Low rates of apoB secretion from HepG2 cells result from reduced delivery of newly synthesized triglyceride to a "secretion-coupled" pool

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Abstract The present study was aimed at defining the roles of intracellular triglyceride pools in apolipoprotein B secretion from HepG2 cells. Oleic acid (0.2 mmol/L) in the medium stimulated both triglyceride synthesis and apolipoprotein B secretion. Stimulation of apolipoprotein B secretion was lost about 30–40 min after oleic acid was removed from the medium, despite the finding that most newly synthesized triglyceride was still present in the cells. This suggested that only a small fraction of newly synthesized triglyceride was transferred to a pool available for assembly of nascent apoB into lipoproteins. Using cell fractionation, we analyzed two triglyceride pools in HepG2 cells: a microsomal pool and a cytoplasmic pool. Oleic acid-induced increases in the microsomal pool were small and short-lived due to secretion; this pool, therefore, is a "secretion-coupled" pool. The large majority of newly synthesized triglyceride was in a cytosolic pool that was not associated with secretion of apoB. Dibutyryl cAMP treatment was associated with a 3-fold increase in the mobilization of the triglyceride droplets. Apolipoprotein B secretion, however, was not increased, suggesting that the amount of triglyceride that entered the "secretion-coupled" pool after hydrolysis and re-esterification of cytoplasmic triglyceride was inadequate to stimulate apolipoprotein B secretion. In summary, the majority of newly synthesized triglyceride, whether derived from exogenous or endogenous fatty acids, is rapidly shifted to a cytoplasmic pool that does not play a regulatory role in apolipoprotein B secretion. The presence of a very small "secretion-coupled" pool of triglyceride in HepG2 cells likely explains the high rates of degradation of nascent apolipoprotein B, and the low rates of secretion of lipid-poor lipoproteins.–**Wu, X., A. Shang, H. Jiang, and H. N. Ginsberg.** Low rates of apoB secretion from HepG2 cells result from reduced delivery of newly synthesized triglyceride to a "secretion-coupled" pool. *J. Lipid Res.* 1996. **37:** 1198–1206.

Supplementary key words apolipoprotein B • triglyceride • HepG2 cells • endoplasmic reticulum • cytoplasma

Studies in primary cultures of rat hepatocytes have demonstrated that triglyceride (TG) exists in two distinct pools in the hepatocytes (1–3): a microsomal pool, which probably resides mainly in the smooth endoplasmic reticulum (ER), and a cytoplasmic pool. These two pools are derived from a single site of TG synthesis that is associated with the cytosolic surface of ER (4, 5). From a topologic point of view, it is reasonable to propose a role for the microsomal pool in the assembly of apolipoprotein B (apoB)-containing lipoproteins. Indeed, the microsomal pool is very active in rat hepatocytes, with a half-life of less than 60 min (2, 6). However, accumulating data have provided evidence that the cytoplasmic TG pool in rat hepatocytes may also be a major contributor to the assembly of very low density lipoproteins (VLDL) TG (2, 7, 8).

HepG2 cells have been used widely as a model of the secretion of apoB-containing lipoproteins. The basal rates of secretion of apoB in HepG2 cells are very low; the majority of the newly synthesized apoB is targeted for rapid intracellular degradation before secretion. ApoB secretion from HepG2 cells, however, demonstrates a good response to the stimulation of lipid synthesis, especially TG. We and others (9–12) have demonstrated that newly synthesized TG plays a important role in the regulation of apoB secretion in HepG2 cells. On the other hand, HepG2 cells do not assemble typical TG-rich lipoproteins; the majority of secreted apoB is associated with particles with a density of LDL and HDL, even in the presence of oleic acid (OA) (13). The lipid-poor nature of these lipoproteins is compatible with studies showing that the smooth ER compartment, which is believed to be the site where the majority of core lipids is added to nascent apoB particle (14), is present in very limited amounts in HepG2 cells (13). Based on these characteristics of HepG2 cells, we carried out studies directed at the following questions. How

Abbreviations: TG, triglyceride; ER, endoplasmic reticulum; VLDL, very low density lipoprotein; OA, oleic acid; BSA, bovine serum albumin.

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does newly synthesized TG distribute between cytosolic and ER pools in HepG2 cells? Which pool, ER or cytoplasmic (or both), is linked to apoB secretion? Do significant quantities of TG transfer from one pool to the other?

