. of measurements on triplicate dishes and are representative of two separate experiments. Significant differences from MEM control: **P* < 0.02, ***P* < 0.001, ****P* < 0.006. (b) For each incubation condition the data in (a) were normalized to the amount of cell-associated ¹²⁵I-LDL measured in cells incubated with MEM. Significant differences from the zero β-VLDL control: **P* < 0.003; ***P* < 0.03. Where not shown, the error bars are contained within the symbols.

Fig. 5. Effect of cholesterol loading of Hep-G2 cells on serum stimulation of HMG-CoA reductase activity

Hep-G2 cells were cultured and cholesterol-loaded with β-VLDL as described in the legend to Fig. 4. (a) After incubation with MEM (○) or MEM + 15 % serum (●) for 5 h at 37 °C the cells were placed on ice and harvested for measurement of HMG-CoA reductase activity as described in the Experimental section. Each point represents the mean ± s.e.m. of three separate experiments with triplicate flasks of cells for each incubation condition. There was a significant difference (*P* < 0.002) between MEM and MEM + serum at all time points. (b) For each incubation condition the data in (a) were normalized to the reductase activity measured in cells incubated with MEM. Differences were not significant (*P* = 0.15) from the zero β-VLDL control. Where not shown the error bars are contained within the symbols.

esterol esters (Table 4). In addition, the level of intracellular [³H]cholesteryl esters was not significantly affected by incubation with MEM + *d* > 1.25. Thus cellular cholesteryl ester synthesis and hydrolysis were not affected by incubation of Hep-G2 cells with MEM + *d* > 1.25. Taken together, these results suggest that the serum-factor-mediated stimulation of LDL receptors and HMG-CoA reductase activities in Hep-G2 cells occurred without any demonstrable alteration in cellular sterol metabolism.

Effect of incubation with MEM + serum on LDL receptor and HMG-CoA reductase activities in cholesterol-loaded cells

It has been reported that LDL receptor activity in primary cultures of human [39–41], rat [38] and pig [37] hepatocytes and Hep-G2 cells [33–36] is relatively insensitive to changes in cell cholesterol content as compared with human skin fibroblasts. To determine if the serum-mediated induction of ¹²⁵I-LDL uptake in Hep-G2 cells is sensitive to changes in the cellular content of

cholesterol, Hep-G2 cells were cholesterol-loaded by incubation with β-VLDL isolated from hypercholesterolaemic rats. Incubation of Hep-G2 cells for 22 h at 37 °C with increasing amounts of rat β-VLDL produced a dose-dependent increase in the content of cholesteryl ester. A maximal increase of 3-fold was achieved at an extracellular β-VLDL concentration of 0.4 mg of cholesterol/ml. No significant changes were observed in the cellular content of unesterified cholesterol. Incubation of Hep-G2 cells with increasing amounts of β-VLDL produced a dose-dependent suppression of ¹²⁵I-LDL uptake in cells incubated with either MEM or MEM + serum (Fig. 4a). However, compared with incubation with MEM alone, incubation with MEM + serum stimulated ¹²⁵I-LDL uptake in the cholesterol-loaded cells. Since the basal activity was lower, the relative increase in ¹²⁵I-LDL uptake was actually greater than in the control cells (Fig. 4b), although the absolute increase was modestly lower. Similarly, cholesterol loading decreased the activity of HMG-CoA reductase in Hep-G2 cells incubated with either MEM or MEM + serum (Fig. 5a). However, the serum-mediated increase in HMG-CoA reductase activity was also

Fig. 6. Time course of the serum-mediated induction of LDL receptor mRNA in Hep-G2 cells

(a) Hep-G2 cells were subcultured in T-75 flasks as described in the Experimental section. On day 3 the cells were washed and each dish was incubated with MEM + 15% calf serum for the indicated time at 37 °C. Total RNA was isolated and selected for poly(A)⁺ RNA and Northern blotting analysis was carried out as described in the Experimental section using 5 μ g of poly(A)⁺ RNA applied to each lane. RNA in the left-hand lanes was incubated with [³²P]cDNA of LDL receptors and β -actin, and that in the right-hand lanes with [³²P]cDNA of LDL receptors and ASGR-H₁. (b) For each sample in (a) the relative amount of LDL receptor mRNA was quantified by scanning densitometry of the autoradiograms and normalized to that of the β -actin and ASGR receptor mRNAs. ●, LDL receptor relative to ASGR-H₁; ○, LDL receptor relative to β -actin.

relatively unaffected by cholesterol loading (Fig. 5b). These data demonstrate that the serum factor(s) is able to induce LDL receptor and HMG-CoA reductase activities even in Hep-G2 cells maximally loaded with cholesterol, further supporting the hypothesis that the induction of LDL receptor activity was not due to an increased need for cellular sterol.

