Cloning and expression of a cDNA encoding a hepatic microsomal lipase that mobilizes stored triacylglycerol

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A microsomal triacylglycerol hydrolase (TGH) was recently purified from porcine liver [Lehner and Verger (1997) Biochemistry **36**, 1861–1868]. To gain further insight into the function of TGH, we have cloned a cDNA encoding TGH from a rat liver cDNA library and generated McArdle RH7777 rat hepatoma cell lines that stably express the rat TGH. The putative protein derived from the cDNA sequence contains a cleavable signal sequence and a catalytic site serine residue present within a pentapeptide motif (GXSXG) that is conserved in all known lipases. TGH-transfected cells showed a 2-fold increase, compared with control cells, in the rate of depletion of prelabelled triacylglycerol stores. Thus, TGH is capable of hydrolysis of stored triacylglycerol. In contrast, the rate of turnover of labelled phosphatidylcholine was similar in both the vector- and TGH-transfected cells. Studies in TGH-transfected cells demonstrated that utilization of intracellular triacylglycerol pools for secretion was approx. 30 % higher than in vector-transfected cells. Whereas phosphatidylcholine secretion was essentially the same in control and TGH-transfected cells, TGH-transfected cells also secreted an approx. 25 % greater mass of triacylglycerol into the medium and had increased levels of apolipoprotein B100 in the very-low-density lipoprotein density range compared with control cells. The results suggest that the microsomal TGH actively participates in the mobilization of cytoplasmic triacylglycerol stores, some of which can be used for lipoprotein assembly.

Key words: glycerolipid synthesis and secretion, McArdle RH7777, triacsin C, very-low-density lipoprotein.

INTRODUCTION

Enzymes catalysing the *de novo* synthesis of triacylglycerol are associated with the endoplasmic reticulum (ER) membranes but very little triacylglycerol is stored in the ER [1,2]. Instead, most newly made triacylglycerol is stored in intracellular droplets in the cytoplasm of the cell. The stored triacylglycerol can be hydrolysed and the derived fatty acids used for metabolic energy. In adipose tissue the liberated fatty acids are secreted into the bloodstream where they are carried by albumin to other tissues to supply energy. The liver secretes some of the stored triacylglycerol, after hydrolysis and resynthesis, as a component of very-low-density lipoprotein (VLDL). It has been estimated that approx. 70 % of the triacylglycerol that is secreted by hepatocytes arises from the triacylglycerol storage pool whereas only 30 % is derived from *de novo* triacylglycerol synthesis [3,4].

Enzymes involved in the hydrolysis of stored triacylglycerol have not been described, with the exception of hormone-sensitive lipase [5]. Beside playing a fundamental role in adipose tissue lipid metabolism and overall energy homoeostasis, hormone-sensitive lipase functions as a cholesteryl ester hydrolase in steroidogenic tissues [6]. However, hormone-sensitive lipase is not expressed in the liver in appreciable quantities. Furthermore, the rate of lipolysis of stored triacylglycerol in liver is not significantly altered by insulin or glucagon [3]. An issue has been raised as to whether the release of fatty acids from intracellular triacylglycerol stores for ketogenesis and for triacylglycerol secretion/recycling is controlled by different lipases [3]. Therefore, it was an important advance when a triacylglycerol hydrolase (TGH) was recently purified from porcine liver microsomes and characterized [7]. The isolated TGH hydrolysed long-, medium- and short-chain triacylglycerols. The enzyme had a pH optimum of 8.5, was insensitive to thiol-directed reagents, but was inactivated by diethyl-*p*-nitrophenyl phosphate and di-isopropyl fluorophosphate indicating that it was a serine esterase. Whether or not this TGH functioned to hydrolyse triacylglycerol in liver was presumed but not demonstrated. To provide information on the function of TGH, we have cloned the cDNA that encodes the rat TGH and stably expressed it in McArdle RH7777 rat hepatoma cells. The studies reported herein unambiguously demonstrate that TGH does indeed hydrolyse stored triacylglycerol in liver and provides the first proof of such an activity in liver.

MATERIALS AND METHODS

Materials

Oleic acid, essentially fatty acid-free BSA, *p*-nitrophenyl fatty acyl esters and Protein A–Sepharose CL 4B were purchased from Sigma (St. Louis, MO, U.S.A.). [9,10-^{3}H]Triolein (28 Ci/mmol) was from Dupont NEN (Boston, MA, U.S.A.). [1,3-^{3}H]glycerol (2.6 Ci/mmol), [U-^{14}C]glycerol (149 mCi/mmol), [9,10-^{3}H]oleic acid (10 Ci/mmol), L-[4,5-^{3}H]leucine (57 Ci/mmol) and ECL Western blotting reagents were obtained from Amersham Canada (Oakville, Ontario, Canada). Dulbecco's modified Eagle's medium (DMEM), sodium pyruvate, penicillin/streptomycin, foetal bovine and horse sera and Geneticin (G-418 sulphate) were from Gibco BRL (Life Technologies Inc., Grand Island, NY, U.S.A.). Triacsin C was purchased from Biomol Research Laboratories,

Abbreviations used: apo, apolipoprotein; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; TGH, triacylglycerol hydrolase; VLDL, very-low-density lipoprotein.

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Inc. (Plymouth Meeting, PA, U.S.A.). All other chemicals and solvents were of reagent or better quality and were obtained from local suppliers. Sheep anti-[human apolipoprotein (apo)B] IgG was from Boehringer-Mannheim.

cDNA cloning and expression

Analysis of porcine liver TGH N-terminal amino acid sequence and internal sequences showed identity of TGH with porcine proline-β-naphthylamidase [8]. Two oligonucleotides were synthesized according to the cDNA sequence of proline-β-naphthylamidase, one corresponding to nucleotide sequence from residues 526 to 565 of the coding region (5′-GCA TCT GGG GAT TCT TCA GCA CAG GGG ATG AAC ACA GCC G-3′) and the other corresponding to the complementary strand of residues 1508 to 1469 (3′-GAG CAA AGT TGG CCC AGT ATT TCA TCA CCA TTT TGC TGA G-5′). These oligonucleotides were used as probes to screen a rat liver cDNA library (kindly provided by Drs. Gabe Kalmar, Department of Chemistry, and Rosemary Cornell, Institute of Biochemistry, Simon Fraser University, British Columbia, Canada). Among the 10 million colonies screened, one positive cDNA clone recognized by both oligonucleotides was isolated after repeated screening, subcloned into a pBluescript II SK$^-$ plasmid (Strategene) and sequenced in both directions.

A full-length cDNA encoding rat TGH was excised from pBluescript II SK$^-$ plasmid by digestion with *Xba*I and *Cla*I, the insert was purified on an agarose gel (Qiagen gel elution kit) and ligated into pBK-CMV phagemid vector (Strategene) that was digested with the same restriction enzymes and purified by agarose gel electrophoresis. The cDNA was ligated and transformed into DH5α competent cells. Colonies containing TGH cDNA were identified, plasmid DNA isolated (Wizzard Miniprep, Promega) and sequenced. Sequences required for prokaryotic expression (lac promoter region) were removed from the pBK-CMV plasmid with or without TGH cDNA insert by digestion with *Nhe*I and *Spe*I. The resultant plasmids were purified by cesium chloride centrifugation.