EXPERIMENTAL PROCEDURES

Material

L-[4,5-^{3}H]leucine (135 Ci/mmol, catalog No. TRK683), [2-^{3}H]glycerol (1.0 Ci/mmol, catalog No. TRA118), and [2-^{14}C]oleic acid, sodium salt (57 mCi/mmol, catalog No. CFA14) were purchased from Amersham Corp. (Arlington Heights, IL). Bovine serum albumin (BSA) (essentially fatty acid-free), oleic acid (sodium salt) (catalog No. 07501), and dibutyryl cAMP (DBc) (catalog No. D0260) were from Sigma Chemical Co. (St. Louis, MO). Protein A-Sepharose CL 4B was obtained from Pharmacia LKB Biotechnology, Inc. (Uppsala, Sweden). Monospecific anti-human apoB antiserum was raised in a rabbit. Minimum essential medium (MEM), nonessential amino acids, sodium pyruvate, and penicillin/streptomycin were from GIBCO laboratories (Grand Island, NY). Fetal bovine serum (FBS) was from Integen (Purchase, NY). Leucine-free MEM was generated from a kit (GIBCO, catalog No. 300 9050 AV). Leupeptin and pepstatin A were from Peninsula Laboratories, Inc. (Belmont, CA). All other chemicals were of the highest purity available.

Growth of HepG2 cells

HepG2 cells, obtained from ATCC, were grown in 35-mm dishes that had been coated with collagen. The cells were maintained in MEM containing 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% FBS. When the cells were about 90% confluent, the medium was changed to serum-free MEM experimental medium as described below.

Labeling of HepG2 cell triglyceride pools and protein synthesis

For most of the intact cell experiments we utilized the following protocol: HepG2 cells were double-labeled with [^{3}H]glycerol (100 μCi/ml) and [^{3}H]leucine (200 μCi/ml) for 2 h in 35-mm dishes in the presence of BSA or OA complexed to BSA (ratio = 2:1). After labeling, some dishes were washed twice with cold PBS and extracted with hexane–isopropanol 3:2 (v/v) for lipid determination. The medium from those cells was used to determine apoB secretion. Other dishes were chased in serum-free medium for various lengths of times followed by a second period of labeling with [^{3}H]leucine (200 μCi/ml) for 2 h. Medium was collected from these latter dishes for determination of apoB secretion. In some experiments, DBc was used to stimulate hydrolysis of cellular TG. Cellular lipids were determined either before or after the second labeling. Each particular

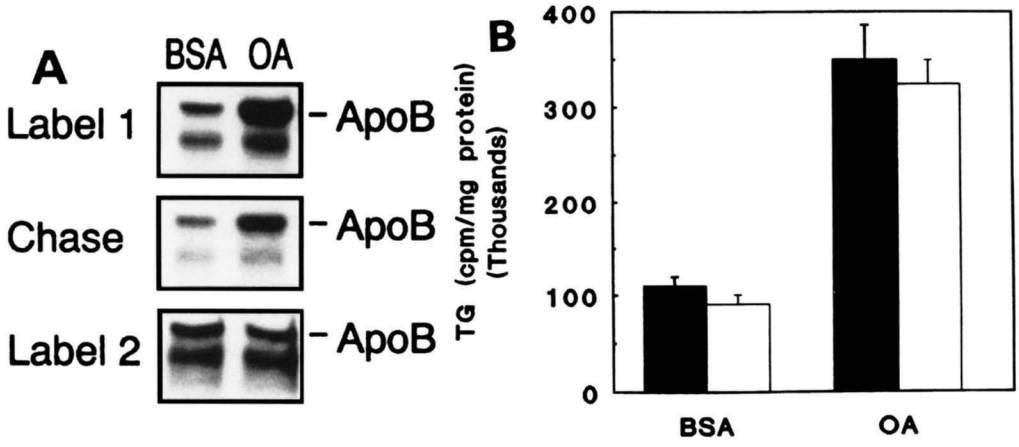


Fig. 1. Effect of OA treatment on apoB secretion (A) and triglyceride synthesis (B). HepG2 cells were double-labeled with [^{3}H]glycerol and [^{3}H]leucine for 2 h in the presence of BSA or OA (0.2 mmol/L). Cells from each treatment group were then divided into two sets. Medium was collected from one set of the cells for determination of apoB secretion (panel A, label 1), and cells were extracted for lipid determination (panel B, solid bars). The rest of the cells were chased in serum-free medium without OA for 40 min, after which time the chase medium was collected to determine apoB secretion (panel A, chase). All of the cells were labeled again with [^{3}H]leucine for an additional 2 h. The medium was collected for determination of apoB secretion (panel A, label 2), and the cells were extracted for lipid determination (panel B, hollow bars). Panel A shows representative results from two experiments. The lower band in the autoradiography is nonspecific protein immunoprecipitated by protein A-Sepharose. Data shown in panel B are expressed as the mean ± SD of six dishes from two experiments for each treatment.

