Lipid balance in HepG2 cells: active synthesis and impaired mobilization

Geoffrey F. Gibbons,[1] Rohit Khurana, Andrew Odwell, and Marilia C. L. Seelaender[2]

Metabolic Research Laboratory, Nuffield Department of Clinical Medicine, University of Oxford, Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE, England

Abstract The human hepatoma cell line HepG2 in culture medium synthesized fatty acids de novo (144 ± 9 nmol fatty acid/mg protein per 24 h) at a rate similar to that observed in freshly prepared rat hepatocytes (192 ± 8 nmol/mg per 24 h) and in primary cultures of rat hepatocytes (165.4 ± 29.3 nmol/mg per 24 h). In HepG2 cells, fatty acid synthesis was inhibited by extracellular oleate (0.75 mM), and, to a lesser extent, by glucagon (10^{-7} M). Insulin (7.8×10^{-8} M) had a mild stimulatory effect. Fatty acid synthesis was not influenced by lipogenic precursors (lactate plus pyruvate), substances which, in rat hepatocytes, had pronounced stimulatory effects. Fatty acid synthesis rates were also unchanged in the presence of prostaglandin E_2 (PGE_2). In general, compared to rat hepatocytes, fatty acid synthesis in HepG2 cells was less sensitive to manipulation of the culture medium in vitro. HepG2 cells had a high capacity for triacylglycerol synthesis from extracellular oleate (469 ± 43 nmol triacylglycerol/mg protein per 24 h) but phospholipid synthesis was relatively low (15.8 ± 0.4% of total glycerolipids). Very little of the above newly synthesized triacylglycerol was secreted as lipoprotein (4.62 ± 0.88 nmol triacylglycerol/mg protein per 24 h) resulting in a large intracellular accumulation of triacylglycerol. This was exacerbated by the absence of any detectable ketogenesis. The secretion of triacylglycerol produced from de novo synthesized fatty acids was also very low in HepG2 compared to that observed in primary cultures of rat hepatocytes. In HepG2, the capacity for triacylglycerol + phospholipid synthesis from exogenous fatty acids was far higher than that from endogenous synthesized fatty acids. Lipoprotein triacylglycerol secretion was inhibited by insulin in HepG2. However, glucagon and PGE_2, which inhibit this process in rat hepatocytes, were without effect. In contrast to rat hepatocytes, most of the lipoprotein triacylglycerol in HepG2 was secreted without prior lipolysis and re-esterification of intracellular triacylglycerol. This reflected a very low overall rate of intracellular triacylglycerol lipolysis in HepG2. These results suggest that, although lipid synthesis is very active in HepG2, there is a defect in lipid mobilization (lipolysis, secretion, and oxidation) that results in excessive intracellular storage of triacylglycerol. **—Gibbons, G. F., R. Khurana, A. Odwell, and M. C. L. Seelaender.** Lipid balance in HepG2 cells: active synthesis and impaired mobilization. *J. Lipid Res.* 1994. **35:** 1801-1808.

Supplementary key words apolipoprotein B • lipoprotein assembly • hormones • fatty acids • ketogenesis • lipolysis

Although the human hepatoma cell line HepG2 is widely used as an in vitro model of human hepatic lipid metabolism (for a review, see ref. 1), there have been no studies of the regulation of fatty acid synthesis or of the reciprocal control of fatty acid disposal via the oxidative and esterification pathways (2). All these factors contribute to the maintenance of hepatic triacylglycerol (TAG) homeostasis. The other important contributory factor in this respect is the rate of TAG secretion as lipoprotein. In this context it is well established that in HepG2, TAG secretion is impaired and that most of the small quantity of secreted TAG is associated with relatively dense, LDL-like particles (3, 4). Quantitatively, there does not appear to be any defect in the secretion of apolipoprotein B (apoB) especially in the presence of extracellular fatty acids (5–7). The reason(s) for this uncoupling of apoB and TAG secretion in HepG2 remains obscure although a defect in the so-called "second stage" transfer of TAG to the nascent lipoprotein has been implicated (8).

There were two major objectives of the present work. First, to determine whether TAG homeostasis in HepG2 is affected by the same hormonal and metabolite influences that regulate hepatic lipid balance in animal models. In particular, as previous studies have suggested a link between fatty acid synthesis de novo and lipid secretion (9–13), we have focused attention on this relationship in HepG2. Second, in view of evidence that intracellular TAG lipolysis may be a prerequisite for a high rate of lipoprotein TAG secretion (14), we have examined the possibility that the low rate of TAG secretion in HepG2 is associated with a defective lipolytic step.

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; apoB, apolipoprotein B; TAG, triacylglycerol; PGE_2, prostaglandin E_2.

[1]To whom correspondence should be addressed.

[2]Present address: Instituto de Ciências Biomédicas, Universidade de São Paolo, Brasil.

MATERIALS AND METHODS

Materials

Cell culture medium (RPMI-1640), Puck's saline A, penicillin, streptomycin, fetal calf serum (heat inactivated), and trypsin-EDTA solution were obtained from Gibco (Paisley, UK). Oleic acid, bovine serum albumin, bovine insulin and tolbutamide were from Sigma (Poole, Dorset, UK). ^3H_2O, [^{3}H]oleic acid, [U-^{14}C]glycerol, and tri[^{14}C]oleoylglycerol were from Amersham International (Little Chalfont, U.K.). Tissue culture flasks (Falcon, T75) were supplied by Becton Dickinson (Cowley, Oxford, U.K.).

