Effect of Fatty Acids on Lipid and Apoprotein Secretion and Association in Hepatocyte Cultures

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ABSTRACT Increasing availability of free fatty acids (FFA) to liver results in enhanced rates of secretion of triglycerides in lipoproteins. However, as FFA uptake increases, triglyceride secretory rates reach a plateau and esterified fatty acids accumulate intracellularly, suggesting that something is limiting lipid transport out of the liver. One possibility could be the limited availability of apoproteins. To test this hypothesis, primary rat hepatocytes in culture were incubated with increasing amounts of FFA (0–2.1 μmol/dish) and the amounts of lipids and apoproteins inside the cells and in culture media were measured; the latter by specific radioimmunoassays. Media also were fractionated on Sepharose 2B and 6B columns and the elution profiles of apoproteins were obtained. With exposure to increasing amounts of free fatty acids, hepatocytes took up more fatty acids and intracellular levels of triglycerides rose (from 71 to 146 μg/mg cell protein). Concomitantly, media triglycerides nearly doubled (31 to 51 μg/mg). Incorporation of [^{3}H]glyceride into cellular and media triglyceride also rose. However, levels of apoproteins A-I, B, C-III$_3$, and E in cells and media were unchanged. The increasing amounts of triglycerides in media were present in larger particles, as demonstrated on gel permeation chromatography. The elution profiles of apoproteins B, C-III$_3$, and E were altered in that a greater proportion of the apoproteins eluted with larger particles. Similar results were obtained when hepatocytes were preloaded with increasing amounts of FFA over 12 h and analyses of cells and media were carried out 8 and 22 h after removal of fatty acids from the media. During loading of cells, accumulation of cellular triglycerides was directly related to media FFA concentrations. During unloading, triglyceride secretory rates were related to cellular triglyceride levels. At higher triglyceride secretory rates larger particles were secreted and a greater proportion of apoproteins was associated with the larger particles, but total amounts of apoproteins in the system did not change. These data lead us to suggest that enhanced rates of apoprotein synthesis need not occur in the response to acute changes in hepatic lipid transport, rather, increased secretion of lipid is brought about by augmented intracellular lipid apoprotein association.

INTRODUCTION

Free fatty acids (FFA) are rapidly removed from the plasma compartment virtually by all tissues (1). In the liver, FFA are converted to fatty acyl CoA, which are then either partially or wholly oxidized or esterified to form triglycerides (TG),[1] phospholipids (PL), and other fatty acyl esters (2). The rate of esterification of fatty acids and the formation of TG in liver depend upon the rate of hepatic uptake of FFA, i.e., esterification rates appear to be nonsaturable (3). Increased formation of lipids results in intracellular storage, lipolysis of excess lipid, and increased secretion of lipid into the circulation via lipoproteins (4). Concomitantly, the output of TG by livers also increases (5–9); however, in contrast with intracellular esterification rates, secretion of TG does reach a plateau. It is not clear whether the limitation on TG secretion is determined by the availability of apoproteins at the site of lipoprotein assembly or at some point beyond, e.g., the storage or secretory processes. However, if availability of apoproteins is important, either increased rates of apoproteins synthesis or the association of more lipids with a given amount of apoprotein, or both would al-

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[1] *Abbreviations used in this paper:* apo A-I, apo B, apo C-III$_3$, and apo E, apoproteins A-I, B, C-III$_3$, and E; B$_L$ and B$_S$, large and small subspecies of apo B; DME, Dulbecco's modified Eagle's medium; LPDS, lipoprotein-deficient serum; PL, phospholipids; TG, triglycerides.

low for increased secretion of lipoprotein-TG by hepatocytes. To discern which, if either, of these two processes occur during an acute stimulus to hepatic TG secretion we studied the effect of oleic acid administration on lipid and apoprotein secretion and lipid protein association in primary rat hepatocyte cultures. Parts of the results have been presented (10).

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 at 9 a.m. and animals were anesthetized 3 h later with sodium pentobarbital (5 mg/100 g). Hepatocytes were prepared according to the method of Berry and Friend (11). Livers were perfused ex situ with oxygenated Krebs-Ringer bicarbonate buffer without Ca^{++} at 37°C, pH 7.4, containing 50 mg of collagenase (Type IA, Sigma Chemical Co., St. Louis, MO). Hepatocytes were plated on a fibronectin matrix (12, 13) at a cell density of $2–3 \times 10^6$ hepatocytes/60-mm dish in arginine-free Dulbecco's modified Eagle's medium (DME, Grand Island Biological Co., Santa Clara, CA) which was supplemented with 0.4 mM *l*-ornithine, 25 or 5 mM glucose, 10 mM Hepes, 100 U/ml penicillin, 100 mg/liter streptomycin, and 7 or 10% lipoprotein-deficient serum (LPDS). 6 h after plating, cells were washed with saline G, and new media of the same composition were added along with various doses of oleic acid complexed to albumin. Equal volumes of sodium oleate (16 mg/ml) dissolved in saline at 70°C were mixed with 20% bovine serum albumin previously defatted (14) at 4°C. The emulsion was quickly filter sterilized through 0.45-μm Millipore filters (Millipore Corp., Bedford, MA). The molar ratio of oleate to albumin was ~18, and between 0 and 80 μl of the suspension was added per dish along with 40–0 μl of 20% albumin to keep the amount of albumin added constant between control and experimental dishes. Considering the albumin content of LPDS present in the media (~0.34 nmol/dish), molar ratios of fatty acids to albumin were between 2.4 and 9.7 at initiation of experiments, depending on the dose of oleic acid added. Fatty acid levels were below 30 μM in the LPDS-containing media. After various incubation periods, media were harvested and cells were detached with Ca^{++}- and Mg^{++}-free saline buffered with phosphate and using a rubber policeman. Dislodged cells were centrifuged at 1,000 *g* for 5 min, and the cellular pellet was resuspended in 0.05 M sodium barbital buffer, 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 in the presence or absence of 0.5% (g/vol) Triton X-100, and supernatants of homogenates were prepared by ultracentrifugation at 100,000 *g* for 60 min.

