Origin of hepatic very-low-density lipoprotein triacylglycerol: the contribution of cellular phospholipid

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When rat hepatocytes were cultured for 24 h in the absence of exogenous fatty acid, the amount of very-low-density lipoprotein (VLDL) triacylglycerol (TAG) secreted (114 ± 14 μg/mg of cell protein) could not be accounted for by the mass of TAG lost from the cells (29 ± 6.1 μg/mg of cell protein) during this period ($n = 12$). Of the balance (85 ± 14 μg/mg; 94 ± 15 nmol/mg), a maximum of only 37 nmol/mg of cell protein of TAG could be accounted for by fatty acids synthesized *de novo*. When labelled exogenous oleate (initial concentration, 0.75 mM) was present in the culture medium, the net gain in cellular plus VLDL TAG (253 ± 38 μg/mg of cell protein per 24 h) was greater than that contributed by the exogenous fatty acid (155 ± 18.2 μg/mg of cell protein, $n = 5$). Again, the balance (98.8 ± 18.2 μg/mg of cell protein per 24 h) was too great to be accounted for by fatty acid synthesis *de novo*. In experiments in which cellular glycerolipids were prelabelled with [9,10(n)-^{3}H]oleic acid, following removal of the labelled fatty acid, there was a net increase in labelled cellular plus VLDL TAG over the next 24 h. That cellular phospholipids are the source of a substantial part of the excess TAG synthesized is supported by the following evidence. (1) The loss of prelabelled cellular phospholipid during culture was greater than could be accounted for by secretion into the medium. (2) During culture of cells prelabelled with 1,2-di-[1-^{14}C]palmitoyl phosphatidylcholine, a substantial amount of label was secreted as VLDL TAG. (3) In pulse–chase experiments, the kinetics of labelled phospholipid turnover were consistent with conversion into a non-phospholipid pool. The enzymology involved in the transfer of phospholipid fatty acids into TAG is probably complex, but the present results suggest that this pathway may represent an important route by which extracellular fatty acids are channelled into VLDL TAG.

INTRODUCTION

One of the major functions of the liver is to package endogenously synthesized triacylglycerol (TAG) into a form which is efficiently transported in the blood plasma. This function is achieved by the assembly of very-low-density lipoprotein (VLDL) in which the hydrophobic TAG is encased in a hydrophilic mantle of the more polar lipids, phospholipid and non-esterified cholesterol. Apolipoprotein B (apoB) orchestrates the overall process by providing the structural scaffolding required for the transfer of lipids during the intracellular construction of VLDL particles. The precise role of apoB during lipid transfer to the incipient VLDL in the secretory apparatus of the cell is currently the subject of intensive investigation (for reviews, see [1,2]) and it is clear that some TAG becomes associated with apoB during its translation and translocation into the lumen of the rough endoplasmic reticulum (ER) [3]. Microsomal TAG transfer protein (MTP) is essential for the addition of at least some TAG at a critical step(s) during lipoprotein assembly and mutations in the 97 kDa subunit of this protein are responsible for abetalipoproteinaemia [4,5]. Although apoB and MTP are required for the secretion of lipoproteins, their co-expression in non-hepatic cells is not sufficient to drive the secretion of large, TAG-rich particles such as VLDL [6,7], and it is possible that bulk lipid addition to maturing VLDL particles requires some other factor(s). Precisely how apoB, MTP and TAG interact during lipoprotein assembly is not known, although it has recently been reported that the complete translocation of apoB is dependent upon the presence of MTP [8]. Overall, the exact sequence of events involved in lipid addition to apoB and the relative importance of the various organelles of the secretory pathway in this process remain controversial [9–12]. Central to a complete understanding of these complex issues is the identification of the immediate metabolic sources of the lipids involved in VLDL assembly and their location within the cell. We have previously provided some evidence that newly-synthesized TAG is not immediately utilized for VLDL production but that it first enters a storage pool, probably located in the cell cytosol [13,14]. An important question here relates to the means by which cytosolic TAG is translocated across the phospholipid bilayer of the ER membrane for incorporation into the core of nascent VLDL particles. The mechanism involved may be more complex than is immediately apparent, since at least 70 % of the cellular TAG which was secreted as VLDL had undergone lipolysis followed by re-esterification of the product fatty acids [14,15]. This observation was based on calculations of the relative dilution of labelled TAG glycerol compared with that of the labelled fatty acid moiety during the secretion of VLDL from cells in which TAG had been prelabelled with [^{3}H]oleate and [^{14}C]glycerol. The observation, therefore, is an empirical one and provides information only about bulk input and output of TAG at each end of the secretory chain. It tells us nothing about the nature of the individual processes involved, the net outcome of which is a relative loss of prelabelled TAG glycerol during the secretion of VLDL. In particular, the individual pool(s) of lipids involved and their location within the cell remain obscure, although recent