Cell culture

HepG2 cells were cultured in 12.0 ml RPMI-1640 (containing L-glutamine (2 mM)), supplemented with fetal calf serum (10%), penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were cultured for 5 days at 37°C in a humidified incubator at which time they formed confluent monolayers. During this period the medium was replaced by fresh medium (containing all supplements) every 48 h. After 5 days, the cells were washed twice with 10.0 ml Puck's saline A and then treated according to different protocols, depending upon the experimental objective(s) (see below). Rat hepatocytes were prepared under sterile conditions (15) and either incubated immediately as fresh suspensions (16) or cultured in 250-ml tissue culture flasks (Falcon, T75). In this case, 12.0 ml of the cell suspension in Waymouth's medium containing supplements and serum (15) was added to each flask and the cells were allowed to attach to the flask for 4 h. After this time, the medium was removed, the cell monolayer was washed with Puck's saline (see above), and 12.0 ml of supplemented Waymouth's medium (without serum) was added.

Isolation of secreted lipoproteins

After culture of HepG2, a mixture of anti-oxidants, antibiotics, and protease inhibitors was added to the cell medium (17). The density of the medium was adjusted to 1.21 g/ml (18) by the addition of solid KBr. The solution was centrifuged at 40,000 rpm (154,000 *g* av) for 16 h at 10°C in a Beckman L8-70 ultracentrifuge using a 50.4 rotor. The resulting supernatant containing the floating lipoproteins was obtained by tube slicing. The fraction containing VLDL was obtained from the culture medium of rat hepatocytes as described previously (15).

Measurement of fatty acid synthesis

Immediately after washing with Puck's saline A (see above) 12.0 ml RPMI medium (without serum) was added to the HepG2 cell monolayer together with hormones and other supplements as detailed in the table legends. Twelve ml of Waymouth's medium was added to the primary cultures of rat hepatocytes. In each case ^3H_2O (2 mCi) was added and the cells were cultured for a further 24 h. After this time, the medium was removed, the monolayer was washed twice with Puck's saline A, and the cells were detached by addition of 4.0 ml of a solution of trypsin and EDTA in Puck's saline A (0.5 g and 0.2 g/liter, respectively. The flasks were incubated for 5 min at 37°C and the resulting cell suspension was transferred to 6.0 ml RPMI-1640 containing 10% fetal calf serum. The cells were pelleted by centrifugation and suspended in 1.0 ml 0.9% saline. The suspension was sonicated and an aliquot was removed for measurement of protein. The remainder was saponified with an ethanolic solution of potassium hydroxide (final concentration, 5.0%) and the non-saponifiable and saponifiable fractions of the cell were extracted as described previously (19). The ^{3}H radioactivity of the saponifiable fraction (containing the fatty acids) was determined by scintillation counting. The labeled fatty acids were also obtained from 1.0 ml of the secreted lipoprotein fraction using the same method. Fatty acid synthesis rates were calculated from the relationship determined by Jungas (20), i.e., 1 μmol of C_{18} fatty acid incorporates 10.34 μmol ^3H_2O.

Determination of [^{3}H]oleate incorporation into cellular and lipoprotein triacylglycerol and phospholipid

Cell monolayers were treated as described above for determination of fatty acid synthesis. However, instead of adding ^3H_2O, at this stage [^{3}H]oleate (0.75 mM; 0.98 × 10^6 dpm/μmol) was added to the medium. The cells were incubated for 24 h and the medium was removed for isolation of the secreted lipoprotein (see above). The cell monolayer was treated as described above and the total lipid fraction was extracted from the sonicated cell suspension by the method of Folch, Lees, and Sloane Stanley (21). The labeled triacylglycerol and phospholipid were purified by thin-layer chromatography (22). The lipid fractions were scraped from the plate and their radioactivities were determined by scintillation counting. The mass of lipids synthesized was calculated from the specific radioactivity of the exogenous labeled oleate and from the stoichiometry of its esterification. The labeled triacylglycerol and phospholipids were also isolated from 1.0 ml of the secreted lipoprotein fraction by the same method as described above. Manipulative losses of lipids were accounted for by addition of a known amount of tri[^{14}C]oleoylglycerol as internal standard.

Determination of intracellular triacylglycerol/fatty acid cycling

Cells were treated as described above. However, in this case, after washing with Puck's saline A, 12.0 ml RPMI-1640 medium, supplemented with glutamine, antibiotics, and fetal calf serum, was added to the monolayer followed by [^{3}H]oleate (0.75 mM; 0.98 × 10^6 dpm/μmol) and, in

addition, [U-^{14}C]glycerol (1.0 mM; 1.83 × 10^5 dpm/μmol). After 16 h, during which time the intracellular triacylglycerol became labeled in both the glycerol and fatty acid moieties, the medium containing the labeled substances was removed and the monolayer was washed twice with Puck's saline A. At this stage the cells from two flasks were harvested as described above, the labeled triacylglycerol was extracted, and the ^{3}H and ^{14}C radioactivities were determined. These are subsequently referred to as the "initial values." To the remaining flasks was added RPMI-1640 medium (12.0 ml) containing glutamine and antibiotics but no serum. Insulin and other additions, as appropriate, were made at this stage. The cells were cultured for a further 24 h during which time the only source of secreted triacylglycerol was the doubly labeled intracellular pool. At the end of this period the medium was removed and retained for isolation of secreted lipoproteins. The cells were harvested and the doubly labeled triacylglycerol fractions from both the cells and the secreted lipoproteins were isolated. The ^{3}H and ^{14}C contents were determined by scintillation counting. These are subsequently referred to as the "final" values.

