Role of Insulin in Lipoprotein Secretion by Cultured Rat Hepatocytes

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ABSTRACT To study the effect of insulin on lipoprotein synthesis and secretion by the liver, apoprotein and lipid levels were measured in primary rat liver cell cultures grown on fibronectin-coated dishes. Triglycerides, phospholipids, apoprotein (apo) B, apo-E, and apo-C-III$_3$ all accumulated in culture media linearly for periods up to 20 h. During incubations, cellular triglyceride contents increased slightly, while cellular apoprotein and phospholipid contents remained constant. In the absence of insulin, rates of accumulation in media of triglycerides, apo-B, apo-C-III$_3$, and apo-E were 2.5±0.3 μg/mg and 33±5, 24±3, and 162±32 ng/mg cell protein per h, respectively. On gel permeation chromatography and density gradient ultracentrifugation, the majority of apoproteins in media were found to be associated with very low density lipoproteins (VLDL) and very little eluted or sedimented with albumin. Incubations in the presence of 50–800 μU/ml of insulin resulted in dose-dependent decreases of triglyceride, phospholipid, apo-B, and apo-E accumulation in the media, paralleled by increases in the cellular contents of these lipoprotein components. The inhibitory effects of insulin on secretion were reversible. Levels of apo-C-III$_3$ and albumin were not affected by insulin. In addition to decreasing secretory rates, the proportion of apo-B, apo-E, and apo-C-III$_3$ associated with VLDL also decreased after the addition of insulin. Concomitantly, the proportion of apo-B eluting with LDL and apo-C-III$_3$, and apo-E eluting near albumin increased. Control experiments, in which exogenous ^{125}I-VLDL or endogenously labeled [^{14}C]VLDL were added to cultures, revealed that the insulin-induced differences in VLDL accumulation and the lipid association of media apoproteins were not due to differences in the processing of VLDL by cells cultured in the presence or absence of insulin. Therefore, it appears that insulin may inhibit the secretion of VLDL perhaps by reducing the intracellular association of lipids and apoproteins.

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INTRODUCTION

Plasma lipoproteins or their precursors are secreted into the circulation by the intestine and the liver (1–7). Hepatic synthesis and secretion of apoproteins and lipoproteins have been studied most frequently in isolated perfused livers or liver slices (8–12). However, these preparations can be maintained in viable conditions only for several hours. They also have other limitations. Liver slices contain damaged cells and therefore it may be difficult to distinguish lipoprotein secretion from leakage. With perfused livers it is laborious to perform the large numbers of replicate experiments needed to demonstrate any statistical differences that may be produced by experimental manipulations. Finally, the in vivo metabolic state of the animal continues to affect the subsequent behavior of both of these liver preparations for the few hours they survive in vitro (9). This is of particular importance in studying hormonal and metabolic effects on the liver because experimental perturbations of hormonal and/or metabolic factors in vivo may provoke alterations in levels of counter regulatory hormones and/or other metabolic intermediates in the plasma. These substances, by interacting with the liver, may modify the actions of the hormone under study over several hours, and thus make interpretations of the effects of single hormones difficult.

Recently, primary rat liver cell cultures have been introduced for studies of lipoprotein synthesis and secretion (13, 14). Although the hepatocytes in culture are one step further removed from the whole organ than the liver perfusion system (e.g., the sinusoidal cells and the space of Disse are not present and bile acids do accumulate in the media [15]), nevertheless, hepatocytes in culture have several attractive features

to study hepatocyte function: The increased longevity of the cells compared with other liver preparations and the presence in cultures of virtually only parenchymal cells may allow studies on the effects of single factors to be interpreted more readily (16). Furthermore, several perturbations can be tested on cells cultured from a single liver, thereby overcoming variations between animals. We therefore have adopted the cultured hepatocyte to investigate possible effects of various factors on lipoprotein secretion. When cultures were first set up, 10 mU/ml of insulin were included in the culture media because insulin was reported to prolong the survival of cultured hepatocytes (14) and to increase glycogen and fatty acid synthesis (13, 17). Although we found apoprotein secretion in the presence of insulin to be linear, >70% of apoprotein (apo)[1] C-III_3 and apo-E in media were not associated with lipoproteins (vs. 25% for hepatic perfusate apoproteins). Because of our findings and the report that insulin inhibits very low density lipoprotein (VLDL)-triglyceride-(TG) secretion by cultured hepatocytes (18), we reasoned that one cause for the ineffective lipid-apoprotein association could be insulin. Therefore, detailed studies of the effects of insulin on apoprotein secretion were performed. At near physiological levels, insulin inhibited the secretion of apoproteins and lipids, and the assembly of triglyceride (TG) with apoproteins. Some of these results have been presented in abstract form (19).

METHODS

Adult male Sprague-Dawley rats weighing 200–250 g (Eldridge Laboratory Animals, Barnhart, MO), housed in a room with a 12-h light cycle (7–19 h), were given water and standard rat chow ad lib. Food was removed (3–4 h before experiments) at 9 a.m., and the animals were anesthetized with sodium pentobarbital (5 mg/100 g). For preparation of cultured cells, livers were perfused ex situ with 50 ml of oxygenated Krebs-Ringer bicarbonate buffer without Ca^{++} at 37°C, pH 7.4, in a Miller-type perfusion apparatus (20) containing a silastic lung (21). After 5 min, 50 mg collagenase (type IA, Sigma Chemical Co., St. Louis, MO) was added to the perfusate and perfusion was continued for 15 min (22). The liver was then transferred to a beaker containing 50 ml of arginine-free Dulbecco's modified Eagle's medium (DME; Gibco Laboratories, Santa Clara, CA) that was supplemented with 0.4 mM *l*-ornithine, 25 mM glucose, 10 mM Hepes, 100 U/ml penicillin, 100 mg/liter streptomycin, and 50 mg/liter gentamycin before use. After removal of the liver capsule with a forceps, the cell suspension was filtered through four layers of gauze. The hepatocytes were separated from debris by centrifugation at 80 *g* for 5 min at 4°C. The resulting pellet was resuspended in DME and washed three times more by centrifugation at 80 *g* for 4 min. Each liver yielded 2–4 × 10^8 cells and 90–95% of the cells excluded Trypan blue dye. After washing, cells were taken up in DME containing antibiotics and 7% lipoprotein-deficient serum (LPDS) prepared from human plasma by ultracentrifugation. The "insulin-free" media contained <5 μU/ml of immunoassayable insulin (23). In some experiments, LPDS was omitted as indicated in the text. 3 ml of the cell suspensions (2–3 × 10^6 cells) were seeded on 60-mm plastic dishes that had been precoated with fibronectin (16, 18, 24, 25). (Dishes were prepared by incubating them with 25 μg fibronectin in 2.5 ml DME for at least 1 h at 37°C). Fibronectin was purified from human plasma by affinity chromatography (26). The product yielded a single band with an apparent mol wt of 250,000 on sodium dodecyl sulfate (SDS) electrophoresis in 3% acrylamide gels (27). 6 h after plating, cells were washed and new media of the same composition were added with the indicated amount of insulin (Regular Iletin II, Eli Lilly & Co., Indianapolis, IN), and experiments were started. After the washes, ~60% of the cells originally seeded remained attached to the plates as judged by protein measurements, and attached cells had polygonal shapes. After incubation periods of up to 30 h, cells were detached by using Ca^{++}- and Mg^{++}-free saline buffered with phosphate and a rubber policeman. Dislodged cells were centrifuged at 1,000 *g* for 5 min, and the cellular pellet was resuspended in 1.5 ml of 0.05 M sodium barbital buffer and 1 mM EDTA, pH 8.6. Benzamidine (1 mM) and phenylmethylsulfonyl fluoride (0.3 mM) were added to cell suspensions and hepatocyte media. Cell suspensions were sonicated at 4°C with a Sonifier cell disrupter (Heat Systems-Ultrasonics, Inc., Plainview, NY). Cellular protein to DNA ratio was 58±5 at the beginning of experiments and did not change during the subsequent experimental period. Typically, >90% of protein was recovered after incubation periods of up to 24 h.