Abbreviations used: VLDL, very-low-density lipoprotein; apoB, apolipoprotein B; TAG, triacylglycerol; ER, endoplasmic reticulum; MTP, microsomal triacylglycerol transfer protein.

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work has provided evidence for the involvement of di- and/or mono-acylglycerols [16]. Earlier work has provided circumstantial evidence for a metabolic role of hepatic phospholipid in VLDL TAG recruitment [17,18] but this topic has not yet been investigated in a systematic way. The present work was designed to provide more information about the metabolic relationships between TAG and phospholipid during the recruitment of lipids for VLDL assembly. One of the findings to emerge is that there is a net production of cellular and VLDL TAG from a source(s) other than that arising from fatty acid synthesis *de novo* and from extracellular fatty acids. This paper presents evidence that this source consists of a pool(s) of cellular phospholipid.

MATERIALS AND METHODS

Preparation of hepatocyte cultures and incubation conditions

Male Wistar rats were fed and housed as described previously [19] and were used for the preparation of hepatocytes when they weighed between 200 and 300 g. Hepatocytes were prepared under sterile conditions [20] and were suspended [$(0.75–1.0) \times 10^6$ cells/ml] in Waymouth's medium MB752/1 supplemented with amino acids, antibiotics and 10% (v/v) heat-inactivated fetal bovine serum [20]. The initial viability of the hepatocytes (determined by Trypan Blue dye exclusion) was greater than 90%. A 3.0 ml portion of this suspension was added to each culture dish and, after 4 h, the cells became attached to the dish as a monolayer. The medium was removed and the cells were washed twice with 3.0 ml of PBS. At this stage 3.0 ml of Waymouth's medium (lacking serum) was added. The medium included, in addition to amino acids and antibiotics, dexamethasone (1 μM), pyruvate (1 mM) and lactate (10 mM). This medium is subsequently referred to as the supplemented medium. To some dishes was added either unlabelled or ^{3}H-labelled oleate to give an initial concentration of 0.75 mM. The specific radioactivity of the ^{3}H-labelled oleate was 0.98×10^6 d.p.m./μmol. Cells were cultured for 24 h under these conditions. In some cases, cells were cultured overnight in the presence of supplemented medium with or without labelled oleate. The medium was removed and replaced with supplemented medium without oleate. The cells were then cultured for a further 24 h. In pulse–chase experiments, cells were cultured overnight in the absence of oleate, pulsed for 0.5 h with trace quantities of [^{3}H]oleate (10 Ci/mmol), and chased for periods of between 1 and 24 h in the presence of 0.75 mM oleate. Details of these experiments are given in the respective Figure legends.

Isolation of secreted VLDL

At the end of the appropriate 24 h culture period, the medium was removed and added to a mixture of antibiotics, anti-oxidants and protease inhibitors [21]. The medium ($d = 1.006$) was centrifuged at 154000 ***g*** (av) for 16 h at 4 °C in a Beckman L8-70 ultracentrifuge using a 50.4 rotor. The resulting supernatant containing the floating VLDL was obtained by tube-slicing.