Calculations

The proportions of lipoprotein triacylglycerol secreted as intact molecules ("en-bloc" secretion) and that secreted after lipolysis and re-esterification of intracellular material were determined as follows. Under the experimental conditions used, all the secreted triacylglycerol originated from the ^{3}H- and ^{14}C-labeled triacylglycerol within the cell. If all the lipoprotein triacylglycerol was secreted "en-bloc" then the "initial" ^{14}C/^{3}H ratio of the intracellular triacylglycerol would be identical to that of the secreted material. Any relative loss of ^{14}C in the latter would indicate lipolysis followed by re-esterification of the ^{3}H-labeled fatty acid with unlabeled glycerol. The extent of the decline in the ^{14}C/^{3}H ratio (i.e., the relative loss of ^{14}C) of the secreted triacylglycerol is a measure of the degree to which lipolysis has occurred prior to secretion and can be used to calculate the proportion of total triacylglycerol that was secreted via this pathway. Similarly, any difference between the "initial" and "final" ^{14}C/^{3}H ratios of the intracellular triacylglycerol will reflect the amount of this lipid that has undergone lipolysis followed by re-cycling of the re-esterified ^{3}H-labeled fatty acids back to the cell, rather than into the secretory pathway. For instance, it may be calculated that the final ^{14}C specific radioactivity of the cellular TAG is x% of the initial value. The amount of unlabeled glycerol that must have entered the TAG pool to achieve this decrease is $(\frac{100}{x} - 1)$ pools (see Table 7). As the total mass of the cellular triacylglycerol pool is known, calculation of the proportion of the pool that is re-cycled in this way can be used to estimate the actual mass of cellular triacylglycerol that has undergone lipolysis followed by re-esterification (14).

Other methods

Protein was determined by the method of Lowry et al. (23). [^{3}H]oleate was complexed with bovine serum albumin (fatty acid-free) by the method of Van Harken, Dixon, and Heimberg (24). The concentration of albumin in the culture medium was 0.5%. The mass of cellular triacylglycerol was determined by the method of Trinder (25) using a test kit supplied by Boehringer-Mannheim.

Statistical analysis

Experiments were carried out on several different occasions, the exact numbers of which (n) are cited in the tables. Significant differences were tested for using a paired or unpaired Student's *t*-test.

RESULTS AND DISCUSSION

Origins of cellular TAG: synthesis de novo and from extracellular fatty acids

In the absence of hormones or of lipogenic precursors, the rate of fatty acid synthesis in HepG2 amounted to 144 ± 9 nmol/mg protein per 24 h. This was similar to that observed in freshly prepared rat hepatocytes which produced 24 ± 1 nmol fatty acid/mg protein per 3 h (192 ± 8 nmol/mg per 24 h) (**Table 1**). Although fatty acid production was stimulated considerably by the lipogenic combination of lactate plus pyruvate in rat hepatocytes, as observed previously (26, 27), a mixture of these substrates had no stimulatory effect in HepG2 (Table 1). In another series of experiments, insulin had a slight but significant stimulatory effect on fatty acid synthesis whereas glucagon was slightly inhibitory (**Table 2**). These effects are qualitatively similar to those observed previously in rat hepatocytes but are not so pronounced quantitatively (27). Prostaglandin E_2 had no effect on fatty acid

TABLE 1. Comparison of fatty acid synthesis de novo in rat hepatocytes and HepG2 cells

	Fatty Acid Synthesis	
Additions to Cells	Fresh Rat Hepatocytes	HepG2
	nmol/mg protein/24 h	
None	192 ± 8	144 ± 9
Lactate + pyruvate	360 ± 18	119 ± 3

Rat hepatocytes and HepG2 cells were incubated for 3 h and 24 h, respectively, in the presence of ^3H_2O (2 mCi) as described in Materials and Methods. A mixture of lactate (10 mM) and pyruvate (1 mM) was added to flasks as indicated. The fatty fraction was isolated from each flask of cells and its radioactivity was determined. Each value is the mean ± SEM of three individual flasks.

TABLE 2. Fatty acid synthesis de novo: effects of hormones and metabolites

Addition(s) to Medium	Fatty Acid Synthesis	% of Control
	nmol/mg protein	
Control	84.9 ± 14.1 (5)	
Insulin	97.6 ± 12.6 (4)	130.0 ± 7.9 (4)[a]
Glucagon	75.0 ± 20.7 (3)	90.8 ± 1.6 (3)[a]
Prostaglandin E_2	83.8 ± 15.5 (3)	85.5 ± 8.3 (3)
Oleate	43.5 (2)	51.2 (2)
Lactate + pyruvate	74.1 ± 11.1 (4)	97.7 ± 4.8 (4)

HepG2 cells were cultured as described in Materials and Methods. ^3H_2O (2 mCi) was added to each flask and the incorporation of ^3H into the fatty acid fraction of the cell was determined. Where appropriate the above additions were present in the following concentration: insulin (7.8×10^{-8} M); glucagon (1×10^{-7} M); prostaglandin E_2 (1×10^{-4} M); oleate (0.75 mM); lactate (10 mM); pyruvate (1 mM). The above values are the mean ± SEM of several independent experiments (numbers in parentheses) each of which were carried out using duplicate flasks.

[a]Significantly different ($P < 0.05$) from the control values.

synthesis in HepG2 (Table 2) and this result is similar to that observed previously in rat hepatocytes (28). In general, fatty acid synthesis in HepG2 appeared to be less sensitive to in vitro manipulations of the type which, in normal rat hepatocytes, produce large variations in the rate at which this lipogenic process occurs. The major source of substrate for de novo lipogenesis in the HepG2 line has not been identified, but in view of the high apparent rate of glycolysis or glycogenolysis (see below) it is probable that glucose in the medium or cellular glycogen makes a major contribution.