Chemical determinations included cellular protein (28), cellular DNA (29), media and cellular phospholipids (PL) (30), media glucose (Beckman glucose analyzer II, Beckman Instruments, Inc., Spinco Div., Palo Alto, CA), media fatty acids (31), media and cellular TG (Triglyceride Kit, Boehringer & Söehne, Mannheim, West Germany), cellular glycogen (32), and media urea (33). Incorporation of [2-^{3}H]glycerol (ICN Pharmaceuticals, Inc., Irvine, CA) was determined on the appropriate thin-layer chromatographic spots of lipid extracts (34, 35). Incorporation of L[4,5-^{3}H]leucine (Research Products International Corp., Mount Prospect, IL) into cellular and media proteins was determined by precipitation of cell homogenates and media with 10% trichloracetic acid (TCA). Rat serum albumin secreted into the media was measured from 10-μl aliquots by immunoelectrophoresis (36). Rat albumin fraction V (N. L. Cappel Laboratories, Cochranville, PA) was used as a standard. Rat plasma VLDL was isolated by fixed angle head ultracentrifugation at $d < 1.006$ (37) and labeled with iodine monochloride (38).

To prepare endogenously labeled VLDL, [^{14}C]acetate (5 μCi/100-mm dish) was added to cultures receiving either 800 μU/ml insulin or no hormone. Media were removed after an incubation period of 16 h, concentrated by ultrafiltration, and subjected to molecular sieving chromatography in Sepharose 6B. Fractions corresponding to VLDL elution volumes were rechromatographed on the same column and dialyzed. Upon thin-layer chromatography, 71 and 67% of total radioactivity comigrated with TG in control and experimental VLDL. The percentages of counts in other lipid fractions were as follows for control and experimental groups, respectively: PL, 3 and 7; unesterified cholesterol, 17 and 20; and cholesterol esters, 8 and 6%. Specific activities

[1] *Abbreviations used in this paper:* apo, apoprotein; DME, Dulbecco's modified Eagle's medium; LPDS, lipoprotein-deficient serum; PL, phospholipid; TG, triglyceride.

were 1,350 dpm/μg VLDL-TG in control and 1,855 dpm/μg VLDL-TG in VLDL of insulin-treated cultures.

Levels of apoproteins were measured by radioimmunoassays. Low density lipoproteins (LDL) used as assay standards and for iodination in apo-B assays (9) were isolated from the plasma of adult male rats by zonal ultracentrifugation (39). Only the faster floating portion of the LDL peak was taken. More than 95% of the protein moiety of this fraction exhibited a mol wt of >200,000 on SDS gel electrophoresis (40). The apo-C-III$_3$ assay described and used previously (41, 42) measured primarily apo-C-III$_3$ and dectects only ~5% of the less sialylated forms of apo-C-III. For the immunoassay of apo-E, the protein was purified by heparin affinity chromatography from delipidated rat high density lipoproteins (HDL) (43), emulsified in complete Freund's adjuvant, and injected into rabbits to generate antisera. Similar preparations of apo-E were iodinated with lactoperoxidase (44) and also used as assay standards. The buffer of the apo-E assay contained 3% bovine serum albumin in 0.05 M sodium barbital and 1 mM EDTA, pH 8.6. Incubation with the primary antibody was in siliconized tubes for 48 h at 4°C, the goat anti-rabbit IgG was added for 16 h for separation of bound and unbound ^{125}I-apo-E. Displacement curves of ^{125}I-apo-E by rat plasma, hepatocyte media, cell homogenates, and irrelevant antigens (apo-A-I, apo-A-IV, and apo-C-III$_3$) are shown in Fig. 1. Apo-E plasma levels of rats on regular rat chow were 18±3 mg/100 ml (mean±1 SD of six pools, each consisting of three animals), which is in agreement with published values (45–47).

Hepatocyte media produced paralleled displacement curves in the apo-B, apo-C-III$_3$, and apo-E assays. To measure cellular apoprotein contents, 1.5–2 ml barbital buffer containing Triton X-100 (0.5%, g/vol) per dish were added to harvested cell suspensions before sonication. Homogenates were centrifuged at 100,000 *g* for 60 min, and supernatants were used for apoprotein measurements. Supernatants contained between 84 and 91% of total cellular protein. Addition of ^{125}I-apo-C-III$_3$, ^{125}I-LDL, and ^{125}I-apo-E before homogenization resulted in recoveries of 94±2, 92±3, and 89±5% of the added radioactivity in the supernatants (mean±1 SD). Omission of Triton X-100 reduced recoveries of ^{125}I-apo-E to 52±10%, whereas recoveries of ^{125}I-apo-C-III$_3$ and ^{125}I-LDL were not affected. Omission of Triton X-100 also reduced the apparent contents by radioimmunoassay of apo-B, apo-C-III$_3$, and apo-E in supernatants of cellular homogenates to 60, 64, and 56%, respectively, of that obtained in the presence of the detergent (mean of two experiments). When Triton-free pellets were extracted with 0.5% Triton X-100, the amount of apo-E, apo-B, and apo-C-III$_3$ in the initial supernates and pellet extracts added up to 95, 109, and 105% of the contents of whole homogenate, respectively. A second extraction of the cell pellets with Triton X-100 yielded <10% of the amount of apoproteins obtained by a single extraction procedure. Thus, single extraction with Triton X-100 appeared to be sufficient for solubilization of immunoreactivity.