Effect of incubation of Hep-G2 cells with MEM + serum on the accumulation of LDL receptor mRNA

In preliminary studies, incubation of Hep-G2 cells with either cycloheximide or actinomycin D produced a dose-dependent inhibition of the serum-factor-mediated induction of [¹²⁵I]-LDL uptake (J. L. Ellsworth & A. D. Cooper, unpublished work). These data indicated that the increase in LDL receptor activity was due to the synthesis of new receptor molecules and not to the activation or subcellular redistribution of a preformed pool of receptors. To investigate whether the serum-factor-mediated induction of LDL receptors is associated with the accumulation of LDL receptor mRNA within the cell, the time course of LDL receptor mRNA accumulation was determined. Incubation with MEM + serum produced a time-dependent increase in the level of LDL receptor mRNA that reached a maximum of 3–5-fold

Fig. 7. Actinomycin D blocks the serum-mediated induction of LDL receptor mRNA

Hep-G2 cells were subcultured in T-75 flasks as described in the Experimental section. On day 3 the cells were washed and each dish was incubated with the indicated concentration of actinomycin D in 100% ethanol or ethanol alone (final ethanol concentration = 1.0%) in a final volume of 1.8 ml of MEM. After 20 min at 37 °C each dish received a 0.2 ml sample of either MEM or calf serum and the cells were incubated for 5 h at 37 °C. Total cellular RNA was isolated and the relative levels of LDL receptor, β -actin and ASGR mRNAs were quantified by slot-blot hybridization using the various [³²P]-labelled cDNA fragments as described in the Experimental section. ●, LDL receptor/ β -actin; ○, LDL receptor/ASGR; ▲, ASGR/ β -actin.

after 2 h and decreased to near baseline levels by 4 h (Figs. 6a and 6b). In contrast, the levels of both β -actin and ASGR H₁ mRNAs remained relatively constant over this time period. As determined by scanning densitometry, the relative increase in LDL receptor mRNA levels at 2 h was nearly identical whether normalized to the level of β -actin or ASGR H₁ mRNA. Furthermore, the serum-factor-mediated increase in LDL receptor mRNA was blocked by incubation of the cells with actinomycin D (Fig. 7). In contrast, relative to the level of β -actin mRNA, actinomycin D had little effect on the level of ASGR mRNA (Fig. 7, ▲). Taken together, these data indicate that the serum-mediated induction of LDL receptor activity is due to an increase in the accumulation of LDL receptor mRNA.

DISCUSSION

The current study demonstrates that LDL receptor expression in Hep-G2 cells can be increased by short-term incubation with a factor(s) present in serum and provides strong support for the hypothesis that the serum-factor-mediated induction of LDL receptors is not simply a response to cholesterol depletion in the cell. This conclusion is supported by the fact that the induction of [¹²⁵I]-LDL uptake occurred when cells were incubated with high concentrations of whole serum, and occurred over a time course in which little change in cholesterol or cholesteryl ester biosynthesis, cholesteryl ester hydrolysis or cholesterol efflux was observed. It also occurred in the absence of measurable changes in cellular cholesterol mass and in cells that had been preloaded with cholesterol. The induction of [¹²⁵I]-LDL uptake was rapid, being easily detected within 4 h of serum addition, was correlated with increased LDL receptor protein in Hep-G2 cell membranes and appeared to be mediated at the level of gene transcription. It was noteworthy that the induction of LDL receptor mRNA was detectable within 1 h, maximal at 2 h and virtually back to control levels before the increase in LDL receptor proteins was apparent.

