Metabolic characteristics of a human hepatoma cell line stably transfected with hormone-sensitive lipase

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Clones of HepG2 cells were selected that stably express the cDNA for hormone-sensitive lipase (HSL). When cells were cultured in the presence of labelled extracellular oleate, accumulation of labelled fatty acid as cellular triacylglycerol (TAG) was significantly lower in the transfectants compared with the wild-type cells. There was no change in the net rate of phospholipid (PL) synthesis. Culture of cells containing isotopically prelabelled TAG resulted in a greater net loss of TAG from the transfectant cells than from the wild-type cells. The excess loss of labelled TAG was primarily due to an increased TAG fatty acid oxidation. Free fatty acid release into the medium was not increased in the transfectants, nor was the very low rate of lipoprotein lipid secretion. Also, there was no increased net

trafficking of fatty acids from TAG into PLs. Changes in the ³H: ¹⁴C ratio of TAG prelabelled with [³H]glycerol and [¹⁴C]oleate suggested that none of excess TAG fatty acid released in the transfectant cells underwent intracellular re-esterification to TAG prior to oxidation. The results suggest that fatty acids mobilized by HSL are directed immediately into the oxidative pathway and are not available for biosynthetic processes. It appears likely, therefore, that intracellular TAG-derived fatty acids which enter the oxidative pathway exist in a different compartment from those that are directed towards synthesis.

Key words: cytosol, endoplasmic reticulum, lipid synthesis, lipolysis, secretion.

INTRODUCTION

Fatty acids released from the storage pool of triacylglycerol (TAG) in the cytosol of hepatocytes may be utilized as substrates for very-low-density lipoprotein (VLDL) TAG synthesis [1–4], for oxidation [5,6], or for re-esterification and cycling back to the intracellular pool [1,7,8]. Little is known, however, of the lipase(s) responsible for mobilization of hepatic cytosolic TAG or the means by which the product fatty acids are targeted either to the endoplasmic reticulum (ER) for esterification and VLDL assembly, or to the mitochondrion for oxidation. In several tissues that are capable of mobilizing neutral lipids, hormone-sensitive lipase (HSL) has been implicated (for a review, see [8]). It is generally agreed, however, that HSL, with the possible exception of the pig [9], is not expressed in mammalian liver [10–12]. Nevertheless, in view of the central role of HSL in other tissues, we determined the fate of fatty acids produced by HSL in a hepatocellular environment. To do this we have expressed HSL in the human hepatocellular cell line HepG2, since the ability of these cells to mobilize endogenous TAG is greatly impaired in comparison with that in primary hepatocytes [13]. Particularly, we wished to determine whether expression of HSL facilitated the lipolytic recruitment of fatty acids from TAG in the cell cytosol, the site of the major TAG storage pool in the liver [3,14,15], for irreversible removal from the hepatocytes as oxidation products or as lipoprotein TAG.

MATERIALS AND METHODS

Materials

All reagents, including organic solvents, were of analytical grade. Materials for tissue culture (RPMI 1640, Dulbecco's modified Eagle's medium, Optimen, Dulbecco's PBS, fetal calf serum, penicillin, streptomycin and geneticin sulphate) were obtained

from Gibco (Paisley, Scotland, U.K.). [¹⁻¹⁴C]Oleate, [²⁻³H]glycerol and ¹²⁵I-Protein A were obtained from Amersham International (Little Chalfont, Bucks, U.K.). [³²P]dCTP and [^{γ-32}P]ATP were obtained from New England Nuclear. BSA (fatty acid free), sodium oleate, glycerol, bovine insulin, Protein A–Sepharose, digitonin, prestained colour electrophoresis markers and proteinase K were from Sigma (Poole, Dorset, U.K.). The triacylglycerol assay kit (GPO-PAP) was obtained from Boehringer Mannheim (Lewes, Sussex, U.K.). A detergent-compatible protein assay kit was obtained from Bio-Rad (Hemel Hempstead, Herts, U.K.). Immobilon P (PVDF) transfer membranes were obtained from Millipore (Watford, Herts, U.K.). 3T3-L1 cells that had been differentiated to adipocytes and the anti-HSL antibody as described in [12] were kind gifts from Ms. Lisa O'Rourke and Dr. Peter Shepherd (Department of Biochemistry and Molecular Biology, University College London, London, U.K.).

Hepatoma transfection

HepG2 cells and McArdle RH7777 (rat hepatoma) cells were obtained from Dr. Philippa Talmud (Rayne Institute, University College Medical School, London, U.K.) and were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum. A construct containing the coding region of the rat HSL sequence (in plasmid pSVL [16]) was linearized at the unique *Pvu*I site present in the β -lactamase gene and was co-transfected with plasmid pK-neo into HepG2 cells (30 % confluent in 6-cm dishes) by the lipofectin procedure (as described by the manufacturers, Gibco). Colonies resistant to geneticin (1000 μ g/ml) were expanded. Genomic DNA was prepared from 5×10^7 cells by lysis with SDS in the presence of proteinase K followed by phenol/chloroform extraction essentially as described by Sambrook et al. [17].

Abbreviations used: VLDL, very-low-density lipoprotein; TAG, triacylglycerol; ER, endoplasmic reticulum; HSL, hormone-sensitive lipase; PL, phospholipid; MAG, monoacylglycerol.