Measurement of cellular and VLDL phospholipids and TAG

After removal of the medium, the cell monolayer was washed with PBS and the cells were removed from the dish and sonicated [22]. Portions of the cell sonicate and of the VLDL-containing fraction were used for the extraction of total lipids [23]. Parts of these extracts were used for the enzymic determination of TAG [24] and phospholipids [25]. Kits from Boehringer–Mannheim were used for these assays (TAG, GPO-PAP; phospholipids, MPR2, respectively). When required, ^{3}H-labelled phospholipids and TAG were purified from the total lipid extract by TLC [26]. In all cases, manipulative losses of TAG were accounted for by the addition of glycerol [^{14}C]trioleate as internal standard. Radioactivity was determined by scintillation counting of the bands obtained after TLC.

Other methods

Cellular protein was measured using the method of Lowry et al. [27]. Oleate bound to BSA (essentially fatty-acid free) was prepared by the method of van Harken et al. [28]. The BSA concentration in the cell medium was 0.5%. All results are presented as the means ± S.E.M. of several different hepatocyte preparations. The exact number used in each experiment is given in the legend to the appropriate Figure or Table. Significant differences were tested by application of the Student's t test to paired or unpaired data.

Materials

Male Wistar rats were obtained from Harlan U.K. Ltd. (Bicester, U.K.). All radiochemicals were from Amersham International (Aylesbury, Bucks., U.K.). Solutions for tissue culture were from Gibco Ltd. (Paisley, Scotland, U.K.). Enzymic assay kits were obtained from Boehringer–Mannheim (Lewes, E. Sussex, U.K.). All other organic chemicals were from Sigma (Poole, Dorset, U.K.).

RESULTS

Hepatocellular TAG balance during the secretion of VLDL

Rat hepatocytes were loaded with TAG by culturing them overnight in a medium supplemented with 0.75 mM oleate [13]. When these cells were subsequently cultured for a further 24 h period in the absence of extracellular fatty acids, the rate of secretion of VLDL TAG was greater than the amount of TAG lost from the cell (Table 1). It would appear, therefore, that a cellular source other than pre-existing TAG was responsible for providing at least some of the TAG secreted into the medium.

Further support for this suggestion was obtained from labelling experiments. For instance, when hepatocytes are cultured in the presence of exogenous ^{3}H-labelled oleate of known specific radioactivity, it is possible to calculate the mass of VLDL and cellular TAG synthesized exclusively from this exogenous source by assuming that the molar specific radioactivity of the newly synthesized TAG is 3-fold greater than that of the fatty acids

Table 1 Cellular TAG balance during the secretion of VLDL

Hepatocytes were cultured overnight in the presence of supplemented Waymouth's medium plus oleate (initial concentration 0.75 mM). The medium was then removed and the cells were washed twice with Dulbecco's PBS (no oleate). Some dishes were then removed to determine the intracellular TAG concentration (initial cellular TAG). To the remaining dishes was added supplemented Waymouth's medium (no oleate) and the cells were cultured for a further 24 h, after which the concentration of cellular TAG (final cellular TAG) and the concentration of VLDL TAG were determined. Each value is the mean ± S.E.M. of twelve independent hepatocyte preparations.

	TAG (μg/mg of protein)
Initial cellular TAG	130 ± 11
Final cellular TAG	101 ± 10
Loss of cellular TAG	29 ± 6.5
VLDL TAG secreted	114 ± 14
Net gain in VLDL TAG	85 ± 14

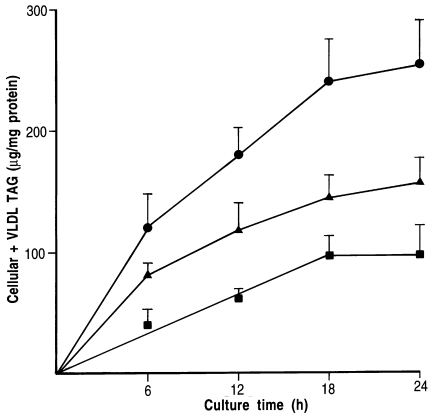

Figure 1 Contribution of exogenous oleate to the total (cell plus VLDL) increase in TAG