If all the fatty acids synthesized by HepG2 from endogenous sources (144 nmol/mg per 24 h) (Table 1) were converted into TAG, the maximum amount potentially arising from this source is 48 nmol and this takes no account of phospholipid synthesis. By contrast, when cells were cultured in the presence of 0.75 mM oleate for 24 h, an average of 469 ± 43 nmol/mg of TAG was obtained from this source (**Table 3**). The capacity for TAG synthesis from the latter, therefore, was clearly much greater than that from endogenous sources via the de novo synthetic route. Neither insulin, glucagon, nor PGE_2 had any effect on the synthesis of cellular TAG from exogenous oleate (Table 3).

Secretion of lipoprotein lipids from HepG2 and from primary hepatocyte cultures

HepG2 cells cultured in the presence of exogenous [^3H]oleate (0.75 mM) synthesized considerably more triacylglycerol (474 nmol/mg protein per 24 h) than did primary cultures of rat hepatocytes (137.9 nmol/mg protein per 24 h) (Table 3). However, in HepG2, of the total, only 4.62 nmol (0.94%) was secreted into the medium. This contrasts with the much higher quantity (47.8% of total synthesized) secreted by primary cultures of rat hepatocytes over the same period of time (Table 3) (29). It could be argued that, in HepG2, the secretion of TAG labeled from fatty acids synthesized de novo differed from that of those labeled from exogenous fatty acids. To check this point, primary cultures of rat hepatocytes and HepG2 cultures were incubated for 24 h with ^3H_2O and the amounts of labeled fatty acids derived from the esterified lipids of the secreted lipoprotein fractions were compared with those remaining within the cell (**Table 4**). Although the total amounts of fatty acids synthesized by HepG2 and primary cultures were very similar, in the former only a small fraction of the total (approx. 4%) was secreted as lipoprotein lipids. This contrasts with the primary hepatocyte cultures in which most (68%) of the newly synthesized fatty acids were secreted.

Effects of hormones on the secretion of lipoprotein triacylglycerol in HepG2 and primary cultures of rat hepatocytes

Addition of insulin (7.8×10^{-8} M) to the medium of HepG2 significantly decreased the secretion of lipoprotein TAG to 66.7% of the control value (Table 3). This is in agreement with several previous observations of the effects of insulin in HepG2 (18, 30–32) and, quantitatively, the effect is similar to that observed in primary cultures of rat hepatocytes (61.7 ± 5.9% of control, Table 3). Thus, proportionately, the effect of insulin is comparable in HepG2 and in primary cultures, despite the very large difference in the absolute rates of TAG secretion. In HepG2, neither glucagon nor PGE_2 had any significant effect on the secretion of lipoprotein TAG (0.87 ± 0.13% and 0.90 ± 0.15% of total, respectively) compared to a

TABLE 3. Effect of insulin on the secretion of lipoprotein triacylglycerol in HepG2 cells and primary cultures of rat hepatocytes

Cell Type and Culture Conditions	n	[^3H]Triacylglycerol		
		Secreted	Cellular	Secreted
		nmol/mg prtein		*% of total*
HepG2				
– Insulin	8	4.62 ± 0.88	469 ± 43	0.94 ± 0.14
+ Insulin	5	2.63 ± 0.58	508 ± 97	0.51 ± 0.05[a]
Primary cultures				
– Insulin	3	63.9 ± 9.6	75.9 ± 24.1	47.8 ± 6.9
+ Insulin	3	39.8 ± 7.2	115.2 ± 11.3	27.2 ± 4.1[a]

HepG2 cells and primary rat hepatocytes were cultured for 24 h with [^3H]oleate (0.75 mM) in the presence or absence of insulin (7.8×10^{-8} M). The medium was removed and the cells were washed twice with Puck's saline. The cellular and secreted lipoprotein-triacylglycerol fractions were isolated and their radioactivities were determined. Each value is the average ± SEM of several independent determinations (n).

[a]Significantly different from the corresponding values observed in the absence of insulin ($P < 0.05$).

TABLE 4. Retention and secretion of newly synthesized fatty acids in primary hepatocyte cultures and in HepG2 cells

Cell Types	Cellular Fatty Acids	Secreted Fatty Acids	Total Fatty Acids
		nmol fatty acids/mg cell protein/24 h	
Primary cultures	52.9 ± 11.1	112 ± 21.6	165.4 ± 29.3
HepG2	144.4 ± 9.2	6.3 ± 1.0	150.7 ± 9.3

Rat hepatocytes and HepG2 cells were cultured for 24 h in the presence of ^3H_2O as described in Materials and Methods. The fatty acid fractions were isolated from the cells and from the medium and their radioactivities were determined. For HepG2, the values are the means ± SEM of three individual flasks. For the primary cultures, the values are the means ± SEM of five individual hepatocyte preparations.

control value of 0.94 ± 0.14%. This contrasts with the potent suppression of VLDL secretion in rat hepatocyte cultures (28, 33, 34). The ineffectiveness of glucagon in this regard supports the earlier work of Pullinger and colleagues (18), and, together with the very small effect on de novo lipogenesis (Table 1), is consistent with the idea of a defective signalling mechanism involving cAMP and/or cAMP-mediated protein kinases in HepG2 (18).

In the above experiment (Table 3) extracellular oleate was present during the period over which the cells were exposed to insulin. To determine whether insulin had a similar effect in HepG2 on the secretion of lipoprotein assembled exclusively from pre-formed intracellular TAG, cells were cultured overnight in the presence of [^{3}H]oleate (0.75 mM). This served to prelabel the intracellular TAG. The cells were then transferred to oleate-free medium and the secretion of labeled TAG was determined in the presence or absence of insulin over the next 24-h period. Although the absolute rate of TAG secretion decreased under these conditions, this process was significantly inhibited by insulin (**Table 5**).

