The apolipoprotein B gene is constitutively expressed in HepG2 cells: regulation of secretion by oleic acid, albumin, and insulin, and measurement of the mRNA half-life

Clive R. Pullinger,[1] Julia D. North, Ba-Bie Teng, Vincent A. Rifici,[2] A. E. Ronhild de Brito, and James Scott[3]

Division of Molecular Medicine, MRC Clinical Research Centre, Watford Road, Harrow HA1 3UJ, United Kingdom

Abstract The objective of this study was to establish conditions whereby apoB secreted from HepG2 cells could be regulated over a wide range, and to determine whether changes of output were correlated with the level of apoB mRNA. The presence of oleate (complexed to 3% albumin at a molar ratio of 1.7:1) resulted in a 3.5-fold stimulation of apoB secretion that was apparent after only 3 h. Insulin halved the rate of apoB output and the inhibition was detectable within the physiological insulin range, but was not apparent until 12–16 h. Albumin in the culture medium had a dose-dependent inhibitory effect on apoB production. Overall, apoB secretion from HepG2 cells was modulated over a 7-fold range. However, when apoB mRNA was assayed by slot-blot hybridization, no change was detectable under any of the conditions that modulated apoB output. Quantitative solution hybridization was used to confirm that oleate did not affect the level of apoB mRNA. Kinetic analysis of the decay of [^{3}H]uridine-labeled apoB mRNA showed that the half-life of apoB mRNA was 16 h. We conclude from these studies that the apoB gene is constitutively expressed in HepG2 cells and that the mechanism of acute regulation of apoB production by these cells must involve co- or post-translational processes. — **Pullinger, C. R., J. D. North, B-B. Teng, V. A. Rifici, A. E. Ronhild de Brito, and J. Scott.** The apolipoprotein B gene is constitutively expressed in HepG2 cells: regulation of secretion by oleic acid, albumin, and insulin, and measurement of the mRNA half-life. *J. Lipid Res.* 1989. **30:** 1065–1077.

Supplementary key words VLDL • LDL • hormones • glucagon • triglycerides • cholesterol • hepatoma • electron microscopy • transcription

The liver secretes triglyceride-rich, very low density lipoproteins (VLDL) that contain apolipoprotein (apo) B, apoE, and the C apolipoproteins. ApoB comprises over 40% of the total protein content of VLDL (1). The rate of VLDL secretion is important in determining the plasma low density lipoprotein (LDL) concentration, and LDL levels correlate with the risk of coronary heart disease. Increased VLDL secretion is responsible in part for the hyperlipidemia that complicates excessive consumption of fat and cholesterol, diabetes mellitus, hypoalbuminemic renal disease, and glucocorticoid excess (2). The factors that control the rate of secretion of apoB-containing lipoproteins from the liver are, therefore, of considerable importance.

In rats VLDL triglyceride secretion is enhanced by sucrose feeding (3,4), by supplying nonesterified fatty acid (NEFA) to perfused livers from fed (4–6) or fasted rats (6), and by oleate in cultured rat hepatocytes (7–10). VLDL cholesterol secretion was stimulated in perfused rat livers (11) and was unaffected in rat hepatocytes (7, 8, 12) when oleate was added to the medium. ApoB secretion is increased by oleate perfusion (6). However, in studies with cultured rat hepatocytes, VLDL apoB secretion is apparently unaffected by the addition of oleate to the medium (8–10). In these experiments the availability of apoB was considered to be rate limiting for VLDL secretion, when NEFA was freely provided. The human hepatoblastoma-derived cell line, HepG2, secretes triglyceride-rich lipoprotein with the density of LDL, the apolipoprotein content of which is almost exclusively apoB (13– 15). With this cell line apoB secretion has been reported to increase 2- fold (13), 4.5-fold (16), only moderately (17), or to be unaffected (15) by oleate. A 2.8-fold stimulation of apoB

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; NEFA, nonesterified fatty acid; ELISA, enzyme-linked immunosorbent assay.

[1]Present address: Cardiovascular Research Institute, School of Medicine, University of California, San Francisco, San Francisco, CA 94143.

[2]Present address: Department of Medicine, Albert Einstein College of Medicine, Bronx, New York 10461.

[3]Corresponding author.

output from HepG2 cells was seen with linoleate (18). Similarly, triglyceride output has been reported to be stimulated by 2- to 2.5-fold (17, 19), and to be unaffected (15) by oleate.

The role of insulin in regulating apoB secretion is important in view of the association between hyperlipoproteinemia and diabetes, since atherosclerosis is a common complication of this disease (20,21). In vivo human studies have shown that insulin increases hepatic VLDL output (22- 25); other studies in humans (26, 27) and the rat (28) suggest that insulin decreases VLDL secretion. Reports on the effects of insulin in vitro are also conflicting. Insulin increased VLDL triglyceride secretion from perfused rat livers (5, 29, 30) and rat hepatocytes (31) in some studies. In other studies using rat liver cells, insulin inhibited both triglyceride (32–36) and apoB (33, 35, 37) secretion. The output of apoB (17) and triglyceride (19) by HepG2 cells has also been shown to be inhibited by insulin.

A variety of other hormones affect hepatic VLDL secretion. Glucagon, which strongly inhibits hepatic lipogenesis, also inhibits hepatic VLDL triglyceride secretion (11, 31, 34, 38). Dexamethasone has been reported to increase the secretion of VLDL triglyceride by rat hepatocytes (36, 39).

Decreased concentration of plasma albumin in renal disease is considered to cause increased VLDL secretion and LDL hypercholesterolemia (40, 41). The apoB synthetic rate is increased in patients with nephrotic syndrome (42). The presence of albumin or other macromolecules in the medium of cultured rat hepatocytes (43) and HepG2 cells (13) has been reported to inhibit apoB output.

Little is known about the underlying mechanisms that bring about changes in hepatic apoB output, and there are very few reports concerning the regulation of apolipoprotein gene expression. The present study was therefore undertaken to establish conditions in HepG2 cells whereby large variations in apoB secretion could be examined for their effect on the level of apoB mRNA; i.e., whether changes in the transcription of the apoB gene or changes in mRNA stability are responsible for controlling apoB secretion by these cells.

METHODS

Materials

Fatty acid-free, bovine serum albumin (BSA), oleic acid, and porcine insulin were purchased from Sigma (Poole, UK). [α-^{32}P]dCTP was supplied by NEN (Stevenage, UK). [5,6-^{3}H]Uridine and [5,6-^{3}H]UTP were supplied by Amersham International (Amersham, UK). Eagle's minimal essential medium (MEM) and nonessential amino acids were from Flow Labs (Rickmansworth, UK). Fetal calf serum (FCS) was from Biological Industries (Israel). Tissue culture flasks (T75) were supplied by Falcon (Cowley, UK).

Cell culture

Human hepatoblastoma cells, HepG2, were supplied by Dr. B. B. Knowles (Wistar Institute of Anatomy and Biology, Philadelphia, PA). They were cultured in MEM plus penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), nonessential amino acids (1% of 100 ×) and 10% heat-inactivated FCS at 37°C in an atmosphere of 95% air–5% CO_2.

The cells were passaged weekly, when 1.5 × 10^6 cells were seeded into 75-cm^2 flasks. For cells used in experiments, the medium was removed after 4 days and replaced with FCS-free medium containing 3% (w/v) of BSA. One day later the medium was replaced with FCS-free medium plus, where appropriate, 3% (w/v) BSA, and various additions. Preparation of oleate/albumin complex was prepared according to the method of Van Harken, Dixon, and Heinberg (44).

Electron microscopy

To examine the effect of oleic acid on HepG2 cell structure, cells were incubated for 24 h in serum-free MEM containing 3% (w/v) albumin in the presence or absence of 0.8 mM oleic acid. Cells were washed with PBS and fixed overnight at 0°C in 3% (w/v) glutaraldehyde, 0.1 mM sodium cacodylate (pH 7.4), and 5% (w/v) sucrose. After three washes of 30 min in 0.1 M sodium cacodylate, the cells were post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, dehydrated through a graded series of acetones and embedded in Spurr resin with epoxypropane as the transitional solvent. Ultrathin sections were cut on a Reichert OMU3 ultratome with a diamond knife. Sections were counter-stained with uranyl acetate and lead citrate and examined in a Phillips EM300 electron microscope operating at 60 kV.

Preparation of lipoprotein fractions from cell conditioned culture medium

After a 24 h incubation (except for time course studies) the medium was removed from the cells and centrifuged for 5 min at 1000 *g* to remove cell debris. Sodium azide (0.02% w/v), EDTA (0.02% w/v), and gentamicin sulfate (0.02% w/v) were added and the density was adjusted to either 1.063 or 1.21 g/ml with solid KBr. After centrifugation for 20 h at 150,000 *g* (Beckman 70.1 Ti rotor, 45,000 rpm) the tubes were sliced and the top 2.0 ml was collected and dialyzed.