Cell culture

Hepatocytes were isolated from male Sprague-Dawley rats (body wt. 125–150 g) fed *ad libitum*, by collagenase perfusion of the liver. The cells were cultured in DMEM containing 15% (v/v) foetal bovine serum [9]. HepG2 cells, obtained from A.T.C.C., were cultured in minimal Eagle's medium containing 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, penicillin (100 units/ml), streptomycin (100 μg/ml) and 10% foetal bovine serum. McArdle RH7777 cells, obtained from A.T.C.C., were cultured in DMEM containing pyruvate, antibiotics, 10% foetal bovine serum and 10% (v/v) horse serum. All cultures were maintained in 100-mm diameter dishes (Corning) at 37 °C in humidified air (89–91% saturation) containing 5% CO_2.

Generation of McArdle RH7777 cell lines stably expressing TGH cDNA

McArdle RH7777 cells were transfected with 10 μg of pBK-CMV plasmid with or without TGH cDNA insert using a calcium precipitation procedure [10]. Cells stably expressing either the empty vector or the vector containing the TGH cDNA were selected for resistance to the antibiotic G-418 (0.08% w/v). Transfected McArdle RH7777 cells were grown in DMEM supplemented with 10% horse serum and 10% foetal bovine serum, penicillin/streptomycin (40 units/ml) and 0.02% (w/v) G-418. Cells were maintained at 37 °C in humidified air containing 5% CO_2.

Preparation of microsomal membranes from McArdle RH7777 cells

Cells from ten 100-mm diameter culture dishes (approx. 50 mg of protein) were harvested into 5 ml of 10 mM Tris/HCl, pH 7.4, containing 250 mM sucrose and 5 mM EDTA. Cells were homogenized with a Polytron and the microsomal membranes were isolated by ultracentrifugation from a post-mitochondrial supernatant [11].

Lipase assay

Lipolytic activities in microsomal membranes (50 μg of protein) isolated from 'mock' transfected (empty vector) and TGH cDNA-transfected McArdle RH7777 cells were assessed using either radiolabelled triolein or a chromogenic substrate (*p*-nitrophenyl laurate) as described earlier [7].

Immunoblot analyses

McArdle RH7777 cells (non-transfected, 'mock' transfected and TGH cDNA transfected) were harvested in PBS and disrupted by brief sonication. Cell homogenates (35 μg of protein) were electrophoresed on an SDS/12% (w/v) polyacrylamide gel, transferred to a nitrocellulose membrane, and the expression of TGH was analysed by blotting with anti-TGH polyclonal antibodies as described previously [7].

Lipid uptake by transfected McArdle RH7777 cells

McArdle RH7777 cells (at approx. 80% confluency in 60-mm diameter culture dishes) were incubated with 2 ml of serum-free DMEM containing 0.5% BSA and 100 μCi [^{3}H]oleic acid for 1 h. The medium was aspirated, the cells were washed with DMEM/0.5% BSA and then the cells were incubated for a chase period with 5 ml of DMEM/0.5% BSA for 2 h. The medium was removed, diluted with DMEM/0.5% BSA and 5 ml aliquots were added to McArdle RH7777 cells stably transfected with either pBK-CMV vector or with pBK-CMV containing TGH cDNA. Aliquots of media (1 ml) were taken after 2, 4 and 6 h of incubation. At the end of the incubations cells were washed with ice-cold PBS, harvested in the same buffer and dispersed by brief sonication. Cellular and media lipids were extracted according to Folch et al. [12] in the presence of non-labelled lipid carriers. The lipids were applied to TLC plates and developed to one-third the height with chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) to separate glycerophospholipids, followed by development in heptane/isopropyl ether/acetic acid (60:40:4, by vol.) to separate neutral lipids. Lipids were made visible by exposure to iodine; bands corresponding to various lipid classes were scraped, and the associated radioactivity was determined by scintillation counting.

Effect of triacsin C on glycerolipid synthesis and secretion by McArdle RH7777 cells

Cells stably transfected with pBK-CMV or pBK-CMV containing rat TGH cDNA were grown to approx. 70% confluency in 60-mm diameter dishes and incubated for 24 h with serum-free DMEM containing 0.1 mM oleic acid complexed with BSA (0.13% final concentration). The medium was aspirated and the cells were incubated for 1 h with DMEM containing 0.1 mM

oleic acid/BSA and various concentrations of triacsin C in DMSO. The final concentration of DMSO was 0.4 %. After a 1 h incubation ± triacsin C, 10 μCi/dish of [^{3}H]glycerol was added. Cells and media were collected after 2 h, lipids were extracted and the radioactivity associated with phosphatidylcholine and triacylglycerol was analysed as described above.

Intracellular lipid turnover in transfected McArdle RH7777 cells

Cells stably transfected with either pBK-CMV or pBK-CMV containing rat TGH cDNA (grown to approx. 70 % confluency) were incubated with serum-free DMEM containing 0.1 mM oleic acid complexed with BSA (0.13 % final concentration) and 20 μCi [^{3}H]glycerol for 15 h. After 13 h, triacsin C (20 μM final concentration) was added. After an additional 2 h labelling, media were removed, cells were washed and incubated for 6 h ± triacsin C (chase). Cells were harvested, lipids were extracted and separated by TLC, and the amount of radioactivity in phosphatidylcholine and triacylglycerol was determined.

Utilization of intracellular triacylglycerol stores for lipoprotein assembly and secretion

Contribution of [^{14}C]glycerol-labelled intracellular triacylglycerol pools in the presence of exogenous oleate: pulse–chase protocol

Cells were incubated for 2 h with 0.375 mM oleic acid/0.5 % BSA to increase the triacylglycerol stores. Cells were then washed and incubated for 1 h with DMEM followed by 2 h with 0.375 mM oleic acid/0.5 % BSA and 0.25 μCi [^{14}C]glycerol. Radioactivity in cellular and media phosphatidylcholine and triacylglycerol was analysed following lipid extraction and TLC as described above.

Secretion of [^{3}H]glycerol-prelabelled intracellular triacylglycerol pools in the absence of oleate

Cells were incubated for 16 h with serum-free DMEM containing 0.375 mM oleic acid/0.5 % BSA and 25 μCi [^{3}H]glycerol, washed and incubated with DMEM in the absence of extracellular oleate for up to 8 h. Radioactivity in cellular and media lipids was analysed.

Lipid secretion from transfected McArdle RH7777 cells

Cells at 60–70 % confluency were washed with DMEM and incubated in the absence of sera for 2 h. One set of dishes was incubated for 4 h with serum-free DMEM containing 0.5 % BSA and 10 μCi [^{3}H]glycerol. The other set of dishes was incubated for 4 h with serum-free DMEM containing 0.375 mM oleic acid/0.5 % BSA and 10 μCi [^{3}H]glycerol. At the end of a 4 h pulse a set of oleate-treated cells was washed with DMEM and incubated for various times with serum-free DMEM containing 0.5 % BSA (chase). Media and cells were collected and lipids were extracted in the presence of non-labelled lipid carriers. Lipids were separated by TLC and made visible by exposure to iodine and the radioactivity associated with phosphatidylcholine and triacylglycerol was determined.