To assess lipoprotein distribution of media apoproteins, incubation media were concentrated fourfold by using a Millipore cell equipped with a pellicon membrane (PTGC 04710) and operated at 5–12 pounds per square inch. Ultrafiltrates did not contain apoprotein immunoreactivity. Alternatively, incubation media were concentrated by using dialysis tubing (Spectrapor, Los Angeles, CA, cutoff 3,500 mol wt) embedded in Sephadex G-100. Use of either procedure resulted in similar apoprotein distribution upon gel filtration (see below). Concentrated media were subjected to ultracentrifugation in discontinuous density gradients (48). After ultracentrifugation, 1-ml aliquots were pipetted

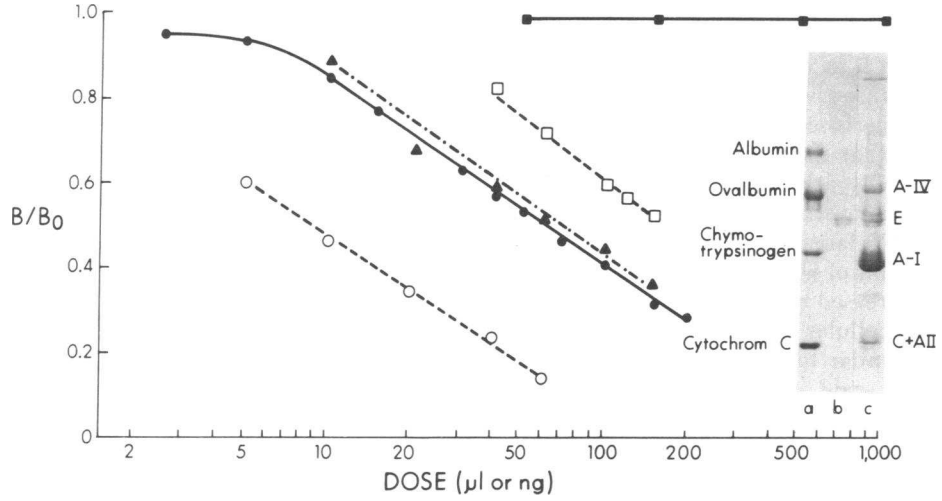

FIGURE 1 Radioimmunoassay displacement curves of ^{125}I-apo-E produced by increasing amounts of apo-E (●, standard, nanograms), plasma (□, 1:1,000 diluted, microliters), hepatocyte media (○, microliters), hepatocyte homogenates (▲, microliters), and apo-A-I, apo-A-IV, and apo-C-III$_3$ (■, nanograms). Plasma was from a carbohydrate-fed rat. Hepatocyte media was obtained after incubation of cells with media for 20 h. Hepatocyte cell homogenate represents the 100,000 *g*, 60-min supernatant of hepatocytes sonicated in the presence of 0.5% (wt/vol) Triton X-100. The insert shows (a) staining patterns of molecular weight standards (cytochrom, cytochrome), (b) apo-E used as standard and for iodination, and (c) apo-HDL used to prepare apo-E in 5–20% polyacrylamide gels after electrophoresis in 0.1% SDS.

off the tubes, dialyzed against EDTA-saline, and analyzed for apoprotein content. The densities of fractions after centrifugation of blank tubes were measured with a precision densitometer (DMA 45, Mettler-Paar, Graz, Austria). Sums of the respective contents of apo-C-III$_3$, apo-B, and apo-E in the various fractions averaged 81, 83, and 87% of the contents of the starting material. Concentrated hepatocyte media also were subjected to molecular sieving chromatography on Sepharose 6B and 2B (Pharmacia, Uppsala, Sweden; 0.15 M saline and 1 mM EDTA, pH 8.2). Columns were calibrated with human VLDL, LDL, HDL$_3$ (49), albumin, and ^{125}iodine. The Sepharose 2B column was calibrated with VLDL of defined flotation rates (50). Average recoveries from the columns of immunoreactivities of apo-B, apo-C-III$_3$, and apo-E were 90, 84, and 88%, respectively. Recovery of cholesterol after chromatography of human plasma averaged 98%.

Apo-B heterogeneity of media lipoproteins was determined by SDS electrophoresis in 3% acrylamide gels (27). For this purpose, the $d < 1.21$ density fraction of media lipoproteins was isolated by fixed angle head ultracentrifugation (37). Lipoproteins were delipidated twice with 50 vol of ethanol/diethylether, (3:1, vol/vol) for 20 h at 4°C, followed by an ether wash. Albumin, cross-linked with dimethyl suberidimate (51), was used as molecular weight standard. Gels were stained and scanned as described (52).

RESULTS

Rates of accumulation and association of lipoprotein components in the absence of insulin. In the absence of insulin, incorporation of [^{3}H]leucine into the total proteins of cells and media, and accumulation of TG, PL, and apoproteins in the media were linear over 20 h (Fig. 2). Cellular TG content increased, but cellular PL and apoprotein contents remained nearly constant. The rate of TG accumulation in the media was 2.5±0.3 μg/mg h (n = five livers). Accumulation rates of apoproteins are given in Table I and are similar to rates found for perfused livers (9, 11, 42).

To demonstrate that the apoproteins found in the media were actively synthesized and secreted by the cultured cells, hepatocytes were exposed to 30 μM cyclohexamide and 8 h later, media and cells were harvested. Incorporation of [^{3}H]leucine into cellular and media proteins decreased by 92 and 76%, respectively. Immunoassayable cellular content of apo-B and apo-C-III$_3$ remained similar to those of control cells, but levels of apo-B and apo-C-III$_3$ in the media decreased by 79 and 83%, respectively. Exposure of cells to 10 μM colchicine for 8 h decreased incorporation of [^{3}H]leucine into media proteins by 35% and accumulation of apo-C-III$_3$ by 55%.

Apoprotein distribution among lipoproteins was examined by density gradient ultracentrifugation and gel permeation chromatography. In the absence of added insulin, the majority of apoproteins appeared to be associated with VLDL particles, on density gradient ultracentrifugation (Fig. 3). The minority of apo-B appeared in the LDL- and HDL-eluting regions and

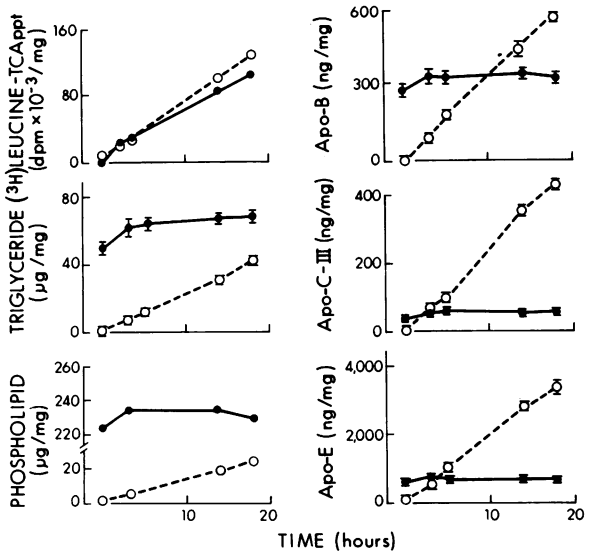

FIGURE 2 Time course of accumulation in culture media of [^{3}H]leucine incorporated into TCA-precipitable (ppt) material, TG, PL, apo-B, apo-C-III$_3$, and apo-E (open symbols). Cellular contents of same components after various periods of incubation are shown in closed symbols. Values are means±1 SD of (3–4 dishes) or means of two dishes per time point. Liver cells were seeded on a fibronectin matrix in the presence of 7% LPDS. The media were changed after 6 h, 5 μCi [^{3}H]leucine was added per dish, and media and cells were harvested at the indicated times.

none was found in the "bottom" sedimenting fraction; <10% of total apo-C-III$_3$ and ~25% apo-E were found with LDL and HDL and 20–25% of each sedimented to the bottom fraction. On gel permeation chromatography (Fig. 4 and Table II), 40–70% of all quantified apoproteins eluted in the VLDL range; 25–50%

TABLE I
Cellular Apoprotein Content and Accumulation Rates of Apoproteins in the Media of Hepatocyte Cultures By Using a Fibronectin Matrix

Apoprotein	Cell (n = 4)	Media (n = 7)	Perfusate*
	ng/ml cell protein	*ng/mg cell protein hour*	*ng/mg cell protein hour*
Apo-B	244±55	33±5	42±19
Apo-C-III$_3$	61±7	24±3	60±27
Apo-E	392±105	162±32	190

* Based on published results (9, 11, 42) recalculated on the basis that 21% of the wet weight of tissue is protein as determined in our laboratory.