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Following digestion with restriction enzymes, the DNA fragments were fractionated on 1% agarose gels and transferred by capillary blotting on to Zeta Probe GT membranes (Bio-Rad). Blots were probed with ^{32}P -labelled cDNA using the random-priming kit obtained from Sigma [17]. Total cellular RNA was extracted using the SV RNA-isolation system as described by the manufacturers (Promega, Southampton, Hants, U.K.). RNA was fractionated on Mops/agarose gels containing 2.2 M formaldehyde [17], and was then transferred on to Hybond N membranes (Amersham) by capillary blotting. Immobilized RNA was probed sequentially with ^{32}P -labelled cDNA for HSL and glyceraldehyde-3-phosphate dehydrogenase under conditions of high stringency [17]. Finally, blots were reprobed with an oligonucleotide complementary to 28 S rRNA (5'-AACGATC-AGAGTAGTGGTATTTCCACC-3'); 0.75 μg was end labelled by incubation with 30 μCi [γ - ^{32}P]ATP and 5 units of T_4 polynucleotide kinase. The product was separated from unincorporated label on a Sephadex G-25 spin column (Pharmacia, St. Albans, Herts, U.K.) and was hybridized with the filter for 4 h at 45 °C in 10 ml of medium containing 5 $\times$ SSPE [where 1 $\times$ SSPE is 0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA], 50% formamide, 0.2% SDS, 10% dextran sulphate, 5 $\times$ Denhardt's solution and 100 $\mu\text{g}/\text{ml}$ sonicated salmon sperm DNA, prepared as in [17]. The filter was washed at 25 °C for 15 min with 2 $\times$ SSPE/0.1% SDS and then for 3 min with 1 $\times$ SSPE/0.1% SDS. Expression of HSL protein was verified by immunoprecipitation followed by immunoblotting. Approx. 5×10^7 cells were disrupted in 1.5 ml of immunoprecipitation buffer [1% Triton X-100/10 mM Tris/5 mM EDTA/50 mM NaCl/50 mM disodium pyrophosphate/50 mM sodium fluoride/0.1 mM sodium orthovanadate (pH 7.4)/1 mM PMSF] by 10 passes through a 21-gauge needle. The samples were incubated at 4 °C for 30 min and then insoluble material was removed by centrifugation in a bench-top centrifuge. The supernatant was pre-cleared by incubation for 2 h with Protein A-Sepharose and centrifugation. Finally, the supernatant was incubated for 16 h with 3 μl of affinity-purified anti-HSL antibody (described in [12]), followed by a 2-h incubation with Protein A-Sepharose. Immune complexes were collected by centrifugation, heated at 80 °C with 4 $\times$ SDS/PAGE loading buffer [18], and subsequently cooled and diluted with 4 vols. of 0.25 M sucrose. The beads were removed by centrifugation and supernatants were resolved on 11% polyacrylamide gels and then electrophoretically transferred on to PVDF membranes as described in [19]. Membranes were blocked with 1% non-fat milk containing 0.1% Tween 20, and then probed sequentially with a 1:2000 dilution of anti-HSL antibody and 10 μCi ^{125}I -Protein A in blocking buffer as described in [19]. In some experiments, cells were disrupted in 0.25 M sucrose/25 mM Tris (pH 7.4) containing 20 $\mu\text{g}/\text{ml}$ water-soluble digitonin with 10 passes through a 21-gauge needle. A soluble fraction was separated from total cellular membranes by centrifugation at 100 000 g for 15 min at 4 °C in the TLA 100.3 rotor of a Beckman bench-top ultracentrifuge. Subsequently, the supernatant fraction was concentrated in a Centrprep 10 cartridge and the membrane and supernatant fractions were immunoprecipitated essentially as described above.

Hepatoma culture

After transfection, all cell lines, including wild-type cells, were maintained in 250-ml tissue culture flasks with 12.0 ml of RPMI 1640 (containing 2 mM L-glutamine) and supplemented with fetal calf serum (10%, v/v), penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$). The medium was changed every 48 h.

When the cells had reached between 50 and 75% confluence, the medium was changed and replaced with fresh RPMI 1640 supplemented with fetal calf serum. Up to this point, geneticin sulphate (500 $\mu\text{g}/\text{ml}$) was present in the medium of the transfected cells. [^{14}C]Oleate (0.75 mM; 224 d.p.m./nmol) and [^3H]glycerol (0.25 mM; 9800 d.p.m./nmol) were added at this stage and the cells were cultured for 16 h. The labelled medium was removed and retained for lipid and lipoprotein analysis. The cell monolayer was washed twice with PBS. Cells from two flasks in each experimental group were harvested for measurement of [^{14}C]oleate and [^3H]glycerol incorporation into cellular glycerolipids. To the remaining flasks in each group was added fresh medium, as above, but lacking serum and labelled substrates. Cells were cultured under these conditions for a further 24 h. The medium was removed for measurement of lipids, lipoproteins and oxidation products. Cells were harvested and stored at -20 °C for lipid analysis.