TABLE 5. Effect of insulin on the mobilization of intracellular triacylglycerol for lipoprotein assembly in HepG2 cells

Addition to Flask	[^{3}H]Triacylglycerol		Secreted Triacylglycerol
	Cellular	Secreted	
	nmol/mg protein		*% of control*
Control	357 ± 73	1.38 ± 0.49	100
Insulin	320 ± 39	0.84 ± 0.38	56.7 ± 7.2 ($P < 0.01$)

Cellular triacylglycerol was pre-labeled by culturing cells for 16 h with [^{3}H]oleate. After removal of the labeled medium, cells were washed twice with Puck's saline A, and fresh unlabeled medium was added (no serum). Cells were incubated for a further 24 h and the ^{3}H radioactivity of the cellular and secreted lipoprotein triacylglycerol was determined. Each value is the average ± SEM of four independent experiments.

Synthesis and secretion of phospholipid by HepG2

In the absence of hormones, HepG2 synthesized 53.2 ± 4.8 nmol of phospholipid from [^{3}H]oleate. This amounted to 15.8 ± 0.4% (n = 6) of the total cellular glycerolipid, a value similar to that observed previously in cultured rat hepatocytes (data not shown). Of this, 2.54 ± 0.57 nmol was secreted in association with lipoprotein, an amount that comprised 51.0 ± 7.4% of the lipoprotein glycerolipids. This contrasts with the very small proportion of labeled glycerolipid arising from phospholipid in VLDL secreted by monolayers of cultured rat hepatocytes (approx. 2%, data not shown). This suggests that there is no defect in the incorporation of phospholipid into the maturing lipoprotein by HepG2 and that the low relative content of TAG in the particle results from a specific defect in the addition of this lipid during the lipoprotein assembly process.

Lipoprotein TAG: secretion "en bloc" or via lipolysis of intracellular TAG?

Comparison of the time-dependent changes in the ^{14}C:^{3}H ratios of cellular and secreted TAG labeled from [^{3}H]oleate and [^{14}C]glycerol permitted calculations of the proportion of the total TAG that had undergone lipolysis followed by re-esterification prior to secretion (see Experimental section for details of the calculation used). In the absence of any addition to the culture medium most (83%) of the TAG was secreted without prior lipolysis (**Table 6**). This contrasts with the relatively low proportion (maximum 30%) of the VLDL TAG secreted without lipolysis in cultured rat hepatocytes (14). Insulin had no significant effect on the proportion of TAG secreted "en bloc" (Table 6).

The validity of this technique depends upon the assumption that a molecule of [^{3}H]oleate, released by lipolysis, is re-esterified to an unlabeled, rather than to a ^{14}C-labeled molecule of glycerol. This assumption is considered reasonable in view of the very large excess of unlabeled, compared to labeled molecules of glyceride glycerol within the cell (the exogenous labeled glycerol

TABLE 6. Proportion of lipoprotein triacylglycerol secreted directly or via lipolysis of intracellular triacylglycerol

Addition to Culture Medium	n	Initial $^{14}C/^{3}H$ Ratio ($\times 10^2$)	Final $^{14}C/^{3}H$ Ratio ($\times 10^2$)	% Loss of ^{14}C (Secretion via Lipolysis)	Secretion without Lipolysis (% of Total)
Control	7	2.48 ± 0.49	2.04 ± 0.38	17.0 ± 2.5	83.0 ± 2.5
Insulin	4	2.46 ± 0.87	1.70 ± 0.47	24.6 ± 7.6	75.4 ± 7.6
Tolbutamide	4	2.50 ± 0.33	2.00 ± 0.35	18.3 ± 3.4	81.7 ± 3.4

Cells were cultured in the presence of [^{14}C]glycerol and [^{3}H]oleate for 16 h. The medium was removed and the monolayer was washed twice with Puck's saline. Cells from two of the flasks were harvested for measurement of the $^{14}C/^{3}H$ ratio of the intracellular triacylglycerol (column 3). To the remaining cells was added fresh medium (without labeled substrates) with or without insulin (7.8×10^{-8} M) or tolbutamide (5 mM). The cells were cultured for a further 24 h after which the secreted lipoprotein fraction was obtained from the medium and the $^{14}C/^{3}H$ radioactivity ratio of its triacylglycerol was determined (column 4). For calculation of the above value, see Materials and Methods. To calculate the data in column 5, each individual initial ratio was paired with the corresponding final ratio in each experiment which was, in all cases, lower. Each value is the mean of several experiments (n), each of which was the average of results obtained from two flasks.

was diluted at least 3000-fold during its incorporation into cellular glyceride glycerol). A second assumption is that the production of unlabeled glycerol (or glycerophosphate) does not become rate-limiting for re-esterification. If this were to be the case, then if lipolysis occurred to a significant extent, labeled fatty acids would accumulate intracellularly. This was not observed and, in most cases, labeled intracellular free fatty acids amounted to no more than 1% of the total label in the neutral lipid fractions. Furthermore, indirect evidence for a high rate of glycolysis (or glycogenolysis) was observed in HepG2 consistent with a rapid flux of carbon through glycerophosphate. This was based on the rate of appearance of lactate in the culture medium (6.46 ± 0.28 μmol/ml per 24 h; mean ± SEM of 9 flasks). This is equivalent to a production of 77.5 μmol lactate per flask over this period (approx. 25 μmol/mg protein). These data suggest that unlabeled glycerophosphate availability is unlikely to be rate-limiting for the re-esterification of any intracellular fatty acids released by lipolysis.