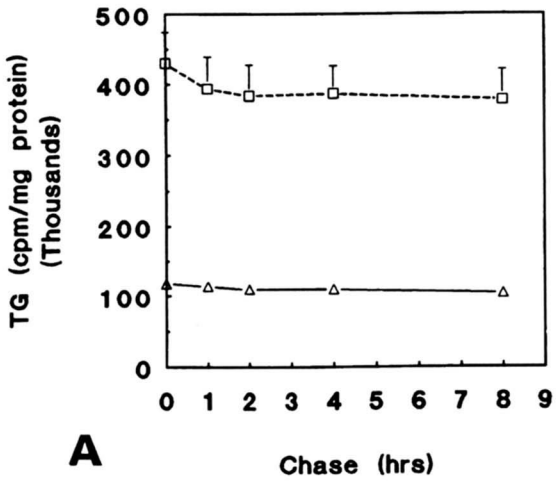
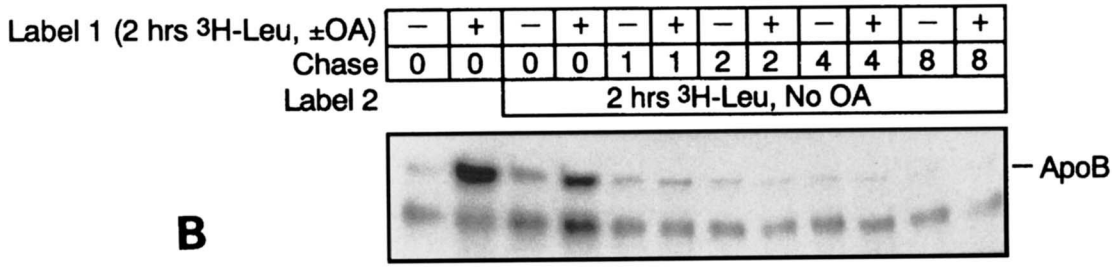


Fig. 2. A very small fraction of newly synthesized TG is coupled with apoB secretion. HepG2 cells were double-labeled with [^{3}H]glycerol and [^{3}H]leucine for 2 h in the presence of BSA or OA, the medium was collected for determination of apoB secretion (panel B, label 1), and cells were chased in the absence of OA for 0, 1, 2, 4, or 8 h, respectively. At each time point of the chase, the cells were divided into two groups. One group was extracted for determination of radiolabeled triglyceride (panel A) and the second group was labeled with [^{3}H]leucine for an additional 2 h to determine the secretion of apoB (panel B, label 2). After chase for 8 h, the majority of the newly synthesized TG was still found in the cells (panel A; (△) BSA; (□) OA). ApoB secretion, however, decreased to control level after chase for 1 h and thereafter (panel B). Data shown in panel A are expressed as the mean ± SD of five dishes from two experiments. Panel B is a representative set of dishes.



intact-cell experiment will be described in detail in the Results section and the figure legends.

For cell fractionation experiments, cells were labeled with [^{3}H]glycerol for 2 h in the presence of BSA or OA, followed by cell fractionation procedures as described previously (15). Briefly, cell pellets were suspended in 0.25 M sucrose, 10 mM HEPES, pH 7.4, and homogenized in a ball-bearing homogenizer. The homogenate was centrifuged 1900 *g* for 10 min, and the supernatant was adjusted to 0.5% BSA and layered on the top of a sucrose step gradient consisting of the following sucrose solutions (from the bottom): 0.5 ml (56%), 1.0 ml (50%), 1.5 ml (45%), 2.5 ml (40%), 2.5 ml (35%), 1.5 ml (30%), 0.5 ml (20%) (15). The gradients were centrifuged at 39,000 rpm at 4°C for 18 h using an SW-40 rotor. Fractions (0.6 ml each) were collected from the bottom of the tube. The endoplasmic reticulum (ER) fractions were collected (fractions 2–6) and pooled. A pure fraction representing cytosolic lipid was recovered from the top of the tube (fractions 16–20). Total recovery, using [^{3}H]TG and [^{3}H]albumin as markers, was greater than 60%; the majority of losses occurred during homogenization. These results compare well with those reported by Borchard and Davis (16) and Higgins and Hutson (17). Purity of the ER fractions was excellent as assessed by assay of glucose-6-phosphatase and galactosyl transferase (15).

Thin-layer chromatography

TG synthesis was determined by thin-layer chromatography (TLC) as described previously (15). Briefly, cell monolayers were extracted with hexane–isopropanol 3:2 (v/v). The solvent was collected and dried under gas nitrogen. To determine TG secretion, the medium was extracted with 20 volumes of chloroform–methanol 2:1 at room temperature for 2 h. Five volumes of water was added to the mixture which was then centrifuged at low speed. The lower phase (containing lipids) was dried under gas nitrogen. The lipid content from both the cell monolayers and the medium was suspended in a small volume of hexane and applied to a TLC plate (silica gel 60, 5506-7 E. Merck, Darmstadt, Germany), which was then developed in hexane–diethyl ether–glacial acetic acid 90:10:1 (v/v/v). The lipid spots were visualized with iodine vapor and counted in a Hydrofluor cocktail (National Diagnostics, Atlanta, GA) in a scintillation counter.