Enzyme-linked immunosorbent assay of apoB (ELISA)

The lipoprotein fractions prepared from the conditioned medium were assayed for apoB using an ELISA method similar to that of Thrift et al. (14). Human LDL prepared by sequential ultracentrifugation (45) was adsorbed (0.5 μg apoB in 100 μl 500 mM Na_2CO_3, pH 9.6, 16 h, 4°C) to the wells of 96-well microtiter plates (Dynatech Laboratory Ltd., Billingshurst, West Sussex, UK). The plates were washed with phosphate-buffered saline (PBS) (8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4 per liter) and blocked for 1 h with 2% BSA in PBS containing 0.05% Tween-20. The plates were washed twice with PBS. Samples and standards (LDL apoB) in the range 0–0.2 μg/100 μl were mixed with a monoclonal antibody (1D1) (46) to apoB (final dilution 1 in 20,000 in PBS/Tween-20) and added to the wells in quadruplicate. The monoclonal antibody was kindly supplied by Dr. Y. L. Marcel and Dr. R. W. Milne. The plates were incubated at 37°C for 2 h. After washing three times with PBS/Tween-20, anti-mouse IgG alkaline phosphatase conjugate (Sigma, Poole, Dorset, UK) was added to each well (1 in 2,000 dilution in 100 μl PBS). The plates were incubated at 37°C for 1 h. Controls with no LDL coating plus first and second antibody were included. After washing three times with PBS, the substrate (*p*-nitrophenyl phosphate, 0.1 mg in 100 μl) was added and the plates were incubated at 37°C for 45 min. The absorbances were read at 405 nm using an automated ELISA plate reader. The displacement curves of standard human LDL were similar to that of the lipoprotein fraction prepared from HepG2 cell conditioned medium (data not shown).

Preparation of total RNA from HepG2 cells

Total RNA was prepared from HepG2 cells using, essentially, the method of Auffray and Rougeon (47). The cells were harvested by washing with a solution of trypsin (2.5 mg/ml) and EDTA (1 mg/ml). After 5 min at 37°C, medium containing 10% heat-inactivated FCS was added to inactivate the trypsin. An aliquot of the cell suspension was taken to estimate protein and DNA content. The cells were pelleted at 500 *g* for 5 min. To the cell pellet was added 10 ml of a solution of urea (6 M), LiCl (3 M), and SDS (0.2%, w/v). The tube was vortexed and the lysed cells were homogenized using an "ultra turax" tissue homogenizer (Janke and Kunkel, FRG) for 2 min in order to shear the cellular DNA. The cell homogenate was passed three times through a 19-gauge needle and then three times through a 21-gauge needle to ensure complete shearing of DNA. At this stage the sample was left on ice overnight. The suspension was centrifuged at 9,000 *g* (av) for 20 min. The supernatant was discarded and the pellet was washed in 5 ml of ice-cold LiCl (3 M). The suspension was again centrifuged at 9,000 *g* (av) for 20 min. The supernatant was discarded and the pellet was dissolved in 3 ml of ice-cold TES (10 mM Tris-HCl; 1 mM EDTA; 0.5% SDS). The solution was extracted with 1.5 ml phenol containing 8-hydroxyquinoline (0.1% w/v) and β-mercaptoethanol (0.2% w/v), and 1.5 ml chloroform–isoamyl alcohol 24:1 (v/v)and finally with chloroform (3 ml). The RNA was precipitated at −70°C after the addition of 0.35 ml sodium acetate (3 M) and 9 ml ethanol. The RNA was pelleted at 9,000 *g* (av) for 20 min. After washing with 80% ethanol (v/v) the RNA was re-pelleted. All traces of 80% ethanol were carefully removed, the pellet was dissolved in TE (10 mM Tris-HCl; 1 mM EDTA) and stored at −70°C. RNA content was estimated by measuring the absorbance at 260 mm and by using an orcinol assay (48).

Northern and slot blotting

For slot-blots, 1-, 4-, and 8- μg amounts of total cellular RNA were denatured and applied to the wells of a "minifold" blotting apparatus (Schleicher and Schuell, FRG) onto nitrocellulose filters. For Northern blots, 10 μg of RNA was run on 0.8% agarose denaturing gels containing formaldehyde (2.2 M) and then transferred to nitrocellulose filters by capillary blotting. Filters were baked at 80°C for 2 h. The filters were prehybridized for 4 h and hybridized overnight at 42°C in 50 % deionized formamide, 5 × SSC (5 × SSC is sodium chloride, 750 mM, and sodium citrate, 75 mM), 100 mM sodium phosphate (pH 7.0), 2 × Denhardt's solution (2 × Denhardt's solution contains 0.04% Ficoll, 0.04% polyvinyl pyrolidone, and 0.04% BSA), 300 μg/ml denatured salmon sperm DNA and 0.2% SDS with a ^{32}P-oligolabeled 6.2 kb apoB cDNA probe (pABF) (49), or with a human β-actin cDNA probe (p69A-23) (50). The specific activity of the probe was 10^9 cpm/μg. Filters were washed twice in 2 × SSC/0.1% SDS at room temperature, twice in 1 × SSC/0.1% SDS at 42°C, once in 1 × SSC/0.1% SDS at 55°C, and once in 0.1 × SSC/0.1% SDS at 55°C. Each wash was for 15 min. The relative quantities of each mRNA in the slot blots were determined by cutting the filters and determining the amount of ^{32}P by liquid scintillation spectrometry.

Measurement of apoB mRNA by DNA-excess solution hybridization

A single stranded ^{32}P-labeled cDNA probe (8 × 10^6 cpm/pmol) was synthesized from a 215-bp SstI/HindIII (nucleotides 9854–10069) (51) apoB cDNA fragment subcloned into the bacteriophage M13 using a 17-nucleotide primer complementary to the M13 polylinker in the presence of 3 μM [α-^{32}P]CTP, 24 μM each of dTTP, dATP, and dGTP, and the Klenow fragment of *E.coli* DNA polymerase. After EcoRI digestion the probe was isolated by

electrophoresis on a 6% polyacrylamide, 8 M urea gel followed by electroelution.

The probe was hybridized with various quantities of total RNA in 50% formamide, 400 mM NaCl, 10 mM PIPES, 1 mM EDTA, for 40 h at 42°C. Following digestion with S1 nuclease to remove excess unhybridized probe, the samples were precipitated with trichloroacetic acid on glass-fiber discs and the radioactivity was measured by liquid scintillation spectrometry. From a knowledge of the length of apoB mRNA (14, 121 bases) (51) and the specific radioactivity of the probe, the mass of apoB mRNA per μg of total RNA was calculated.

Incorporation of [5,6-^{3}H]uridine into HepG2 cell RNA

HepG2 cells were seeded into 75-cm^2 flasks (as described above). Four days later 1 mCi of [5,6-^{3}H]uridine was added in 10 ml of serum-free medium containing 3% albumin. The cells were cultured for 20 h and the medium was removed; the cells were washed with PBS and fresh medium containing 5 mM uridine was added to prevent reutilization of label. The incubations were then continued for the time periods indicated for Fig. 9 when the cells were harvested and RNA was prepared as described above.

Preparation of [^{3}H]apoB cRNA

Tritium-labeled sense transcripts of apoB cRNA were prepared using a TransProbe kit supplied by Pharmacia (Milton Keynes, UK). The pGem 3 plasmid used contained a 2384-bp SalI to XbaI insert (nucleotides 5290 to 7674) (51). This was linearized with SmaI and KpnI and transcribed with T7 RNA polymerase at 37°C for 30 min in the presence of 20 μCi of [5,6-^{3}H]UTP. The yield was 200 ng of RNA with a specific activity of 6.6 × 10^7 dpm/μg RNA.

Filter hybridization of [^{3}H]RNA

Aliquots of the ^{3}H-labeled HepG2 cell RNA were analyzed for [^{3}H]apoB mRNA by hybridization to nitrocellulose filters containing linearized, immobilized, denatured DNA according to the method of Rodgers, Johnson, and Rosen (52). Filters contained 2 μg of pABI (49) 2 μg of pABF (49), and 2 μg of pSB9 (an exon 26 genomic subclone containing a 5826-bp SalI to BamHI insert; nucleotides 5290 to 11116) (51). Thus, each filter contained DNA spanning the whole length of the apoB cDNA. Control filters contained 3 μg of pUC8 and 3 μg of pAT153. Filters were prehybridized for 16 h at 42°C using the same hybridization buffer as reported above for Northern and slot blotting. Aliquots (25 μg) of the ^{3}H-labeled HepG2 cell RNA were added and the hybridizations were carried out at 42°C for 50 h. Separate hybridizations were set up containing 25 μg of unlabeled HepG2 cell RNA plus 3.4 × 10^5 dpm of ^{3}H-labeled apoB cRNA. The filters were washed twice with 2 × SSC containing 0.1% SDS at room temperature for 15 min. They were incubated for 30 min at 37°C in 2 × SSC containing 10 μg/ml RNaseA and 1 μg/ml RNaseT1. After two further washes in 2 × SSC/0.1% SDS at room temperature for 15 min, the filters were washed once in 1 × SSC/0.1% SDS at 42°C for 30 min and finally in 1 × SSC/0.1% SDS at 65°C for 15 min. The amount of ^{3}H was determined by liquid scintillation spectrometry.

Extraction of total lipids from HepG2 cells and lipoprotein fraction from conditioned medium

Cell pellets and lipoprotein fractions were extracted by the addition of 20 ml of chloroform–methanol 2:1 (containing 10 μg/l of butylated hydroxytoluene) according to the method of Folch, Lees, and Sloane Stanley (53).