Chemical determinations included cellular protein (15), media and cellular PL (16), media glucose (Beckman glucose analyzer II, Beckman Instruments, Inc., Palo Alto, CA), media fatty acids (17), media and cellular TG (Triglyceride Kit, C. F. Boehringer and Sons, Mannheim, West Germany) and media β-hydroxybutyrate and acetoacetate (18, 19). Incorporation of [2-^{3}H]glycerol and [1-^{14}C]acetate (ICN Pharmaceuticals, Inc., Irvine, CA) into cell and media lipids was determined by analyses of appropriate spots following thin-layer chromatography of lipid extracts (20, 21). 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% trichloroacetic acid (TCA). Rat serum albumin accumulating in the media was measured from 10- to 15-μl aliquots by immunoelectrophoresis (22). Rat albumin fraction V (Cappel Laboratories, Cochranville, PA) was used as a standard.

Levels of apoproteins (apo) B, C-III_3, A-I, and E were measured by radioimmunoassays (23–27). Supernatants of cellular pellets, obtained by sonication in the presence of 0.5% Triton X-100 and subsequent ultracentrifugation at 100,000 *g* for 60 min, and culture media both produced parallel displacement curves when compared with purified standards. The validity of these assays in the hepatocyte culture system has been described (12, 27).

To assess lipoprotein distribution of media apoproteins, incubation media were concentrated three- to fourfold by using a Millipore cell (Millipore Corp.) equipped with a pellicon membrane (PTGC 04710) and operated at 5–12 psi. Alternatively, incubation media were concentrated by using dialysis tubing (Spectrapor, mol wt cut-off 3,500, Los Angeles, CA) embedded in Sephadex G-100. Concentrated hepatocyte media were subjected to molecular sieving chromatography on Sepharose 6B and 2B (Pharmacia, Uppsala, Sweden). Columns were eluted with 0.15 M saline, containing 1 mM EDTA, pH 8.2. Columns were calibrated with human very low density lipoprotein (VLDL), low density lipoprotein (LDL), high density lipoprotein $(HDL)_3$, albumin, and ^{125}iodine. The Sepharose 2B column was calibrated with VLDL of defined flotation rates and Stokes radii (28). Recoveries of immunoreactivity ranged from 80 to 105% of immunoreactivity loaded onto the columns.

Incorporation of [^{3}H]leucine into media apo B subspecies was determined by sodium dodecyl sulfate (SDS) electrophoresis in 3% polyacrylamide gels (29). After incubation of hepatocytes with 80 μCi [^{3}H]leucine/100-mm dish for 16 h in the presence or absence of added oleic acid, media lipoproteins were isolated at a density of 1.21 g/ml by ultracentrifugation (30). 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 (31) was used as molecular weight standard. Gels were sliced into 5-mm pieces, digested with 30% hydrogen peroxide, and radioactivity was determined.

RESULTS

The time course of TG accumulation in media and cells in response to supplementation with fatty acid was studied first (Fig. 1). After the addition of a single dose of 1.8 μmol of oleic acid per dish, fatty acid levels in the media decreased at rates resembling first order kinetics, indicating the occurrence of cellular uptake. TG accumulation in the media of these cells was linear and exceeded that of control cells by 50%. Cellular TG content increased by 100%. Addition of a second dose of 1.8 μmol of oleic acid 5 h after the initial dose resulted in peak fatty acid levels of 800 μM and fatty acid uptake thereafter. Cellular TG content increased further, but TG accumulation in the media did not exceed the accumulation rates achieved by cultures that had received only one dose of fatty acid. Thus,

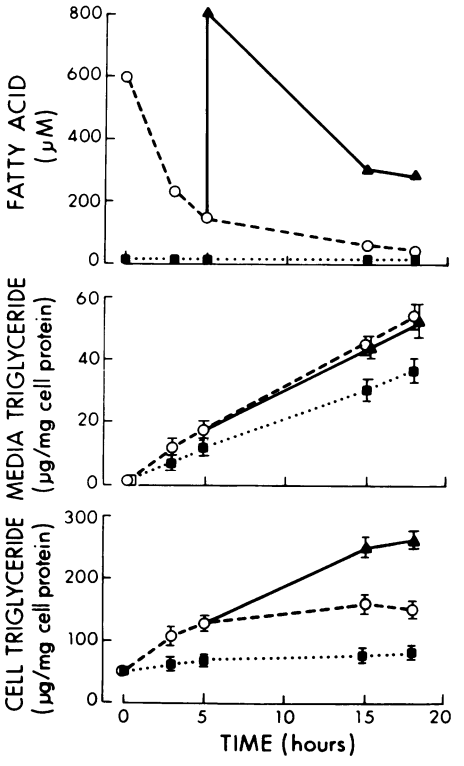

FIGURE 1 Effect of one dose of oleic acid (1.8 μmol/dish, - - -) and a second dose (——) 5 h after the first dose on the time course of media fatty acid levels (upper panel), media (middle panel), and cell triglyceride (lower panel). Control cultures, receiving no fatty acids, are shown by the dotted line. Media contained 23 mM glucose.