In rat liver, much more intracellular TAG was lipolyzed than was required to meet the demands for VLDL secretion. Thus, most of the derived fatty acids were simply re-esterified and returned to the cell cytosol (14). Under normal conditions, this fatty acid/TAG "cycling" amounted to a turnover of 1.4 pools of total cellular TAG per 24 h. Using a similar calculation (see Materials and Methods), it could be estimated that under similar conditions in HepG2, only 0.201 pools (20%) of intracellular TAG was lipolyzed and re-esterified during a 24-h period (**Table 7**). This represents a 7-fold decrease compared to that which occurs in normal rat hepatocytes. If lipolysis of intracellular TAG is an obligate step in the mobilization of intracellular lipid for the assembly of normal VLDL particles, as seems likely (14, 35, 36), the relative lack of lipolytic activity in HepG2 may explain the inability of this cell line to assemble normal-size TAG-rich lipoproteins. To test this hypothesis, we studied the effects of tolbutamide on the secretion of lipoprotein TAG in HepG2. In rat hepatocytes, this substance inhibits intracellular TAG lipolysis and suppresses the secretion of VLDL (14). If this is only a minor pathway in the recruitment of the small quantity of lipoprotein TAG in HepG2, it might be expected that tolbutamide would not inhibit TAG secretion to any great extent. This turned out to be the case and although tolbutamide led to a further slight suppression in the already

TABLE 7. Intracellular triacylglycerol turnover in HepG2 cells: effect of tolbutamide

Addition to Culture Medium	n	Initial $^{14}C/^{3}H$ Ratio (×2)	Final $^{14}C/^{3}H$ Ratio (×2)	Relative Decrease in ^{14}C Specific Radioactivity	Entry of Unlabeled Glycerol (% of original pool)	Cell Triacylglycerol (μg/mg protein)	Triacylglycerol Turnover (μg/mg protein)
Control	7	2.48 ± 0.49	2.11 ± 0.43	16.3 ± 3.7	20.1 ± 5.4	310 ± 64.5	62.5
Tolbutamide	4	2.50 ± 0.33	2.16 ± 0.29	13.5 ± 3.5	16.2 ± 4.5	231 ± 74.0	37.4

Cells were cultured in the presence of [^{14}C]glycerol and [^{3}H]oleate for 16 h. The medium was then removed and the monolayer was washed twice with Puck's saline. Cells from two of the flasks were harvested for measurement of the relative specific radioactivity (^{3}H an ^{14}C) of the cellular triacylglycerol (column 3). To the remaining flasks was added fresh medium (without labeled substrates) with or without tolbutamide (5 mM). The cells were cultured for a further 24 h after which the final relative specific radioactivity of the cellular triacylglycerol was determined (column 4). The relative loss of ^{14}C (compared to ^{3}H) during the 24-h culture period (column 5) was calculated by comparing the values in column 3 (initial) with those in column 4 (final). To calculate the data in column 5, each individual initial value was paired with its corresponding final value in each experiment. The entry of unlabeled glycerol into the cell triacylglycerol pool required to achieve the relative change in ^{14}C specific radioactivity was then calculated (column 6, see also Materials and Methods). Triacylglycerol turnover (column 8) was calculated by multiplying the pool size (column 7) by the extent of dilution by unlabeled glycerol (column 6). Each value is the mean of several experiments (n), each of which is the average of two flasks.

TABLE 8. Effect of tolbutamide on the secretion of lipoprotein-associated triacylglycerol and phospholipid

Addition to Flask	Secretion of Lipoprotein Lipid	
	Triacylglycerol	Phospholipid
	nmol/mg protein	
Control	2.25 ± 0.70	1.72 ± 0.54
Tolbutamide (5 mM)	1.84 ± 0.54	1.53 ± 0.45

The triacylglycerol and phospholipid of HepG2 cells were labeled overnight by culturing in the presence of [^{3}H]oleate. After washing the cells, the secretion of pre-labeled lipids was determined over the next 24 h period. Each value is the average ± SEM of three independent experiments, each of which contained duplicate flasks.

low rate of intracellular lipolysis (Table 7), it did not significantly affect the total amount of TAG secreted (**Table 8**) or the proportion of this material that was secreted "en bloc" (Table 6).

Although the fractional rate of intracellular TAG turnover was low, calculation of the absolute rate of intracellular TAG lipolysis suggests that sufficient ester is hydrolyzed to maintain a higher rate of TAG secretion than is actually observed (Table 7). Even if all this lipolyzed TAG were re-esterified and secreted, the resulting rate of TAG output would remain much lower than that observed in rat hepatocytes (34).