Hepatocytes were cultured for a total of 24 h in the presence of [^{3}H]oleate (0.75 mM; 0.98×10^6 d.p.m./μmol). At each time point dishes were removed for the measurement of cellular and VLDL TAG mass and radioactivity. The contribution of exogenous oleate to the increase in TAG mass was calculated from its specific radioactivity in the cell medium. Each point is the mean $\pm$ S.E.M. of values obtained in six independent hepatocyte preparations. Key: ●, total increase; ▲, increase due to exogenous oleate; ■, increase not due to exogenous oleate.

present in the medium. If this exogenous pool of fatty acids is the only source of cellular and VLDL TAG synthesized over a given period, then the increase in the mass of TAG calculated on this basis, and that measured directly using an enzymic assay, should be identical. In reality, of course, labelled fatty acid from the exogenous source will be diluted with unlabelled material derived from endogenous sources so that the specific radioactivity of the effective precursor pool will be lower than that of the exogenous oleate. Thus the extent of the difference between the total measured mass increase in TAG and that calculated on the basis of the specific radioactivity of the exogenous oleate will reflect the contribution to total TAG synthesis from a 'non-oleate' source. Comparisons of the calculated and actual increases in the mass of cellular and VLDL TAG were, therefore, made at various times after culturing hepatocytes in the presence of ^{3}H-labelled oleate (specific radioactivity 0.98×10^6 d.p.m./μmol) for a total of 24 h (Figure 1). It is clear that, at all points studied, the increase in the actual total mass of TAG (measured enzymically as the sum of the cellular and VLDL material) was considerably in excess of that which could be calculated as arising exclusively from exogenous oleate. The contribution of the 'non-oleate' source of TAG was linear for up to 18 h of culture, at which point it accounted for 43 % of the actual increase in total TAG (Figure 1).

How much TAG is synthesized *de novo*?

An obvious candidate for the unlabelled source of TAG described above is that produced from fatty acids synthesized *de novo* from small lipogenic precursors derived from carbohydrate oxidation or from lactate and pyruvate present in the culture medium. Our previous studies have suggested, however, that the contribution from this source in cultured rat hepatocytes is relatively small. For instance, in the absence of exogenous oleate, an average of 165 nmol of fatty acids was synthesized per 24 h per mg of cell protein [29]. Of these fatty acids, at least 33 % can be accounted for by incorporation into cellular phospholipid fatty acids [30], leaving a balance of 110 nmol per mg of protein per 24 h for

Table 2 Distribution of prelabelled cellular TAG during the secretion of VLDL

Hepatocytes were cultured overnight in the presence of [^{3}H]oleate (0.75 mM; 0.98×10^6 d.p.m./μmol). The medium was removed and the cells were washed twice with Dulbecco's PBS. At this stage, hepatocytes from several dishes were used to determine the incorporation of label into cellular TAG (line 1). Hepatocytes on the remaining dishes were cultured for a further 24 h in the absence of oleate. At the end of this period, the amounts of TAG label remaining within the cells (line 2) and appearing as secreted VLDL (line 4) were determined. Each value is the mean $\pm$ S.E.M. of between nine and eleven independent preparations.

Line		$10^{-3} \times$ TAG (d.p.m./mg of cell protein)
1	Initial cellular TAG label	199.0 $\pm$ 20.8
2	Final cellular TAG label	129.5 $\pm$ 13.4
3	Loss of cellular TAG label	70.0 $\pm$ 13.3
4	VLDL TAG label secreted	186.7 $\pm$ 33.5

incorporation as TAG fatty acids. This amount is equivalent to 37 nmol of TAG per mg of protein per 24 h. It should be recognized that this is a maximum value for synthesis *de novo*, which will be suppressed considerably when exogenous fatty acids are added to the medium [31]. The quantity of TAG synthesized *de novo*, therefore, is clearly insufficient to account for the net gain of 85 $\pm$ 14 μg of TAG (94 $\pm$ 15 nmol of TAG assuming an average molecular mass of 900 Da) which occurred per mg of cell protein over a 24 h period (Table 1). Neither can the calculated figure for synthesis *de novo* account for the shortfall of 98.8 $\pm$ 18.2 μg (109.7 $\pm$ 20.2 nmol) of TAG per mg of cell protein per 24 h in the experiment described in Figure 1, in which endogenous fatty acid synthesis was most likely suppressed by the presence of exogenous oleate.