In the past, most examples of regulation of LDL receptor activity could be ascribed to changes in cellular cholesterol metabolism [1]. For instance, in cultured cells incubated in media containing whole serum, LDL receptor expression represents a balance between at least two opposing factors that regulate the cell cholesterol balance: the lipoprotein (VLDL + LDL)-induced suppression and the HDL-mediated induction of LDL receptor gene transcription. HDL and VLDL and other cholesterol acceptors can induce LDL receptor activity by promoting cholesterol efflux [54,55]. In the present study an increase in the amount of radiolabelled cholesterol present in the cell media with cells incubated in the presence of whole serum was observed. Interestingly, this occurred without a change in the mass of cellular cholesterol, indicating that even after 22 h of incubation the [^3H]mevalonate-labelled pool in Hep-G2 cells is not in equilibrium with total cellular pool of cholesterol. The efflux of cholesterol was due to the presence of HDL, since it was eliminated by removal of the $d = 1.063\text{--}1.25$ lipoproteins. However, an HDL-induced decrease in cellular cholesterol does not appear to be responsible for the serum-factor induced LDL receptor activity, since induction of ^{125}I -LDL uptake was not diminished by the use of the $d > 1.25$ fraction of serum that did not promote efflux. These data are consistent with our previous report [32] where we observed that incubation of Hep-G2 cells with the HDL fraction ($d = 1.063\text{--}1.210$) of human serum did not increase the total processing of ^{125}I -LDL. Finally, since Hep-G2 cells synthesize and secrete lipoproteins [56], a portion of the radiolabelled cholesterol entering the medium is probably due to lipoprotein secretion. Thus the conclusion that the stimulatory effect of the serum factor on LDL receptor activity is not due to enhanced cholesterol efflux appears to be sound.

When a cell is induced to grow, particularly in preparation for cell division, new membranes must be synthesized, and this requires additional cholesterol. This could result in the movement of cholesterol from some regulatory internal pool to the membrane and thus stimulate LDL receptor activity. Such a process cannot be completely excluded in the present study, since it has not been possible to identify or isolate this putative regulatory pool of sterol. However, several of our findings argue against this sequence of events. First, there was little evidence of cell growth during the time course of these studies, as demonstrated by the lack of change in cell number, protein content, DNA synthesis [32], ASGR activity or level of mRNA for β -actin. As reported previously [32], however, with longer-term incubations (24–48 h) some of these parameters did ultimately change, consistent with the concept that the serum factor is inducing cell growth. Secondly, in the present study no changes were observed in cellular cholesteryl ester metabolism. In general the cellular cholesterol ester pool changes rapidly in concert with other changes in cholesterol metabolism [57]. Finally, when the cells were preloaded with a large amount of cholesterol the induction of LDL receptor activity by the serum factor still occurred, although the magnitude of ^{125}I -LDL uptake was somewhat diminished. Thus the most simple hypothesis is that, in response to a stimulus that initiates the process of cell growth, there is activation of a group of genes that provide the necessary materials for cell growth and replication, and this begins before there is an actual depletion or shortage of the material, presumably in this case cholesterol.

This hypothesis is also consistent with our observations that HMG-CoA reductase activity was stimulated by incubation with the serum factor, even though cholesterol synthesis was not increased over the time period of these experiments. Further, the induction of HMG-CoA reductase activity also occurred in cholesterol-loaded cells. Most probably, the additional mevalonate being generated may be used for the biosynthesis of

non-sterol isoprenoids that are now recognized to be critical for cell replication, particularly isoprenylated proteins (such as p21^{ras} proteins) and others (such as the nuclear membrane protein lamin B) [8]. However, a serum-factor-induced increase in isoprenylation must be confined to a subset of cellular proteins, since incubation of Hep-G2 cells with the serum factor had no significant effect on [^{14}C]acetate incorporation into the bulk of cell protein. Alternatively, if the concentration of HMG-CoA is limiting under our experimental conditions, as has been observed in rat hepatocytes stimulated with insulin and glucagon [58], HMG-CoA reductase activation may be necessary to provide additional mevalonate in order to maintain a constant level of cellular cholesterol biosynthesis in the serum-factor-stimulated cells. However, this explanation is relatively unlikely, since no significant changes were observed in [^{14}C]acetate incorporation into tri- and di-acylglycerol and non-esterified fatty acid in cells incubated with serum-containing medium.