Immunoprecipitation

Immunoprecipitation of apoB in medium and cell lysate was carried out as described previously (10). Briefly, samples were mixed with NET buffer (150 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L Tris-HCl, pH 7.4, 0.5% Triton X-100, and 0.1% SDS) and an excess

amount of anti-apoB antiserum, and the mixture was incubated for 10 h at 4°C. Protein A-Sepharose CL 4B was added to the mixture; the incubation was continued for an additional 3 h; and the beads were extensively washed with NET buffer. ApoB was extracted from the protein A pellet by adding sample buffer (0.125 mol/L Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% mercaptoethanol) and boiling for 4 min. An aliquot of the sample was run on SDS-PAGE (3–10% gradient). The gel was treated with Autofluor (National Diagnostics, Atlanta, GA) and, after drying, was exposed to a film (Kodak, X-OMAT AR) at -80°C. Quantitation of apoB was determined by scanning the film with a densitometer.

RESULTS

Effect of OA on apoB secretion is rapidly reversed by removal of OA from the medium, although the major portion of newly synthesized TG is still found in the cells

When HepG2 cells were double-labeled with [^{3}H]leucine and [^{3}H]glycerol for 2 h in the presence or absence of 0.2 mM OA, both apoB secretion (**Fig. 1A,** label 1; 100 ± 18% vs. 470 ± 66%, BSA vs. OA, n = 6, $P < 0.01$) and TG synthesis (Fig. 1B, solid bars; 113,232 ± 13,298 cpm/mg protein vs. 343,245 ± 43,876 cpm/mg protein, BSA vs. OA, n = 6, $P < 0.01$) were stimulated significantly by OA. Even after OA was removed from the medium, the cells that had been treated with OA continued to secrete more apoB than the BSA-treated cells for another 30–40 min (100 ± 12% vs. 279 ± 25%, BSA vs. OA, n = 6, $P < 0.01$) (Fig. 1A, chase). When all of the cells were incubated for an additional 2 h with fresh BSA-containing medium and [^{3}H]leucine, but without additional OA, the amount of apoB secreted by the cells originally treated with OA was the same as that secreted by the cells that had never received OA (Fig. 1A, label 2, 100 ± 19% vs. 89 ± 26%, BSA vs. OA, n = 6, $P > 0.05$). The loss of the OA-stimulated increase in apoB secretion from cells during the second labeling period with BSA alone occurred despite the fact that there was little change in cellular TG compared to the original OA treatment period (Fig. 1B, OA; 327,432 ± 4011 cpm/mg protein vs. 343,245 ± 43876 cpm/mg protein, hollow bar vs. solid bar, $P > 0.05$). As there was much more TG in the cells treated initially with OA compared to those always incubated in BSA alone (Fig. 1B; OA hollow bar vs. BSA hollow bar), it appeared that this increased TG pool could not stimulate apoB secretion (Fig. 1A, label 2). Even when cells were incubated initially with 0.4 or 0.8 mmol/L OA (with concomitant further increases in the cell TG pools), apoB secretion returned to baseline (BSA-only conditions) during the second 2-h labeling period, despite the continued presence of a large intracellular TG pool (data not shown).

The second band present in all lanes in Fig. 1A, which migrated more rapidly than apoB, is an unidentified protein that is nonspecifically precipitated from the medium by our procedure. This protein was also present in our earlier studies (10–12, 15).

In a second series of experiments, HepG2 cells were double-labeled with [^{3}H]glycerol and [^{3}H]leucine for 2 h with or without OA. One set of dishes was harvested for determination of apoB secretion and cell TG. The rest of the dishes were chased in serum-free medium without OA for 0, 1, 2, 4, and 8 h. After each chase period, the cells were subdivided into two groups. One group of the cells was extracted for lipids and the second group was labeled again with [^{3}H]leucine without OA for 2 h to determine the secretion of apoB. As shown in **Fig. 2A,** even after chase for 8 h, the majority of the labeled TG synthesized during the first 2-h incubation period was still present in the cells. Furthermore, the cells that had been treated with OA during the initial 2

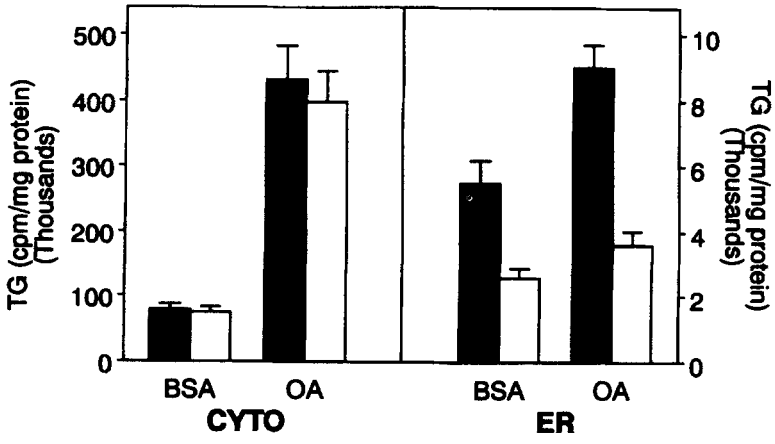


Fig. 3. The secretion-coupled TG pool was very small but very active in HepG2 cells. HepG2 cells were labeled with [^{3}H]glycerol for 2 h in the presence of BSA or OA and chased in serum-free medium for 0 or 30 min, after which the cytoplasmic (cyto) and ER pools were isolated for determination of radiolabeled triglyceride. At 0 min (solid bars) and 30 min (open bars), the ratios of cytoplasmic TG to ER TG were calculated. Results are presented as the means ± SD of three dishes. This experiment was performed three times with similar results. Note the different scales for TG synthesis in cyto and ER.