Results represent mean±1 SD (n = number of livers, 3–5 dishes/liver).

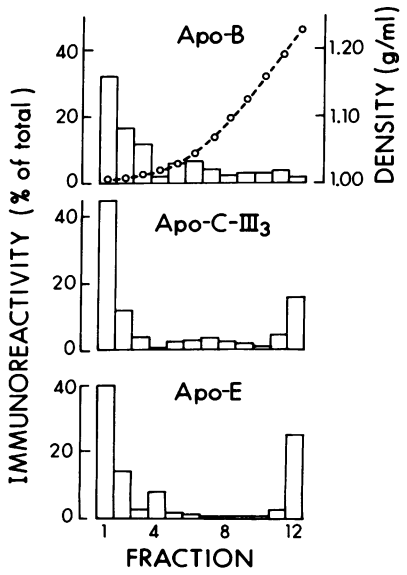

FIGURE 3 Distributions of apoproteins in hepatocyte media after density gradient ultracentrifugation. Hepatocytes were seeded on dishes precoated with fibronectin in the absence of added insulin. 6 h after plating, media were changed and cultures were incubated for an additional 18 h. Apoproteins were quantified by radioimmunoassays.

of apo-B and 10–20% of apo-C and apo-E eluted with LDL; ~20% of apo-B and 15–40% of apo-C and apo-E eluted with HDL; none of apo-B, 30% of apo-C-III$_3$, and <25% of apo-E eluted beyond albumin. In a separate experiment, upon density gradient centrifugation of plasma, 85% of apo-E was associated with lipoproteins and 15% of total apo-E appeared at the bottom of the tubes. This confirms the findings of others (53) that ultracentrifugation causes dissociation of apo-E from lipoproteins. However, upon gel permeation chromatography of plasma, none of the apo-E was found beyond albumin, rather all of the apo-E eluted with lipoproteins. In the same experiment, all of the plasma apo-C-III$_3$ remained associated with lipoproteins after both methods of lipoprotein separation. Therefore, it is unlikely that the elution of media apoproteins beyond HDL was due to artifacts of separation.

Effects of insulin on rates of accumulation of lipoprotein components. Increasing doses of insulin produced dose-dependent decreases in rates of accumulation in media of apo-B, apo-E, TG, and PL. In the experiment presented in Table III, at a dose of 800 μU/ml insulin, concentrations of TG, apo-B, and apo-E in the media were decreased to 56±6, 69±14, and

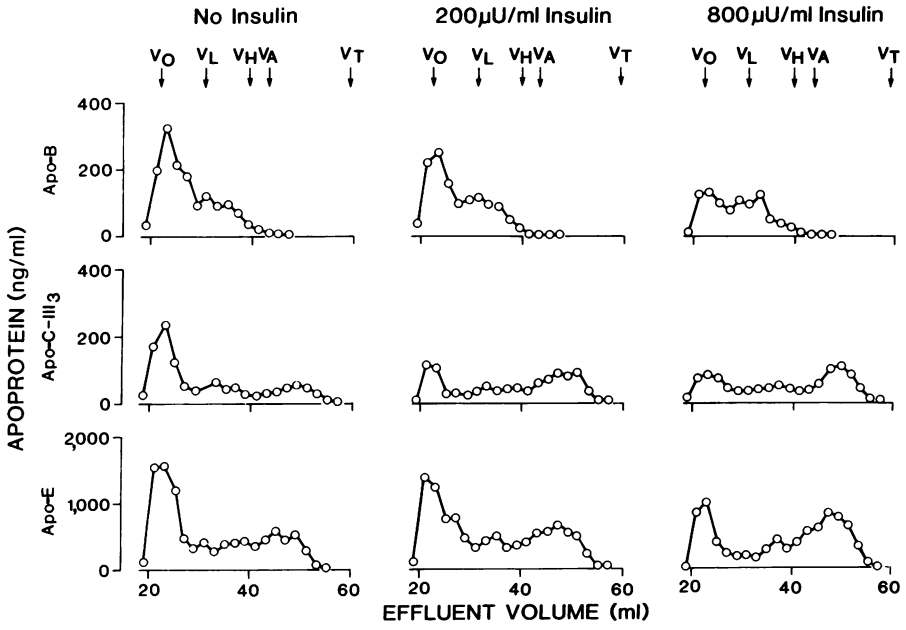

FIGURE 4 Effect of increasing doses of insulin on apoprotein distributions in hepatocyte culture media. Hepatocytes were seeded on a fibronectin matrix, media were changed 6 h later, the indicated amounts of insulin were added, and incubation was continued for another 16 h. Media from four dishes (2.1±0.2 mg cell protein/dish) were concentrated and applied to molecular sieving chromatography on Sepharose 6B. Apoproteins of indicated fractions were quantified by radioimmunoassays. The elution volumes of human VLDL (V_O), human LDL (V_L), human HDL$_3$ (V_H), bovine serum albumin (V_A), and ^{125}I (V_T) are indicated by arrows.

TABLE II

Effects of Insulin on the Distribution of Apoproteins in the Media of Cultured Hepatocytes

		Apoproteins											
		Apo-B				Apo-C-III$_3$				Apo-E			
Experiment	Insulin dose	VLDL	LDL	HDL	HSA	VLDL	LDL	HDL	HSA	LDL	LDL	HDL	HSA
	μU/ml						%						
A	0	43	27	29	—	40	16	11	33	71	10	13	6
	50	30	38	31	—	27	14	12	47	50	16	24	10
	200	24	40	36	—	21	18	12	48	43	21	24	11
	800	28	42	31	—	21	20	14	45	17	15	44	23
B	0	62	23	15	—	52	14	12	21	50	9	16	25
	200	57	27	16	—	30	11	17	42	42	11	17	30
	800	47	32	21	—	28	11	17	44	28	4	18	49
C	0	62	33	4	—	48	9	7	35	ND	ND	ND	ND
	800	24	58	17	—	30	17	3	50	ND	ND	ND	ND

The apoprotein contents of the column chromatographic fractions of Fig. 4 were used to derive the results presented above. Human VLDL eluted between volumes 20 and 28 ml, LDL 30 and 34 ml, HDL 36 and 42 ml, and albumin (HSA) 44 and 56 ml. For convenience, elution of media apoproteins are reported for the same pooled fractions. Results are sums of apoprotein contents of the respective pooled fractions divided by the total apoprotein recovered from the column × 100. Each experiment represents a cell preparation from one liver.

ND, not determined.