synthesis of TG was stimulated by the second addition of fatty acid, but saturation of TG secretion already had been reached by the first dose. In the same experiment, levels of apo B and apo C-III$_3$ in the media increased linearly with time, but no differences in accumulation rates were observed between control and fatty acid-treated cultures (Fig. 2). (Apo C-III$_3$ levels in the media increased linearly with time at a rate of 26±3 ng/mg cell protein per hour.)

The responses of media and cell TG levels to increases in media FFA concentration were determined by adding 0–2.1 μmol of oleic acid/dish to hepatocyte cultures (Table I). With increasing doses of fatty acid, cellular TG content increased. Maximal TG accumulation into media was achieved by addition of 1.5 μmol of oleic acid/dish. Incorporation of [^{14}C]acetate into media and cell TG decreased as a function of fatty acid dose administered, indicating a decrease in endogenous fatty acid synthesis or a decrease in the incorporation of endogenous FA into TG. [^{3}H]glycerol incorporation into TG was increased in response to fatty acid treatment. In a similar 16-h experiment (not shown in Table I), where [^{3}H]glycerol was added along with oleic acid at the beginning of the experiment (rather than 4 h before the end as in Table I), [^{3}H]glycerol in media TG averaged 62 dpm/μg cell protein in dishes containing no added oleate, and 340 dpm/μg in dishes containing 2 μmol of oleate/dish; respective cellular TG radioactivities were 52 and 805 dpm/μg cell protein. Cellular and media apoprotein contents, as well as rates of accumulation of albumin, were not increased by administration of oleic acid. At the highest dose of FA administered, uptake of FA as calculated from the differences of the FA dose added and media FA levels at harvesting, exceeded the amount of FA found in media and cellular TG by 44%, indicating that oleate also had entered other major pathways of FA utilization. This also was demonstrated in a subsequent experiment, conducted in 23 mM glucose (Table II). Accumulation of ketone bodies was positively correlated with the dose of fatty acid administered, partially accounting for the difference in FA uptake and the amount of fatty acid present in media and cell TG. Here too, addition of FA increased accumulation of media TG, and the maximal accumulation rates were similar to those found in low glucose media. Accumulation of apoproteins was not affected. Increased accumulation of media TG in response to oleic acid supplementation (1.6–2.0 μmol/dish) was a consistent finding and amounted to 162±15% (n = 5) of accumulation in control hepatocyte cultures.

In the experiments described we utilized ratios of FA to albumin that were above the physiologic range to achieve maximal lipid synthesis by hepatocytes,

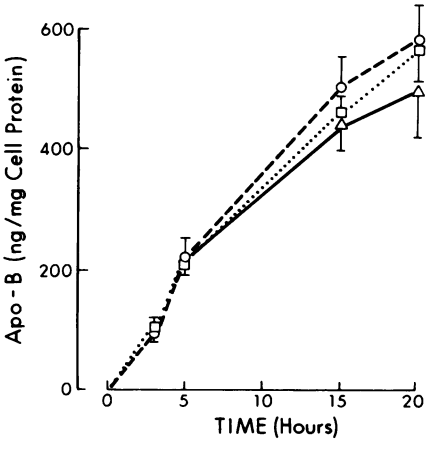

FIGURE 2 Accumulation of apo B as determined by radioimmunoassay in hepatocyte media of control cultures (□), after addition of 1.8 μmol of oleic acid/dish at 0 h (○), and supplementation with oleic acid at 0 and 5 h (△). Experimental conditions are as in Fig. 1.

TABLE I

Effect of Oleic Acid Administration on Lipid and Apoprotein Levels in Primary Rat Hepatocyte Cultures

	Oleic acid added				
			μmol/dish		
	0	0.53	1.06	1.6	2.1
Cell protein (*mg/dish*)	2.0±0.1	1.9±0.2	2.0±0.3	1.8±0.2	2.0±0.2
FA levels (*mM*)	0.02	0.03	0.03	0.07	0.09
Cell TG (*μg/mg*)	71±2	90±6*	93±7*	125±14*	146±7*
Media TG (*μg/mg*)	31±3	42±1*	44±4*	56±4*	51±2*
[^{3}H]Glycerol in cell TG (*dpm* × 10^{-3}/*mg*)	151±25	271	ND	400	542±115
[^{3}H]Glycerol in media TG (*dpm* × 10^{-3}/*mg*)	15.1±1.6	28	ND	33	28.5±2.0
[^{14}C]Acetate in cell TG (*dpm* × 10^{-3}/*mg*)	4.6±0.5	4.4	ND	3.0	1.8±0.4
[^{14}C]Acetate in media TG (*dpm* × 10^{-3}/*mg*)	8.3±0.7	4.3	ND	1.9	0.3±0.1
Apo B in cells (*ng/mg*)	232±10	245±18	222±16	253±55	220±33
Apo B in media (*ng/mg*)	411±48	455±26	377±143	435±51	361±10*
Apo C-III$_3$ in cells (*ng/mg*)	56±3	61±7	57±7	64±4	52±4
Apo C-III$_3$ in media (*ng/mg*)	383±11	390±24	360±63	411±16	342±8*
Albumin in media (*μg/mg*)	28±2	27±1	26±3	27±2	23±2*