Analytical methods

Aliquots of the cell medium were used for extraction of labelled and unlabelled lipids using the method of Folch et al. [20]. In some cases, the total lipoprotein fraction of the medium was obtained by adjusting the density to 1.21 g/ml with solid KBr. The medium was centrifuged for 16 h in the outer ring of a Beckman 50.4 rotor at 149 000 g and the floating lipoprotein fraction was obtained by tube slicing. Lipids were obtained from this fraction by Folch extraction [20]. Individual classes of labelled lipids [fatty acids, phospholipid (PL), TAG] were separated by TLC [21]. Fatty acid oxidation from prelabelled cellular TAG was determined by removing an aliquot of the medium and measuring total ^{14}C content by scintillation counting. The contribution of secreted lipids to the measured ^{14}C label was negligible (see below). Another aliquot of the medium was acidified with 60% perchloric acid for measurement of acid-soluble ketone bodies [22]. Measurement of $^{14}\text{CO}_2$ production was carried out in modified tissue culture flasks fitted with a removable inner well containing 2.0 ml of 3 M KOH. Flasks were sealed with a 'Subaseal' rubber cap. At the end of the culture period, 60% perchloric acid was introduced using a syringe. Released $^{14}\text{CO}_2$ was trapped as $\text{KH}^{14}\text{CO}_2$ in the inner well by shaking the flasks gently at room temperature for 1 h. Radioactivity was determined by scintillation counting.

Cellular lipids were extracted using the method of Folch et al. [20]. Aliquots of the total lipid fractions were removed for enzymic measurement of the mass of cellular TAG using the GPO-PAP assay kit (see the Materials and methods section). Individual lipids doubly labelled with ^3H and ^{14}C were separated from the remainder of the total-lipid fraction by TLC [21]. Bands containing these lipids were scraped from the plate and assayed by scintillation counting. Prior to addition to the cells, oleate was bound to BSA (essentially fatty acid-free) by the method of Van Harken et al. [23]. Cellular protein was determined using the method of Lowry et al. [24]. Lactate dehydrogenase was assayed as described in [25].

Calculation of intracellular TAG lipolysis and re-esterification

When cellular TAG is prelabelled with [^3H]glycerol and [^{14}C]oleate followed by culture of cells in the absence of extracellular fatty acid, *de novo* synthesis of unlabelled TAG might be expected to decrease the initial ^3H -specific radioactivity of cellular TAG to exactly the same extent as that for ^{14}C . Any differential change in the specific radioactivity of the two isotopes implies that the [^3H]glycerol and [^{14}C]oleate moieties must, at some stage, have become metabolically distinct during the subsequent

culture period, a process which required lipolysis followed by re-esterification. For instance, in a typical experiment, in the wild-type cells, the initial ^{14}C -specific activity of cellular TAG was 624 ± 46 d.p.m./ μg and this declined only slightly to 560 ± 33 d.p.m./ μg (87.9 % of initial) during a subsequent 24-h culture period. The ^3H -specific activity, on the other hand, decreased from 1053 ± 238 to 625 ± 103 d.p.m./ μg (59.3 % of initial). The extent of the decrease in the $^3\text{H}:^{14}\text{C}$ specific-activity ratio is a measure of the extent to which a relatively unlabelled glycerol-containing moiety has entered the glyceride glycerol pool and, thus, reflects the degree of TAG lipolysis and re-esterification. In Table 1 (see below), the $^3\text{H}:^{14}\text{C}$ specific-activity ratio of cellular TAG of the wild-type cells declined from 1.49 to 1.03. The latter value was 68.9 % of the former. In general, if the specific-activity ratio decreased to $\chi\%$ of the initial value, it may be calculated that the amount of excess unlabelled glycerol that has entered the cellular TAG pool to account for this decrease is $(100/\chi) - 1$ pools. This is equivalent to the fractional turnover (lipolysis and re-esterification) of cellular TAG per 24 h.

Statistical analysis

All values are presented as the means $\pm$ S.E.M. of several independent experiments, each carried out at a different time. In each experiment, flasks were cultured in duplicate at each time point and the values averaged. Significant differences were obtained using a paired Student's *t* test.

RESULTS

Preliminary experiments were carried out to examine the rate of endogenous lipid mobilization in the rat hepatoma cell line, McArdle RH7777. This line has been used more widely than HepG2 for transfection studies of lipoprotein secretion and might, therefore, be preferable for our current investigation of lipase expression. Cellular TAG was prelabelled by overnight culture in the presence of [^{14}C]oleate and [^3H]glycerol. Following removal of the labelled medium and washing the cells free of labelled substrate, the hepatocytes were cultured in fresh medium, without labelled substrates, for a further 24 h. As shown in Table 1, the amount of labelled TAG secreted from these cells amounted to little more than 1 % of the total cellular pool. This proportion was very small compared with that secreted by primary cultures of rat hepatocytes under similar conditions, which amounted to

Table 1 Triacylglycerol synthesis, turnover and secretion in McArdle RH7777 cells

Cellular TAG was prelabelled with [^3H]glycerol and [^{14}C]oleate. Labelled cells were then cultured for 24 h in the absence of labelled substrate and the secretion of labelled TAG associated with lipoproteins of density < 1.21 g/ml was measured. The ^3H and ^{14}C specific radioactivities of the cellular TAG at the beginning and the end of the 24-h culture period were measured and the $^3\text{H}:^{14}\text{C}$ ratios calculated. These values were used to calculate the fractional turnover of cellular TAG (see the Materials and methods section). The results are the means $\pm$ S.E.M. of four flasks of cells.