Triacylglycerol and phosphatidylcholine mass secretion from transfected McArdle RH7777 cells

The experimental design for determination of the mass of triacylglycerol and phosphatidylcholine secreted was identical to that described above except that cells were grown in 100-mm diameter dishes and media from three dishes were combined. Lipids were separated by TLC and the mass of phosphatidylcholine was determined [13]. The mass of triacylglycerol was determined according to Snyder and Stephens [14] using trioleoylglycerol as an authentic standard.

Determination of intracellular and secreted triacylglycerol mass from primary rat hepatocytes, wild-type McArdle RH7777 and HepG2 cells

Freshly prepared primary rat hepatocytes seeded at (5–6) × 10^6 cells/60-mm diameter dish, McArdle RH7777 cells at 80 % confluency and confluent HepG2 cells were incubated for 12 h in their respective growth media that were supplemented with 0.375 mM oleate/0.5 % BSA. Cells were then washed with DMEM and incubated for 2 h with DMEM containing 0.375 mM oleate/0.5 % BSA. The media were collected and triacylglycerol mass was analysed (triacylglycerol secretion at 2 h of supplementation with oleate). Cells were washed and incubated for 2 h with DMEM in the absence of oleate. Medium was removed, cells washed and fresh DMEM replenished. The procedure was repeated every 2 h. Media and cell lipids were extracted and triacylglycerol mass determined as described above.

ApoB secretion

Cells stably transfected with either pBK-CMV or pBK-CMV containing rat TGH cDNA grown to approx. 70 % confluency in 60-mm diameter dishes were incubated for 16 h with serum-free DMEM. Cells were then incubated for 2 h with DMEM containing 0.375 mM oleic acid/0.5 % BSA, washed for 1 h with leucine-free media ± oleic acid/BSA and incubated for 2 h with 2 ml of either leucine-free DMEM containing 0.5 % BSA and 250 μCi of [^{3}H]leucine, or leucine-free DMEM containing 0.375 mM oleic acid/0.5 % BSA and 250 μCi [^{3}H]leucine. Media were removed and briefly centrifuged to remove cellular debris. To culture media (0.9 ml containing 1 mM final concentration of benzamidine) was added 0.1 ml of 10 × immunoprecipitation buffer [1.5 M NaCl, 0.5 M Tris/HCl, pH 7.4, 50 mM EDTA, 5 % (v/v) Triton X-100, 1 % (w/v) SDS][15]. Anti-(human apoB) IgG (10 μl) was added. The mixture was incubated for 12 h at 4 °C, then 50 μl of Protein A–Sepharose was added and the mixture was incubated for 3 h. The beads were pelleted by brief centrifugation, washed three times with an excess of immunoprecipitation buffer, then 0.1 μg of rat VLDL protein was added, followed by electrophoresis sample buffer [125 mM Tris/HCl, pH 6.8, 4 % SDS, 20 % (v/v) glycerol, 10 % (v/v) β-mercaptoethanol, 0.02 % Bromophenol Blue] [15]. Samples were boiled and electrophoresed through 5 % (w/v) polyacrylamide gels containing 0.1 % SDS. Gels were silver stained [16] and bands corresponding to apoB48 and apoB100 were excised, dissolved at 60 °C in 0.2 ml of 60 % (v/v) perchloric acid followed by 0.4 ml of 30 % (v/v) hydrogen peroxide [17], and the radioactivity associated with apoB48 and apoB100 was determined using Hionic-Fluor scintillation cocktail (Packard Instrument Co., Meriden, CT, U.S.A.).

For detection of apoB in VLDL, density centrifugation was performed according to Chung et al. [18]. To 1.2 ml of media (containing 1 mM benzamidine) were added 100 μl of freshly prepared rat plasma and 0.7 g KBr (4 M final concentration of KBr, density 1.3 g/ml). The mixture was placed into 5.5 ml Quick-Seal centrifuge tubes (Beckman), carefully overlayed with 0.9 % NaCl and centrifuged for 45 min at 416000 ***g*** (65000 rev./min; VTi 65.2 rotor). Fractions of 0.5 ml were collected from the bottom of the tubes and the densities were determined. Top

fractions containing VLDL had a density of < 1.013 g/ml. Fractions were adjusted to 0.9 ml with water and apoB was immunoprecipitated and analysed as described above.

Other methods

The 7.5 mM oleic acid/10% (w/v) BSA stock solution was prepared by dissolving fatty acid-free BSA in DMEM. The solution was heated to 56 °C, added to 0.106 g oleic acid, stirred until the solution clarified and sterilized by filtration.

Protein concentration was determined with the Bio-Rad Protein Assay kit using BSA as a protein standard.

RESULTS

Secretion of triacylglycerol from primary hepatocytes, McArdle RH7777 and HepG2 cells

Cultured hepatocytes most frequently used to study lipoprotein assembly and secretion were analysed for triacylglycerol mass secretion after generation of a large intracellular triacylglycerol store. When the media were supplemented with extracellular oleic acid all three hepatocyte cultures secreted appreciable amount of triacylglycerol into the media (Figure 1). Primary rat hepatocytes appeared to have secreted approx. 30% more triacylglycerol per mg of cell protein than the rat hepatoma McArdle cells, and approx. 280% more triacylglycerol than the human hepatoma HepG2 cells. Upon the removal of oleic acid from the media the primary hepatocytes continued secreting relatively constant levels of triacylglycerol for at least 8 h, while in both McArdle and HepG2 cells attenuation of triacylglycerol secretion was observed. The attenuation of triacylglycerol secretion in McArdle and HepG2 cells occurred despite the presence of large intracellular triacylglycerol stores (343 ± 55, 196 ± 43 and 436 ± 37 μg triacylglycerol/mg of cell protein in primary rat hepatocytes, McArdle and HepG2 cells respectively) still present at 8 h post-withdrawal of oleic acid from the media. This indicates that primary hepatocytes can apparently utilize fatty acids and/or partial acylglycerols for resynthesis and assembly of lipoprotein triacylglycerol. These results are consistent with those observed by others [3,4]. On the other hand, both lines of hepatoma cells appear to be deficient in the mobilization of the storage pool, and the secreted triacylglycerol is primarily derived from the newly synthesized pool in these cell lines. The lack of mobilization (lipolysis/re-esterification) of triacylglycerol stores for VLDL assembly in HepG2 cells has also been demonstrated previously [15,19] and reviewed [20–22]. However, the data presented in Figure 1 suggest, for the first time to our knowledge, that McArdle cells appear to be deficient in the mobilization of stored triacylglycerol for VLDL secretion.

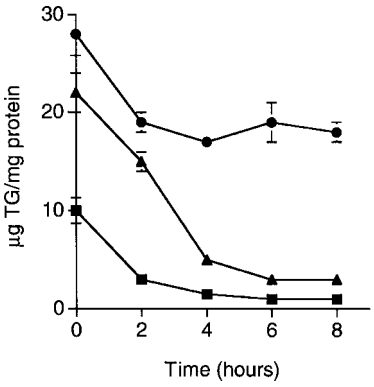

Figure 1 Triacylglycerol secretion from cultured hepatocytes

Primary rat hepatocytes (●), McArdle RH7777 (▲) and HepG2 (■) cells were incubated for a total of 14 h with 0.375 mM oleic acid complexed with BSA. Media were replaced with fresh oleate/BSA for the last 2 h of incubation. Time point 0 h corresponds to the amount of triacylglycerol (TG) secreted during this last 2 h of incubation. Cells were washed and incubated up to 8 h in the absence of extracellular oleate. Media were removed and replenished every 2 h. Media from three dishes were combined, lipids were extracted and the triacylglycerol mass was analysed. Values are means ± S.D. from triplicate experiments. Error bars for several determinations are too small to be seen.