A number of other examples of regulation of LDL receptor activity that may not be preceded by a change in sterol content using cultured human granulosa cells, have been published [16,59]. In addition, Cuthbert and co-workers [60,61] reported that the early induction (≤ 2 h) of LDL receptor gene transcription in lymphocytes by incubation with lipoprotein-deficient serum was independent of protein synthesis and was not regulated by exogenous sterols. In contrast, continued LDL receptor gene transcription detected after longer (24 h) incubations in the absence of sterols required ongoing protein synthesis and gene transcription, and was down-regulated by exogenous sterols. Taken together, these findings and those of the present study indicate that factors other than cellular sterols may also regulate LDL receptor gene expression.

Factors such as platelet-, endothelial cell- and fibroblast-derived growth factors produce a time-dependent stimulation of DNA synthesis, cholesterol synthesis and LDL receptor activity in cultured human fibroblasts and vascular smooth muscle cells [21–24]. In some instances the induction of LDL receptors appears to be independent of changes in cholesterol metabolism; for example, PDGF can stimulate LDL receptor activity in the presence of high extracellular concentrations of LDL [23,24] and in the absence of measurable changes in cellular cholesterol biosynthesis [23]. However, recent evidence [62] from studies of LDL receptor activity in PDGF-stimulated fibroblasts transfected with plasmids bearing a portion of the LDL receptor gene 5'-flanking sequence from -76 to -35 suggests that growth factor induction of LDL receptor protein abundance and gene transcription is mediated by this region, which also modulates the response to a change in cell sterol balance. In preliminary studies (J. L. Ellsworth & A. D. Cooper, unpublished work) we have excluded PDGF, basic fibroblast growth factor, insulin, insulin-like growth factors-I and -II, transferrin, thrombin, 17β -oestradiol and tri-iodothyronine as the modulator of the serum factor effect we are describing.

In the liver, hormones such as insulin, thyroxine and oestrogen increase LDL receptor activity [19,20,26]. The induction of hepatic LDL receptors by oestrogen occurred under conditions in which the liver content of cholesteryl ester was increased 2-fold and the non-esterified and esterified cholesterol content of liver plasma membranes was increased by 130% and 400% respectively [63,64]. Thus increased LDL receptor expression occurred in the face of increases in plasma membrane and total cellular cholesterol. These observations support the data presented in the present paper, which suggest there are signals that can stimulate LDL receptor gene transcription without being preceded by an alteration in cell cholesterol content. It will be of interest to learn how this response is transduced. It is possible that there is alteration of a subpool of cholesterol or

another sterol which then mediates transcription by the mechanism previously described for sterols [8]. Alternatively, there may be another mediator(s) which affects the LDL receptor and HMG-CoA reductase genes directly. This could occur at the same *cis*-acting regulatory sites as the sterol-mediated effect, such as SRE-1 [8], or it could occur at novel sites. In this regard, it is important to note that a number of other genes, such as *c-fos* [65], respond to serum and/or growth factors by an induction of gene transcription. Elements within these genes that respond to serum and the *trans*-acting proteins that bind to these regions have been identified and isolated [66]. Whether the LDL receptor gene contains similar serum-responsive elements is unknown.