65±10% of controls (mean±1 SD, n = 3). Compatible results of several other experiments are summarized in Fig. 5. The amounts of total cellular protein and PL were not affected by insulin. However, there were insulin dose-dependent increases in cell-associated TG, apo-B, and apo-E, but since increases in cellular apoprotein levels did not compensate for decreasing rates of secretion, total mass of apo-B and apo-E in the sys-

TABLE III

Effect of Insulin on Cellular Contents and Accumulation of Lipids and Apoproteins into the Media

	Insulin added *μU/ml*			
	0	50	200	800
Cell				
Protein, *mg/dish*	1.65±0.1	1.71±0.1	1.68±0.2	1.69±0.2
TG, *μg/mg cell protein**	65±4	74±7	77±4‡	80±7‡
PL, *μg/mg cell protein**	190±5	201±14	201±21	200±45
Apo-B, *ng/mg cell protein*§	200±36	255±12	250±19	270±18‡
Apo-C-III$_3$, *ng/mg cell protein*§	63±5	64±6	58±7	70±8
Apo-E, *ng/mg cell protein*§	254±22	350±25‡	369±13‡	353±35‡
Media				
TG, *μg/mg cell protein**	46±2	41±2‡	39±3‡	26±4‡
PL, *μg/mg cell protein**	24±2	21±2	21±3	17±3‡
Apo-B§	512±26	399±19‡	372±22‡	358±10‡
Apo-C-III$_3$§	352±26	371±27	351±14	350±17
ApoE§	2,007±402	1,908±173	1,742±311	1,311±195‡

* Values were not corrected for amounts of PL present in LPDS in the media.

‡ $P < 0.05$.

§ Insulin was added 6 h after plating, incubation period was 16 h. Results from one liver preparation are means±SD of 2–4 dishes per data point (see also Fig. 5).

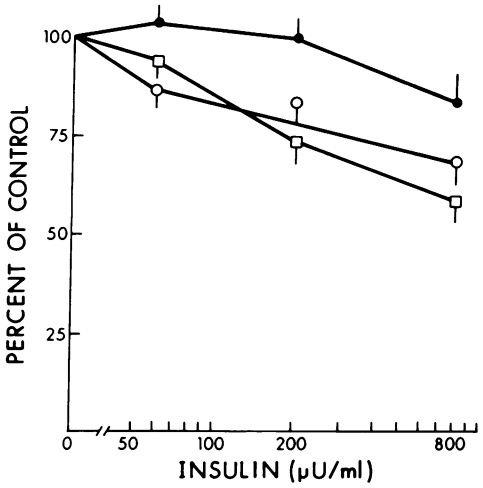

FIGURE 5 Insulin-induced inhibition of the accumulation of apoproteins in hepatocyte culture media. Results of experiments using four livers are summarized here. For each liver, each data point was determined in three dishes. Means±SE are shown. See Table III for a sample experiment in which typical absolute values are provided. O, apo-B; □, apo-E; and ●, apo-C.

tem decreased with insulin treatment. Cell and media levels of apo-C-III$_3$ were not greatly affected by insulin. At 800 μU/ml of insulin, apo-C-III$_3$ accumulation was 84±14% (n = 4) of zero insulin values.

While TG concentrations fell in response to insulin in media in each of four experiments (Table III and Fig. 5), the TG content of the entire system increased by 6% in one experiment, whereas in the three other experiments no significant change of TG mass was observed. The consistent change in partitioning of TG between media and cells due to insulin also was demonstrated in another series of experiments. 10 μCi of [2-^{3}H]glycerol was added to dishes 14 h after the administration of insulin. 2 h later, media and cells were harvested and the ratios of [^{3}H]glycerol incorporated into media and cell TG were determined. In control dishes receiving no insulin, the ratio of disintegrations per minute was 0.21±0.01. Administration of 50, 200, and 800 μU/ml insulin decreased the ratio to 0.16±0.02, 0.14±0.02, and 0.10±0.01 (mean±1 SD), and radioactivity associated with TG in the entire system was 13.4±1.7, 14.6±3.5, 17.0±1.2, and 16.3±3.3 dpm 10^3/mg cell protein, respectively.

To determine whether these inhibiting effects of insulin on the secretion of lipoprotein components were specific or merely the result of changes in the metabolic viability of cells, various other cell functions were studied. Media glucose concentrations were unchanged by the hormone (range of values were 402–410 mg/dl). Incorporation of [^{3}H]leucine into cellular and media proteins over 15 h remained constant (Table IV) and comparable with levels without insulin. Albumin accumulated in the media at unchanged rates. Urea accumulation was slightly decreased only at the highest insulin dose. Glycogen content of cells decreased by >60% over 15 h in the absence of insulin, but declined to a lesser extent in the presence of insulin, thus establishing the effectiveness of the hormone.

Effects of insulin on the association of lipoprotein components. Insulin decreased not only the concentrations of lipoprotein components in media but it also affected their distribution among lipoproteins. To demonstrate this, media of cells cultured in DME in the absence of LPDS with and without insulin were filtered in a column of Sepharose 6B. The A_{280} profile of the secreted products was altered by insulin (Fig. 6). Since absorbance due to light scattering is not easily converted to mass, TG and PL concentrations were determined in column fractions. Lipids were decreased, but only in the VLDL elution range, by >50% (Table V). In a second experiment, where only TG mass was determined, nearly identical results were obtained.

Distributions of the apoproteins among lipoprotein fractions in media also were altered by insulin in a dose-dependent fashion. On column chromatography (Fig. 4 and Table II), with the addition of insulin, the amounts of apo-B, apo-C-III$_3$, and apo-E eluting in the VLDL region decreased, while the proportions of apo-C-III$_3$ and apo-E eluting near albumin increased. The amount of apo-B eluting in the VLDL region decreased, while apo-B eluting in the LDL-HDL region stayed constant. Insulin administration also increased the proportions of apo-E in the bottom fraction upon density gradient centrifugation (Fig. 7).

Insulin could have altered lipid-apoprotein association in media by changing hepatocyte uptake or degradation of lipoproteins. To study this possibility, ^{125}I-VLDL (isolated from rat plasma and radioiodinated) was added to cultures. At all doses of ^{125}I-VLDL added, 92–100% of added radioactivity was recovered from the dishes (Table VI). However, the proportions of ^{125}I precipitable by TCA were significantly smaller in dishes that contained hepatocytes, indicating that hepatocytes did degrade VLDL. (Calculated rates were ~0.7 μg VLDL protein/20 h, which is 15% of the amount of VLDL protein secreted over the same time period.) Indeed, similar preparations of hepatocytes express high affinity binding sites for rat VLDL and HDL (unpublished observations). But no substantial differences in TCA precipitability of ^{125}I-VLDL were found between control and insulin-treated cultures, indicating that insulin did not affect overall ^{125}I-VLDL degradation. To assess whether exposure to cells al-