Incubation was for 16 h in DME (see Methods), 5 mM glucose, and 7% LPDS. Results are expressed as micrograms, nanograms, or disintegrations per minute per milligram of cell protein and are means±SD of triplicate dishes or means of duplicate dishes. [^{3}H]glycerol (10 μCi/dish) and [^{14}C]acetate (2.5 μCi/dish) were added 12 h after or at the start of the incubation. ND, not determined.

* $P < 0.05$ of control dishes.

TABLE II

Effect of Oleic Acid Administration on Levels of Ketone Bodies, Lipids, and Apoproteins in Primary Rat Hepatocyte Cultures

	Oleic acid added		
		mol/dish	
	0	0.9	1.8
Protein (*mg/dish*)	2.4±0.2	2.5±0.2	2.4±0.4
FA levels (*mM*)	0.023	0.025	0.035
Cell TG (*μg/mg*)	65±8	101±5*	120±9*
Media TG (*μg/mg*)	39±2	52±3*	60±4*
Cell PL (*μg/mg*)	162±18	ND	167±35
Media PL (*μg/mg*)	16±3	17±4	19±3
TCA-precipitable [^{3}H]leucine‡			
In cells (*cpm* × 10^{-3}/*mg*)	37±8	ND	36±6
In media (*cpm* × 10^{-3}/*mg*)	53±8	ND	50±8
Glucose (*mg/100 ml media*)	422	426	420
β-OH-butyrate (*μmol/mg*)	1.185±0.118	1.338±0.105	1.449±0.035*
Acetoacetate (*μmol/mg*)	0.353±0.025	0.538±0.025*	0.599±0.114*
Apo B in media (*ng/mg*)	407±53	369±54	396±37
Apo E in media (*ng/mg*)	2,761±177	2,637±350	2,661±340
Apo A-I in media (*ng/mg*)	127±13	149±12	133±42

Incubations were for 16 h in DME, 23 mM glucose, and 7% LPDS. Results, expressed as nanograms, micrograms, micromoles, or counts per minute per milligram of cell protein unless specified otherwise, are means±SD of triplicate dishes or means of duplicate dishes. ND, not determined.

* $P < 0.05$ of control.

‡ Dishes received 5 μCi [^{3}H]leucine at 0 time.

because uptake and intracellular esterification of fatty acids has been shown to become reduced by lowering FA to albumin ratio in medium (3). To study effects of FA on lipid and apoprotein synthesis and secretion at conditions that may closer reflect the in vivo situation, three doses of the fatty acid albumin suspension (40 μl each) were added in 8-h intervals. The molar ratio of oleic acid to albumin was <4.5 over the entire experiment. Accumulation of media TG increased to 172% of control cells, cellular TG content increased to 256%, but accumulation of media apo B and apo C-III$_3$ was similar in control and experimental dishes. Cellular contents of these apoproteins were also not affected by oleic acid administration.

Since apoprotein mass as determined by radioimmunoassay did not parallel the increases of media lipids, changes in the lipid association of media apoproteins must have occurred. To characterize these changes in greater detail, media of control and experimental dishes were subjected to molecular sieving chromatography and apoprotein contents were determined in column effluents. In the media of control dishes, apo B appeared in two peaks corresponding to elution volumes of VLDL and LDL with trailing in the HDL region (Fig. 3). Apo C-III$_3$ and apo E each appeared as major peaks in the VLDL region, but immunoreactivity also was present in the LDL-HDL elution volumes and a distinct peak was found beyond the elution volume of albumin. Apo A-I eluted as an asymmetric peak near albumin. Administration of fatty acid changed the elution patterns of apo B, apo C-III$_3$, and apo E, in that a greater majority of these apoproteins now was found in the VLDL region, and the proportions eluting at or beyond the LDL region decreased. Elution profiles of apo A-I were minimally affected by oleic acid supplementation. The redistribution of apoproteins was a consistent finding and the relative proportions of apo B found in the VLDL fraction increased by 34±10% (n = 3 liver preparations) following fatty acid administration. Distribution of TG in column fractions was also determined. In control cells, the proportions of TG found in VLDL, LDL, and HDL elution volumes were 70, 17, and 13%, respectively. In experimental cells these values were 82, 14, and 4%, respectively.