Measurement of triglyceride, cholesterol, and phospholipid

The phospholipid content of the HepG2 cells and the triglyceride content of the cells and lipoprotein fractions were determined on the lipid extracts using kits supplied by BCL (Lewes, East Sussex, UK). The unesterified and esterified cholesterol content of the cells was determined by gas–liquid chromatography after thin-layer chromatography of the total lipid extracts as described previously (54).

Measurement of cell-mediated insulin degradation

Insulin was labeled with ^{125}I as previously described (55) and 200,000 cpm was added to flasks of HepG2 cells with the appropriate concentration of unlabeled insulin. Aliquots of the medium were precipitated with trichloroacetic acid on glass-fiber discs and the amount of radioactivity remaining was determined. The half-life for the removal of insulin from the medium was of the order of 20–24 h (data not shown). To account for this degradation, an additional amount of insulin, equivalent to the initial amount, was added 6 h after the start of each experiment.

Other analytical methods

Protein was measured by the method of Lowry et al. (56) and DNA by the method of Schneider (48).

Statistical treatment of results

All values are presented as means ± SEM. Statistical significance was tested by paired *t*-test on logarithmically transformed data.

RESULTS

Conditioned medium from HepG2 cells was collected over a period of 48 h. The d < 1.063 g/ml fraction was prepared and the triglyceride and apoB contents were de-

termined. There was a linear output of both components over this period (data not shown). In subsequent experiments the d < 1.21 g/ml fraction was prepared, because we found that some apoB (18% of the total) appeared in the d 1.063–1.21 g/ml fraction (C. Pullinger and J. North, unpublished observations).

Substances studied for effect on apoB secretion

A major objective of the study was to identify factors that regulate apoB secretion from HepG2 cells. HepG2 cells were grown for 24 h in serum-free medium containing 3% albumin (w/v) supplemented with each of the test substances. **Table 1** lists the substances found to perturb apoB secretion. Oleate stimulated apoB secretion. Albumin and insulin inhibited secretion. No other substance examined had a significant effect on apoB secretion (**Table 2**). Results with oleate, albumin, and insulin are described in detail.

Effect of albumin and oleate on apoB secretion

The amount of apoB secreted by HepG2 cells over 24 h under different conditions that affected secretion is summarized in Table 1. HepG2 cells grown for 24 h in serum-free medium supplemented with 3% albumin (w/v) showed a 47% decrease in the amount of apoB secreted into the culture medium compared to cells grown in serum-free medium alone. Supplementation of the culture medium with 0.8 mM oleate bound to 3% albumin increased apoB secretion by on average 3.5-fold. In ten individual experiments the range was 1.5- to 7.5-fold. Triglyceride secretion was stimulated 2.6-fold by oleate (**Table 3**).

Effect of insulin on apoB secretion

Insulin at a concentration of 10^{-8}M was used to supplement HepG2 cells grown with 3% albumin alone or with 3% albumin plus 0.8 mM oleate. In the presence of insulin the amount of apoB secreted over 24 h was decreased by 55% when oleate was present in the medium and 52% in the absence of oleate. Overall apoB secretion could be varied by more than 7-fold by stimulation with oleate and inhibition by insulin in the absence of oleate (Table 1).

TABLE 1. Effects of insulin, albumin, and oleate on apoB secretion by HepG2 cells

Additions			
Oleate 0.8 mM	Insulin 10^{-8} M	Albumin 3% (w/v)	ApoB Output
			μg/mg cell protein/24 h
+	–	+	1.02 ± 0.27[a]
–	–	–	0.55 ± 0.19[b]
+	+	+	0.45 ± 0.10[c]
–	–	+	0.29 ± 0.07
–	+	+	0.14 ± 0.04[b]

Cells were preincubated for 24 h in MEM plus BSA (3%). The medium was changed to MEM plus the above additions and the cells were incubated for a further 24 h. The medium was removed and assayed by ELISA for apoB as described in Methods. Results are mean ± SEM of at least nine experiments for each condition.

[a] $P < 0.001$ as compared to albumin control.

[b] $P < 0.01$ as compared to albumin control.

[c] $P < 0.001$ as compared to oleate.

TABLE 2. Other substrates, hormones, and drugs tested for their effect on apoB secretion from HepG2 cells

Substance	Percent of Control[a]
Lactate + pyruvate (10 mM + 1 mM)	138 ± 19 (9)
Mevalonate (1 mM)	138.3 ± 16.8 (7)
Glucagon (10^{-8} M)	88.7 ± 10.4 (7)
8-Br cAMP (0.5 mM)	87.3 (2)
Dexamethasone (10^{-6} M)	100.1 ± 15.4 (7)
Triiodothyronine (10^{-8} M)	113.9 ± 8.1 (5)
25-Hydroxycholesterol (10 μg/ml)	124.0 ± 16.2 (5)
Mevinolin (1 μM)	143.5 ± 15.6 (4)
Sandoz 58-035 (5 μg/ml)	122.2 ± 11.0 (6)
Calcium ionophore (10^{-7} M)	100.3 ± 6.5 (3)
A23187 (10^{-6} M)	66.8 ± 9.6 (3)

The conditions were the same as in Table 1. Albumin (3%, w/v) was present throughout.

[a] The results are expressed as a percentage of the amount secreted by cells in paired flasks incubated in albumin alone ± SEM. The numbers of separate experiments are in parentheses.

Dose-responses for albumin, oleate, and insulin

In a dose-response experiment, albumin 0.5% (w/v) decreased apoB secreted over 24 h from HepG2 cells by 54% (**Fig. 1**). The inhibition was maximal (83% decrease) at 1.5% (w/v) albumin. No further decline was observed with 3 or 5% albumin. Ovalbumin 3% (w/v) and dextran-80 3% (w/v) also suppressed apoB secretion (Fig. 1). Albumin (3% solution) was used in all subsequent experiments to allow a low fatty acid to albumin ratio (molar ratio of 1.7:1 in the presence of 0.8 mM oleate) in those incubations containing oleate. ApoB secretion was examined at oleate concentrations between 0.02 mM and 0.8 mM (**Fig. 2**). Maximal stimulation of apoB secretion was achieved at 0.1 mM. In the presence of 0.8 mM oleate large lipid droplets were found to accumulate in the cytoplasm of HepG2 cells (**Fig. 3**). Extraction of total HepG2 cellular lipid from oleate-treated and untreated cells showed a 7-fold increase in cellular triglyceride (Table 3). There was no change in cellular phospholipid or unesterified cholesterol. The 2-fold increase observed in cellular cholesteryl ester was not statistically significant.

An inhibition of apoB secretion of 32% was observed at 10^{-9}M insulin, which is within the physiological concentration range (**Fig.4**). The greatest inhibition of 59% was seen at an insulin concentration of 10^{-7}M.

TABLE 3. Effects of oleate on HepG2 cell triglyceride secretion and cellular lipids

Additions	Cellular Lipids				Lipoprotein Triglyceride
	Triglyceride	Nonesterified Cholesterol	Esterified Cholesterol	Phospholipid	
	μg/mg cell protein				*μg/mg cell protein/24 h*
3% Albumin	39.6 ± 1.5	14.1 ± 1.0	4.9 ± 1.6	87.0 ± 5.7	1.29 ± 0.41
3% Albumin + 0.8 mM oleate	284.7 ± 1.2[a]	15.7 ± 1.5	10.3 ± 4.0	103.1 ± 8.8	3.38 ± 1.60[b]

Confluent monolayers of HepG2 cells were incubated for 24 h in serum-free medium containing BSA (3% w/v). The medium was then changed to BSA ± 0.8 mM oleate and the cells were incubated for a further 24 h. The cells were harvested, the lipoprotein fraction was prepared, and the cellular lipids and lipoproteins were assayed for lipid as described in Methods. The values are the mean ± SEM. Three flasks were assayed for cellular lipids and five for lipoprotein triglyceride.

[a] $P < 0.001$.

[b] $P < 0.03$.

Time courses for albumin, oleate, and insulin effects

The effects of albumin, oleate, and insulin were examined over a 24-h time period. A decrease in output of apoB in the presence of 3% (w/v) albumin compared to no albumin control was detectable between 15 h and 24 h (**Fig. 5**). In contrast, there was a rapid stimulation of apoB secretion in the presence of 0.8 mM oleate, which was seen after only 3 h. The inhibitory effect of 10^{-8}M insulin was detected between 4 and 16 h (**Fig. 6**).

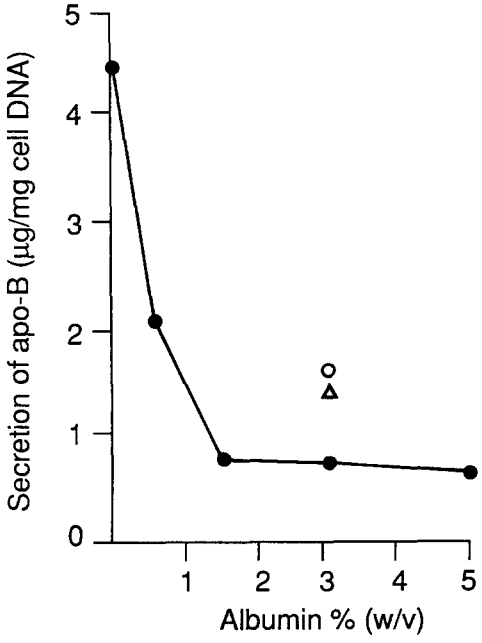

Fig. 1. Effect of albumin, dextran and ovalbumin on the secretion of apoB from HepG2 cells. Cells were incubated for 24 h in serum-free MEM. The medium was then changed to serum-free MEM plus 0–5% (w/v) albumin and 3% (w/v) dextran 80 or 3% (w/v) ovalbumin. After 24 h the medium was removed and the cells were harvested. The d < 1.21 g/ml lipoprotein fraction was prepared by ultracentrifugation and assayed for apoB by ELISA as described in Methods. The cells were assayed for DNA content; (●) albumin; (○) ovalbumin; (△) dextran 80.