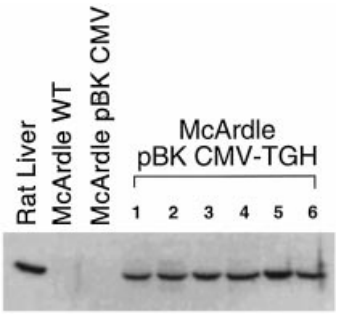

Figure 2 TGH expression in McArdle RH7777 cells

Homogenate proteins (35 μg) from McArdle cells or rat liver were electrophoresed on a 0.1% SDS/10% polyacrylamide gel, transferred on to a nitrocellulose membrane and immunoblotted with an affinity-purified anti-porcine TGH polyclonal antibody. Each of the six McArdle pBK-CMV-TGH-lanes was derived from a separate clone of TGH expressing McArdle cells.

Isolation of the cDNA encoding rat liver TGH

Sequence analysis of N-terminal amino acid residues and of polypeptide fragments obtained from proteolytic digestion of purified porcine TGH [7] showed identity with proline-β-naphthylamidase [8], which shares homology with rat liver [23], rat kidney [24], rabbit liver [25,26] and human liver [27,28] carboxylesterases. Two oligonucleotide probes were synthesized according to internal cDNA sequences of proline-β-naphthylamidase and used to screen a random-primed rat liver cDNA library. A cDNA clone encoding a 568 amino acid protein was isolated. Sequence alignment showed identity with rat liver carboxylesterase pI 6.1 (Genbank accession number X51974) [23]. The putative protein derived from the cDNA contained domains for a signal sequence in agreement with the results of Robbi et al. [23], and a motif for a catalytic site serine, GESAG(G), which is conserved in all known lipases [29]. The deduced sequence thus agreed with that of a protein that would be targeted to the ER and that would have lipolytic activity.

Functional expression of TGH cDNA

To confirm that the cloned cDNA encoded for a protein with triacylglycerol lipase activity, the cDNA was stably expressed in McArdle RH7777 rat hepatoma cells. Wild-type McArdle cells do not synthesize TGH (Figure 2). Immunoblotting of cell homogenates, prepared from rat TGH cDNA-transfected McArdle cells, indicated that a single protein of apparent molecular mass of 60 kDa was recognized by the polyclonal antibody raised against native porcine TGH (Figure 2). The expressed polypeptide in the McArdle cells exhibited the same mobility in SDS/polyacrylamide gels as did the rat liver enzyme, suggesting that TGH in McArdle cells has undergone similar post-translational modifications (signal sequence cleavage and

Table 1 Lipolytic activity in McArdle RH7777 cells

Lipolytic activities in microsomal fractions prepared from vector only- and rat TGH cDNA-transfected McArdle RH7777 cells were determined as described previously [7]. The values are means ± S.D. from two independent experiments performed in duplicate. CMV, McArdle cells transfected with pBK CMV vector; TGH, McArdle cells transfected with vector containing TGH cDNA.

Clone	*p*-Nitrophenyl-laurate (nmol *p*-nitrophenyl/mg of protein per min)	Triolein (nmol oleic acid/mg of protein per min)
CMV	1.8 ± 0.2	0.12 ± 0.03
TGH 5	7.1 ± 0.3	0.73 ± 0.1
TGH 4	3.8 ± 0.2	0.36 ± 0.08
TGH 3	3.9 ± 0.1	0.40 ± 0.05

glycosylation). Immunogold electron microscopy of the transfected McArdle cells showed that the enzyme was associated with ER membranes, with lipid droplets, but was not detected in cytosol, Golgi stacks, mitochondria or nuclei (results not shown).

Microsomal membranes prepared from cells transfected with rat TGH cDNA (clone 5) were enriched approx. 4-fold in hydrolytic activity towards *p*-nitrophenyl laurate and 6-fold in hydrolysis of trioleoylglycerol, compared with microsomal membranes isolated from control cells transfected with vector alone (Table 1). Hence, the rat TGH cDNA encodes an enzyme with lipolytic activity. The lipolytic activity in the other stably transfected clones varied from 2- to 3-fold enrichment compared with the control cells (Table 1).

Lipid synthesis, turnover and secretion studies presented in this manuscript were done with clone 5. Several experimental protocols (secretion and turnover of labelled lipids) were also performed with clones 3 and 4. Similar results were observed among all clones tested.

Lipid synthesis and turnover in McArdle RH7777 cells

A competitive inhibitor of acyl-CoA ligase, triacsin C [30], was used to determine whether cells transfected with rat TGH cDNA have increased mobilization (hydrolysis) of stored triacylglycerol. The inhibition of acyl-CoA formation by triacsin C should attenuate conversion of fatty acids, released by the lipase action on the stored triacylglycerol, to their activated form and decrease a 'futile cycle' of lipolysis/re-esterification. Thus, net lipolysis of triacylglycerol stores should become more apparent. Intracellular triacylglycerol stores were increased by incubation of cells with oleic acid, hence, concentrations of triacsin C that would result in sufficient inhibition of glycerolipid synthesis were determined. At 0.375 mM extracellular oleate, triacsin C (20 μM) did not block glycerolipid synthesis. Higher concentrations of the inhibitor were toxic to the cells. Hence, as a compromise, a lower concentration of extracellular oleate (0.1 mM) was chosen that did not yield the same levels of intracellular triacylglycerol, but which allowed an assessment of the rate of turnover of cellular lipids in the presence of triacsin C. The decrease of the incorporation of radiolabelled glycerol into phosphatidylcholine and triacylglycerol was linear with increasing concentrations of triacsin C (results not shown). In the presence of 0.1 mM oleic acid, 20 μM triacsin C decreased the incorporation of radiolabelled glycerol into triacylglycerol by approx. 50 % and phosphatidylcholine by 25–35 % (Table 2). Interestingly, whereas secretion of labelled triacylglycerol was correspondingly inhibited 30–50 % by triacsin C (Table 2), levels of secreted labelled phosphatidylcholine were not affected (Table 2).

Table 2 Inhibition of phosphatidylcholine and triacylglycerol *de novo* synthesis by triacsin C

Cells were preincubated for 1 h with DMEM containing 0.1 mM oleate/0.13 % BSA ± 20 μM triacsin C. [^{3}H]Glycerol (10 μCi) was then added to each dish and the cells were incubated for an additional 2 h. Cells and media were collected and the radioactivities in phosphatidylcholine and triacylglycerol were analysed as described in the Materials and methods section. Results are means ± S.D. from triplicate experiments.