TABLE IV
Effects of Insulin on Various Cell Functions

Incubation time, *h*	0	—	6	—	—	15	—
Insulin added, *μU/ml*	0	0	200	800	0	200	800
Media							
[^{3}H]Leucine-TCA ppt, *dpm* × 10^{-3}/*mg cell protein*	2.2±0.3	23±7	23±6	25±4	57±8	54±8	50±7
Albumin, *μg/mg cell protein*	ND	12±2	14±2	12±1	25±2	27±4	23±3
Urea, *μg/mg cell protein*	35±3	ND	ND	ND	297±10	322±16	264±14*
Cell							
[^{3}H]Leucine-TCA ppt, *dpm* × 10^{-3}/*mg cell protein*	0.4±0.2	25±4	23±5	24±3	42±7	40±5	40±6
Glycogen, *μg/mg cell protein*	128±11	ND	ND	ND	44±12	54±14	64±9*

* $P < 0.05$. Media did not contain LPDS. Results are from one liver, three dishes per treatment, except for glycogen where there were five dishes per treatment; means±SD.
ND, not determined; ppt, precipitate.

tered the distribution of ^{125}I radioactivity in VLDL, media were chromatographed on Sepharose 6B and TCA-precipitable counts were quantified (Fig. 8). Media taken from dishes without any cells and media of hepatocyte cultures with or without insulin yielded similar elution patterns of TCA-precipitable ^{125}I-VLDL. The constancy of elution profiles, especially the lack of increase of TCA-precipitable ^{125}I-materials eluting near albumin, argues against the elaboration of "lipid-free" apoproteins during degradation of ^{125}I-VLDL. Media also were chromatographed on Sepharose 2B. Here too the elution profiles of ^{125}I-VLDL appeared to be unaffected whether or not hepatocytes were incubated in the presence of insulin (not shown). To examine radioactivity in ^{125}I-VLDL apoproteins by another technique, media also were analyzed by SDS polyacrylamide gel electrophoresis (10% gels, ref. 37); areas corresponding to apo-B and apo-E were cut out and counted. The respective ratios of counts (^{125}I-apo-B/^{125}I-apo-E) were 1.4, 1.3, and 1.4, in media exposed to no cells and in media taken from insulin-untreated and -treated hepatocytes, indicating no selective uptake of these apoproteins by cells. Thus, insulin appears not to have influenced the hepatocyte uptake of VLDL isolated from plasma. It is therefore unlikely that insulin exerted its effect on the association of apoproteins with lipids in media by altering the hepatocyte uptake or catabolism of VLDL.

In another set of experiments, we examined whether nascent VLDL not processed in plasma in vivo, was metabolized differently by insulin-treated and non-insulin-treated hepatocytes. Endogenously labeled VLDL was prepared in hepatocyte cultures. Labeling

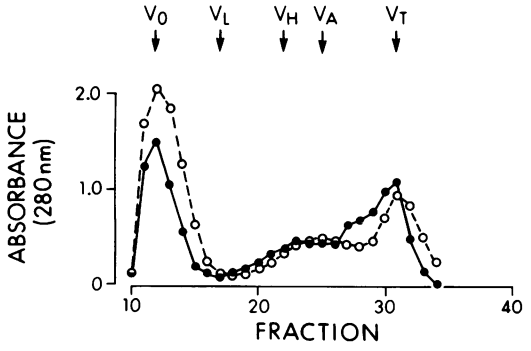

FIGURE 6 Absorbance at 280 nm of media of control cultures (○) and cultures treated with 800 μU/ml insulin (●). Dishes were incubated for 16 h in the absence of LPDS. Media obtained from 32 mg of cell protein were concentrated and chromatographed on Sepharose 6B. Each column fraction contained 2 ml. Elution volumes are as in Fig. 4.

TABLE V
Effect of Insulin on Media Lipids

	TG				PL			
Insulin	VLDL	LDL	HDL	HSA	VLDL	LDL	HDL	HSA
μU/ml								
0	231	29	23	<15	32	12	16	<10
800	108	51	29	<15	12	10	10	<10

Media of cultures incubated for 16 h were chromatographed on Sepharose 6B (Fig. 6). Results are expressed as micrograms lipid per 10 mg cell protein recovered in column fractions corresponding to elution volumes of VLDL, LDL, HDL, and HSA. For this experiment cells were grown in DME in the absence of LPDS.

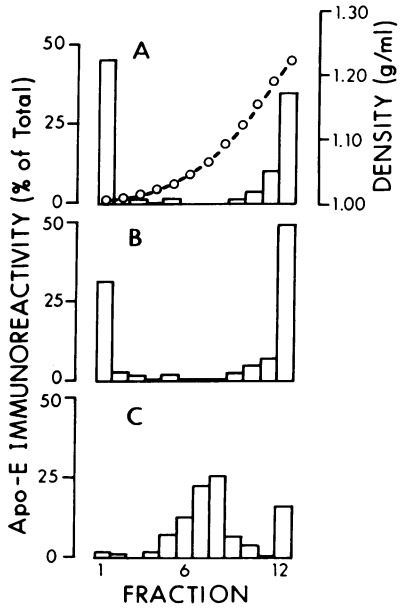

FIGURE 7 Distribution of apo-E immunoreactivity after (A) gradient centrifugation of 2.5 ml media of control cultures, (B) cultures supplemented with 800 μU/ml insulin, and (C) normal rat plasma shown for comparison.

was carried out with [^{14}C]acetate, both in the presence and absence of insulin, and ^{14}C-labeled VLDL isolated from both kinds of cultures were incubated with both

TABLE VI
Effect of Insulin on Degradation of ^{125}I-VLDL in Primary Rat Hepatocyted Cultures

	Radioactivity recovered				
		Hepatocyte culture without insulin		Hepatocyte culture with 800 μU/ml insulin	
^{125}I-VLDL added	Control media no cells	Media	Cell	Media	Cell
μg VLDL protein/dish		%			
0.7	92	89	6	87	6
	(91)	(67)	(89)	(63)	(88)
1.4	97	92	5	96	4
	(88)	(69)	(92)	(67)	(89)
2.8	96	88	4	90	4
	(87)	(72)	(91)	(71)	(88)
7.0	95	89	3	91	3
	(87)	(73)	(90)	(76)	(89)

Numbers in parentheses represent the percentages of radioactivity precipitable by 10% TCA. Specific activity of VLDL was 50 cpm/ng protein; from 3.5×10^4 to 3.5×10^5 cpm/dish were added. 96% of ^{125}I-VLDL counts were initially TCA precipitable. Cultures or control media were incubated with ^{125}I-VLDL for 20 h.