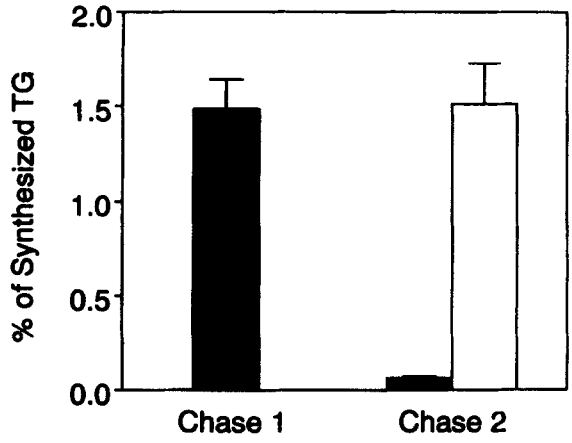


Fig. 4. The secretion-coupled TG pool was tightly linked to the ongoing TG synthesis. HepG2 cells were labeled with [^{3}H]glycerol for 1 h, after which cells were divided into two groups. One group was extracted to determine [^{3}H]TG synthesis; the second was washed and chased in serum-free medium for 1 h. The chase medium (chase 1) was collected to determine [^{3}H]TG secretion (solid bar). The cells were then labeled with [^{14}C]OA for 1 h and divided into two groups: one used to measure [^{14}C]TG synthesis and one chased for an additional 1 h. The chase medium (chase 2) was collected to determine the secretion of [^{3}H]TG and [^{14}C]TG (open bar). Data for TG secretion are expressed as the percentage of labeled TG synthesized and are the means ± SD of three dishes. This experiment was performed twice with similar results.

h labeling maintained much higher levels of radiolabeled TG throughout the 8-h chase (OA compared to BSA, n = 5, $P < 0.01$ at each point). This was true for TG mass as well, which increased 20–30% after incubation of cells with OA for 2 h (data not shown). However, the presence of a large, newly synthesized pool of TG in the cells initially treated with OA had a short-lived effect on apoB secretion. Thus, Fig. 2B demonstrates that although the initial stimulation of apoB by OA (label 1: 2 lanes, far left;, 100 ± 22% vs. 726 ± 83%, BSA vs. OA, n = 5, $P < 0.01$) was maintained during the second labeling (label 2: chase 0; 100 ± 16% vs. 284 ± 21%, BSA vs. OA, n = 5, $P < 0.01$), stimulation of apoB secretion was no longer observed after chase for 1 h (n = 5, $P > 0.05$ at each point, BSA vs. OA). These results indicate that although cell TG remained significantly increased for several hours after OA treatment, it had only a transient effect on apoB secretion. In addition, the stability of the cellular TG pool over 8 h indicated that while a small component of the newly synthesized TG pool was initially secreted with apoB, the major portion was not linked to apoB secretion.

A large cytoplasmic TG pool is not linked to apoB secretion

In order to determine the distribution and turnover rate of newly synthesized TG in HepG2 cells, we carried out cell fractionation experiments. HepG2 cells were first labeled with [^{3}H]glycerol for 2 h in the presence of either BSA or OA, and chased in serum-free medium for either 0 min or 30 min, followed by cell fractionation to isolate cytoplasmic and ER TG pools. The results are shown in **Fig. 3** (note the different scales for the left and right panels). In BSA-treated cells the ratio of the cytoplasmic pool to the ER pool was 14:1 at 0 min chase (BSA, cyto vs. ER, solid bars); this ratio was increased to 30:1 at 30 min chase due to loss of ER TG (BSA, cyto vs. ER, hollow bars). OA treatment had a greater effect on the cytoplasmic TG pool than on the ER TG. Thus, in OA-treated cells, the ratio of the cytoplasmic pool to the ER pool was 75:1 at 0 min chase (OA, cyto vs. ER, solid bars); the ratio was increased to 190:1 at 30 min chase due to the loss of ER TG (OA, cyto vs. ER, hollow bars). These results suggest that the distribution of newly synthesized TG in HepG2 cells is markedly imbalanced in favor of a cytoplasmic pool. In addition, the very small ER pool of TG diminished rapidly during the 30-min chase period indicating, based on the results in Figs. 1 and 2, that it was tightly coupled with apoB secretion in both BSA- and OA-treated cells. The cytoplasmic pool, on the other hand, did not appear to be associated with apoB secretion.