Parameter	Value
Cellular [^{14}C]TAG (d.p.m. $\times 10^{-3}$ /mg of protein)	305 ± 67
Lipoprotein [^{14}C]TAG (dpm $\times 10^{-3}$ /mg of protein)	2.61 ± 0.23
Lipoprotein [^{14}C]TAG (% of cellular [^{14}C]TAG)	1.04 ± 0.29
Initial cellular TAG $^3\text{H}:^{14}\text{C}$ ratio	1.79 ± 0.22
Final cellular TAG $^3\text{H}:^{14}\text{C}$ ratio	0.61 ± 0.09
Final cellular TAG $^3\text{H}:^{14}\text{C}$ ratio (% of initial)	34.4 ± 5.3
Fractional TAG turnover (pools/24 h)	2.12 ± 0.48

Figure 1 Southern blots of untransfected and transfected cells

HepG2 cells were transfected with plasmid pSVL-HSL, which was linearized at the unique *PvuII* site. Genomic DNA was isolated from both transfected (HS2D) and untransfected (UT) cells and, following restriction digestion, was blotted on to nitrocellulose and probed with radiolabelled rat HSL cDNA. Digestion with *EcoRI* yielded a major hybridizing band (A), consistent with the anticipated size (3.9 kb) in the transfected cells, and also two larger cross-hybridizing fragments in the untransfected cells (arrows). Lanes 1 and 2 show a 48-h exposure, where endogenous gene fragments are visible in the untransfected cells. Lanes 3 and 4 show a shorter exposure (4 h) to confirm that the intense hybridization due to the transfected gene in lane 2 derive from a single band. Double digestion with *PstI* and *EcoRV* (lanes 5 and 6) yielded three fragments (B, C and D) of the expected sizes in the transfected cells (3.1, 0.93 and 0.56 kb respectively), and three endogenous bands (arrows) in the untransfected cells. One of these was similar in size to band B but the relative intensities confirm that band B derives from the transfected plasmid.

50 % of the cellular pool [1]. Efficient secretion of VLDL TAG in rat hepatocytes was accompanied by a high rate of TAG lipolysis and re-esterification, as indicated by the relative decrease in the specific radioactivity of the labelled glycerol moiety of TAG compared with that of the labelled fatty acid moiety [1,7]. To determine whether, as seemed to be the case in HepG2 [13], the low rate of TAG secretion in McArdle cells was associated with a low intracellular TAG turnover, we measured the relative decrease in the specific radioactivity of the [^3H]glycerol-prelabelled cellular TAG over the 24-h period during which TAG was being secreted. This was reflected by the difference between the initial and final $^3\text{H}:^{14}\text{C}$ specific-activity ratios of the cellular TAG (Table 1). In contrast to HepG2, a substantial rate of TAG recycling was apparent in the McArdle cells, approximately equal to that observed previously in primary hepatocytes [1]. It seems clear, therefore, that a normal rate of TAG lipolysis and re-esterification is not invariably associated with a high rate of TAG secretion. This observation is of some importance in interpreting the results from the HepG2 transfectants described below. The high rate of endogenous TAG recycling in the wild-type McArdle cells clearly limited their potential for lipase-gene expression studies. All subsequent transfection studies were therefore carried out in HepG2 cells, in which both TAG recycling and TAG secretion are decreased [13].

HepG2 colonies arising from co-transfection of HSL and neo-cDNAs were expanded and analysed for the presence of integrated copies of the *HSL* gene by Southern blotting of restriction-digested DNA. Digestion of genomic DNA from clone HS2D gave a single band of $\approx 4\text{kDa}$, which hybridized

Figure 2 Northern blots of transfected and untransfected cells

Total cellular RNA (5 µg) from untransfected (UT) or HSL-transfected (HS2D) HepG2 cells was fractionated on 1% denaturing agarose gels, and transferred on to nylon membranes. The blot was probed sequentially with (i) rat HSL cDNA (lanes 1 and 2), an oligonucleotide complementary to 28 S rRNA (lanes 3 and 4) and cDNA encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH, lanes 5 and 6). Lanes 1 and 2 show an 18-h exposure to autoradiography film, lanes 3–6 were visualized with a Fuji PhosphorImager. HSL transcripts were detected only in the transfected cells, whereas the control RNAs were present at comparable levels in the transfected and untransfected cells. Some faster-migrating products were revealed in both UT and HS2D RNA with the 28 S rRNA probe (broken arrow) representing either cross reaction with 18 S rRNA or cleavage of the 28 S species.

with the HSL cDNA while *EcoRV/PstI* double digestion yielded the expected three fragments (Figure 1). The presence of HSL transcripts was confirmed by probing Northern blots of total cellular RNA HS2D (Figure 2). It was previously shown that transcripts from vector pSVL-HSL generate enzymically active HSL in COS cells [16]. To verify that the HSL mRNA is translated in HepG2 cells, lysates were immunoblotted with an anti-HSL antibody. A polypeptide of the expected size was detected in transfected but not untransfected HepG2 cells (Figure 3). The 88-kDa polypeptide was also present in differentiated 3T3-L1 adipocytes, which express HSL. In other experiments (results not shown) it was verified that the antibody was present in excess throughout the procedure. Since the total protein concentrations in the lysates from the transfectants and the 3T3-L1 cells were similar, the results suggest that the concentration of HSL protein in the transfectants was comparable with that in 3T3-L1 cells. When cells were homogenized and resolved into soluble and total membrane fractions, the majority of the immunoreactive protein was associated with the membrane fraction. This was not due to incomplete homogenization, since > 98% of lactate dehydrogenase activity was present in the soluble fraction.