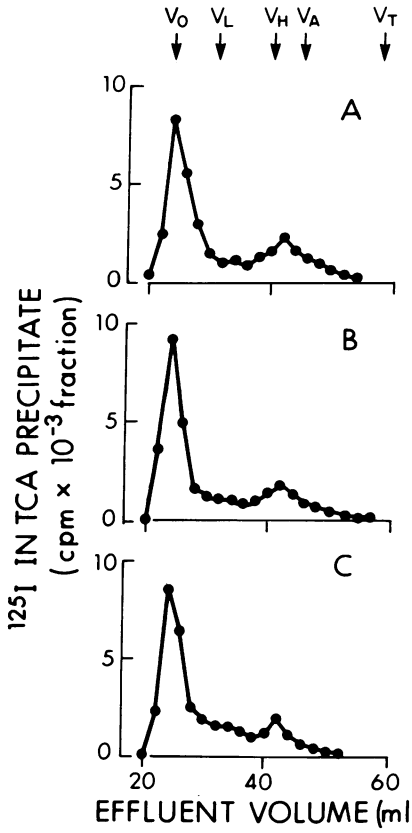

FIGURE 8 Effect of incubation of ^{125}I-VLDL with cultured hepatocytes on its chromatographic elution profile; 2.8 μg of ^{125}I-VLDL protein (50 cpm/ng protein sp act) were added per culture dish and 20 h later 2-ml aliquots of media were analyzed by gel permeation chromatography in Sepharose 6B. Radioactivity in the column effluent was determined after precipitation by 10% TCA. ^{125}I-VLDL was incubated (A) with culture media in the absence of cells; (B) with hepatocytes without insulin, and (C) with hepatocytes and 800 μU/ml insulin. Elution volumes are as in Fig. 4.

insulin-treated and non-insulin-treated hepatocytes. Under all conditions, media radioactivity decreased with time and cell-associated radioactivity increased (Fig. 9). Recovery of added [^{14}C]VLDL radioactivity in media and cellular lipids at 0 h was 95 and 82% at 12 h, suggesting that some of the labeled lipid that had been taken up by cells was converted to nonlipid extractable compounds. [^{14}C]VLDL obtained either from insulin- or non-insulin-treated cultures disappeared a little more rapidly from media containing 800 μU/ml of insulin. This was expected because the concentration of VLDL in media of insulin-treated cells was lower than non-insulin-treated media and therefore the dilution of a given amount of added [^{14}C]VLDL by secreted VLDL was lower. When uptake of VLDL was calculated by taking these differences into account

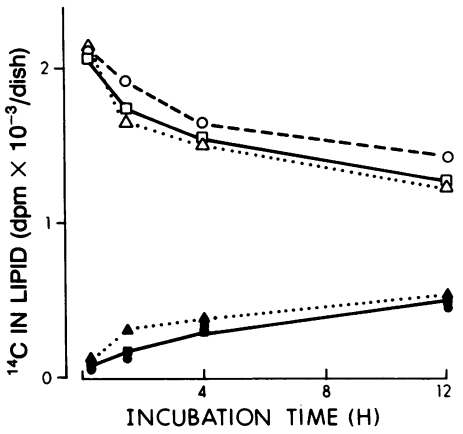

FIGURE 9 Time course of media (open symbols) and cell-associated (filled symbols), lipid-extractable radioactivity after addition of endogenously labeled [^{14}C]VLDL. ○, control VLDL prepared from cultures in the absence of insulin was incubated with control cultures; □, control VLDL incubated with cultures receiving 800 μU/ml insulin; △, VLDL prepared from cultures receiving 800 μU/ml insulin and incubated with cultures supplemented with 800 μU/ml insulin. Incubation was in 30-mm dishes containing 1.1 mg of cell protein in 1.5 ml of media.

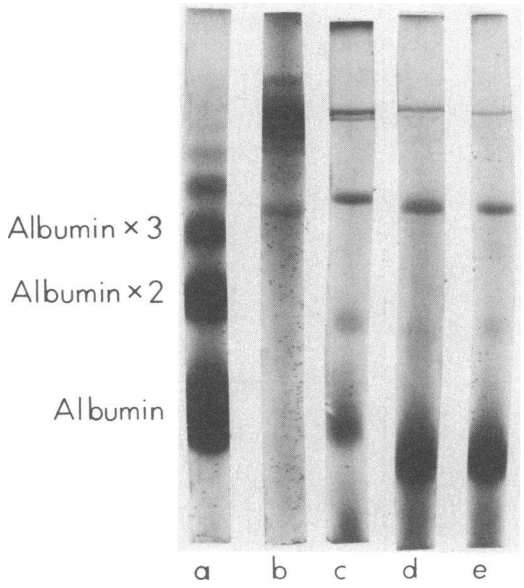

FIGURE 10 Apo-B subspecies of hepatocyte media lipoproteins. SDS electrophoresis in 3% acrylamide gels of (a) molecular weight standards, (b) rat plasma LDL, (c) rat plasma VLDL, (d) lipoproteins of hepatocyte media not containing insulin, and (e) lipoproteins of hepatocyte media containing 800 μU/ml insulin. Plasma LDL was isolated by zonal ultracentrifugation, plasma VLDL (d = 1.006) and hepatocyte media (d < 1.21) were isolated by fixed angle head ultracentrifugation. Hepatocyte media were harvested after an incubation period of 20 h. Albumin was cross-linked by using dimethylsuberimidate.

(see Appendix), uptake of VLDL-TG over the 12-h incubation period was 3.2 and 2.6 μg/mg cell protein in control and insulin-treated cultures. Uptake of VLDL-TG labeled in insulin-treated cultures by cells cultured in 800 μU/ml insulin was 2.7 μg VLDL-TG/mg cell protein. These values may only represent an approximation, since calculations were based on the assumption that VLDL-lipids are taken up at the same rate. However, uptake studies with endogenously labeled VLDL (Fig. 9) are compatible with uptake studies using exogenously labeled VLDL (Table VI) and strongly suggest that the decreased accumulation of VLDL in media after insulin supplemented reflects decreased secretion rather than increased uptake of secreted VLDL. Similar conclusions were reached by Durrington et al. (18) who studied uptake of VLDL by hepatocytes by using endogenously labeled [^{3}H]-VLDL.

In contrast with its effect on apoprotein association in media, insulin treatment did not affect the relative proportions of apo-B subspecies in the lipoproteins of the media (Fig. 10). 84% of the apo-B both in insulin-treated and insulin-free cultures consisted of the smaller B-48 subspecies and 16% of apo-B mass appeared in the larger B-100 subspecies (as determined by integration of dye uptake after electrophoresis in 0.1% SDS, ref. 51).

Finally, the reversibility of the observed hormonal effects was tested (Table VII). After exposure of cells to 800 μU/ml insulin for 15 h, cells were washed three times over a period of 30 min. These washes reduced the immunoassayable content of insulin per dish more than 600-fold (data not shown). Accumulation of TG in the media of these cultures during a subsequent incubation period of 8 h was similar to accumulation rates in dishes that had not been exposed to added insulin. Furthermore, ultracentrifugal distribution of

TABLE VII
Reversibility of Insulin Effects on Triglyceride Secretion

Insulin added		TG	
Preincubation	Incubation	Media	Cell
U/ml		*g/mg cell protein*	
0	0	18.9±1.9	75±7
0	800	13.8±2.0	84±6
800	0	19.9±2.7	76±8

Cells were washed three times after the preincubation period of 15 h. Incubation period was 8 h. During preincubation with 800 μU/ml, insulin decreased the accumulation of TG in the media to 67% of controls. Results are means±SD (n = 4 dishes).

apo-C-III$_3$ in the media of these cultures also was similar to cultures that had not been preincubated with added insulin (not shown). Thus, the observed effect of insulin on lipid secretion and lipid apoprotein association appeared to be reversible.

DISCUSSION

In cells cultured in insulin-free media on fibronectin-coated dishes, apo-B, apo-C-III$_3$, and apo-E accumulated linearly in culture media over 20 h at rates that were similar to values for perfused livers (9, 11, 42). Since <15% of secreted VLDL was degraded, it is likely that rates of accumulation approach rates of secretion.