In further experiments, HepG2 cells were first labeled with [^{3}H]glycerol in the presence of OA (0.2 mmol/L) for 1 h. One set of the cells was extracted to determine total [^{3}H]TG synthesis, the second set of cells was washed and chased in serum-free medium for 1 h and the chase medium was collected to determine the secretion of [^{3}H]TG. The cells that were chased were then incubated with OA (0.2 mmol/L) and [^{14}C]OA for another 1 h, and then half were used to measure [^{14}C]TG synthesis and half were chased again in serum-free medium for 1 h. This chase medium was collected to determine secretion of both [^{3}H]TG and [^{14}C]TG. We observed that 1.5% of newly synthesized [^{3}H]TG was secreted during the first chase period (**Fig. 4,** chase 1). During the second chase period, a similar percentage of newly synthesized [^{14}C]TG was secreted. In contrast, less than 0.1% of [^{3}H]TG was secreted during the second chase period (Fig. 4, chase 2). These data provide further support for the conclusion that only newly synthesized TG was associated with the "secretion-coupled" pool in the ER; very little preexisting TG (synthesized 2 h earlier) was available for transfer to that pool.

Stimulated hydrolysis of the cytoplasmic TG pool is not associated with increased apoB secretion

The experiments described above indicated that the majority of TG in HepG2 cells was in a cytosolic pool whose turnover was very slow and that very little newly synthesized TG was transferred to a "secretion-coupled" pool. We sought to determine whether increases in the

turnover of the cytosolic pool would increase its potential to regulate apoB secretion. To do this, HepG2 cells were double-labeled with [^{3}H]glycerol and [^{3}H]leucine for 2 h in the presence of BSA or OA, after which one set of the cells was extracted for lipid determination and the medium was collected for determination of apoB secretion. The rest of the cells were chased in serum-free medium for 40 min. After chase, cells were labeled again with [^{3}H]leucine for another 2 h in the presence of either BSA or DBc. DBc stimulates the mobilization of TG droplets in rat hepatocytes (18), which, in turn, should increase intracellular fatty acid availability for re-esterification and new TG synthesis. After labeling, the cells were extracted for lipids, and the medium was collected to determine apoB secretion. ApoB secretion was stimulated from cells treated with OA during the first 2 h labeling (**Fig. 5A,** label 1; 100 ± 8% vs. 665 ± 78%, BSA vs. OA, n = 6, $P < 0.01$) and during the chase period (Fig. 5A, chase; 100 ± 13% vs. 243 ± 20%, BSA vs. OA, n = 6, $P < 0.01$). DBc caused a significant increase in mobilization of labeled TG in both BSA- and OA-treated cells, indicating hydrolysis had occurred (Fig. 5B, BSA: 4401 ± 325 cpm/mg protein vs. 9836 ± 1611 cpm/mg protein; OA: 13102 ± 1331 cpm/mg protein vs. 43395 ± 5210 cpm/mg protein; without (solid bar) vs. with (hollow bar) DBc treatment, n = 6, $P < 0.01$ in each case). ApoB secretion, however, was not stimulated during DBc treatment (Fig. 5A, label 2, BSA: 100 ± 18% vs. 108 ± 14; OA: 100 ± 23 vs. 103 ± 12; -DBc vs. +DBc, n = 6, $P > 0.05$ in each case). Although we cannot rule out the possibility that DBc treatment simply did not stimulate hydrolysis and re-esterification of cell TG adequately, these results are compatible with limited transfer of newly synthesized TG into the "secretion-coupled" ER pool.

DISCUSSION

ApoB-containing lipoprotein assembly seems to occur in a stepwise process, particularly in rat hepatocytes (14, 19–21), although this opinion is not accepted by all investigators (22). In a proposed two-step model, apoB binds to a small quantity of TG cotranslationally to form a primordial lipid-poor lipoprotein particle in rough ER. This particle then moves to smooth ER where it fuses with a TG-rich droplet to form a TG-rich apoB-containing lipoprotein particle. An alternative model has the additional core TG being added in less discrete steps as the primordial particle traverses through the lumen of rough and smooth ER. Both of these models are dependent on the addition of significant quantities of TG to the newly translocated, primordial apoB-lipoproteins in the ER lumen. In particular, the addition of signifi-