Concluding remarks

The rate of TAG synthesis in HepG2 is comparable to that observed in normal rat hepatocytes. This is the case irrespective of whether the TAG is synthesized de novo, from small precursors, or from extracellular fatty acids. Again, like rat hepatocytes, the capacity for TAG synthesis from the latter source is much greater than that from the former. It seems unlikely, therefore, that an impairment of lipogenesis restricts the availability of TAG for the assembly of lipoproteins in HepG2. It is the mobilization of intracellular lipid which, in HepG2, appears to be impaired compared to that which occurs in rat liver. It has previously been shown, for instance, that the capacity for fatty acid oxidation by HepG2, as measured by the activity of carnitine palmitoyl transferase (CPT), is very low (37). It is of possible relevance that, in FAO hepatoma cells, fatty acid oxidation is prevented by hypersensitivity of CPT to inhibition by malonyl-CoA (38). Consistent with this, in the present work, in three independent experiments we were unable to detect any ketone body formation when HepG2 cells were cultured in the presence of oleate (0.75 mM) for 24 h. The lower limit of detection was 0.05 μmol/mg protein. In the present work under identical conditions, rat hepatocytes synthesized 2.4 μmol of ketone bodies (acetoacetate plus β-hydroxybutyrate) per mg protein. This inability to mobilize intracellular TAG also extends to a defective lipolysis and may account for the inability of this cell line to recruit sufficient TAG for the assembly of normal sized VLDL particles. Some of the TAG that becomes associated with apolipoprotein B does so during translocation into the lumen of the ER (39, 40). It has been suggested, however, that most of the TAG normally becomes associated with the incipient lipoprotein particle at some later stage (8) and that this step is absent in HepG2. Whether both of these TAG-loading operations require lipolysis and re-esterification is not known. Although the microsomal TAG transfer protein is essential for the assembly of TAG-rich lipoproteins (41), the precise details of its functional role remain obscure. Finally, any comparison of the assembly and secretion of lipoproteins by HepG2 and rat hepatocytes should take account of the fact that in the latter model a large proportion of the particles secreted contain apoB-48 rather than apoB-100. We cannot rule out the possibility that this, in itself, may impose differences in the means of recruitment of intracellular TAG for lipoprotein assembly.

We would like to thank Mrs. M. Barber for typing the manuscript. M. Seelaender was supported by a grant from the government of Brazil. This work was supported by a project grant from the Medical Research Council (U.K.). G. F. G. is a member of the External Staff of the Medical Research Council at the University of Oxford.

Manuscript received 11 October 1993 and in revised form 27 March 1994.

REFERENCES

1. Javitt, N. B. 1990. HepG2 cells as a resource for metabolic studies: lipoprotein, cholesterol, and bile acids. *FASEB J.* **4:** 161–168.
2. McGarry, J. D., and D. W. Foster. 1980. Regulation of hepatic fatty acid oxidation and ketone body production. *Annu. Rev. Biochem.* **49:** 395–420.
3. Thrift, R. N., T. M. Forte, B. E. Cahoon, and V. G. Shore. 1986. Characterization of lipoproteins produced by the human liver cell line, HepG2, under defined conditions. *J. Lipid Res.* **27:** 236–250.
4. Ellsworth, J. L., S. K. Erickson, and A. D. Cooper. 1986. Very low and low density lipoprotein synthesis and secretion by the human hepatoma cell line Hep-G2: effects of free fatty acid. *J. Lipid Res.* **27:** 858–874.
5. Dixon, J. L., S. Furukawa, and H. N. Ginsberg. 1991. Oleate stimulates secretion of apolipoprotein B-containing lipoproteins from HepG2 cells by inhibiting early intracellular degradation of apolipoprotein B. *J. Biol. Chem.* **266:** 5080–5086.
6. Furukawa, S., N. Sakata, H. N. Ginsberg, and J. L. Dixon. 1992. Studies of the sites of intracellular degradation of apolipoprotein B in HepG2 cells. *J. Biol. Chem.* **267:** 22630–22638.
7. Dixon, J. L., and H. N. Ginsberg. 1993. Regulation of hepatic secretion of apolipoprotein B-containing lipoprotein: information obtained from cultured liver cells. *J. Lipid Res.* **34:** 167–179.
8. Spring, D. J., L. W. Chen-Liu, J. E. Chatterton, J. Elovson, and V. N. Schumaker. 1992. Lipoprotein assembly. Apolipoprotein B size determines lipoprotein core circumfer-