The net incorporation of [14 C]oleate into cellular TAG was significantly decreased in the HS2D transfectants compared with the wild-type cells (355000 ± 42000 versus 497000 ± 39000 d.p.m./mg of protein respectively, $P < 0.02$). In contrast, there was no change in the incorporation of label into cellular PLs (158300 ± 13700 versus 157300 ± 11700 d.p.m./mg of protein respectively, $n = 11$). Further culture of cells in which the cellular TAG had been prelabelled with [14 C]oleate showed a net loss of [14 C]-labelled TAG from both the wild-type cells and the HSL transfectants. However, the loss was much greater in the transfected cells (Table 2). Measurement of changes in the cellular TAG mass showed a similar pattern. In each cell type,

Figure 3 HSL protein is expressed in transfected (HS2D) HepG2 cells

Lysates from untransfected (UT) HepG2 cells, HS2D transfectants and differentiated 3T3-L1 adipocytes were adjusted to similar total protein concentrations and then analysed by immunoblotting with an antibody against HSL, as described in the Materials and methods section. A band of the expected size was observed in the transfected cells and the adipocytes (lanes 2 and 3). HS2D transfectants were also fractionated into a cytosolic supernatant fraction (SN) and a total membrane pellet (PELL). When equivalent amounts of each fraction (proportional to their relative quantities within the intact cell) were analysed, the immunoreactive band was found primarily in the membrane pellet (lanes 4 and 5).

the loss of [14 C] label was always greater than the loss of TAG mass ($P < 0.05$ in a paired t test). There was no net loss of labelled PL from either cell type. Indeed, there was a significant increase in the amounts of label associated with PL in both the wild-type cells ($P < 0.05$) and the HSL transfectants ($P < 0.05$) during the 24-h culture period. In the wild-type cells, this transfer accounted for about 35% of the TAG that disappeared from the cells; the corresponding value for the HSL transfectants was about 15%. In absolute terms, the amount of TAG fatty acids directed into PLs was somewhat higher in the wild-type cells compared with the transfected cells. It was, therefore, apparent that HSL over-expression selectively mobilized stored TAG in HepG2 and irreversibly removed it from the cell. This process can be assumed to have been responsible for the decreased net accumulation of cellular TAG during the prelabelling period.

We next investigated the fate of the TAG fatty acids, which disappeared and which were not incorporated into PL. As mentioned above, this loss was far greater in the transfectants than in the wild-type cells. The increased loss in the transfectants could not be explained by an increase in the release of unesterified fatty acid into the medium (wild-type cells, 2344 ± 953 d.p.m./flask; transfectants, 1372 ± 128 d.p.m./flask; $n = 4$). The absolute amounts of labelled non-esterified fatty acids remaining with the cell were also similar in each case (28592 ± 2691 and 29585 ± 3390 d.p.m./flask for the wild-type and transfectants respectively, $n = 4$). Measurement of the total amounts of [14 C] label released into the medium after 24-h culture of the prelabelled cells showed that this was increased in the HSL transfectants compared with that in the wild-type cells (156000 ± 12500 and 92800 ± 8600 d.p.m./flask respectively, $P < 0.01$). These amounts represented $53.1 \pm 4.3\%$ and $28.3 \pm 4.3\%$ respectively of the corresponding initial amounts of

Table 2 Net removal of cellular TAG fatty acids from wild-type and HSL-transfected HepG2 cells

Cellular TAG was prelabelled with [^{14}C]oleate (224 d.p.m./nmol) for 16 h. Cells were then cultured for a further 24 h in the absence of labelled substrates. The mass and ^{14}C content of the cellular TAG was determined at the beginning (0 h) and the end (24 h) of the 24-h culture period. The values are the means $\pm$ S.E.M. of eight flasks in each group. Values marked ** and *** for the HS2D transfectants are significantly different ($P < 0.01$ and $P < 0.001$ respectively) from the corresponding value for the wild-type cells.

	Triacylglycerol ($\mu\text{g}/\text{flask}$)			Triacylglycerol (^{14}C d.p.m. $\times 10^{-3}/\text{flask}$)		
	0 h	24 h	% Change	0 h	24 h	% Change
Wild-type cells	548 $\pm$ 73	334 $\pm$ 67	-35.5 $\pm$ 5.3	362 $\pm$ 63	218 $\pm$ 47	-45.1 $\pm$ 4.9
HS2D transfectants	386 $\pm$ 31	104 $\pm$ 8**	-71.1 $\pm$ 2.9***	242 $\pm$ 35	53 $\pm$ 6**	-78.1 $\pm$ 1.8***