Relationship between labelled TAG and labelled phospholipid

A similar net gain, in this case of labelled TAG, occurred when hepatocytes were cultured overnight with [^{3}H]oleate to prelabel the cellular TAG. When the cells were subsequently transferred to a medium lacking [^{3}H]oleate, the loss of labelled TAG from the cell was insufficient to account for the amount of label secreted as VLDL TAG and this resulted in a net increase in the total (i.e. cell plus VLDL) TAG label compared with that present in the cell at the start of the culture period (Table 2). It is important to note that the labelled cellular lipids present at the beginning of the culture period were the sole source of label both for that remaining within the cell and that secreted during culture. The amount of labelled unesterified fatty acids present in the cell at the start of the culture period was relatively low (13400 $\pm$ 4100 d.p.m./mg of cell protein) and constituted only 6.7 % of the amount of label initially present in the cellular TAG. This was clearly insufficient to account for more than a small proportion of the total increase in labelled TAG.

A clue as to the possible source of some of this 'extra' TAG came from the observation that the loss of prelabelled phospholipid from the cell was much greater than that which appeared as phospholipid in VLDL during a subsequent 24 h culture period. For instance, in one series of experiments, whereas there was a disappearance of 26700 $\pm$ 3100 d.p.m./mg of cell protein in labelled cellular phospholipid, only 6400 $\pm$ 1200 d.p.m./mg of this appeared as phospholipid associated with VLDL. The amounts of labelled phospholipid associated with the VLDL infranate were very small. Thus, in contrast with the increase in the mass (Table 1; Figure 1) and radioactivity (Table 2) associated with total TAG during a 24 h culture period in the absence of

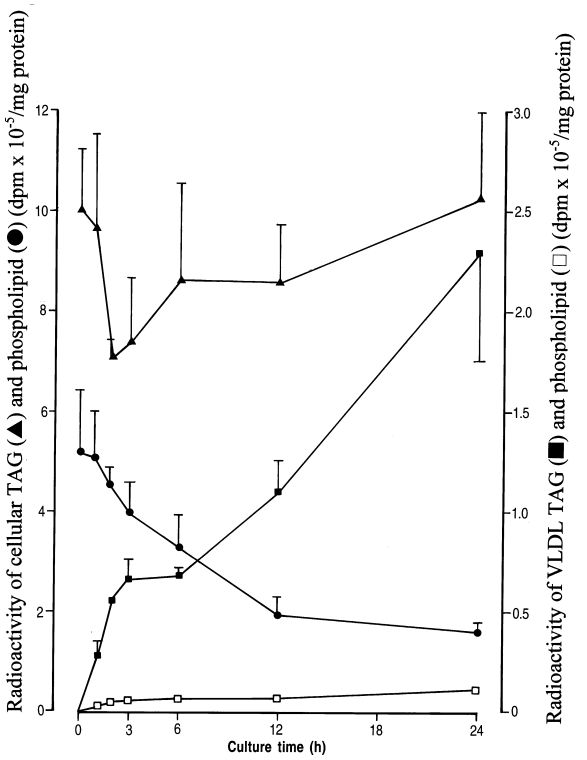

Figure 2 Relationship between cellular and VLDL lipids following a pulse–chase with [^{3}H]oleate

Hepatocytes were cultured overnight with supplemented Waymouth's medium. No oleate was present. The medium was changed and the cellular lipids were pulse-labelled for 0.5 h with [^{3}H]oleate (10 μCi; 10 Ci/mmol) bound to BSA [0.05% (w/v) final concentration in culture medium]. The labelled medium was rapidly removed, the cells were washed twice with ice-cold PBS and supplemented Waymouth's medium was added. The cellular label was then chased by the addition of 0.75 mM oleate for 0, 1, 2, 3, 6, 12 or 24 h. At each time point two dishes were removed for determination of incorporation of label into the VLDL- and cellular phospholipids and TAG. The average measurements from two dishes were taken for each experiment. Each point represents the mean $\pm$ S.E.M of three individual hepatocyte preparations.

oleate under the same conditions, the amount of labelled phospholipid (cell plus VLDL) declined.