Effect of albumin, oleate, and insulin on apoB mRNA

The second major objective of these studies was to examine the effect of those substances that perturb the secretion of apoB on the level of mRNA. The relative levels of apoB mRNA were compared under the five different conditions found to perturb secretion. The integrity of apoB mRNA was demonstrated by Northern hybridization (**Fig. 7**). No differences in apoB mRNA levels were demonstrated with the different treatments found to alter apoB secretion. The results were confirmed by quantitative RNA slot-blot hybridization. Duplicate blots were hybri-

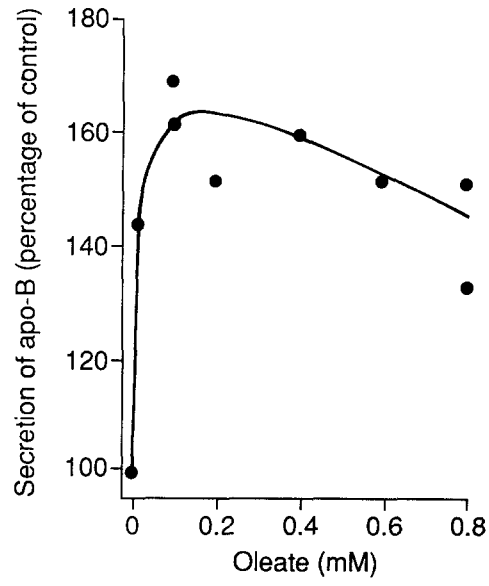

Fig. 2. Effect of oleic acid on the secretion of apoB from HepG2 cells. Cells were incubated for 24 h in serum-free MEM plus 3% albumin. The medium was changed to serum-free MEM containing 3% albumin plus 0.02–0.8 mM oleate. After 24 h the medium was removed and assayed for apoB as described in Fig. 1.

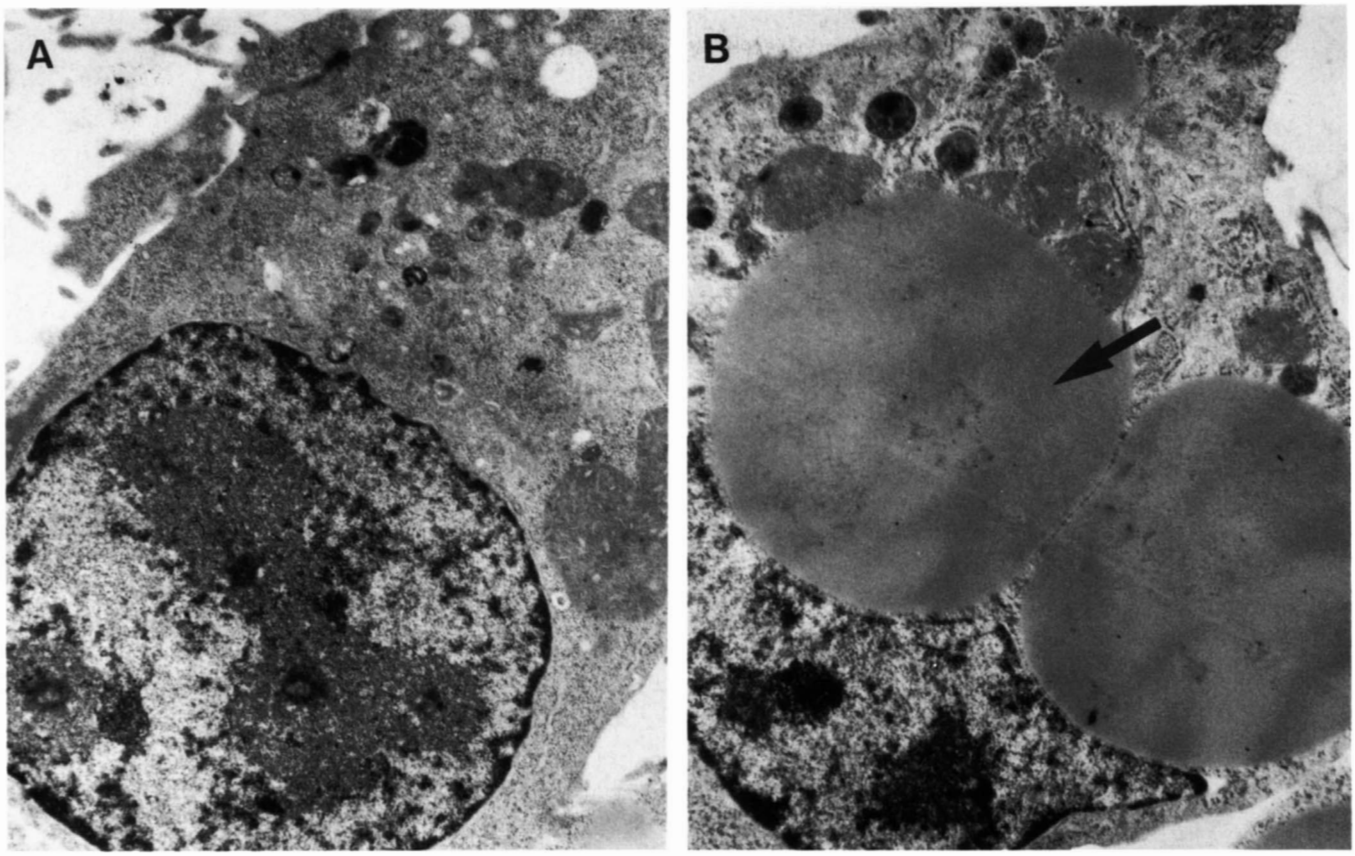

Fig. 3. Electron micrography of HepG2 cells. (A) cells incubated in serum-free MEM plus 3% (w/v) albumin. (B) cells incubated for 24 h in 0.8 mM oleate complexed to 3% (w/v) albumin and showing the presence of large lipid droplets (arrowed). Magnification × 10,000.

dized to ^{32}P-labeled apoB cDNA and to actin cDNA. **Fig. 8** shows typical blots. Under all conditions the relative apoB mRNA levels were close to control values, with the exception that there was a small (16%) decrease in apoB mRNA in the presence of insulin (10^{-8} M) and oleate (0.8 mM) compared to oleate alone (**Table 4**). Although this small change was statistically significant ($P < 0.05$), it cannot account for the 2.3-fold difference in apoB secretion between these two conditions. When the apoB mRNA levels were normalized to the level of actin mRNA, the results were unchanged except for a slightly higher SEM.

That the effect of oleate on apoB output was not accompanied by changes in apoB mRNA was confirmed using DNA excess solution hybridization to measure the apoB mRNA content of the cells. The concentration of apoB mRNA determined on six separate preparations of RNA was 90.1 ± 15.0 (SEM) ng per mg total RNA for cells incubated with 3% albumin alone and 68.1 ± 11.0 from cells incubated with 3% albumin plus oleate (0.8 mM). This difference was not statistically significant and is in the wrong direction to account even partially for the 3.5-fold difference in apoB secretion between these two conditions.

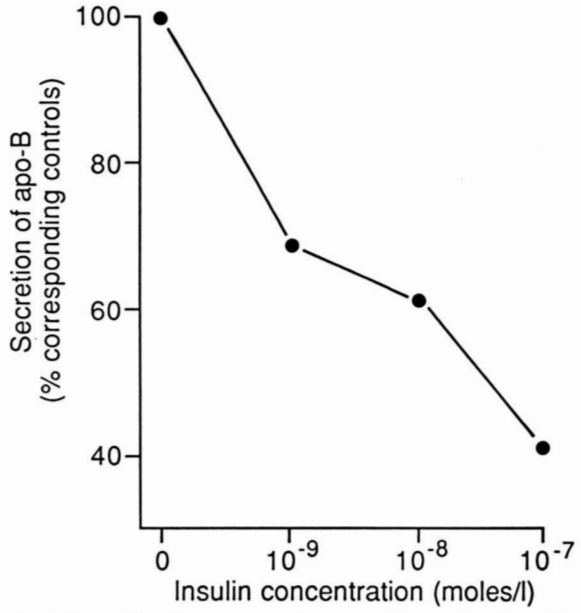

Fig. 4. Effect of insulin concentration on apoB secretion from HepG2 cells. Cells were incubated for 24 h in serum-free MEM plus 3% (w/v) albumin. The medium was changed to serum-free MEM containing 3% albumin plus 0-10^{-7}M insulin. After 24 h the apoB content of the medium was determined as described in Fig. 1.