Immunoassay of apo B may considerably underestimate apo B mass, since part of apo B may be masked in VLDL (31–33). The proportion of B_L and B_S (apo B large and small, the major apo B subspecies present in rat lipoproteins [34–36]), also may determine quantitation by immunoassay. Quantitation of apo B secretion rates was therefore attempted also by measuring incorporation of [^{3}H]leucine into proteins co-migrating with apo B on SDS gel electrophoresis. Following fatty acid treatment (5.3 μmol/100-mm dish) media apo B radioactivity was 988 cpm/mg cell protein (control dishes, 945 cpm/mg cell protein). The ratio of radioactivity between B_L to B_S was 0.59 in experimental dishes and 0.56 in control dishes. Thus, increased availability of fatty acids did not cause significant stimulation of [^{3}H]leucine incorporation into apo B and the relative proportions of B_L and B_S were not altered.

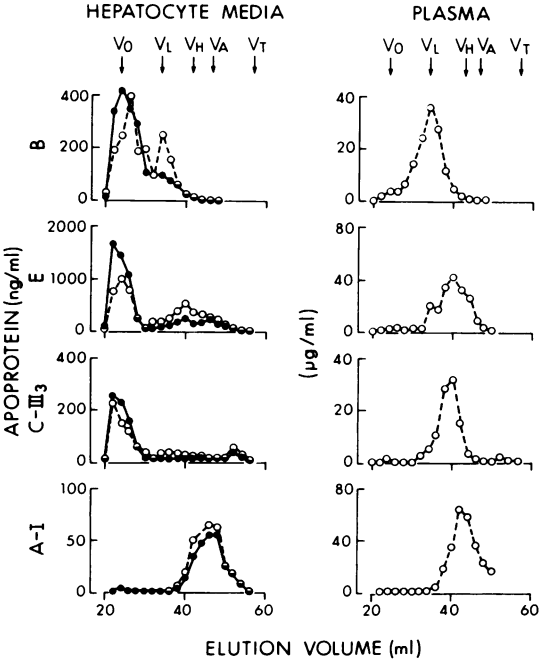

FIGURE 3 Apoprotein distribution following chromatography on Sepharose 6B in control media (O) and after administration of oleic acid (●). Hepatocytes were seeded on a fibronectin matrix, media were changed 6 h later, 1.7 μmol of oleic acid/dish was added, and incubation was continued for 16 h in 23 mM glucose. Media from five dishes were concentrated and aliquots corresponding to 10 mg of cell protein were applied to molecular sieving chromatography on Sepharose 6B. Apoproteins of indicated fractions were quantified by radioimmunoassays. The elution volume of human VLDL (V_O), human LDL (V_L), human HDL$_3$ (V_H), bovine albumin (V_A), and ^{125}I (V_T) are indicated by arrows. Elution profiles of apoproteins in rat plasma are shown on the right for comparison. Profiles of apo B, apo E, and apo A-I were obtained from the same plasma; apo C-III$_3$ was measured in a different plasma chromatographed on the same column.

To test the possibility that larger VLDL were secreted in response to fatty acid, a response that would enhance the ability of the hepatocyte to secrete more lipid with any given amount of apoprotein, media of cells incubated with [^{3}H]glycerol were chromatographed on Sepharose 2B. Administration of fatty acid not only increased the amount of [^{3}H]glycerol in lipid (see above), but also caused the appearance of label

in elution volumes corresponding to particles with larger Stokes radii (Fig. 4).

The fate of intracellular TG accumulating during exposure of cells to fatty acid was studied next (Fig. 5). Cells were preincubated for 12 h with increasing doses of FFA (0–2.2 μmol). The accumulation of TG by cells during the 12 h depended on the FFA concentration of the media (see 0 h, Fig. 5). Cells then were further incubated in the absence of added FFA for 8 and 22 h. During this time, cellular TG fell in all but the 0-dose dishes, whereas media TG rose. Rates of TG accumulation in media were directly related to TG contents of cells at the end of preincubation. The increased accumulation of media TG could only partially account for the fall in cellular TG, which indicates intracellular lipolysis of TG. Apo B accumulation in media of control (0 μmol FFA) and experimental cells (2.2 μmol/dish) was similar. In a similar but separate experiment, column chromatography revealed increased lipid association of apo B in media taken from cells which had been preloaded with TG: Note the redistribution of apo B immunoreactivity into the VLDL region (Fig. 6).

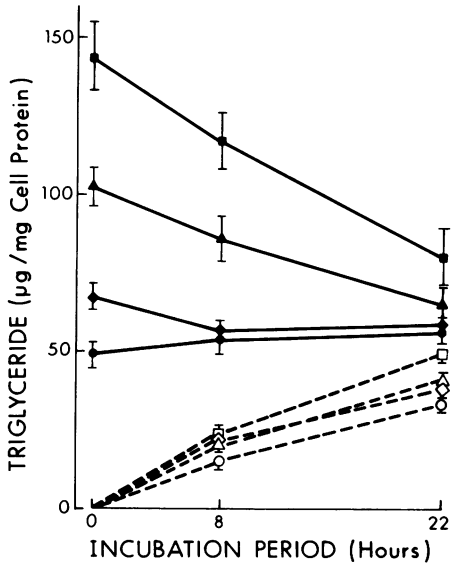

FIGURE 5 Time course of cell and media TG in hepatocyte cultures preincubated with oleic acid. 6 h after plating, media was changed and dishes received 2.2 μmol oleic acid (□), 1.1 μmol (△), 0.55 μmol (◇), or no fatty acid (○) for 12 h. Media was then removed, hepatocytes were washed with saline G, and the TG contents of some dishes was determined (0 h), other dishes were incubated further in the absence of fatty acid for an additional 8 and 22 h. Values are mean±SD for triplicate dishes. Solid lines are intracellular and dashed lines media contents of TG.