Pulse–chase studies

To investigate the above relationship in greater detail, cellular phospholipids and TAG were pulse-labelled by culturing hepatocytes in the presence of trace amounts of highly labelled [^{3}H]oleate for 0.5 h. During a subsequent chase period in the presence of unlabelled oleate, although there was an initial decline in the intracellular content of labelled TAG at 2 h ($P < 0.05$), this did not subsequently persist and was slowly reversed (Figure 2). At the end of the 24 h chase period, therefore, there was no significant decline in the initial cellular content of labelled TAG. Nevertheless, a considerable quantity of labelled TAG appeared in the VLDL fraction of the medium over this period. The secretion pattern of labelled VLDL appeared to be biphasic (Figure 2). In contrast to the behaviour of cellular labelled TAG, there was a continuous decline in the amount of labelled phospholipid remaining within the cell. This label did not appear as VLDL phospholipid, which, at 24 h, did not constitute more than 5% of the total labelled VLDL lipid. The amount of labelled phospholipid appearing in the VLDL infranate was negligible. The overall pattern of the changes observed in these experiments

Table 3 Redistribution of labelled cellular and VLDL lipids after a 24 h chase of cellular lipids pulse-labelled with [^{3}H]oleate for 0.5 h

For details, see the legend to Figure 2. At 24 h, the radioactivities of the cellular and VLDL TAG were added, as were those of the respective phospholipid fractions. These were compared with the initial cellular radioactivity of each lipid at the end of the pulse-label (time zero). Values marked * are significantly different ($P < 0.01$) from the initial values.

Time (h)	Total TAG ($10^{-4} \times$ d.p.m./mg of protein)	Total phospholipid ($10^{-4} \times$ d.p.m./mg of protein)
0	100.4 $\pm$ 12.7	51.5 $\pm$ 12.5
24	126.3 $\pm$ 13.6*	17.9 $\pm$ 1.6*

Table 4 Redistribution of label during culture of cells containing pre-labelled lipids

Cells were cultured for 4 h with labelled oleate (0.75 mM; 0.95×10^6 d.p.m./μmol). The medium was removed and the cells were washed twice with PBS. Some dishes were removed for measurement of incorporation of radiolabel into cell lipids (0 h). The remainder were cultured for a further 24 h in the absence of exogenous oleate and the cells and VLDL were harvested. The results are expressed as $10^{-4} \times$ d.p.m. per mg of cell protein ($n = 3$).

Fraction	Time	TAG	Phospholipid	Fatty acid	Cholesteryl ester
Cells	0 h	21.61 $\pm$ 0.56	7.13 $\pm$ 0.40	0.53 $\pm$ 0.07	0.25 $\pm$ 0.01
Cells	24 h	15.43 $\pm$ 0.29	3.26 $\pm$ 0.03	0.38 $\pm$ 0.01	0.63 $\pm$ 0.02
VLDL	24 h	15.22 $\pm$ 0.99	0.74 $\pm$ 0.03	0.09 $\pm$ 0.01	0.21 $\pm$ 0.01