	Incorporation of [^{3}H]glycerol (10^{-3} × d.p.m./mg of cell protein)			
	Triacylglycerol		Phosphatidylcholine	
	CMV	TGH	CMV	TGH
Cells				
Control	115.5 ± 6.9	102.6 ± 2.3	104.1 ± 6.3	119.1 ± 19.6
Triacsin C	69.7 ± 4.5	52.7 ± 2.0	84.8 ± 0.8	75.9 ± 3.0
Media				
Control	19.9 ± 1.3	14.1 ± 0.9	6.5 ± 0.2	6.3 ± 0.1
Triacsin C	12.6 ± 0.9	7.5 ± 0.3	6.1 ± 1.2	6.0 ± 0.1

To determine whether cells expressing rat TGH cDNA exhibited increased turnover of stored triacylglycerol, we preincubated cells with oleic acid in the presence of [^{3}H]glycerol for 15 h to radiolabel the intracellular triacylglycerol pool. For the last 2 h of preincubation (at 13 h), triacsin C (20 μM final concentration) was added to the medium of half the dishes. During the subsequent 6 h chase period with a new medium ± triacsin C, 30–34 % of the label in cellular phosphatidylcholine was depleted in both control and TGH cDNA-transfected cells, and this turnover was relatively unaffected by the presence of the inhibitor (Figure 3). In cells transfected with vector alone approx. 35 % of radiolabelled triacylglycerol was depleted and triacsin C did not increase the amount of label triacylglycerol depleted. In contrast, cells transfected with rat TGH cDNA had degraded 45 % and 60 % of the labelled triacylglycerol after 6 h in the absence and presence of triacsin C, respectively (Figure 3). The depletion of labelled intracellular triacylglycerol in TGH-transfected cells in the presence of triacsin C was approx. double that in cells transfected with vector alone. These results indicate that the expressed TGH had access to stored triacylglycerol pools and increased its mobilization (hydrolysis).

Lipid uptake by transfected McArdle RH7777 cells

Prior to detailed studies on lipid secretion, we determined whether secreted lipids would undergo re-uptake by McArdle cells. Cells were incubated with radiolabelled oleic acid for 1 h to label intracellular glycerolipids. Cells were washed and incubated for 2 h with serum-free DMEM/0.5 % BSA. The media, which contained secreted labelled lipids, were subsequently added to McArdle cells transfected either with vector alone or with rat TGH cDNA. Radioactive lipids present in media at various times of incubation were analysed. Exogenously added phosphatidylcholine and triacylglycerol were not taken up by either vector- or rat TGH cDNA-transfected McArdle cells during the 6 h incubation (results not shown). We, therefore, assume that in subsequent experiments net secretion of glycerolipids, without re-uptake, is observed.

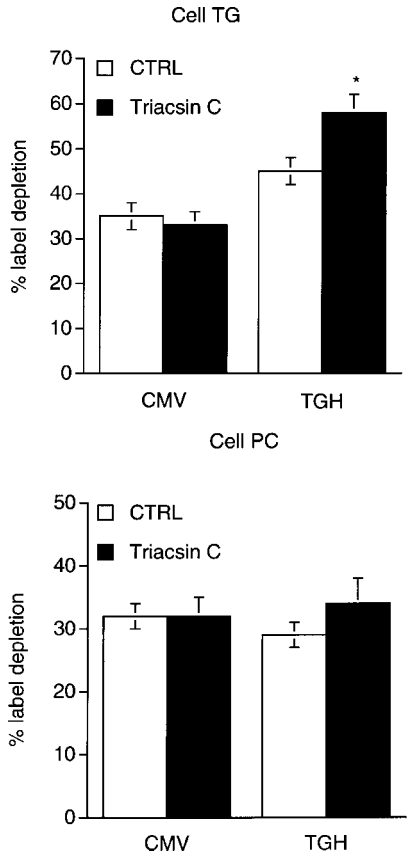

Figure 3 Intracellular triacylglycerol (top) and phosphatidylcholine (bottom) turnover in McArdle RH7777 cells

Cells stably transfected with either vector alone (CMV) or with rat TGH cDNA (TGH) were incubated with 0.1 mM oleic acid and [^{3}H]glycerol for 15 h and treated with or without (control) triacsin C (20 μM) during the last 2 h. Cells were washed and a set of dishes was collected (pulse). The remaining dishes were incubated for 6 h $\pm$ triacsin C. Radioactivity associated with cellular phosphatidylcholine and triacylglycerol at the end of the pulse and chase periods was determined. Values are expressed as the percentage of intracellular label depleted during the 6 h chase period and are means $\pm$ S.D. from triplicate experiments. *Significant difference between CMV and TGH in the presence of triacsin C at $P < 0.03$.

Utilization of intracellular triacylglycerol stores for secretion by TGH-transfected McArdle RH7777 cells

The results from triacsin C turnover studies (Table 2) suggested that the TGH-transfected cells secreted less labelled triacylglycerol into the media than the vector only-transfected cells (19.9 versus 14.1 d.p.m. $\times 10^{-3}$ and 12.6 versus 7.5 d.p.m. $\times 10^{-3}$ per mg of cell protein in control and triacsin C-treated cells respectively) even though the incorporation of the radiolabelled glycerol into cellular triacylglycerol was not significantly different. There was no difference in the label of secreted phosphatidylcholine between the two clones. This suggested that either the TGH-transfected cells secrete less triacylglycerol into the media, or that the labelled glycerol mixes with the unlabelled glycerol (or partial acylglycerol) released by the action of TGH on the unlabelled intracellular triacylglycerol pool, and this results in the secretion of triacylglycerol with lower specific radioactivity from the TGH-transfected cells. To differentiate between these possibilities, we utilized pulse–chase protocols to determine whether TGH-expressing cells utilize stored triacylglycerol for secretion more efficiently than do vector-transfected cells. Initially, the stored triacylglycerol pool in the McArdle cells was increased by preincubation of the cells with 0.375 mM oleic acid for 2 h. Cells were washed and incubated for 2 h with [^{14}C]glycerol in the presence of oleic acid. The rationale for the experimental protocol is that secreted newly synthesized [^{14}C]triacylglycerol is of high specific radioactivity whereas the triacylglycerol that is directed to a storage pool where it is mixed with pre-existing triacylglycerol would be of a lower specific ^{14}C radioactivity than the newly made triacylglycerol. Any secreted triacylglycerol resulting from mobilization/hydrolysis of the intracellular pool and subsequent re-synthesis by re-esterification of the partial glycerides would, therefore, have a lower specific radioactivity than the triacylglycerol to which the storage pool does not contribute. Table 3 shows that [^{14}C]triacylglycerol secreted from the cells transfected with the pBK-CMV vector alone was more labelled than that secreted from the TGH-transfected cells. While both vector only- and TGH cDNA-transfected cells secreted similar amounts of [^{14}C]phosphatidylcholine, the TGH cDNA-transfected cells secreted significantly less [^{14}C]triacylglycerol after 2 h than did the control cells. These results suggest that TGH-expressing cells mobilized intracellular triacylglycerol stores and utilized some of the glycerol/partial acylglycerols for re-esterification and secretion. Similar results were obtained with clones 3 and 4 of TGH-transfected cells.

Table 3 Secretion of [^{14}C]glycerol-labelled lipids from transfected McArdle RH7777 cells

Cells were incubated for 2 h with 0.375 mM oleic acid to increase intracellular triacylglycerol levels, washed, incubated for 1 h and then pulsed for 2 h with [^{14}C]glycerol in the presence of oleic acid as described in the Materials and methods section. Incorporation of [^{14}C]glycerol into cellular and secreted triacylglycerol and phosphatidylcholine was analysed. Values are the means $\pm$ S.D. from triplicate experiments. * Significant difference between TGH and CMV at $P < 0.016$.