	Phospholipid (^{14}C d.p.m. $\times 10^{-3}/\text{flask}$)			Fatty acid (^{14}C d.p.m. $\times 10^{-3}/\text{flask}$)		
	0 h	24 h	% Change	0 h	24 h	% Change
Wild-type cells	97.3 $\pm$ 19.9	146.3 $\pm$ 22.8	+61.3 $\pm$ 8.3	29.5 $\pm$ 2.9	28.6 $\pm$ 2.7	-7.8 $\pm$ 3.5
HS2D transfectants	107.2 $\pm$ 15.6	136.8 $\pm$ 22.1	+20.6 $\pm$ 8.7**	50.4 $\pm$ 4.0***	29.6 $\pm$ 3.4	-42.8 $\pm$ 5.0***

Table 3 Fatty acid oxidation by wild-type and HSL transfected HepG2 cells

Cellular TAG was prelabelled overnight by culturing cells for 16 h in the presence of [^{14}C]oleate (224 d.p.m./nmol). Following removal of the labelled medium and addition of fresh medium, cells were cultured for a further 24 h. The production of ^{14}C -labelled ketone bodies and $^{14}\text{CO}_2$ during this period was determined as described in the Materials and methods section. The above values are the means $\pm$ S.E.M. of six flasks in each group. Values marked * and ** for the HS2D transfectants are significantly different ($P < 0.05$ and $P < 0.01$ respectively) from the corresponding value for the wild-type cells.

	Ketone bodies		CO_2	
	d.p.m./flask	% of cell TAG	d.p.m./flask	% of cell TAG
Wild-type cells	51 800 $\pm$ 1200	17.0 $\pm$ 3.4	28 200 $\pm$ 7600	10.1 $\pm$ 2.4
HS2D transfectants	100 100 $\pm$ 7900**	36.9 $\pm$ 6.6*	48 500 $\pm$ 4300*	23.2 $\pm$ 1.0**

labelled TAG in each cell type. The contribution of labelled lipids associated with secreted lipoprotein of density < 1.21 g/ml to these total amounts was very low and variable in both types of cell. For TAG, this amounted to 168 ± 84 and 141 ± 63 d.p.m./flask for the wild-type and transfected cells respectively. For PL, the corresponding values were 393 ± 54 and 633 ± 75 d.p.m./flask respectively.

Since labelled lipids comprised only a very small fraction of label in the medium, the remainder of the measured label represents oxidation products (CO_2 and ketone bodies) of the prelabelled cellular lipids. Release of labelled CO_2 by acidification of the conditioned medium decreased, but by no means completely removed, label from the medium. The lipid-derived acid-soluble label remaining after this procedure is associated with ketone bodies [22] and it can be seen from Table 3 that labelled ketone-body production was substantially greater in the HSL transfectants than in the wild-type HepG2. In a further series of experiments in which $^{14}\text{CO}_2$ production was measured directly, Table 4 shows that this was also substantially increased in the HSL transfectants, in terms both of absolute quantities, and as a percentage of the original cellular [^{14}C]TAG from which it was derived.

In primary hepatocytes, there is an active cycle of cellular TAG lipolysis and re-esterification of the product fatty acids. If

Table 4 Fractional turnover (lipolysis/re-esterification) of cellular TAG

Cellular TAG was prelabelled with [^3H]glycerol (9800 d.p.m./nmol) and [^{14}C]oleate (224 d.p.m./nmol) for 16 h. The medium was then replaced by fresh medium in the absence of labelled substrates and the cells cultured for a further 24 h. The $^3\text{H}:^{14}\text{C}$ specific-activity ratio was determined at the beginning (0 h) and at the end (24 h) of the 24-h culture period and the fractional TAG turnover calculated as described in the Materials and methods section. The above values are the means $\pm$ S.E.M. for eight flasks in each group.

	$^3\text{H}:^{14}\text{C}$ Specific-activity ratio		Ratio at 24 h (% of 0 h)	Fractional TAG turnover (pools/24 h)
	0 h	24 h		
Wild-type cells	1.49 $\pm$ 0.13	1.03 $\pm$ 0.09	68.9 $\pm$ 0.9	0.45
HS2D transfectants	1.18 $\pm$ 0.11	0.77 $\pm$ 0.08	65.2 $\pm$ 1.5	0.53

cellular TAG is prelabelled with [^3H]glycerol and [^{14}C]fatty acid, this process results in a decline in the relative specific activity of the cellular ^3H -labelled TAG during a subsequent culture period, owing to the entry of a large amount of a relatively unlabelled glycerol-containing moiety into the glyceride glycerol pool. To determine whether the mobilization of intracellular TAG resulting from HSL transfection also gave rise to a significant TAG/fatty acid cycle, we determined the relative decline in the ^3H -specific radioactivity of wild-type cells and HSL transfectants in which the cellular TAG had been prelabelled with [^3H]glycerol and [^{14}C]oleate. Table 4 shows that, compared with the wild-type, there was no increase in the relative loss of [^3H]glycerol (as measured by the change in the $^3\text{H}:^{14}\text{C}$ specific-activity ratio). In both cases, it could be calculated that approx. 0.4–0.5 pools of intracellular TAG were recycled over a 24-h period.