resulted in a net gain of 26.1 $\pm$ 2.6% ($P < 0.01$) in the total labelled TAG present in the combined cell plus VLDL fractions, compared with that initially present in the cell TAG at the end of the labelling period. By contrast, the total amount of label associated with the combined phospholipid fractions declined to 37.3 $\pm$ 6.0% ($P < 0.01$) of the initial value (Table 3). There was little significant cellular re-uptake of labelled VLDL lipids secreted into the medium. This was checked by addition of VLDL containing labelled TAG (100 5000 d.p.m.), phospholipid (7000 d.p.m.) and non-esterified fatty acid (10 600 d.p.m.) to unlabelled cell cultures for 24 h. At the end of this period, the amount of labelled cellular TAG was relatively very small (71 080 $\pm$ 5930 d.p.m.), although relatively larger quantities were associated with cellular phospholipid (5260 $\pm$ 993 d.p.m.) and non-esterified fatty acid (4500 $\pm$ 950 d.p.m., $n = 3$) Nevertheless, total uptake of label amounted to only about 8% of the added label. During the chase period, the mass of cellular phospholipid remained constant whilst its specific radioactivity showed a continuous decline (results not shown). This pattern is suggestive of a rapid turnover of phospholipids in which labelled phospholipid fatty acids present at the end of the 0.5 h pulse were chased out of the phospholipid fraction and replaced by unlabelled oleate entering the cell from the extracellular medium. The results of other experiments showed that the labelled fatty acids derived from the phospholipid fraction did not accumulate within the cell as non-esterified fatty acids, nor were they secreted as such with the VLDL (Table 4). The remaining alternatives are that the labelled fatty acids are either oxidized or re-esterified to a non-phospholipid acylglycerol. The concomitant increase in the total TAG label over this period (Table 1) suggests that the latter is the most likely explanation. The possibility of a contribution of labelled fatty acids from cholesteryl ester to the net increase in TAG fatty acid label was also investigated. In this respect, the amount of label in cholesteryl ester was insufficient

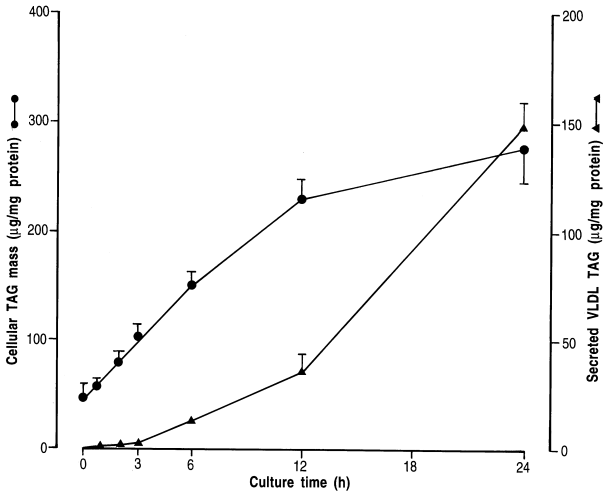

Figure 3 Relationship between the mass of cellular and secreted VLDL TAG during the culture of hepatocytes in the presence of 0.75 mM oleate

Hepatocytes were cultured overnight with supplemented Waymouth's medium in the absence of oleate. The medium was changed and [^{3}H]oleate was added for 0.5 h (see legend to Figure 2). After removal of the labelled medium, the cells were washed and the cells were cultured for various times in the presence of 0.75 mM oleate. At each time point, two dishes were removed and the mass of TAG in the cells and in the secreted VLDL was determined. Each point is the mean ± S.E.M of three independent hepatocyte preparations.

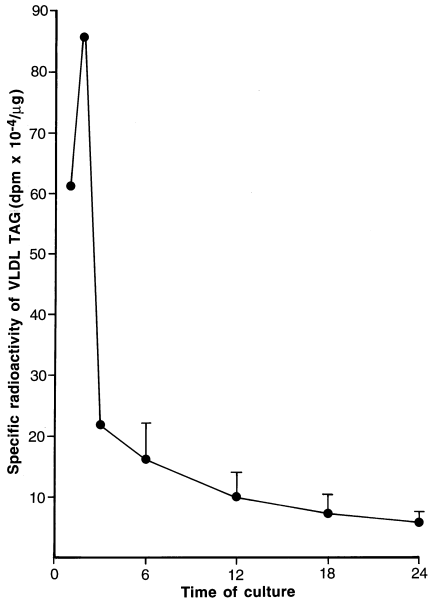

Figure 4 Specific radioactivity of VLDL TAG following a pulse-label with [^{3}H]oleate