	Incorporation of [^{14}C]glycerol ($10^{-3} \times$ d.p.m./mg of cell protein)	
	Phosphatidylcholine	Triacylglycerol
Cells		
CMV	131.4 $\pm$ 9.6	120.3 $\pm$ 7.6
TGH	136.2 $\pm$ 11.8	146.7 $\pm$ 7.9
Media		
CMV	1.07 $\pm$ 0.05	6.30 $\pm$ 0.66
TGH	1.03 $\pm$ 0.02	4.11 $\pm$ 0.41*

The results in Table 3 could be explained by decreased secretion of lipid mass from the TGH-transfected cells. This possibility was investigated by measuring the mass of triacylglycerol and phosphatidylcholine secreted from the respective cell lines. Cells were incubated with 0.375 mM oleate for 4, 8 or 16 h. The amount of oleate incorporated into cellular triacylglycerol was similar in both cell lines, reached near maximum within 4 h of incubation and remained relatively constant for at least 16 h. Therefore, a 4 h preincubation with oleate was selected for increasing the mass of triacylglycerol in the two cell types. The medium was changed and cells incubated for another 4 h in the absence of oleate. The TGH-expressing cells did not secrete less triacylglycerol into the medium (Figure 4). On the contrary, it appeared that the amount of triacylglycerol secreted might be increased, but this did not reach statistical significance. The amount of phosphatidylcholine secreted was the same (Figure 4). Hence TGH-expressing cells actually secrete more triacylglycerol in the absence of extracellular oleate, perhaps because more triacylglycerol is available for the assembly into lipoproteins

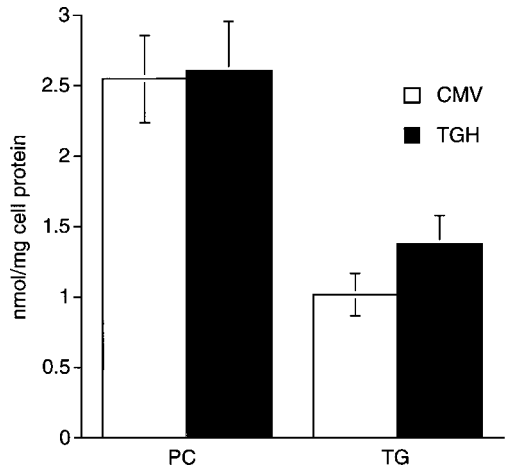

Figure 4 Glycerolipid secretion from transfected McArdle RH7777 cells

Cells transfected with vector alone (CMV) or with TGH cDNA (TGH) were incubated with 0.375 mM oleic acid in serum-free media for 4 h, washed and incubated for 4 h with serum-free media. Media from three 100-mm diameter dishes were combined, lipids were extracted and the mass of triacylglycerol and phosphatidylcholine secreted was determined. Values are means ± S.D. from triplicate experiments.

Table 4 Lipid synthesis and secretion from McArdle RH7777 cells

Cells were preincubated with serum-free medium for 2 h, then incubated for 4 h with [^{3}H]glycerol in the absence (− OA) or presence (+ OA) of exogenous oleic acid (0.375 mM). Some dishes that were initially incubated with oleic acid were incubated for an additional 4 h in the absence of oleic acid (+ OA; − OA). Cells and media were harvested and radiolabelled lipids were analysed. Values are the means ± S.D. from triplicate experiments. PC-CMV, phosphatidylcholine in vector only-transfected cells; PC-TGH, phosphatidylcholine in TGH-transfected cells; TG-CMV, triacylglycerol in vector only-transfected cells; TG-TGH, triacylglycerol in TGH-transfected cells. *Significant differences between TGH and CMV at $P < 0.05$.

	Incorporation of [^{3}H]glycerol (10^{-3} × d.p.m./mg of cell protein)		
	− OA	+ OA	+ OA; − OA
Cellular lipids			
TG-CMV	151.0 ± 5.4	1958.9 ± 118.5	1015.4 ± 110.6
TG-TGH	133.4 ± 5.1	1967.4 ± 240.0	859.9 ± 36.8
PC-CMV	636.4 ± 6.6	1410.3 ± 109.6	820.5 ± 69.9
PC-TGH	588.4 ± 34.5	1164.8 ± 90.81	847.7 ± 30.9
Media lipids			
TG-CMV	19.0 ± 3.0	318.6 ± 42.1	48.5 ± 8.5
TG-TGH	12.3 ± 0.5*	299.3 ± 53.6	71.7 ± 5.2*
PC-CMV	11.5 ± 1.4	33.9 ± 1.8	21.0 ± 1.7
PC-TGH	9.5 ± 0.2	26.6 ± 2.7	20.0 ± 1.7

following lipolysis and re-esterification of the triacylglycerol storage pool.

In another approach, we labelled the cells with [^{3}H]glycerol for 4 h which would enhance the amount of labelled triacylglycerol in the storage pool. Vector only- and TGH-expressing cells incorporated [^{3}H]glycerol into phosphatidylcholine and triacylglycerol at similar levels (Table 4). In the absence of extracellular oleic acid more [^{3}H]glycerol is incorporated into phosphatidylcholine than triacylglycerol (phosphatidylcholine to triacylglycerol ratio of approx. 4). Four hour incubations of McArdle cells with oleic acid increased the rate of [^{3}H]glycerol incorporation into triacylglycerol approx. 13-fold and into phosphatidylcholine by 2-fold. Moreover, oleic acid treatment resulted in a 17– 24-fold increase in secretion of labelled triacylglycerol in pBK-CMV- and TGH-transfected cells respectively. During subsequent incubation without oleic acid and in the absence of [^{3}H]glycerol, the turnover of labelled cellular triacylglycerol and phosphatidylcholine is evident. During this time period more of the labelled triacylglycerol that was secreted would be derived from the storage pool of triacylglycerol. If this were enhanced by the expression of TGH, we would expect to observe more radioactivity in the secreted triacylglycerol as is reported in Table 4.

Despite the presence of labelled intracellular triacylglycerol stores, secretion of labelled triacylglycerol, compared with when oleate was present, was decreased 6.5- and 4-fold in pBK-CMV- and TGH-transfected cells respectively, even though there was clearly an approx. 50 % turnover of the labelled triacylglycerol during the 4 h incubation. Similarly the amount of labelled phosphatidylcholine secreted was decreased even though a significant amount of labelled phosphatidylcholine degraded in the cells during the 4 h incubation. Thus, removal of extracellular oleate resulted in decreased levels of both labelled phosphatidylcholine and triacylglycerol secretion despite the presence of large labelled stores of triacylglycerol and phosphatidylcholine. Apparently, McArdle RH7777 cells, like HepG2 cells [15,31–33] require active *de novo* synthesis of lipids for efficient lipoprotein assembly and secretion.

Incubation of McArdle cells with 0.375 mM oleic acid for 4 h increased cellular triacylglycerol mass approx. 7-fold from 5–6 nmol/mg of cell protein to 35–40 nmol/mg of cell protein in both control and TGH cDNA-transfected cells. The intracellular levels of phosphatidylcholine were approx. the same (70 nmol/mg of protein) in the presence or absence of extracellular oleate. Thus, the results in Table 4 do not seem to result from a difference in the specific radioactivity of cellular triacylglycerol in the two types of cells.