DISCUSSION

Hepatocytes in culture secrete lipoproteins (37, 38) and retain the ability to increase intracellular TG formation in response to fatty acid uptake (4). In this study we demonstrate increased rates of accumulation of TG in media in response to increased availability of fatty acids (Tables I and II, Fig. 1). The increased rates of accumulation probably reflect increased secretion, since reuptake of TG in control hepatocytes is ~10–15% (27), whereas accumulation of TG follow-

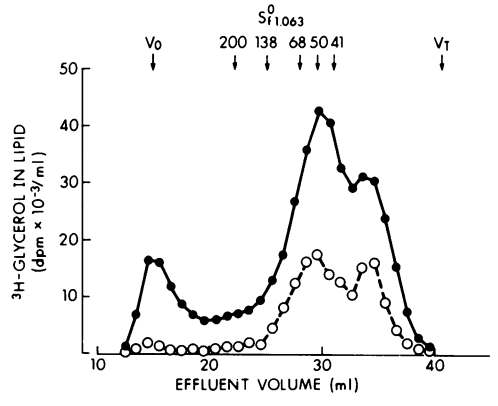

FIGURE 4 Effect of oleic acid on incorporation of [^{3}H]glycerol into media lipoprotein-lipid. Hepatocyte cultures received 20 μCi [^{3}H]glycerol and either 2.1 μmol oleic acid and 8 μg albumin (●) or 8 μg albumin alone per dish (○). Incubations were for 17 h in media containing 5 mM glucose. 2 ml of the media of control and of experimental dishes were chromatographed on a Sepharose 2B column, calibrated with human VLDL fractions of defined peak flotation rates (S_f) (28). V_O refers to the elution volume of human chylomicrons; V_T, elution volume of ^{125}I. Radioactivity was determined in lipid extracts (20).

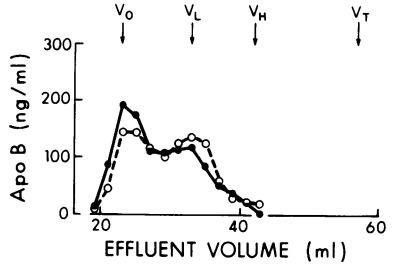

FIGURE 6 Apo B distribution in culture media of hepatocytes following preincubation with oleic acid. Cultures were preincubated with 2.1 μmol oleic acid/dish for 16 h (●) or without fatty acid (○). Cells were then washed, and incubated for another 12 h in the absence of oleic acid, after which period media were concentrated and subjected to chromatography on Sepharose 6B. Chromatographic fractions were analyzed for apo B. Media corresponding to 11.6 mg of cell protein (control) and 12.8 mg cell protein (experimental) were chromatographed. Data were normalized to 12 mg of cell protein. The elution volumes of human VLDL (V_O), human LDL (V_L), human HDL$_3$ (V_H), and ^{125}I (V_T) are indicated by arrows.

ing fatty acid administration was increased by 56%. Rates of accumulation of apoproteins were not increased in parallel with TG (Tables I and II, Figs. 2 and 3). Intracellular levels of apoproteins remained constant (Table I), and incorporation of [^{3}H]leucine into apo B subspecies was also unchanged, suggesting that increased apoprotein synthesis was not necessary for the augmentation of TG transport from cells. Compatible findings have been reported recently on the effect of fatty acids on TG and apolipoprotein synthesis in cultured hepatocytes (39). Incubation with 1 mM oleic acid for 4 h resulted in two- to threefold increases in media VLDL-TG accumulation, but apoprotein secretion as quantified by incorporation of labeled amino acids into protein was not affected by oleic acid administration (39). We also show that the association of media apoproteins with lipids is altered by oleic acid supplementation of media. A greater proportion of the secreted apoproteins was associated with larger lipoproteins indicating that enhanced lipid secretion was accomplished by augmented intracellular lipid association of apoproteins and by secreting larger particles. Even at maximal rates of TG accumulation intracellular apoprotein levels were similar to control cells, which suggests that intracellular apoprotein processing is not accelerated. Siuta-Mangano et al. (40) concluded from studies on the effect of tunicamycin on VLDL assembly that VLDL apoproteins may assemble with and carry into the plasma variable amounts of lipids. Exposure of estrogen-induced chick hepatocytes to 0.1 μg/ml tunicamycin not only inhibited incorporation of [^{3}H]glucosamine into apo B by 98%, but incorporation of [^{3}H]leucine into VLDL apoprotein was also reduced to 60%, whereas incorporation of [^{3}H]palmitate into VLDL lipid was not affected. Thus, glycosylation of apo B was not required for assembly of VLDL and changes in the lipid association of VLDL occurred as reflected by the increased glycerolipid-to-protein ratio observed. Secretion of larger particles may also result from disproportionate increases of core and surface lipids (3). Increases of media PL were less pronounced than increases of media TG upon incubation with oleic acid (Table II).