DISCUSSION

TAG in the cytosolic storage pool is the immediate source of TAG utilized for the assembly of VLDL in the hepatocyte [14,26,27]. Transfer of the bulk of TAG from the cytosol to the site(s) of VLDL assembly in the secretory apparatus of the cell is generally thought to entail a process of lipolysis and re-esterification of the product fatty acids [1–4,27,28]. VLDL TAG is

recruited via this route irrespective of whether particles are assembled with apoB₁₀₀ or with apoB₄₈ [28]. The inability of HepG2 to assemble and secrete mature TAG-rich VLDL particles has been attributed to a defective mobilization of cytosolic TAG [13,26,27] resulting from a lack of TAG lipolytic activity [13]. The lipase(s) responsible for VLDL TAG recruitment in normal hepatocytes has not been identified. It is known, however, that the relevant lipase activity is expressed at a level substantially higher than that required to meet the needs of VLDL TAG output and that the excess fatty acids produced are re-esterified and recycled back to the cell cytosol [1,29]. It is possible, therefore, that a level of regulation may occur downstream of cytosolic TAG lipolysis. This could involve switching the proportion of re-esterified fatty acid that enters the secretory pathway on the one hand, or the recycling pathway on the other. Insulin appears to exert its regulatory effect on the secretion of VLDL at this level [1,29]. The present results using McArdle cells also support the idea that active lipolysis and re-esterification of intracellular TAG is not the only prerequisite for efficient TAG secretion. However, the high rate of TAG recycling in McArdle cells (Table 1) indicates a potential to secrete VLDL-like particles, and small quantities of VLDL are secreted under some circumstances [30–32]. In contrast, HepG2 cells invariably secrete small, dense, lipid-depleted particles irrespective of the culture conditions employed.

HSL is generally thought not to be expressed in mammalian liver [10–12] and the present work also confirms that it was not expressed at a detectable level in untransfected HepG2 cells (Figures 2 and 3). Thus in normal liver it seems unlikely that HSL has any physiological role in the regulation of hepatic lipid storage and secretion, and we predict that a distinct lipase will fulfil this function. However, since HSL is one of the few enzymes with an established role in mobilizing cytosolic neutral lipids, the major aim of the present study was to determine whether it could function in a hepatocellular environment and, if so, whether the mobilized fatty acids could be utilized to drive an increase in lipoprotein TAG output. Alternative possible fates include re-esterification to TAG, conversion to PL, secretion as non-esterified fatty acids, as in adipose tissue, or oxidation to ketone bodies and CO₂. Whereas expression of a heterologous enzyme can never yield information on the normal physiological regulation of any of these processes, it may help to elucidate the targeting and compartmentalization of fatty acids within the cell.

Our results suggest that over-expression of HSL substantially depletes the endogenous stores of preformed TAG in HepG2 (Table 2). In adipocytes, the major product of HSL is monoacylglycerol (MAG), which is then further hydrolysed to glycerol by a MAG lipase (EC 3.1.1.23) [33,34] that is expressed ubiquitously in liver [35,36]. The present results also suggest that MAG lipase is also active in the transfected HepG2 since it was calculated that there was no accumulation of fatty acid label in MAG. Thus although it is difficult to separate PLs from MAG completely by TLC, it is possible to calculate the predicted ³H:¹⁴C ratios for PLs and MAG from the values for TAG in Table 4. Thus, the wild-type TAG ³H:¹⁴C ratio at 0 h is 1.49 ± 0.13 and this represents a [³H]glycerol:[¹⁴C]oleate molar ratio of 1:3. The molar ratio in PL is 1:2. Thus it may be calculated that the ³H:¹⁴C ratio in PL would be 2.26. We actually observed a ³H:¹⁴C ratio of 2.30 ± 0.12, which is almost identical to the calculated value, suggesting that most, if not all, of the label corresponds to PL. Furthermore, using a similar calculation, we can predict that if all the label were associated with MAG (with a 1:1 molar ratio of [³H]glycerol:[¹⁴C]oleate), then the ³H:¹⁴C ratio would be 4.47. This is clearly much higher than that which we actually observe. Similar calculations for the HS2D

transfectants predict a ³H:¹⁴C ratio in PL and MAG of 1.78 and 3.54 respectively. The PL band from TLC has a ³H:¹⁴C ratio of 1.67 ± 0.09, which is very close to the calculated value for pure PL. The ³H:¹⁴C ratios of neither the wild-type PL band nor the HS2D PL band increase during the 24-h culture of prelabelled cells, as would be expected if a significant amount of MAG was accumulating in either of these bands. These labelling patterns suggest that HSL functions in tandem with MAG lipase in the transfected HepG2. The end result of this dual activity is the complete hydrolysis of TAG to fatty acids which then enter the oxidative pathway.