The tissue specificity of the serum-mediated effect on Hep-G2 cells is also of interest. In cultured fibroblasts, the addition of whole serum, and certainly the addition of serum plus the $d < 1.063$ fraction of serum, decreases rather than increases the number of LDL receptors. Thus the effect on LDL receptors in Hep-G2 cells is somewhat unique to this liver-derived tissue. Others have noted sluggish down-regulation of LDL receptors by exogenous LDL in liver-derived cells. Whereas incubation of fibroblasts with as little as 20–40 μg of LDL protein/ml for 24 h results in a 75–85 % decrease in LDL binding [33,42], incubation of pig [37], rat [38] or human [39–41] hepatocytes or Hep-G2 cells [33–36] with 100–200 μg of LDL protein/ml for 24–48 h decreased LDL receptor activity by only 24–65 %. These findings are not limited to studies of LDL receptor activity in cultured cells, since the hepatic catabolism of LDL *in vivo* in rats [67] and hamsters [68], whose serum level of LDL was elevated 6–10-fold by a constant infusion of LDL, was only partially down-regulated. Interestingly, the clearance of LDL by the adrenal gland was decreased to a greater extent than transport by the liver at comparable serum LDL concentrations [68]. The residual hepatic clearance of LDL observed in these studies was not due solely to LDL-receptor-independent transport since, at the maximum serum LDL concentration employed, the hepatic LDL-receptor-mediated clearance of LDL was 6–8 times that of methylated-LDL [68]. Similar observations have been made by Nenseter *et al.* [69] who reported that, although the uptake of LDL by hepatic parenchymal cells of rabbits was decreased by 70 % by feeding a cholesterol-rich diet, the clearance of LDL by hepatocytes was still 2–3 times that of methylated-LDL. A relative insensitivity of hepatic LDL receptor activity to dietary cholesterol was also observed by Reagan *et al.* [70], who found that, despite a greater than 90 % decrease in hepatic sterol synthesis, there was no significant decrease in LDL-receptor-mediated clearance of ^{125}I -tyramine cellobiose-LDL by the liver in pigeons fed a cholesterol-rich diet. Taken together, these studies indicate that receptor-mediated transport of LDL by the liver is relatively insensitive to the serum concentration of LDL cholesterol. Since the liver is exposed to physiological concentrations of LDL *in vivo*, LDL transport in the face of saturating concentrations of LDL may be indicative of sterol-independent regulation of hepatic LDL receptor activity. Defining the mechanism for such regulation will be of considerable importance in understanding those factors that govern the plasma level of LDL cholesterol.

We thank Jonathan Diekman for excellent technical assistance, Jeanne Gill for her help in preparation of the manuscript, Dr. M. Shia and Dr. H. Lodish for kindly providing the ASGR clone pSA1, and Dr. L. Kedes for the β -actin clone pHF β A-3' UT-HF. We also thank the members of the G-3 for their thoughtful suggestions and helpful criticisms. This work was supported in part by grants DK38318 and DK36659 (A.D.C.) from the National Institutes of Health, a Grant-In-Aid from the American Heart Association, California Affiliate (J.L.E.), a grant (DK38707) from the Stanford Digestive Disease Center, USPHS, National Institutes of

Health (J.L.E.), and a grant from the Biomedical Research Support Program BRSG S07 RR005513, National Institutes of Health.