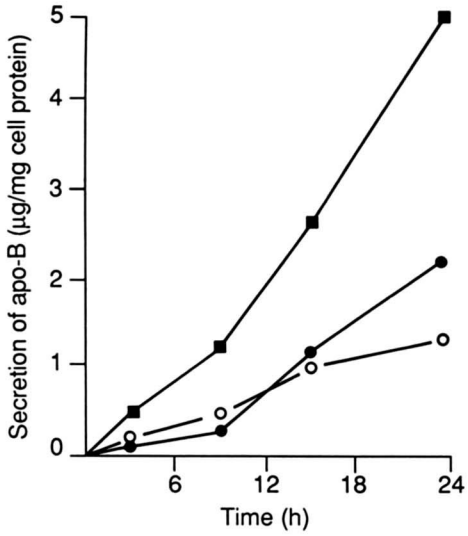

Fig. 5. Time course of the effects of oleate and albumin an apoB secretion from HepG2 cells. Experimental conditions were as described in Fig. 2. Cells and medium were harvested at various times over 24 h to determine the apoB content of the conditioned medium. Cells were incubated in serum-free MEM alone (●); plus 3% albumin (○); plus 0.6 mM oleate complexed to 3% albumin (■).

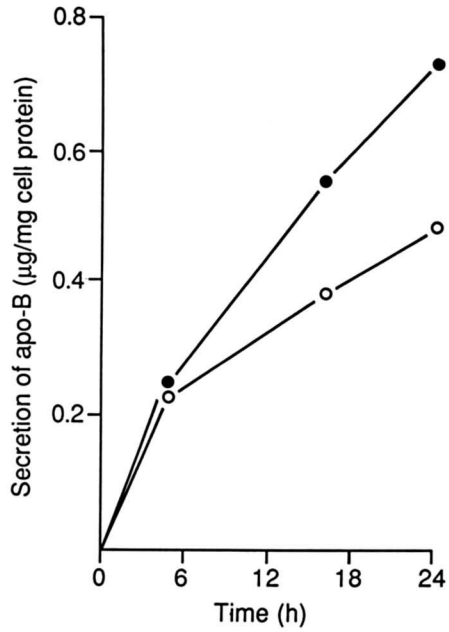

Fig. 6. Time course of the effect of insulin on apoB secretion from HepG2 cells. Conditions were as described in Fig. 4. Cells and medium were harvested at various times over 24 h to determine the apoB content of the conditioned medium. Cells incubated in serum-free MEM plus 3% albumin alone (●); plus 10^{-8} M insulin (○). The values are the mean of duplicate assays of two separate flasks.

ApoB mRNA half-life

In order to measure the apoB mRNA half-life in HepG2 cells, the cellular RNA was labeled with [^{3}H]uridine and the stability of the [^{3}H]apoB mRNA was determined. **Fig. 9** shows the decay curve over the 30-h chase period. If a first order decay process is assumed, the half-life of apoB mRNA is 11.1 h as determined by linear regression analysis. The doubling time of HepG2 cells in these studies was found to be 39 h. When adjusted for cell doubling as described by Rodgers et al. (52), the half-life

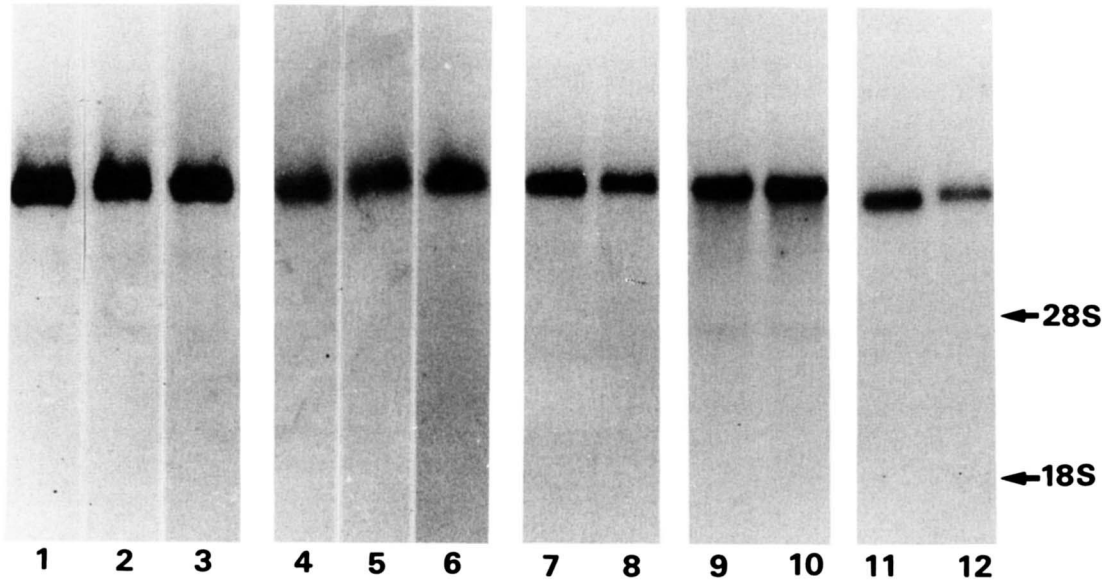

Fig. 7. Northern hybridization analysis of total HepG2 isolated from control (3% albumin) cells (lanes 1,4,7,9 and 11); from cells incubated with 0.8 mM oleate (lanes 2,5,8,10 and 12); and from cells incubated with 10^{-8} M insulin (lanes 3 and 6). Results shown are from five separate experiments. Cell culture conditions were as described in Fig. 2 and Fig. 4. Ten μg of RNA was electrophoresed through 0.8% agarose, 2.2 M formaldehyde gels. The positions of 28S and 18S ribosomal RNA is shown. RNA was transferred to nitrocellulose and hybridized with a ^{32}P-labeled human apoB cDNA.

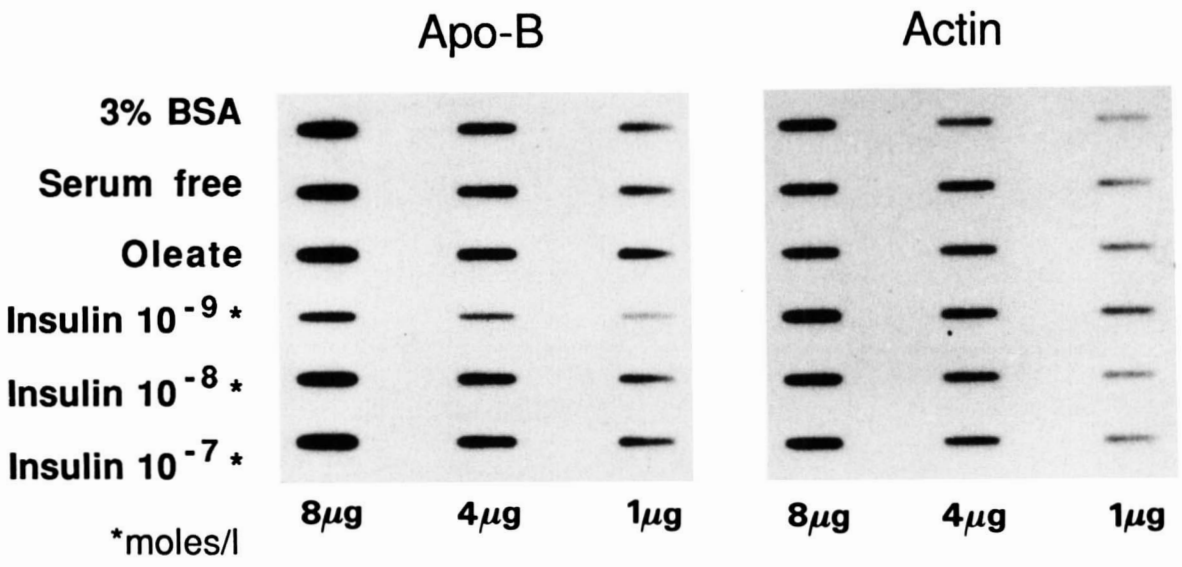

Fig. 8. Slot-blot hybridization analysis of total RNA isolated from HepG2 cells that had been incubated under a variety of conditions as described in Methods. Three amounts of RNA (1, 4, and 8 μg) were blotted per sample onto duplicate nitrocellulose filters. One filter was hybridized with a ^{32}P-labeled human apoB cDNA and the other with a ^{32}P-labeled human β-actin cDNA.

was 15.6 h. The degree of hybridization of the [^{3}H]apoB cRNA to the filters in these experiments was 10.3%.

DISCUSSION

The present study was undertaken to establish conditions by which apoB secretion from HepG2 cells could be varied, and to ascertain whether under these conditions apoB gene transcription or mRNA stability was affected. Cells stimulated with oleate or inhibited with insulin showed a 7-fold difference in apoB output with no change in apoB mRNA levels. This suggests that in HepG2 cells expression of the apoB gene is constitutive and that co- or post-translational mechanisms must be responsible for the short-term regulation of apoB secretion. This conclusion is strongly supported by the observation that the half-life of apoB mRNA in HepG2 cells is 16 h.

An increase in apoB output in the presence of oleate was observable at the lowest concentration used (20 μM) and was apparent in the presence of 0.6 mM oleate within 3 h. This effect could be accounted for by increased translational efficiency of apoB mRNA. Alternatively, the mechanism may involve decreased intracellular degradation of apoB, i.e., increased utilization of the apoB that was synthesized.