With increasing availability of fatty acid in the media, TG formed in excess of the secretory capacity and accumulated intracellularly (Tables I and II, Fig. 1). Estrogen-induced chick hepatocytes in culture also accumulated TG intracellularly, when exposed to oleic acid (4). TG-rich vesicles were recognized as the morphologic correlates of enhanced intracellular TG accumulation and are thought to function as temporary TG stores when the secretory capacity is exceeded (4). In our studies, intracellular TG stores fell rapidly upon removal of fatty acid. 22 h after the media change, 70% of excess TG was catabolized or secreted. TG secretion from preloaded cells exceeded the rates of control (not preloaded) cells, but apoprotein accumulation was not affected during emptying of TG stores. As with experiments in Tables I and II, increased lipid association of apo B was demonstrated in media (Fig. 6).

It is possible that apoprotein synthesis did not increase in these experiments because hepatocytes in culture lost their ability to respond to increased fatty acid uptake with increased apoprotein synthesis. However, cultured chick liver cells do respond to thyroid hormone with increased synthesis of fibrinogen and lipoproteins 6 h after hormone administration (41), a time interval greatly exceeded in our studies (up to 40 h). Furthermore, failure to induce increased apoprotein accumulation following administration of oleic acid also has been observed in hepatic perfusates (25). In these studies, apo C-III$_3$ and apo B accumulation was not enhanced by addition of oleic acid to perfusates, but apo C-III$_3$ accumulation was augmented in perfusates of livers taken from animals fed a high carbohydrate diet for 2 wk. Similarly, hepatocyte cultures obtained from carbohydrate-fed animals retain increased accumulation rates of apo E and apo C-III$_3$ (10). It would therefore appear likely that hepatocytes in culture can retain their potential for augmenting protein and apoprotein synthesis when provided with the proper stimulus. The fact that increasing FA availability does not result in coordinated synthesis of TG and apoproteins suggests that these processes are not tightly coupled. Other factors may be required to coordinate stimulation, such as hormones. Nevertheless, the ability of apoproteins to interact with differing amounts of lipid intracellularly may be of fundamental importance in the regulation of lipid transport from the liver, since the liver may adapt to short-term changes in needs for lipid transport without the need of stimulating *de novo* apoprotein synthesis.

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REFERENCES

1. Fredrickson, D. S., and R. S. Gordon, Jr. 1958. Transport of fatty acids. *Physiol. Rev.* 38:585–630.
2. Mayes, P. A., and J. M. Felts. 1967. Regulation of fat metabolism in the liver. *Nature (Lond.).* 215:716–718.
3. Ontko, J. A. 1972. Metabolism of free fatty acids in isolated liver cells: factors affecting the partition between esterification and oxidation. *J. Biol. Chem.* 247:1788–1800.
4. Mooney, R. A., and D. M. Lane. 1981. Formation and turnover of triglyceride rich vesicles in the chick liver cell. *J. Biol. Chem.* 256:11724–11733.
5. Heimberg, M., M. G. Wilcox, G. D. Dunn, W. F. Woodside, K. J. Breen, and C. Soler-Argilaga. 1974. Studies