REFERENCES

- Goldstein, J. L. & Brown, M. S. (1977) *Annu. Rev. Biochem.* **46**, 897–930
- Attie, A. D., Pittman, R. C. & Steinberg, D. (1982) *Hepatology* **2**, 269–281
- Cooper, A. D. (1985) *Gastroenterology* **88**, 192–205
- Kesaniemi, Y. A., Witztum, J. L. & Steinbrecher, U. P. (1983) *J. Clin. Invest.* **71**, 950–959
- Pittman, R. C. & Steinberg, D. (1984) *J. Lipid Res.* **25**, 1577–1585
- Spady, D. K., Turley, S. D. & Dietsch, J. M. (1985) *J. Lipid Res.* **26**, 465–472
- Nicolosi, R. J., Stucchi, A. F., Kowala, M. C., Hennessy, L. K., Hegsted, D. M. & Schaefer, E. J. (1990) *Atherosclerosis* **10**, 119–128
- Goldstein, J. L. & Brown, M. S. (1990) *Nature (London)* **343**, 425–430
- Südhof, T. C., Russell, D. W., Brown, M. S. & Goldstein, J. L. (1987) *Cell* **48**, 1061–1069
- Südhof, T. C., Van Der Westhuyzen, D. R., Goldstein, J. L., Brown, M. S. & Russell, D. W. (1987) *J. Biol. Chem.* **262**, 10773–10779
- Dawson, P. A., Hofmann, S. L., Van Der Westhuyzen, D. R., Südhof, T. C., Brown, M. S. & Goldstein, J. L. (1988) *J. Biol. Chem.* **263**, 3372–3379
- Brown, M. S., Kovanen, P. T. & Goldstein, J. L. (1979) *Recent Prog. Horm. Res.* **35**, 215–257
- Nonomura, K., Obara, T. & Strott, C. A. (1986) *Endocrinology (Baltimore)* **118**, 653–660
- Faust, J. R., Goldstein, J. L. & Brown, M. S. (1977) *J. Biol. Chem.* **252**, 4861–4871
- Golos, T. G., Soto, E. A., Tureck, R. W. & Strauss, J. F., III. (1985) *J. Clin. Endocrinol. Metab.* **61**, 633–638
- Golos, T. G. & Strauss, J. F., III. (1985) *J. Biol. Chem.* **260**, 14399–14402
- Savion, N., Laherty, R., Lui, G.-M. & Gospodarowicz, D. (1981) *J. Biol. Chem.* **256**, 12817–12822
- Chait, A., Bierman, E. L. & Albers, J. J. (1979) *J. Clin. Invest.* **64**, 1309–1319
- Salter, A. M., Fisher, S. C. & Brindley, D. N. (1987) *FEB Lett.* **220**, 159–162
- Salter, A. M., Fisher, S. C. & Brindley, D. N. (1988) *Atherosclerosis* **71**, 77–80
- Davies, P. F. & Kerr, C. (1982) *Biochim. Biophys. Acta* **712**, 26–32
- Chait, A., Ross, R., Albers, J. J. & Bierman, E. L. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4084–4088
- Chait, A., Ross, R. & Bierman, E. L. (1988) *Biochim. Biophys. Acta* **960**, 183–189
- Witte, L. D., Cornicelli, J. A., Miller, R. W. & Goodman, D. S. (1982) *J. Biol. Chem.* **257**, 5392–5401
- Chait, A., Bierman, E. L. & Albers, J. J. (1979) *J. Clin. Endocrinol. Metab.* **48**, 887–889
- Semenkovich, C. F. & Ostlund, R. E., Jr. (1987) *Biochemistry* **26**, 4987–4992
- Ranganathan, S., Harmony, J. A. K. & Jackson, R. L. (1982) *Biochem. Biophys. Res. Commun.* **107**, 217–224
- Stein, O., Leitersdorf, E. & Stein, Y. (1985) *Arteriosclerosis* **5**, 35–44
- Filipovic, I. & Buddecke, E. (1986) *Biochem. Biophys. Res. Commun.* **136**, 845–850
- Filipovic, I. & Buddecke, E. (1986) *Biochim. Biophys. Acta* **876**, 124–132
- Eckardt, H., Filipovic, I., Hasilik, A. & Buddecke, E. (1988) *Biochem. J.* **252**, 889–892
- Ellsworth, J. L., Brown, C. & Cooper, A. D. (1988) *J. Cell. Physiol.* **135**, 213–223
- Havekes, L. M., Schouten, D., de Wit, E. C. M., Cohen, L. H., Griffioen, M., van Hinsbergh, V. W. M. & Princen, H. M. G. (1986) *Biochim. Biophys. Acta* **875**, 236–246
- Fuki, I. V., Preobrazhensky, S. N., Misharin, A. Yu., Bushmakina, N. G., Menshikov, G. B., Repin, V. S. & Karpov, R. S. (1989) *Biochim. Biophys. Acta* **1001**, 235–238
- Wu, G. Y., Wu, C. H., Rifici, V. A. & Stockert, R. J. (1984) *Hepatology* **4**, 1190–1194
- Havekes, L. M., de Wit, E. C. M. & Princen, H. M. G. (1987) *Biochem. J.* **247**, 739–746