Previously, several studies on the metabolic regulation of apoB gene expression have been performed in experimental animals. Certain strains of mice fed high-fat diets were shown to have increased levels of circulating apoB, but the hepatic and intestinal levels of apoB mRNA were either unchanged or lower (57). These observations were interpreted as the result of differences in lipoprotein catabolism under different dietary conditions or between strains of mice. These results are also consistent with the present work which suggests that hepatic apoB secretion is regulated by post-transcriptional mechanisms that could give rise, in vivo, to different plasma apoB levels. Reports of studies on the effect on apoB mRNA levels of long-term feeding of cholesterol are contradictory. In rabbits, cholesterol feeding, which gives rise to high levels of circulating β-VLDL, was associated with a decrease in hepatic apoB mRNA and a highly variable increase in intestinal apoB mRNA (58). In contrast, in rats hepatic apoB

TABLE 4. ApoB mRNA levels in HepG2 cells incubated in the presence of insulin, albumin, and oleate

Additions			
Oleate 0.8 mM	Insulin 10^{-8} M	Albumin 3% (w/v)	ApoB mRNA[a]
			% of albumin control
+	−	+	93 ± 7 (12)
−	−	−	105 ± 11 (5)
+	+	+	78 ± 8 (5)[b]
−	−	+	100 (14)
−	+	+	95 ± 10 (5)

Incubation conditions were the same as in Table 1. Total cellular RNA was assayed by probing slot-blots with ^{32}P-labeled apoB cDNA as described in Methods.

[a]Results are expressed as a percentage of albumin control ± SEM, with the number of experiments for each condition in parentheses.

[b]$P < 0.05$ as compared to oleate control.

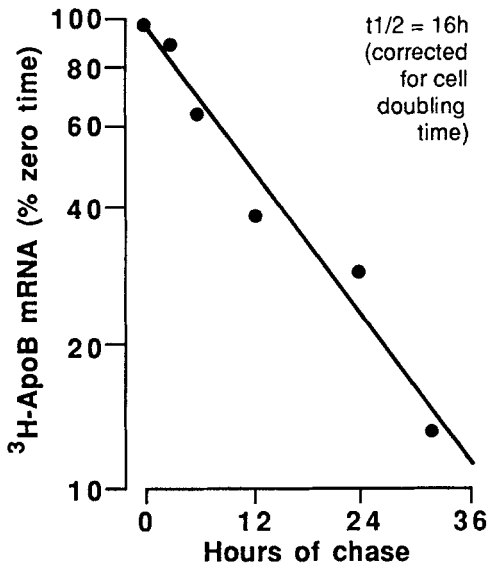

Fig. 9. Semi-log plot showing the decay of [^{3}H]apoB mRNA in HepG2 cells. Cells were incubated with [^{3}H]uridine for 20 h and then chased for various times up to 30 h. Total RNA was isolated and hybridized to filter-immobilized apoB cDNA. For details see Methods.

mRNA content was increased several-fold and the intestinal levels were unchanged by cholesterol feeding (59). In addition, apoB mRNA was found to be increased 6-fold in the livers of patients with the rare recessive disease abetalipoproteinemia compared with control levels (60). All of these changes in liver apoB mRNA content could reflect long-term adaptations to altered intrahepatic lipid metabolism.

The amount of apoB that was secreted by HepG2 cells in the present study is comparable with that secreted by rat hepatocytes (9, 37) and previously reported for HepG2 cells (14). It has been shown that the total intracellular pool of apoB is equal to only 13–14% of the amount secreted into the medium by rat hepatocytes over 16 h (37). The amount of triglyceride secreted by HepG2 cells (see Table 3) is much less than that secreted by rat liver. Three laboratories have reported (8, 11, 33) that rat hepatocytes under control conditions secrete between 27 and 69 μg triglyceride per mg cell protein in 24 h, which is similar to the rate for perfused rat livers (4–6). Even in the presence of oleate, the amount of triglyceride secreted by HepG2 cells is an order of magnitude less than for control rat hepatocytes. However, the difference in rates of triglyceride secretion between HepG2 cells and rat hepatocytes is unlikely to explain the 7-fold accumulation of triglyceride under these circumstances, which results in the large lipid droplets observed by electron microscopy, since rat hepatocytes also accumulate large amounts of triglyceride in the presence of oleate (9).

Thrift et al. (14) have suggested that the low triglyceride content of the lipoproteins secreted by HepG2 cells may be a consequence of the small amount of smooth ER (SER) in these cells; the ER membranes are the site of lipid biosynthesis and nascent lipoprotein assembly. This low amount of SER may be reflected in the residence time of apoB in the ER of HepG2 cells, which is shorter (61) than in isolated rat hepatocytes (62). Together these observations suggest that HepG2 cells do not properly reflect the ER phase of lipoprotein assembly and that some aspects of the regulation of lipoprotein assembly and output found in the liver may be defective in HepG2 cells.

The 3.5-fold stimulation of apoB output observed with oleate in the present study agrees with two other reports with this cell line (13, 16). Dashti and Wolfbauer (17) reported only a moderate (28%) increase in apoB output as a result of incubating HepG2 cells with oleate. However, they compared the rate of secretion in the presence of oleate plus albumin with that in basal medium, i.e., in the absence of fetal calf serum or albumin. At the concentration used (1.5%) albumin inhibits apoB secretion by 50% as shown in the present work and by others (13). Hence a reinterpretation of these results indicates a 2.5-fold stimulation in the presence of oleate plus albumin compared to albumin alone. The increased levels of apoB seen in the medium of HepG2 cells incubated with oleate are not due to a decrease in the uptake and degradation of apoB (16).

ApoB secretion from perfused livers from fasted rats is increased by oleate to the same degree as with HepG2 cells, but oleate leads to a small decline in apoB output from livers from fed animals (6). No effect was seen with rat hepatocyte cultures (8–10).

The metabolic effects of insulin deficiency or resistance, which result in diabetes mellitus, are complex. The liver responds to insulin deficiency per se and to the metabolic consequences of decreased peripheral uptake of triglyceride. In a recent review on the hyperlipidemia of diabetes, Gibbons (21) has pointed out that diabetics with raised VLDL, triglyceride, or LDL cholesterol have elevated apoB levels. Insulin promotes the conversion of dietary carbohydrate to a form utilizable by the liver for lipogenesis as well as stimulating hepatic lipogenesis directly. The clearance of VLDL by adipose tissue lipoprotein lipase and possibly also the ultimate removal of VLDL remnants are promoted by insulin (21). There is a decrease in the fractional catabolic rate for VLDL apoB in patients with noninsulin-dependent diabetes mellitus (NIDDM) (63), the most common form of diabetes in humans. However, the elevated VLDL levels associated with NIDDM have many causes and overproduction of both VLDL triglyceride and VLDL apoB, as well as decreased uptake, are considered to be contributing factors (63). The increased synthesis of VLDL triglyceride by the liver in NIDDM is partly due to the greater availability of substrate for triglyceride synthesis in the form of plasma glucose and nonesterified fatty acid (NEFA) (63, 64). In addition, it has been suggested that the elevated VLDL triglyceride

secretion rates seen in patients with NIDDM may be due in part to the high levels of circulating insulin often seen in individuals with NIDDM. These elevated insulin levels were shown in an early study (24) to be responsible for the stimulation of VLDL production in NIDDM. However, it is difficult from these studies to separate direct from indirect effects of insulin. Other workers have failed to detect a correlation between insulin levels and the rate of VLDL secretion (63). It has, therefore, been postulated that the overproduction of VLDL in NIDDM is associated in some way with insulin resistance (21, 63) rather than with the plasma level of insulin itself. This would be consistent with the observations made in vitro. Insulin has been shown to inhibit VLDL triglyceride (32–36), VLDL cholesterol (34), and VLDL apoB (33, 35, 37) secretion from rat hepatocytes. In the present work, and as reported by others (17), insulin inhibited the output of apoB from HepG2 cells. If, in vivo, the liver becomes resistant to the normal insulin inhibition of VLDL secretion, overproduction of VLDL could result. The role of insulin, as Sparks and colleagues (65) have recently postulated, may be twofold: to maintain levels of hepatic apoB secretion chronically at basal concentrations, and at higher levels in the acute postprandial state to inhibit VLDL output.

That the decreased accumulation of lipoprotein in the presence of insulin is due to a lower rate of secretion rather than to a higher rate of uptake and degradation has been shown by other workers (32, 33). That is, insulin had no effect on the rate of lipoprotein degradation.

Raised plasma LDL cholesterol levels are often found in cases of chronic renal disease and it is considered that this is due to increased hepatic VLDL secretion caused by the associated hypoalbuminemia (40, 41). The present study confirms previous reports (13, 43) that the presence of macromolecules such as albumin, ovalbumin, and dextran in the medium of cultured hepatic cells decreases apoB secretion in a dose-dependent fashion. While it is possible that albumin acts as a sink for fatty acid, Davis and colleagues (43) have provided evidence against this. In addition, dextran and ovalbumin bind fatty acids with a low affinity. Another possible explanation is that the effects are explained by colloid osmotic pressure, but this has been rejected by the same group of workers, who found no impairment of the rate of albumin secretion in the presence of macromolecules (43). The absence of any change in apoB mRNA levels when HepG2 cells were incubated in the presence of albumin again indicates that post-transcriptional mechanisms are responsible for the difference in the rate of apoB output under these conditions.