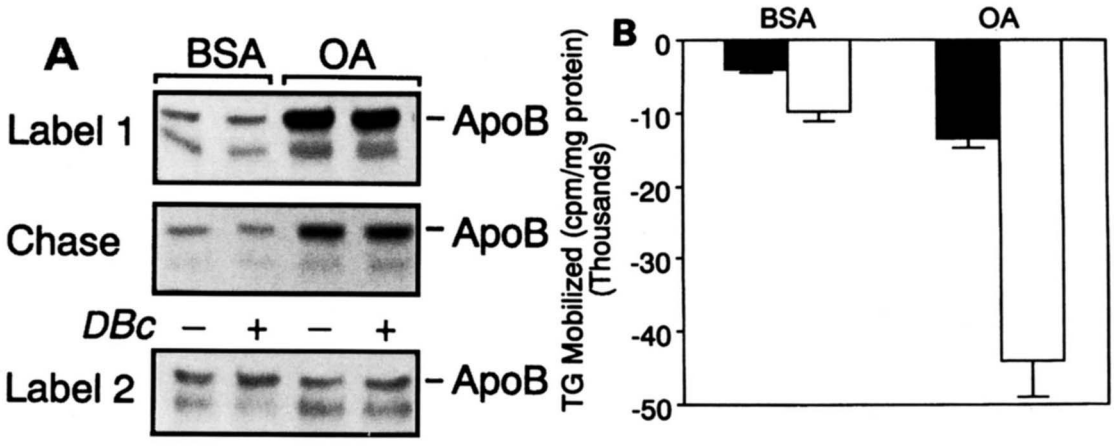


Fig. 5. Stimulated mobilization of cytoplasmic TG pool was not associated with increased apoB secretion. Cells were double-labeled with [^{3}H]glycerol and [^{3}H]leucine for 2 h in the presence of BSA or OA. Cells from each treatment were then divided into two groups. One group was extracted for determination of radiolabeled TG. Medium was collected from the second group which was then chased in serum-free medium without OA for 40 min. Medium was again collected after which cells were labeled with [^{3}H]leucine for additional 2 h in the absence or presence of DBc. All three groups of media were used for determination of apoB secretion. Cells were extracted to determine radiolabeled TG. Mobilization of cytoplasmic TG pool was estimated from the reduction of intracellular labeled TG between the end of the first and the end of the second labeling period. ApoB secretion was increased during the initial 2 h treatment with OA (panel A, label 1) and during the chase period (panel A, chase). During the second labeling period (panel A, label 2), the effects of the earlier OA incubation were no longer present, and addition of DBc did not change apoB secretion when compared to control treatment (-DBc) in cells that had been treated with either BSA or OA during the label 1 period. This lack of effect of DBc was evident despite the fact that TG mobilization was increased 3-fold by addition of DBc (panel B, hollow bars vs. solid bars). The results in panel A are representative of the experiments. The results in panel B are the means ± SD of six dishes pooled from three experiments.

cant quantities of TG after the translocation of nascent apoB is compatible with the demonstration that TG synthesis in the rat hepatocyte takes place predominantly in the smooth ER (4, 5, 14). Newly synthesized TG, however, can enter either a pool near its site of synthesis on the surface of the ER or enrich the cytoplasmic TG pool. The distribution of TG between these two pools may determine the amount of TG available for lipoprotein assembly. The observation that in freshly isolated rat hepatocytes, the ratio of cytoplasmic TG to microsomal TG is 4:1 in fed and 5:1 in fasting animals (3) is indicative of significant ongoing transfer of TG from cytoplasmic pool to the ER pool in these cells, consistent with their ability to secrete TG-rich VLDL under both conditions.

In contrast, the lipid-poor apoB particles secreted from HepG2 cells are thought to be the product of the early addition of TG in the rough ER, with little or no further significant addition of core lipids prior to secretion. The differences in lipoprotein assembly between HepG2 cells and rat hepatocytes were discussed in a recent review by Gibbons (23). To better understand both the high degree of early degradation of nascent apoB and the apparent absence of the second (or later) step(s) of TG assembly into apoB lipoproteins in HepG2 cells, we carried out a series of experiments to characterize TG metabolism in those cells, with a particular focus on the link between TG metabolism and apoB secretion. Our results indicate that: *1)* newly synthesized TG is transferred predominantly from the site of synthesis to a cytoplasmic TG pool; *2)* the cytoplasmic TG pool is large but does not regulate apoB secretion; and *3)* changes in a small microsomal TG pool are associated with changes in apoB secretion.

We found that the ratio of newly synthesized TG in the cytoplasm to that in the ER fraction was approximately 14:1 in the basal state, and 75:1 after incubation of cells with OA. These data, particularly the increase in the ratio after stimulation of new TG synthesis by OA, are consistent with a scheme in which newly synthesized TG in HepG2 cells is transferred predominantly from the site of synthesis to a cytoplasmic pool, with minimal accumulation in microsomes. This scheme receives further support from the observation by other investigators that smooth ER is essentially absent in HepG2 cells (13): thus, the transfer of new TG into the ER is limited to the rough ER. Indeed, Glaumann, Bergstrand, and Ericsson (6) reported that TG synthetic activity was associated with smooth as well as rough ER. The latter is, by far, the major ER compartment in HepG2 cells. However, despite being synthesized on the cytoplasmic surface of the rough ER, very little TG appears to be transferred into the lumen which is believed to be the site of association of TG with newly synthesized apoB through the action of microsomal triglyceride transfer protein (MTP) (24, 25). The limited transfer of newly synthesized TG into this active ER pool, as reflected in the very high ratio of cytoplasmic TG to microsomal TG, appears to be the reason that so little nascent apoB is targeted for secretion in HepG2 cells (9–12). Indeed, we observed that less than 2% of newly synthesized TG was secreted in control HepG2 cells during a 1-h chase. This is consistent with the recent findings by Gibbons et al. (26).