37. Pangburn, S. H., Newton, R. S., Chang, C.-M., Weinstein, D. B. & Steinberg, D. (1981) *J. Biol. Chem.* **256**, 3340–3347
38. Salter, A. M., Bugaut, M., Saxton, J., Fisher, S. C. & Brindley, D. N. (1987) *Biochem. J.* **247**, 79–84
39. Kosykh, V. A., Preobrazhensky, S. N., Ivanov, V. O., Tsibulsky, V. P., Repin, V. S. & Smirnov, V. N. (1985) *FEBS Lett.* **183**, 17–20
40. Havekes, L. M., Verboom, H., de Wit, E., Yap, S. H. & Princen, H. M. G. (1986) *Hepatology* **6**, 1356–1360
41. Edge, S. B., Hoeg, J. M., Triche, T., Schneider, P. D. & Brewer, H. B., Jr. (1986) *J. Biol. Chem.* **261**, 3800–3806
42. Brown, M. S. & Goldstein, J. L. (1975) *Cell* **6**, 307–316
43. Weisgraber, K. H., Innerarity, T. L. & Mahley, R. W. (1978) *J. Biol. Chem.* **253**, 9053–9062
44. Ellsworth, J. L., Cooper, A. D. & Kraemer, F. B. (1986) *J. Lipid Res.* **27**, 1062–1072
45. Maltese, W. A. & Aprille, J. R. (1985) *J. Biol. Chem.* **260**, 11524–11529
46. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
47. Ausubel, F. A., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (eds.) (1990) *Current Protocols in Molecular Biology*, Greene Publishing and Wiley-Interscience, New York
48. Yamamoto, T., Davis, C. G., Brown, M. S., Schneider, W. J., Casey, M. L., Goldstein, J. L. & Russell, D. W. (1984) *Cell* **39**, 27–38
49. Erba, H. P., Gunning, P. & Kedes, L. (1986) *Nucleic Acids Res.* **14**, 5275–5294
50. Spiess, M. & Lodish, H. F. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6465–6469
51. Langridge, J., Langridge, P. & Bergquist, P. L. (1980) *Anal. Biochem.* **103**, 264–271
52. Ellsworth, J. L., Kraemer, F. B. & Cooper, A. D. (1987) *J. Biol. Chem.* **262**, 2316–2325
53. Cooper, A. D., Nutik, R. & Chen, J. (1987) *J. Lipid Res.* **28**, 59–68
54. Jackson, R. L., Gotto, A. M., Stein, O. & Stein, Y. (1975) *J. Biol. Chem.* **250**, 7204–7209
55. Oram, J. F., Albers, J. J., Cheung, M. C. & Bierman, E. L. (1981) *J. Biol. Chem.* **256**, 8348–8356
56. Ellsworth, J. L., Erickson, S. K. & Cooper, A. D. (1986) *J. Lipid Res.* **27**, 858–874
57. Suckling, K. & Stange, E. F. (1985) *J. Lipid Res.* **26**, 647–671
58. Gibbons, G. F., Bjornsson, O. G. & Pullinger, C. R. (1984) *J. Biol. Chem.* **259**, 14399–14405
59. Toaff, M. E., Schleyer, H. & Strauss, J. F., III. (1982) *Endocrinology (Baltimore)* **111**, 1785–1790
60. Cuthbert, J. A., Russell, D. W. & Lipsky, P. E. (1989) *J. Biol. Chem.* **264**, 1298–1304
61. Cuthbert, J. A. & Lipsky, P. E. (1990) *J. Lipid Res.* **31**, 2067–2077
62. Mazzone, T., Basheeruddin, K., Ping, L. & Schick, C. (1990) *J. Biol. Chem.* **265**, 5145–5149
63. Keeffe, E. B., Scharschmidt, B. F., Blankenship, N. M. & Ockner, R. K. (1979) *J. Clin. Invest.* **64**, 1590–1598
64. Davis, R. A., Kern, F., Jr., Showalter, R., Sutherland, E., Sinensky, M. & Simon, F. R. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4130–4134
65. Rollins, B. J. & Stiles, C. D. (1989) *Adv. Cancer Res.* **53**, 1–32
66. Norman, C., Runswick, M., Pollock, R. & Treisman, R. (1988) *Cell* **55**, 989–1003
67. Carew, T. E., Pittman, R. C. & Steinberg, D. (1982) *J. Biol. Chem.* **257**, 8001–8008
68. Spady, D. K., Bilheimer, D. W. & Dietschy, J. M. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3499–3503
69. Nenseter, M. S., Blomhoff, R., Drevon, C. A., Kindberg, G. M., Norum, K. R. & Berg, T. (1988) *Biochem. J.* **254**, 443–448
70. Reagan, J. W., Jr., Miller, L. R. & St. Clair, R. W. (1990) *J. Biol. Chem.* **265**, 9381–9391

Received 28 December 1990/13 May 1991; accepted 21 May 1991