ence. *J. Biol. Chem.* **267:** 14839–14845.
9. Davis, R. A., J. R. Boogaerts, R. A. Borchardt, M. Malone-McNeal, and J. Archambault-Schexnayder. 1985. Intrahepatic assembly of very-low-density lipoproteins. *J. Biol. Chem.* **260:** 14137–14144.
10. Windmueller, H. G., and A. E. Spaeth. 1985. Regulated biosynthesis and divergent metabolism of three forms of hepatic apolipoprotein B in the rat. *J. Lipid Res.* **26:** 70–81.
11. Yamamoto, M., I. Yamamoto, Y. Tanaka, and J. A. Ontko. 1987. Fatty acid metabolism and lipid secretion by perfused livers from rats fed laboratory stock and sucrose-rich diets. *J. Lipid Res.* **28:** 1156–1165.
12. Baum, C. L., B-B. Teng, and N. O. Davidson. 1990. Apolipoprotein B messenger RNA editing in the rat liver. *J. Biol. Chem.* **265:** 19263–19270.
13. Gibbons, G. F. 1990. Assembly and secretion of hepatic very-low-density lipoprotein. *Biochem. J.* **268:** 1–13.
14. Wiggins, D., and G. F. Gibbons. 1992. The lipolysis/esterification cycle of hepatic triacylglycerol. *Biochem. J.* **284:** 457–462.
15. Bartlett, S. M., and G. F. Gibbons. 1988. Short and longer-term regulation of very-low-density lipoprotein secretion by insulin, dexamethasone and lipogenic substrates in cultured hepatocytes. *Biochem. J.* **249:** 37–43.
16. Pullinger, C. R. and G. F. Gibbons. 1983. The relationship between the rate of hepatic sterol synthesis and the incorporation of [^{3}H] water. *J. Lipid Res.* **24:** 1321–1328.
17. Edelstein, C., and A. M. Scanu. 1986. Precautionary measures for collecting blood destined for lipoprotein isolation. *Methods Enzymol.* **128:** 151–155.
18. Pullinger, C. R., J. D. North, B-B. Teng, V. A. Rifici, A. E. Ronhild de Brito, and J. Scott. 1989. The apolipoprotein B gene is constitutively expressed in HepG2 cells: regulation of secretion by oleic acid, albumin, and insulin, and measurement of the mRNA half-life. *J. Lipid Res.* **30:** 1065–1078.
19. Gibbons, G. F., C. P. Attwell Thomas, and C. R. Pullinger. 1986. The metabolic route by which oleate is converted into cholesterol in rat hepatocytes. *Biochem. J.* **235:** 19–24.
20. Jungas, R. L. 1968. Fatty acid synthesis in adipose tissue incubated in tritiated water. *Biochemistry.* **7:** 3708–3717.
21. Folch, J., M. Lees, and G. H. Sloane Stanley. 1956. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
22. Goldfarb, S., T. A. Barber, M. A. Pariza, and T. D. Pugh. 1978. Lipid synthesis and ultrastructure of adult rat hepatocytes during their first twenty-four hours in culture. *Exp. Cell. Res.* **117:** 39–46.
23. 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.
24. van Harken, D. R., C. W. Dixon, and M. Heimberg. 1969. Hepatic lipid metabolism in experimental diabetes. V. The effect of concentration of oleate on metabolism of triglycerides and on ketogenesis. *J. Biol. Chem.* **244:** 2278–2285.
25. Trinder, P. 1969. Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. *Ann. Clin. Biochem.* **6:** 24–30.
26. Gibbons, G. F., and F. J. Burnham. 1991. Effect of nutritional state on the utilization of fatty acids for hepatic triacylglycerol synthesis and secretion as very low density lipoprotein. *Biochem. J.* **275:** 87–92.
27. Gibbons, G. F., C. R. Pullinger, and O. G. Björnsson. 1984. Changes in the sensitivity of lipogenesis in rat hepatocytes to hormones and precursors over the diurnal cycle and during longer-term starvation of donor animals. *J. Lipid Res.* **25:** 1358–1367.
28. Björnsson, O. G., J. D. Sparks, C. E. Sparks, and G. F. Gibbons. 1992. Prostaglandins suppress VLDL secretion in primary rat hepatocyte cultures: relationships to hepatic calcium metabolism. *J. Lipid Res.* **33:** 1017–1027.
29. Duerden, J. M., and G. F. Gibbons. 1990. Storage, mobilization and secretion of cytosolic triacylglycerol in hepatocyte cultures. The role of insulin. *Biochem. J.* **272:** 583–587.
30. Dashti, N., and G. Wolfbauer. 1987. Secretion of lipids, apolipoproteins, and lipoproteins by human hepatoma cell line, HepG2: effects of oleic acid and insulin. *J. Lipid Res.* **28:** 423–436.
31. Byrne, C. D., N. P. J. Brindle, T. W. M. Wang, and C. N. Hales. 1991. Interaction of non-esterified fatty acid and insulin in control of triacylglycerol secretion by HepG2 cells. *Biochem. J.* **280:** 99–104.
32. Byrne, C. D., T. W. M. Wang, and C. N. Hales. 1992. Control of HepG2-cell triacylglycerol and apolipoprotein B synthesis and secretion by polyunsaturated non-esterified fatty acids and insulin. *Biochem. J.* **288:** 101–107.
33. Hermier, D., P. Hales, and D. N. Brindley. 1991. Biphasic effects of glucagon and cyclic AMP on the synthesis and secretion of lipids by rat hepatocytes. *Biochem. J.* **279:** 705–709.
34. Björnsson, O. G., J. M. Duerden, S. M. Bartlett, J. D. Sparks, C. E. Sparks, and G. F. Gibbons. 1992. The role of pancreatic hormones in the regulation of lipid storage, oxidation and secretion in primary cultures of rat hepatocytes. Short- and long-term effects. *Biochem. J.* **281:** 381–386.
35. Bar-On, H., P. S. Roheim, O. Stein, and Y. Stein. 1971. Contribution of floating fat triglyceride and of lecithin towards formation of secretory triglyceride in perfused rat liver. *Biochim. Biophys. Acta.* **248:** 1–11.
36. Francone, O. L., A-D. Kalopissis, and G. Griffaton. 1989. Contribution of cytoplasmic storage triacylglycerol to VLDL-triacylglycerol in isolated rat hepatocytes. *Biochim. Biophys. Acta.* **1002:** 28–36.
37. Agius, L. 1988. Metabolic interactions of parenchymal hepatocytes and dividing epithelial cells in co-culture. *Biochem. J.* **252:** 23–28.
38. Guzmán, M., and M. J. H. Geelen. 1993. Regulation of fatty acid oxidation in the liver. *Biochim. Biophys. Acta.* **1167:** 227–241.
39. Borén, J., L. Graham, M. Wettesten, J. Scott, A. White, and S-V. Olofsson. 1992. The assembly and secretion of apoB-100-containing lipoproteins in HepG2 cells. *J. Biol. Chem.* **267:** 9858–9867.
40. Pease, R. J., G. B. Harrison, and J. Scott. 1991. Co-translational insertion of apolipoprotein B into the inner leaflet of the endoplasmic reticulum. *Nature.* **353:** 448–450.
41. Wetterau, J. R., L. P. Aggerbeck, M. E. Bouma, C. Eisenberg, A. Munck, M. Hermier, J. Schmitz, G. Gay, D. J. Rader, and R. E. Gregg. 1992. Absence of microsomal triglyceride transfer protein in individuals with abetalipoproteinaemia. *Science.* **258:** 999–1001.