The results in Table 3 and Table 4 and Figure 3 and Figure 4 indicated that TGH-transfected cells secreted more triacylglycerol from the storage pools than did the cells transfected with vector alone. Additional support for this conclusion was obtained when we incubated cells with oleic acid and [^{3}H]glycerol for 16 h, rather than 4 h, in order to produce stored triacylglycerol with higher specific radioactivity than the *de novo* made triacylglycerol. Subsequently, the cells were washed and incubated in the absence of oleate for up to 8 h. There was an approx. 25 % increase in secretion of stored [^{3}H]triacylglycerol from TGH-transfected cells that was linear between 2 and 8 h of incubation (results not shown). Together the data strongly suggest that TGH-transfected cells mobilized and secreted more stored triacylglycerol compared with vector-transfected cells.

ApoB secretion by McArdle RH7777 cells

Cells were incubated with 0.375 mM oleic acid for 2 h, washed for 1 h ± oleic acid and incubated with [^{3}H]leucine ± 0.375 mM oleic acid for an additional 2 h. ApoB was immunoprecipitated from the media and the amount of labelled apoB secreted was analysed. Both vector only- and rat TGH cDNA-transfected cells appeared to secrete similar levels of labelled apoB48 and apoB100 regardless of the presence of extracellular oleate (Table 5).

To determine whether the increased secretion of triacylglycerol by TGH-expressing cells altered the overall density distribution of secreted apoB-containing lipoproteins, media from cells incubated with [^{3}H]leucine were subjected to density centrifugation and fractions corresponding to VLDL densities were analysed

Table 5 ApoB secretion from McArdle RH7777 cells

McArdle cells transfected with vector alone or with vector containing the cDNA for TGH were incubated with serum-free media for 16 h. Cells were then incubated for 2 h with DMEM containing 0.375 mM oleic acid/0.5% BSA, washed for 1 h with leucine-free media ± oleic acid/BSA and incubated for 2 h with leucine-free media ± oleic acid/BSA containing [^{3}H]leucine. Media were collected, and the total apoB and VLDL apoB isolated by density gradient centrifugation were immunoprecipitated. VLDL isolated from rat plasma was added to immunoprecipitants, ApoB48 and apoB100 were separated by SDS/PAGE and made visible by silver staining, and the radioactivity measured as described in the Materials and methods section. Values are the means ± S.D. from triplicate experiments. *Significant difference between TGH and CMV at $P < 0.02$.

	Incorporation of [^{3}H]leucine ($10^{-3} \times$ d.p.m./mg of cell protein)			
	Total apoB		VLDL apoB	
	CMV	TGH	CMV	TGH
Minus oleic acid				
ApoB48	39.14 ± 4.5	45.52 ± 3.1	1.24 ± 0.2	1.22 ± 0.15
ApoB100	84.04 ± 6.3	92.57 ± 7.2	23.31 ± 2.1	28.97 ± 2.2
Plus oleic acid				
ApoB48	38.17 ± 3.2	40.76 ± 2.9	8.55 ± 0.6	10.63 ± 2.2
ApoB100	74.51 ± 5.8	84.85 ± 6.3	28.26 ± 2.3	40.62 ± 3.4*

for apoB. Although oleate supplementation did not alter the total amount of apoB secreted, oleate did increase the proportion of apoB (B48 + B100) in the VLDL density range by 50% and 70% in the vector only- and rat TGH cDNA-transfected cells, respectively (Table 5). Oleate-induced shifts from high-density to very-low-density lipoprotein have been reported in other studies in McArdle cells [30]. The major effect of oleate was on apparent lipidation of apoB48 which increased in the VLDL density range 7-fold and 9-fold in the vector only- and TGH cDNA-transfected cells respectively. In the absence of oleate TGH-transfected cells secreted approx. 25% more apoB100 in the VLDL density than control cells, while the amount of secreted apoB48 was similar. This suggests that the mobilization and re-esterification pathway contributed mainly to the lipidation of apoB100. In the presence of oleate TGH-transfected cells showed increased lipidation of apoB48 (24%) and apoB100 (44%, $P < 0.02$).

DISCUSSION

This study provides evidence that TGH is involved in the hydrolysis of stored cytoplasmic triacylglycerol in hepatic cells. Moreover, the data demonstrate that some of the (acyl)glycerol produced by this lipolysis can be reacylated to triacylglycerol and secreted as a component of lipoproteins.

TGH and hydrolysis of the cytoplasmic triacylglycerol storage pool

Although hepatocytes are known to accumulate triacylglycerol in intracellular storage droplets, a lipase responsible for mobilization of these triacylglycerol stores in liver has not been previously demonstrated. Initially, it was thought that lysosomal acid lipase might participate in triacylglycerol lipolysis [34], however, chloroquine (an inhibitor of lysosomal enzymes) treatment did not inhibit lipolysis [3]. A more likely candidate for triacylglycerol hydrolysis would be a lipase found in close association with intracellular lipid droplets as is the case with hormone-sensitive lipase in adipose tissue. However, liver does not seem to express appreciable quantities of this enzyme. Moreover, hepatic triacylglycerol lipolysis is resistant to inhibition by insulin [3], which suggests that a lipase other than hormone-sensitive lipase is responsible for the mobilization of the stored triacylglycerol. Involvement of hepatic and lipoprotein lipases is also unlikely because these enzymes enter the secretory route after their synthesis and would not be expected to have access to cytoplasmic triacylglycerol.

The results presented in this paper show that TGH fulfills the requirements of an enzyme involved in the hydrolysis of stored triacylglycerol in liver. Moreover, the subcellular localization of TGH with elements of the ER that are in contact with these droplets and the developmental expression of TGH further support a role for TGH in the mobilization of droplet triacylglycerol [35]. TGH is absent from HepG2 human hepatoma cells which do not mobilize triacylglycerol from stored droplet pools [15,19]. Although McArdle cells do not express TGH, it is apparent that the vector-transfected cells are also able to turnover cellular triacylglycerol. Thus, at least one more triacylglycerol hydrolase or transacylase activities that are different from TGH appear to be present in McArdle cells.

Approx. 30–35% of the glycerol backbone of *de novo* made phosphatidylcholine and triacylglycerol in McArdle cells is depleted after 6 h incubations in the absence of an exogenous supply of fatty acids. In TGH-transfected cells TGH appears to be responsible for an increased hydrolysis of triacylglycerol. However, lipase(s)/transacylase(s) responsible for the remaining triacylglycerol hydrolysis have not yet been identified. After treatment of McArdle cells with triacsin C, both vector- and TGH cDNA-transfected cells secreted decreased levels of radiolabelled triacylglycerol. Thus, the acyl-CoA ligase inhibitor attenuated secretion of triacylglycerol derived from lipolysis of stored triacylglycerol by preventing re-esterification of released partial acylglycerols. Secretion of prelabelled phosphatidylcholine was not significantly affected by triacsin C, indicating that phosphatidylcholine was secreted without prior lipolysis and re-esterification. In both pBK-CMV- and TGH-transfected cells a much greater loss of cellular glycerol-labelled triacylglycerol (and phosphatidylcholine) was observed that was not accounted for by the amount of the label secreted. Upon analysis of the distribution of the label during the chase after prelabelling cellular triacylglycerol pools with glycerol it was observed that the radioactivity disappeared from the organic material (lipid) and appeared in the aqueous phase in the medium. It is possible that the secreted water-soluble product is glycerol that was not re-utilized for re-esterification since the chase was carried out in the absence of an exogenous supply of oleic acid and the *de novo* synthesis of fatty acids in McArdle cells may not have been sufficient. The fate of fatty acid released by lipolysis but that could not be efficiently re-esterified in the presence of triacsin C is not currently known.