In addition to insulin we have examined other hormones that affect lipid and lipoprotein metablism for their effect on apoB secretion. Glucagon is known to inhibit VLDL triglyceride output by rat hepatocytes (11, 31, 34) and perfused rat livers (38). It was, therefore, surprising that apoB secretion from HepG2 cells was not inhibited by this hormone or with 8-Br cAMP. An explanation for this may be in the finding that hepatoma cells can be unresponsive to glucagon and cAMP in the absence of phosphodiesterase inhibitors (66).

Despite other reports that dexamethasone stimulates VLDL triglyceride output from rat hepatocytes, we could find no effect of this drug on apoB secretion from HepG2 cells. Triiodothyronine has also been reported to stimulate fatty acid and cholesterol synthesis in cultured rat hepatocytes (67) and hypothyroid rats had reduced rates of hepatic apoB synthesis (68). Administration of triiodothyronine to HepG2 cells had no effect on the apoB secretion rate. Lactate plus pyruvate has been shown to stimulate VLDL triglyceride output from rat hepatocyte suspensions (11), but did not cause a significant increase in apoB secretion from HepG2 cells. However, Bartlett and Gibbons (39) have recently shown that the effect of lactate plus pyruvate on VLDL triglyceride output in cultured rat hepatocytes is dependent on the presence of dexamethasone.

Mevinolin and 25-hydroxycholesterol, which inhibit cholesterogenesis, and mevalonate, which has the opposite effect, all tended to increase apoB secretion, but these results did not reach statistical significance. The drug Sandoz 58-035, which inhibits cholesterol esterification, had the same effect.

It has been reported that the calcium ionophore A23187 at a concentration of 10^{-6} M caused increased secretion of apoB from the human adenocarcinoma cell line CaCo-2 (69). In the present study we found no effect of A23187 on apoB output from HepG2 cells at a concentration of 10^{-7} M and a 33.2% decrease at 10^{-6}M. This decrease was due to significant cell death at this concentration of the drug.

In the present study we have shown that apoB mRNA in HepG2 cells has a long half-life of 16 h. This is consistent with the failure to detect changes in the apoB mRNA level under conditions that caused a variation in apoB secretion from these cells over a 7-fold range. We conclude that the apoB gene is not subject to short term regulation of its rate of transcription.

This work was supported by the Medical Research Council. B-B. T. is a Canadian Heart Association fellow. We are grateful to Ms. Paula Medina for her technical assistance and to Dr. S. Venkatesan for assaying the cellular cholesterol levels. We would also like to thank Dr. Richard Pease for his helpful interest in this work. We also thank Ms. Lesley Sargeant for typing the manuscript.

Manuscript received 1 December 1988 and in revised form 23 January 1989.

REFERENCES

1. Sparks, J. D., and C. E. Sparks. 1985. Apolipoprotein B and lipoprotein metabolism. *Adv. Lipid Res.* **21:** 1–46.

2. Brown, M. S., and J. L. Goldstein. 1983. The hyperlipoproteinemias and other disorders of lipid metabolism. *In* Harrison's Principles of Internal Medicine. R. G. Petersdorf, R. D. Adams, E. Braunwald, K. J. Isselbachet, J. B. Martin, and J. D. Wilson, editors. McGraw Hill Book Co., New York. 547-559.
3. Davis, R. A., S. C. Engelhorn, S. H. Pangburn, D. B. Weinstein, and D. Steinberg. 1979. Very low density lipoprotein synthesis and secretion by cultured rat hepatocytes. *J. Biol. Chem.* **254:** 2010-2016.
4. Yamamoto, M., I. Yamamoto, Y. Tanaka, and J. A. Ontko. 1987. Fatty acid metabolism and lipid secretion by perfused livers from rats fed laboratory stock and sucrose-rich diets. *J. Lipid Res.* **28:** 1156-1165.
5. Topping, D. L., and P. A. Mayes. 1982. Insulin and non-esterified fatty acids. *Biochem J.* **204:** 433-439.
6. Salam, W. H., H. G. Wilcox, and M. Heimberg. 1988. Effects of oleic acid on the biosynthesis of lipoprotein apoproteins and distribution into the very-low-density lipoprotein by the isolated perfused rat liver. *Biochem J.* **251:** 809-816.
7. Dashti, A., W. J. McConathy, and J. A. Ontko. 1980. Production of apolipoproteins E and A-I by rat hepatocytes in primary culture. *Biochem. Biophys. Acta.* **618:** 347-358.
8. Davis, R. A., and J. R. Boogaerts. 1982. Intrahepatic assembly of very low density lipoproteins. *J. Biol. Chem.* **257:** 10908-10913.
9. Patsch, W., T. Tamai, and G. Schonfeld. 1983. Effect of fatty acids on lipid and apoprotein secretion and association in hepatocyte cultures. *J. Clin. Invest.* **72:** 371-378.
10. Coleman, R. A., E. B. Haynes, T. M. Sand, and R. A. Davis. 1988. Developmental coordinate expression of triacylglycerol and small molecular weight apoB synthesis and secretion by rat hepatocytes. *J. Lipid Res.* **29:** 33-42.
11. Goh, E. H., and M. Heimberg. 1979. Relationship between activity of hepatic 3-hydroxy-3-methylglutaryl-coenzyme A reductase and secretion of very-low-density-lipoprotein cholesterol by the isolated perfused liver in the intact rat. *Biochem. J.* **184:** 1-6.
12. Gibbons, G. F., and C. R. Pullinger. 1987. Regulation of hepatic very-low-density lipoprotein secretion in rats fed on a diet high in unsaturated fat. *Biochem J.* **243:** 487- 492.
13. Rash, J. M., G. H. Rothblat, and C. E. Sparks. 1981. Lipoprotein apolipoprotein synthesis by human hepatoma cells in culture. *Biochim. Biophys. Acta.* **666:** 294-298.
14. Thrift, R. N., T. M. Forte, B. E. Cahoon, and V. G. Shore. 1986. Characterization of lipoproteins produced by the human liver cell line, HepG2, under defined conditions. *J. Lipid Res.* **27:** 236-250.
15. Ellsworth, J. L., S. K. Erickson, and A. D. Cooper. 1986. Very low and low density lipoprotein synthesis and secretion by the human hepatoma cell line Hep-G2: effects of free fatty acid. *J. Lipid Res.* **27:** 858-874.
16. Boström, K., J. Borén, M. Wettesten, A. Sjöberg, G. Bondjers, O. Wiklund, P. Carlsson, and S-O. Olofsson. 1988. Studies on the assembly of apoB-100-containing lipoproteins in HepG2 cells. *J. Biol. Chem.* **263:** 4434-4442.
17. Dashti, N., and G. Wolfbauer. 1987. Secretion of lipids, apolipoproteins, and lipoproteins by human hepatoma cell line, HepG2: effects of oleic acid and insulin. *J. Lipid Res.* **28:** 423-436.
18. Erickson, S. K., and P. E. Fielding. 1986. Parameters of cholesterol metabolism in the human hepatoma cell line, Hep-G2. *J. Lipid Res.* **27:** 875-883.
19. Wang, S. R., M. Pessah, J. Infante, D. Catala, C. Salvat, and R. Infante. 1988. Lipid and lipoprotein metabolism in Hep G2 cells. *Biochim. Biophys. Acta.* **961:** 351-363.
20. Steiner, G. 1981. Diabetes and atherosclerosis. An overview. *Diabetes.* **30 (suppl 2):** 1-7.
21. Gibbons, G. F. 1986. Hyperlipidaemia of diabetes. *Clin Sci.* **71:** 477-486.
22. Farquhar, J. W., A. Frank, R. C. Gross, and G. M. Reaven. 1966. Glucose, insulin, and triglyceride responses to high and low carbohydrate diets in man. *J. Clin. Invest.* **45:** 1648-1656.
23. Reaven, G. M., R. L. Lerner, M. P. Stern, and J. W. Farquhar. 1967. Role of insulin in endogenous hypertriglyceridemia. *J. Clin. Invest.* **46:** 1756-1767.
24. Olefsky, J. M., J. W. Farquhar, and G. M. Reaven. 1974. Reappraisal of the role of insulin in hypertriglyceridemia. *Am. J. Med.* **57:** 551-560.
25. Reaven, G. M., and M. S. Greenfield. 1981. Diabetic hypertriglyceridemia. Evidence for three clinical syndromes. *Diabetes.* **30 (suppl. 2):** 66-75.
26. Pietri, A. O., F. L. Dunn, S. M. Grundy, and P. Raskin. 1983. The effect of continuous subcutaneous insulin infusion on very-low-density lipoprotein triglyceride metabolism in type 1 diabetes mellitus. *Diabetes.* **32:** 75-81.
27. Rosenstock, J., G. L. Vega, and P. Raskin. 1988. Effect of intensive diabetes treatment on low-density lipoprotein apolipoprotein B kinetics in type 1 diabetes. *Diabetes.* **37:** 393-397.
28. Alcindor, L-G., R. Infante, C. Soler-Argilaga, and J. Polonovski. 1973. Effect of a single insulin administration on the hepatic release of triglyceride into the plasma. *Biochim. Biophys. Acta.* **306:** 347-352.
29. Topping, D. L., and P. A. Mayes. 1972. The immediate effects of insulin and fructose on the metabolism of the perfused liver. *Biochem. J.* **126:** 295-311.
30. Laker, M. E., and P. A. Mayes. 1984. Investigations into the direct effects of insulin on hepatic ketogenesis, lipoprotein secretion and pyruvate dehydrogenase activity. *Biochim. Biophys. Acta.* **795:** 427-430.
31. Beynen, A. C., H. P. Haagsman, L. M. G. Van Golde, and M. J. H. Geelen. 1981. The effects of insulin and glucagon on the release of triacylglycerols by isolated rat hepatocytes are mere reflections of the hormonal effects on the rate of triacylglycerol synthesis. *Biochim. Biophys. Acta.* **665:** 1-7.
32. Durrington, P. N., R. S. Newton, D. B. Weinstein, and D. Steinberg. 1982. Effects of insulin and glucose on very low density lipoprotein triglyceride secretion by cultured rat hepatocytes. *J. Clin. Invest.* **70:** 63-73.
33. Patsch, W., S. Franz, and G. Schonfeld. 1983. Role of insulin in lipoprotein secretion by cultured rat hepatocytes. *J. Clin. Invest.* **71:** 1161-1174.
34. Pullinger, C. R., and G. F. Gibbons. 1985. Effects of hormones and pyruvate on the rates of secretion of very-low-density lipoprotein triacylglycerol and cholesterol by rat hepatocytes. *Biochim. Biophys. Acta.* **833:** 44-51.
35. Patsch, W., A. M. Gotto, Jr., and J. R. Patsch. 1986. Effects of insulin on lipoprotein secretion in rat hepatocyte cultures. *J. Biol. Chem.* **261:** 9603-9606.
36. Mangiapane, E. H., and D. N. Brindley. 1986. Effects of dexamethasone and insulin on the synthesis of triacylglycerols and phosphatidylcholine and the secretion of very-low-density lipoproteins and lysophosphatidylcholine by monolayer cultures of rat hepatocytes. *Biochem. J.* **233:** 151-160.
37. Sparks, C. E., J. D. Sparks, M. Bolognino, A. Salhanick, P. S. Strumph, and J. M. Amatruda. 1986. Insulin effects on apolipoprotein B lipoprotein synthesis and secretion by primary cultures of rat hepatocytes. *Metabolism.* **35:** 1128-1136.