In the absence of insulin, the majority of the measured apoproteins appeared to be associated with lipoproteins (Tables II and V, Figs. 4 and 7), but appreciable amounts of apo-C and apo-E appeared not to be associated with lipoproteins. The distribution of apoproteins among lipoproteins in culture media was comparable with their distributions in liver perfusates as judged by molecular sieving chromatography and gradient ultracentrifugation. Hepatocyte cultures contain bile acids but hepatic perfusates do not. Therefore, it is unlikely that accumulation of bile acids into the culture media in any way affected the distribution of apoproteins in the media. This conclusion also is supported by the nearly identical chromatographic pattern of exogenous ^{125}I-VLDL added to culture dishes containing cells and media, and dishes containing media alone (Fig. 8). Furthermore, gel permeation chromatographic profiles of media apoproteins harvested after a 4-h incubation period were nearly identical with patterns produced by media harvested after 16 h of incubation (data not shown). Bile acids are thought to accumulate linearly with time at rates of 0.05 nmol/mg protein h (15). Yet the higher bile acid concentrations presumably reached by 16 h of incubation apparently did not alter apoprotein distribution. Indeed, the concentration of bile acids even at 16 h would be expected to be well below their critical micellar concentration.

Insulin at near physiologic levels produced a dose-dependent inhibition of accumulation of TG, PL, total apo-B, B-100 and B-48, and apo-E in the culture media, and concomitantly increased the cellular contents of TG and apoproteins. Since insulin did not affect the hepatic degradation of ^{125}I-VLDL significantly (Table VI) and intracellular levels of apoproteins rose (Table III), decreased accumulation rates probably reflect decreased rates of secretion of these components into the media. Total amounts of the above lipoprotein components in the system fell, suggesting either that synthesis rates also fell, or that intracellular degradation of nonsecreted apoproteins rose. We cannot distinguish between these possibilities. Intracellular and media levels of apo-C-III$_3$ were not affected.

It should be noted that the presence of two or three apo-B subspecies in rat plasma, hepatic perfusates, and hepatocyte media (54–56) presents a potential source of error in assessing accumulation rates of apo-B by our radioimmunoassay. The LDL used as a standard in the assay contained 95% B-100 and 5% B-48. In contrast, hepatocyte culture media contained only 15% B-100. Furthermore, part of the immunoreactivity of apo-B may be masked in rat VLDL as was reported for apo-B in human VLDL (57, 58). Although immunological crossreactivity between various apo-B subspecies in the rat has been demonstrated that could mitigate these effects (refs. 54, 55, and unpublished observations), our assay nevertheless could underestimate the accumulation of apo-B. Despite these limitations, the experiments clearly demonstrate that increasing doses of insulin decreased the mass of secreted apo-B (and the proportion of apo-B associated with VLDL). The assays for apo-C-III$_3$ and apo-E do not have these potential problems.

The present results confirm and extend the observations of Durrington et al. (18) who noted in a similar cultured hepatocyte system that insulin produced a dose-dependent inhibition of VLDL-TG secretion. Inhibitory effects on secretion were noted at glucose concentrations ranging between 5 and 25 mM. The present experiments were carried out at 25 mM glucose where maximal inhibition of TG secretion was noted (18). Both mass and radioactivity data clearly demonstrated inhibition of secretion of TG. These results of course do not exclude stimulation of de novo fatty acid synthesis by insulin, which makes a small contribution to TG mass under favorable substrate conditions (59).

The insulin-induced inhibition of secretion of lipoprotein components was accompanied by altered associations of lipids and apoproteins in media. The amounts and proportions of apo-C-III$_3$ and apo-E eluting with lipoproteins decreased, and the proportions eluting near albumin increased. The relative amounts of apo-B eluting with VLDL fell and apo-B eluting with LDL rose. It is assumed that the apo-B, apo-C, and apo-E eluting in the lipoprotein range are associated with the respective lipoproteins. The molecular associations, if any, of the apo-C and apo-E eluting near albumin are unknown. The detailed characterization of the products of the cultured hepatocytes is the subject of future reports. The finding of apo-B in the elution position of LDL is compatible with the demonstrated ability of liver to secrete LDL-like particles directly (60–62).

Control experiments in which ^{125}I-VLDL hepatocyte processing was measured indicated that decreased

VLDL accumulation and the altered lipid association of media apoproteins could not have arisen from insulin-induced postsecretory processing of VLDL. Therefore, it is likely that insulin inhibited VLDL secretion, perhaps by reducing the lipid apoprotein association at some intracellular step before secretion. The disparate effects of insulin on individual apoproteins suggests that insulin may affect the assembly of lipoprotein particles, perhaps by inhibiting the association of lipid with apoproteins. However, this is an area that needs further investigation.

The pattern of inhibition produced by insulin differs from the inhibition of VLDL secretion produced by increased medium viscosity (25). In the latter experiments, accumulation of apo-C-III$_3$ and apo-B in the medium was inhibited equally and to a greater extent than the accumulation of apo-E, and there was no accumulation of TG or apoproteins within hepatocytes. The individual effects of the two inhibitors suggest that each acts via a different mechanism and neither acts as a general inhibitor of cell function.

There is disagreement on the role of insulin in the regulation of VLDL or TG secretion by the liver. Both stimulation (62–65) and inhibition (66–68) of TG or VLDL secretion have been reported. Problems inherent in ascertaining the unmodified effects of insulin in vivo are numerous as counter regulatory hormones and changes to metabolite concentration may make interpretations difficult. Differing degrees of insulin sensitivity or resistance may further confound in vivo observations. It will be interesting to see whether the effects of insulin reported in this paper are insulin receptor mediated.

APPENDIX

Uptake of VLDL-TG/mg cell protein over the 12-h period of observation was calculated by the formula:

$$\frac{dpm_{tn} - dpm_{tn+1}}{dpm_{tn}} \times \left[\frac{(TG_{tn} + TG_{tn+1}) \times 0.8}{2} + \frac{(dpm_{tn} + dpm_{tn+1}) \times 0.7}{2 \times \text{specific radioactivity}} \right] \times \frac{1}{\text{milligram cell protein/dish}},$$

where dpm_{tn} or dpm_{tn+1} is disintegrations per minute in media/dish at times (t)n or $n + 1$ ($n = 0$, 90, 240; $n + 1 = 90$, 240, 720 min); TG_{tn} or TG_{tn+1} is mass of TG in media/dish at time n or $n + 1$; TG masses at given time intervals (0–90, 90–240, and 240–720 min) were calculated from accumulation rates. Protein is mass cellular protein/dish. The factor 0.8 is based on the observation that 80% of TG was found in VLDL in non-insulin-treated cultures. Although the proportion of TG in VLDL in insulin-treated cultures was less, it was assumed that 80% of TG is in particles capable of competing with exogenous VLDL. The factor of 0.7 was included to correct for the proportion of disintegrations per minute present in TG. Uptake over the entire period of observation was obtained by summing the uptake over the three time periods (0–90, 90–240, and 240–270 min).

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