The intracellular site of re-esterification of (acyl)glycerol released by lipolysis of triacylglycerol stores is not known. Gibbons and Wiggins [21,22] have proposed that there might be at least two separate sites of triacylglycerol synthesis within the ER network. In addition to a site on the cytosolic side of the ER, it has been postulated that the lipolytic products (monoacylglycerols, diacylglycerols and fatty acids) might traverse the ER and undergo re-esterification on the lumenal surface of the ER [36]. A recent report on the presence of a lumenal form of diacylglycerol acyltransferase is consistent with this hypothesis [37]. However, acyl-CoAs, which are required substrates for diacylglycerol acyltransferase, are made on the cytosolic surface of the microsomal membrane and cannot translocate across the membrane readily [38,39]. Therefore, a mechanism for an active transport of acyl-CoAs across the bilayer would have to exist. The presence of a microsomal acylcarnitine acyltransferase

activity has been reported [40–42]. However, there is no evidence that acylcarnitines can serve directly as acyl donors for monoacylglycerol or diacylglycerol acyltransferases. Alternatively, an enzyme acyl-CoA acyltransferase that might serve a translocase role has been purified from intestinal microsomes. Immunoblotting with a polyclonal antibody raised against the purified enzyme showed its presence also in liver and other tissues [43]. Removal of this enzyme from solubilized intestinal microsomal preparations by immunoabsorption resulted in decreased neutral lipid synthesis [44] and it has been suggested that the enzyme may function as an acyl-CoA binding subunit of a hetero-oligomeric triacylglycerol synthetase complex or as an acyl-CoA translocase [43]. Immunoblotting of McArdle RH7777 microsomal fractions using anti-(acyl-CoA acyltransferase) polyclonal antibodies indicated that acyl-CoA acyltransferase is expressed but in substantially lower levels than observed in rat liver microsomes and this observation has been confirmed by measuring acyl-CoA acyltransferase enzymic activity (W. Gao and R. Lehner, unpublished results). It is thus possible that the re-acylation pathway is also deficient in these cells. The potential participation of a recently identified acyl-CoA-independent microsomal diacylglycerol transacylase [11] in the re-esterification of diacylglycerols released by lipase action on triacylglycerol stores also cannot be presently excluded.

TGH and lipoprotein secretion

The synthesis of triacylglycerol and secretion of this lipid into the circulation represents a major route of delivery of fatty acids to various tissues in mammals. The amount of triacylglycerol secreted by the liver depends upon the nutritional state of the animal [45]. Incubation of rat hepatocytes with fatty acid increases synthesis and secretion of triacylglycerol. However, the majority of this newly synthesized triacylglycerol is not directly assembled into VLDL and secreted. Rather, triacylglycerol synthesized from extracellularly supplied fatty acids enters a cytoplasmic storage pool. The size of this pool might be a determining factor in secretion of triacylglycerol-rich lipoproteins from primary rat hepatocytes [3,46]. It has been demonstrated [3,34,47] that a significant proportion of the VLDL-triacylglycerol is derived from the cytoplasmic storage pool and recruitment of the pool of triacylglycerol involves lipolysis followed by re-esterification. It has been estimated that at least 70 % of the secreted VLDL-triacylglycerol might be derived via this route [3]. These initial observations have been confirmed in later work [4,36,48].

A role for TGH in mobilization of stored triacylglycerol is supported by the results presented in this paper, which demonstrate that McArdle cells that overexpress TGH utilize more stored triacylglycerol for secretion than vector-transfected cells. Secondly, the TGH expressing cells secrete 25 % more mass of triacylglycerol and this is further reflected by a significant increase in the amount of lipoprotein associated with particles that float at the density of VLDL. Although it appears that the expression of TGH activity in clone TGH 5 (Table 1) is comparable to that observed in rat liver microsomes, this activity is probably deceiving as only certain hepatic cells express this TGH [35] and the rat liver microsomes are from a mixture of these cells. However, every McArdle cell expresses TGH. Thus McArdle cells actually seem to under-express TGH when compared with the liver cells that express this enzyme.

Secretion of VLDL-sized particles from McArdle RH7777 cells depends on the supply of extracellular fatty acids [49,50,51], where apoB48 appeared to be preferentially recruited for assembly of VLDL [51]. Mass analysis of secreted triacylglycerol from primary cultured hepatocytes that had been preincubated with oleate showed that even after removal of extracellular oleate, hepatocytes continued to secrete triacylglycerol at an enhanced rate for at least 8 h (Figure 1). In contrast, in McArdle cells triacylglycerol secretion was attenuated to near control levels within 8 h after removal of oleate despite the presence of large intracellular triacylglycerol content (Figure 1). In separate experiments, glycerolipid synthesis and secretion was studied in McArdle cells incubated with radiolabelled glycerol in the presence or absence of oleic acid. Oleic acid stimulated *de novo* synthesis of phosphatidylcholine approx. 2-fold and of triacylglycerol approx. 13-fold, and secretion 3-fold for phosphatidylcholine and 17–20-fold for triacylglycerol (Table 4). However, oleic acid treatment did not increase the percentage of total label in lipids that was secreted. This means, that the overall percentage of radioactivity of secreted versus intracellular lipid remained essentially the same (2.5 % for phosphatidylcholine and 13 % for triacylglycerol). This percentage remained the same for phosphatidylcholine during subsequent chase in the absence of oleic acid, indicating continual secretion of the preformed lipid, while the percentage for triacylglycerol decreased to 5 %, suggesting that the majority of the prelabelled lipid has exited the secretion-competent pool. The TGH expressing McArdle cells continued to secrete a significantly higher proportion of radiolabelled triacylglycerol when compared with vector only-transfected cells. This suggests that the (acyl)glycerol moiety mobilized by the action of TGH undergoes re-acylation back to triacylglycerol and at least some of this triacylglycerol is assembled into lipoprotein and secreted into the media.

The labelling experiments reported in this study were done with labelled glycerol since it is rapidly utilized when added to cells and allows one to follow the glycerol backbone of the lipids. We also attempted to label the glycerol lipids with radioactive oleate and trace the fate of the labelled lipids. However, the fatty acids were incorporated into the unesterfied fatty acid pool, acyl-CoA, cholesteryl ester and phospholipids, therefore making the origin of any labelled fatty acid in secreted triacylglycerol difficult to identify. The high concentration of oleic acid (0.375 mM) during the pulse and chase periods made the chase of radioactive fatty acid into lipids slow compared with glycerol. Thus, whereas the studies with labelled glycerol suggest that a significant amount of secreted triacylglycerol originated from the storage pool of triacylglycerol, we could not be certain that much of the secreted triacylglycerol was derived from oleate labelled triacylglycerol.

The present studies and our recent paper [35] clearly identify TGH as an enzyme involved in the mobilization of cellular triacylglycerol and provides evidence that some of the mobilized triacylglycerol is secreted. Since McArdle cells do not express TGH but can turn over triacylglycerol (and phosphatidylcholine), the presence of other lipases or transacylases is indicated.

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