38. Shaheen, O., D. W. Morgan, H. G. Wilcox, W. G. Keyes, and M. Heimberg. 1982. Modulation by thyroid status of the actions of glucagon and dibutyryl adenosine 3′, 5′-monophosphate on metabolism of free fatty acids by the isolated perfused rat liver. *Endocrinology.* **110:** 1740–1748.
39. Bartlett, S. M., and G. F. Gibbons. 1988. Short- and longer- term regulation of very-low-density lipoprotein secretion by insulin, dexamethasone and lipogenic substrates in cultured hepatocytes. *Biochem. J.* **249:** 37–43.
40. Chan, M. K., J. W. Persaud, L. Ramdial, Z. Varghese, P. Sweney, and J. F. Moorhead. 1981. Hyperlipidaemia in untreated nephrotic syndrome, increased production or decreased removal? *Clin. Chim. Acta.* **117:** 317–323.
41. Marsh, J. B. 1984. Lipoprotein metabolism in experimental nephrosis. *J. Lipid Res.* **25:** 1619–1623.
42. Joven, J., L. Masana, C. Villabona, and C. Martí. 1987. Increased synthesis of apoprotein-B-LDL in the nephrotic syndrome. *Eur. J. Clin. Invest.* **17:** A10.
43. Davis, R. A., S. C. Engelhorn, D. B. Weinstein, and D. Steinberg. 1980. Very low density lipoprotein secretion by cultured rat hepatocytes. *J. Biol. Chem.* **255:** 2039–2045.
44. Van Harken, D. R., C. W. Dixon, and M. Heimberg. 1969. Hepatic lipid metabolism in experimental diabetes. *J. Biol. Chem.* **244:** 2278–2285.
45. 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.
46. Milne, R. W., L. Blanchette, R. Théolis, Jr., P. K. Weech, and Y. L. Marcel. 1987. Monoclonal antibodies distinguish between lipid-dependent and reversible conformational states of human apolipoprotein B. *Molec. Immunol.* **24:** 435–447.
47. Auffray, C., and F. Rougeon. 1980. Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. *Eur. J. Biochem.* **107:** 303–314.
48. Schneider, W. C. 1957. Determination of nucleic acids in tissues by pentose analysis. *Methods Enzymol.* **3:** 680–684.
49. Knott, T. J., R. J. Pease, L. M. Powell, S. C. Wallis, S. C. Rall, Jr., T. L. Innerarity, B. Blackhart, W. H. Taylor, Y. Marcel, R. Milne, D. Johnson, M. Fuller, A. J. Lusis, B. J. McCarthy, R. W. Mahley, B. Levy-Wilson, and J. Scott. 1986. Complete protein sequence and identification of structural domains of human apolipoprotein B. *Nature.* **323:** 734–738.
50. Moos, M., and D. Gallvitz. 1983. Structure of two human β-actin-related processed genes one of which is located next to a simple repetitive sequence. *EMBO J.* **2:** 757–761.
51. Knott, T. J., S. C. Wallis, L. M. Powell, R. J. Pease, A. J. Lusis, B. Blackhart, B. J. McCarthy, R. W. Mahley, B. Levy-Wilson, and J. Scott. 1986. Complete cDNA and derived protein sequence of human apolipoprotein B-100. *Nucleic Acids Res.* **14:** 7501–7503.
52. Rodgers, J. R., M. L. Johnson, and J. M. Rosen. 1985. Measurement of mRNA concentration and mRNA half-life as a function of hormonal treatment. *Methods Enzymol.* **109:** 572–592.
53. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
54. Venkatesan, S., K. J. Simpson, and T. J. Peters. 1988. Hepatic cholesterol synthesis and esterification in rats after chronic ethanol feeding. *Clin. Sci.* **74:** 407–412.
55. Rideout, J. M., G. D. Smith, C. K. Lim, and T. J. Peters. 1985. Comparative study of several phase systems for the high-performance liquid chromatographic separation of monoiodinsulins. *Biochem. Soc. Trans.* **13:** 1225–1226.
56. 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.
57. Lusis, A. J., B. A. Taylor, D. Quon, S. Zollman, and R. C. LeBoeuf. 1987. Genetic factors controlling structure and expression of apolipoproteins B and E in mice. *J. Biol. Chem.* **262:** 7594–7604.
58. Kroon, P. A., J. A. DeMartino, G. M. Thompson, and Y-S. Chao. 1986. Molecular cloning of partial cDNAs for rabbit liver apolipoprotein B and the regulation of its mRNA levels by dietary cholesterol. *Proc. Natl. Acad. Sci. USA.* **83:** 5071–5075.
59. Matsumoto, A., H. Aburatani, Y. Shibasaki, T. Kodama, F. Takaku, and H. Itakura. 1987. Cloning and regulation of rat apolipoprotein B mRNA. *Biochem. Biophys. Res. Commun.* **142:** 92–99.
60. Lackner, K. J., J. C. Monge, R. E. Gregg, J. M. Hoeg, T. J. Triche, S. W. Law, and H. B. Brewer, Jr. 1986. Analysis of the lipoprotein B gene and messenger ribonucleic acid in abetalipoproteinemia. *J. Clin. Invest.* **78:** 1707– 1712.
61. Boström, K., M. Wettesten, J. Borén, G. Bondjers, O. Wiklund, and S-O. Olofsson. 1986. Pulse-chase studies of the synthesis and intracellular transport of apolipoprotein B-100 in Hep G2 cells. *J. Biol. Chem.* **261:** 13800–13806.
62. Borchardt, R. A., and R. A. Davis. 1987. Intrahepatic assembly of very low density lipoproteins. *J. Biol. Chem.* **262:** 16394–16402.
63. Howard, B. V. 1987. Lipoprotein metabolism in diabetes mellitus. *J. Lipid Res.* **28:** 613–628.
64. Reaven, G. M. 1987. Non-insulin-dependent diabetes mellitus, abnormal lipoprotein metabolism, and atherosclerosis. *Metabolism.* **36 (suppl. 1):** 1–8.
65. Sparks, J. D., C. E. Sparks, M. Bolognino, A. M. Roncone, T. K. Jackson, and J. M. Amatruda. 1988. Effects of nonketotic streptozotocin diabetes on apolipoprotein B synthesis and secretion by primary cultures of rat hepatocytes. *J. Clin. Invest.* **82:** 37–43.
66. Wong. S. S. C., and P. T. L. Woo. 1987. Stimulation by glucagon and adenosine-3′, 5′-cyclic monophosphate of protein degradation in Reuber H35 hepatoma monolayers. *Int. J. Biochem.* **19:** 443–447.
67. Gnoni, G. V., M. J. H. Geelen, C. Bijleveld, E. Quagliariello, and S. G. van den Bergh. 1985. Short-term stimulation of lipogenesis by triiodothyronine in maintenance cultures of rat hepatocytes. *Biochem. Biophys. Res. Commun.* **128:** 525–530.
68. Davidson, N. O., R. C. Carlos, M. J. Drewek, and T. G. Parmer. 1988. Apolipoprotein gene expression in the rat is regulated in a tissue-specific manner by thyroid hormone. *J. Lipid Res.* **29:** 1511–1522.
69. Hughes, T. E., J. M. Ordovas, and E. J. Schaefer. 1988. Regulation of intestinal apolipoprotein B synthesis and secretion by Caco-2 cells. *J. Biol. Chem.* **263:** 3425–3431.