on the regulation of secretion of the very low density lipoprotein and on ketogenesis by the liver. *In* Regulation of Hepatic Metabolism. F. Lundquist and N. Tygstrup, editors. Munksgaard, Copenhagen. 119–143.
6. Kohout, M., B. Kohoutova, and M. Heimberg. 1971. The regulation of hepatic triglyceride metabolism by free fatty acids. *J. Biol. Chem.* 246:5067–5074.
7. Heimberg, M., I. Weinstein, G. Dishmon, and A. Dunkerley. 1962. The action of carbon tetrachloride on the transport and metabolism of triglycerides and fatty acids by the isolated perfused rat liver and its relationship to the etiology of fatty liver. *J. Biol. Chem.* 237:3623–3627.
8. Rose, H., M. Vaughan, and D. Steinberg. 1964. Utilization of fatty acids by rat liver slices as a function of medium concentration. *Am. J. Physiol.* 206:345–356.
9. Schonfeld, G., and B. Pfleger. 1971. Utilization of exogenous free fatty acids for the production of very low density lipoprotein triglyceride by livers of carbohydrate-fed rats. *J. Lipid Res.* 12:614–621.
10. Patsch, W., S. Franz, and G. Schonfeld. 1981. Synthesis and secretion of apolipoproteins in primary rat liver cell cultures. *Arteriosclerosis.* 1:385*a*. (Abstr.)
11. Berry, M. N., and D. S. Friend. 1969. High yield preparation of isolated rat liver parenchymal cells. *J. Cell Biol.* 43:506–520.
12. Yedgar, S., D. B. Weinstein, W. Patsch, G. Schonfeld, F. E. Casanada, and D. Steinberg. 1982. Viscosity of culture medium as a regulator of synthesis and secretion of very low density lipoproteins by cultured hepatocytes. *J. Biol. Chem.* 257:2188–2192.
13. Durrington, P. N., R. S. Newton, D. B. Weinstein, and D. Steinberg. 1982. The effects of insulin and glucose on very low density lipoprotein triglyceride secretion by cultured rat hepatocytes. *J. Clin. Invest.* 70:63–73.
14. Chen, R. F. 1967. Removal of fatty acids from serum albumin by charcoal treatment. *J. Biol. Chem.* 242:173–181.
15. 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.
16. Bartlett, G. R. 1958. Phosphorus assay in column chromatography. *J. Biol. Chem.* 234:466–468.
17. Stajner, A., and J. Suva. 1977. The determination of nonesterified fatty acids in blood serum using a stable cupric reagent. *J. Clin. Chem. Clin. Biochem.* 15:513–514.
18. Williamson, D. H., J. Mellanby, and H. A. Krebs. 1962. Enzymatic determination of D(−)-β-hydroxybutyric acid and acetoacetic acid in blood. *Biochem. J.* 82:90–96.
19. McGarry, J. D., M. J. Guest, and D. W. Foster. 1970. Ketone body metabolism in the ketosis of starvation and alloxan diabetes. *J. Biol. Chem.* 245:4382–4390.
20. 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.
21. Nelson, G. J. 1972. Quantitative analysis of blood lipids. *In* Blood Lipids and Lipoproteins: Quantitation, Composition and Metabolism. G. J. Nelson, editor. Wiley-Interscience, New York. 25–74.
22. Laurell, C. B. 1972. Electroimmunoassay. *Scand. J. Clin. Lab. Invest.* 29(Suppl. 124):21–37.
23. Witztum, J. L., and G. Schonfeld. 1978. Carbohydrate diet induced changes in very low density lipoprotein composition and structure. *Diabetes.* 27:1215–1229.
24. Schonfeld, G., N. Grimme, and D. Alpers. 1980. Detection of apolipoprotein C in human and rat enterocytes. *J. Cell Biol.* 86:562–567.
25. Patsch, W., and G. Schonfeld. 1981. The degree of sialylation of apoC-III is altered by diet. *Diabetes.* 30:530–534.
26. Schonfeld, G., M. S. Frick, and A. P. Bailey. 1976. Measurement of apolipoprotein A-I in rat high density lipoprotein and in rat plasma by radioimmunoassay. *J. Lipid Res.* 17:25–30.
27. Patsch, W., S. Franz, and G. Schonfeld. 1983. Role of insulin in lipoprotein secretion by cultured rat hepatocytes. *J. Clin. Invest.* 71:1161–1174.
28. Patsch, W., J. R. Patsch, G. M. Kostner, S. Sailer, and H. Braunsteiner. 1978. Isolation of subfractions of human very low density lipoproteins by zonal ultracentrifugation. *J. Biol. Chem.* 253:4911–4915.
29. Kane, J. P., D. A. Hardman, and H. E. Paulus. 1980. Heterogeneity of apolipoprotein B: isolation of a new species from human chylomicrons. *Proc. Natl. Acad. Sci. USA.* 66:651–656.
30. 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.
31. Davies, G. E., and G. R. Stark. 1970. Use of dimethyl suberidimate, a cross linking reagent, in studying the subunit structure of oligomeric proteins. *Proc. Natl. Acad. Sci. USA.* 66:651–656.
32. Schonfeld, G., W. Patsch, B. Pfleger, J. L. Witztum, and S. W. Weidman. 1979. Lipolysis produces changes in the immunoreactivity and cell reactivity of very low density lipoproteins. *J. Clin. Invest.* 64:1288–1297.
33. Schonfeld, G., and W. Patsch. 1980. The interactions of lipoproteins with cellular receptors. *In* Drugs Affecting Lipid Metabolism. R. Fumagally, D. Kritchevsky, and R. Paoletti, editors. Elsevier/North-Holland Inc., New York. 37–52.
34. Krishnaiah, D. V., L. F. Walker, J. Borensztajn, G. Schonfeld, and G. S. Getz. 1980. Apolipoprotein B variant derived from rat intestine. *Proc. Natl. Acad. Sci. USA.* 77:3806–3810.
35. Elovson, J., Y. O. Huang, N. Baker, and R. Kannan. 1981. Apolipoprotein B is structurally and metabolically heterogeneous in the rat. *Proc. Natl. Acad. Sci. USA.* 78:157–161.
36. Wu, A. L., and H. G. Windmueller. 1981. Variant forms of plasma apolipoprotein B: Hepatic and intestinal biosynthesis and heterogeneous metabolism in the rat. *J. Biol. Chem.* 256:3615–3618.
37. Tarlow, D. M., P. A. Watkins, R. E. Reed, R. S. Miller, E. E. Zwergel, and M. D. Lane. 1977. Lipogenesis and synthesis and secretion of very low density lipoprotein by avian liver cells in non-proliferating monolayer: hormonal effects. *J. Cell. Biol.* 73:332–353.
38. 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.
39. Davis, R. A., and J. R. Boogaerts. 1982. Intrahepatic assembly of very low density lipoproteins. Effect of fatty acids on triacylglycerol and apolipoprotein synthesis. *J. Biol. Chem.* 257:10908–10913.
40. Siuta-Mangano, P., D. R. Janero, and M. D. Lane. 1982. Association and assembly of triglyceride and phospholipid with glycosylated and unglycosylated apoproteins of very low density lipoprotein in the intact liver cell. *J. Biol. Chem.* 257:11463–11467.
41. Hertzberg, K. M., J. Pindyck, M. W. Mosesson, and G. Grieninger. 1981. Thyroid hormone stimulation of plasma protein synthesis in cultured hepatocytes. *J. Biol. Chem.* 256:563–566.