None of the excess net loss of TAG could be accounted for by an increased lipoprotein TAG or PL secretion. The lack of increase in TAG secretion is unsurprising in view of the observation that fatty acids released by lipolysis were not re-esterified to TAG and recycled back to the storage pool. In rat hepatocytes, such a cycle led to a relative decline in the specific radioactivity of [³H]TAG prelabelled with [³H]glycerol and [¹⁴C]oleate [37]. In the present work, some intracellular TAG/fatty acid recycling occurred in the wild-type HepG2 cells as evidenced by a small decline in the relative ³H-specific activity of the doubly labelled cellular TAG (Table 4). Quantitatively, this was similar to that observed previously [13] and much lower than that observed in primary cultures of rat hepatocytes [1,28]. However, there was no enhancement of TAG/fatty acid cycling in the HSL transfectants, as evidenced by the similarity of the decrease in the relative [³H]TAG-specific activity (as measured by changes in the ³H:¹⁴C ratio). Therefore, it appears that fatty acids released intracellularly as a result of HSL activity do not access the site(s) on the ER responsible for esterification of exogenous fatty acids to TAG. If this is so it would fully support the previous suggestion that fatty acids of exogenous and endogenous origin are compartmentalized separately within the cell and that their fates are metabolically distinct [7]. Indeed it was predicted previously [7] that the lipase responsible for directing endogenously derived fatty acids to the secretory pathway would need to be specifically targeted to the ER to ensure that the endogenous fatty acid pool remains separate from newly acquired fatty acids. HSL is targeted to lipid droplets in adipocytes, and shows some tendency to associate with intracellular membranes in transfected HepG2 cells (Figure 3). However, under some conditions, HSL also associates with a particulate fraction in 3T3-L1 adipocytes [38]. However, it does not have specific targeting information to direct it to the ER. In contrast, a candidate lipase proposed by Lehner and Verger [39] is both directed to the ER and is translocated to the lumen.

It might be argued that re-esterification of the fatty acid products is a more realistic criterion than VLDL secretion by which to assess whether a particular transfected lipase gene has any relevance in the process of VLDL assembly. This is because it cannot be predicted with certainty that TAG secretion would be increased in HepG2 cells even if the correct lipase was expressed. Whereas it is reported widely that HepG2 cells are not deficient in apolipoprotein B synthesis (for reviews, see [40,41]), and we previously observed appropriate levels of microsomal triglyceride-transfer protein [42], this does not exclude the possibility that other, as yet undefined, proteins that are required for the production of TAG-rich lipoproteins are also deficient in HepG2 cells. If this proved to be the case then appropriately transfected HepG2 cells might resemble McArdle cells, where high rates of endogenous TAG lipolysis and resynthesis may be uncoupled from efficient TAG secretion.

Although there is general agreement that intracellular TAG undergoes lipolysis prior to re-esterification and secretion, there is some debate about the principal lipolytic products. Previous

work [2] has suggested that lipolysis to diacylglycerol and MAG predominates over complete lipolysis. In contrast, Bar-On et al. [43] observed that complete lipolysis precedes the reassembly of secreted VLDL TAG in perfused liver, similar to previous findings in cultured primary hepatocytes. Further, mass-isotopomer-distribution analysis of hepatic [^{13}C]glycerol metabolism has confirmed the lipase-dependent formation of unlabelled glycerol from endogenous TAG in perfused livers and in rats and monkeys *in vivo* [44]. Thus measurement of changes in the relative specific activities of glyceride glycerol in liver preparations *in vitro* is a valid measurement of intracellular TAG lipolysis and re-esterification under these conditions. Accordingly, HepG2 cells transfected with the relevant lipase might be expected to behave similarly, so that TAG lipolysis and re-esterification would be readily apparent in the assay currently employed.

Apart from re-esterification to TAG, which is the major pathway in primary hepatocytes, mobilized fatty acids may be esterified to PLs. In both the wild-type and the HSL-transfected hepatocytes some of the labelled TAG that disappeared from the cell during the subsequent 24-h culture period appeared in PL. The active interconversion of TAG and PL in the liver has been noted previously [43,45]. Igal et al. have provided evidence that the transfer of triacylglycerol fatty acids to PL fatty acids is defective in neutral lipid storage disease (resulting in an increased cycling of fatty acids to the TAG pool [46,47]).

In contrast to glycerolipid synthesis, it is apparent that the fatty acids mobilized by HSL are efficient substrates for oxidation to ketone bodies and CO_2 (Table 3). In fact, HSL is probably responsible for mobilizing stored TAG from an intracellular storage pool in cardiomyocytes prior to mitochondrial oxidation to meet energy requirements [48–50]. The lipase normally responsible for the endogenous oxidative/ketogenic pathway in liver is unknown, despite its obvious importance in the regulation of hepatic lipid balance. Despite earlier suggestions [5,6] that lysosomal acid lipase was involved in the recruitment of endogenous TAG for ketogenesis, it is now assumed that lysosomal lipase is utilized only for the metabolism of exogenous TAG, which enters the cell via a receptor-mediated pathway [51]. Our results suggest that a soluble cytosolic enzyme similar to HSL would be in the appropriate location to mobilize endogenous TAG for oxidation in the liver.

In conclusion, the lipase responsible for recruitment of cytosolic TAG for lipoprotein assembly remains elusive. Lysosomal acid lipase seems an unlikely candidate [1,39]. Lehner and Verger [39] have proposed the participation of an enzyme with an N-terminus identical to proline-(β)-naphthylamidase, a member of the liver carboxylesterase class of enzymes. However, current evidence for such a role for this enzyme remains largely circumstantial [52]. Additionally, Lehner and Verger [39] speculate that the enzyme may be absent in McArdle RH7777 hepatocytes, although our data make it clear that these cells are capable of recycling TAG (Table 1) and thus might be expected to express the appropriate lipase. The present study demonstrates that it is possible to assess the consequences of lipase transfection on the major routes of fatty acid trafficking in HepG2 cells and this approach will subsequently be extended to assess the function of various lipases expressed in normal liver following transfection into HepG2 cells.

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