The lack of transfer of newly synthesized TG into a "secretion-coupled" ER pool might be explained by a lack of interaction of newly synthesized TG with MTP or insufficient MTP activity in HepG2 cells. Neither of these possibilities seems likely. First, the increase in apoB secretion observed after OA incubation indicates that if total TG synthesis is increased, there is increased availability of TG for lipoprotein assembly. Thus, when more TG was available, more TG was secreted. Second, HepG2 cells have adequate amounts of MTP, at least equal to that present in rat hepatocytes (27, 28). In addition, transfection of these cells with MTP cDNA does not increase apoB secretion (D. Gordon, personal communication). It seems more likely that the physical limitation of the acceptor organelle (smooth ER) in HepG2 cells is the basis of the marked disparity between cytoplasmic and microsomal TG pool in these cells. Independent of the mechanism, our results indicate that the secretion of relatively lipid-poor apoB-containing lipoproteins from HepG2 cells is not due to impaired synthesis of TG, but rather to the predominant transfer of newly synthesized TG into inactive cytoplasmic pool. This is also consistent with the studies of Gibbons et al. (26) in which only TG synthesis and secretion were determined.

Our results also suggest that the large cytoplasmic TG pool does not have the ability to regulate apoB secretion. Thus, after most newly synthesized TG is diverted into cytoplasmic droplets, very little of this pool appears to be available at any later time for secretion with apoB. This is a rather surprising finding as studies by other investigators in rat hepatocytes have found that the cytoplasmic TG pool, via lipolysis and reesterification, is available for VLDL assembly (2, 29). In fact, the cycle of hydrolysis and reesterification is able to maintain VLDL secretion from rat hepatocytes for several hours after removal of exogenous fatty acids from the media (2). As we have demonstrated in the present studies, this extended period of lipidation of VLDL, after depletion of exogenous fatty acids, does not occur in HepG2 cells.

We believe that the lack of regulation of apoB secretion by the cytoplasmic TG pool is due to the slow mobilization of the pool as suggested in the studies by Gibbons and co-workers (26). Although we were able to

increase modestly the mobilization of cytosolic TG with DBc, this still resulted in the hydrolysis of a very small portion of the TG pool, with only a small increase in cellular fatty acids that could be available for reesterification. The combination of DBc and a mitochondrial fatty acid beta-oxidation inhibitor, pentenoic acid, was also unable to stimulate enough new TG synthesis to increase apoB secretion in HepG2 cells (data not shown). This result is consistent with the findings by Gibbons et al. (26) indicating that mitochondrial beta-oxidation in HepG2 cells is extremely low. If a threshold of reesterification must be reached before newly synthesized TG can target apoB away from degradation, the resistance of the cytoplasmic TG pool to hydrolysis in HepG2 cells would explain the inability of DBc to alter apoB secretion. Indeed, work by Pullinger et al. (9) suggested that the cAMP-linked signalling pathway is defective in HepG2 cells, in contrast to rat hepatocytes (18). The slow turnover of cytoplasmic TG, together with the unbalanced distribution of any newly synthesized TG between the cytoplasmic and ER pools, would surely limit the mass of the "secretion-coupled" pool.

Finally, the present studies, together with our previous studies (11, 12), indicate that the microsomal TG pool, although quite small, is the key regulator of apoB secretion. We found that less than 2% of newly synthesized TG was in the secretion-coupled pool. This is similar to the demonstration by Gibbons and co-workers (26) that only a very small portion (less than 1%) of newly synthesized TG is detected in the medium of HepG2 cells (26). In contrast, almost half of the newly synthesized TG is secreted from rat hepatocytes (26). The central role of the small microsomal TG pool in HepG2 cells is further demonstrated by the significant increase in apoB secretion during OA treatment, despite the modest effect on the ER pool. Thus, in the absence of efficient transfer of cytoplasmic TG to the "secretion-coupled" pool, any new source of TG for this pool seems to stimulate apoB secretion. When we inhibit TG synthesis, apoB secretion is inhibited (11, 12). At the opposite end of the metabolic spectrum, the efficient and continuous transfer to the ER lumen of large quantities of TG, derived from ongoing hydrolysis and reesterification of cytoplasmic TG droplets, is the likely reason that short-term addition of OA does not significantly increase apoB secretion in primary hepatocytes isolated from fed rats (30, 31).

In summary, HepG2 cells, like rat hepatocytes, distribute newly synthesized TG between the cytosol and the ER. In distinction to rat hepatocytes, the microsomal TG pool is significantly smaller than the cytosolic pool in HepG2 cells. This small pool is, however, closely associated with apoB secretion. Also distinct from rat hepatocytes, the large cytoplasmic TG pool in HepG2 cells is very inactive and is not associated with the regulation of apoB secretion. Although we believe that physical limitations of the HepG2 cells, particularly the near absence of smooth ER, account for the differences between these cells and rat hepatocytes, we cannot rule out the absence of a specific molecule that might be involved in regulating the distribution of newly synthesized TG between the ER and cytoplasm.

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