Hepatocytes were cultured overnight in the absence of oleate. The medium was removed and supplemented Waymouth's medium was added. Cells were pulse-labelled for 0.5 h with [^{3}H]oleate, after which the medium was removed and the cells washed twice with ice-cold PBS. Supplemented Waymouth's medium containing 0.75 mM oleate was added and the cells cultured for further periods of 1–24 h. At the end of each period two dishes were removed for determination of the mass and radioactivity of the secreted VLDL TAG. Each point represents the mean ± S.E.M of three individual hepatocyte preparations, except for the 1, 2 and 3 h time points. At these points, in two of the experiments, the specific radioactivity of the VLDL TAG was too high for accurate measurement, since the secreted TAG mass was barely detectable. In these cases, the values are from one experiment only.

to contribute significantly to the overall transfer of labelled fatty acids (Table 4). It is clear from Table 4 that the increase in the total label of TAG over the 24 h period could not be completely accounted for by loss of label from phospholipid. In other experiments we have noted a significant but variable incorporation of [^{3}H]oleate into cellular diacylglycerol at the end of the labelling period, and it is possible that this contributes to the increase in the amount of labelled TAG at the end of the following 24 h. In general, the pattern of transfer of label observed in Figure 2 simply represents the beginning and the end of a complex and, as yet, obscure pathway of intracellular lipid transfer involved in the recruitment of lipids for the assembly of VLDL.

An important question relating to this mechanism is whether VLDL TAG is derived directly from newly-synthesized TAG produced in the ER from exogenous fatty acids (direct route) or whether the newly-synthesized TAG first enters the cytosolic compartment prior to recruitment at some later stage (indirect route). Our previous results have provided some evidence for the predominance of the latter route and suggest that the direct secretion of newly-synthesized TAG is a relatively minor pathway for the formation of VLDL [13,14]. On the other hand, it has been suggested that newly-synthesized TAG passes into the cytosol of the cell only when the secretory route is saturated with TAG produced by a large increase in the availability of extracellular fatty acids [32]. The studies described above are of relevance to this controversial issue. For instance, if most of the trace quantity of highly labelled TAG newly-synthesized from the exogenous [^{3}H]oleate had entered the secretory pathway directly, this material should have been chased out of the cell very rapidly because the assembly and secretion of VLDL particles is complete within 30–40 min. However, only 7.3 ± 0.3 % of the initial cellular label was released as VLDL during the first 3 h of the chase period (Figure 2). This suggested that only a relatively small proportion of the total cellular newly-synthesized TAG had immediately entered the secretory pool during the labelling period. By contrast, a much larger proportion of the cellular TAG was secreted only at a later stage, between 6 h and 24 h after the pulse, indicating that this TAG had been sequestered prior to secretion, probably in the cytosol of the cell.

In the experiment described by Figure 2, the mass of VLDL secreted during the chase period was also measured (Figure 3). Comparison of the patterns of secretion of VLDL TAG radioactivity on the one hand (Figure 2) and TAG mass on the other (Figure 3) may also provide some clues as to the metabolic pathways by which TAG is made available for the synthesis of VLDL. For instance, despite the fact that only a small proportion of the cell TAG label was secreted during the first 2 h of the chase period, the material which appeared in the medium as VLDL during this period had a relatively high specific radioactivity due to the low mass of TAG involved (Figure 4). This might be explained by the presence of a small amount of highly labelled nascent VLDL, the TAG of which had been synthesized immediately from the small proportion of extracellular trace [^{3}H]oleate which, after esterification, had passed directly into the secretory lumen of the cell rather than into the cell cytosol. The initial phase (0–2 h) of the biphasic pattern of labelled VLDL TAG secretion (Figure 2) may, thus, reflect the release of this small mass of highly labelled nascent VLDL. The initial, rather steep, decline in the cell TAG radioactivity during this period (Figure 2) may represent the loss of that proportion of cellular label which was originally present as nascent VLDL in the secretory apparatus at the end of the pulse. After a lag period (2–6 h), the final phase of the secretion of VLDL was associated

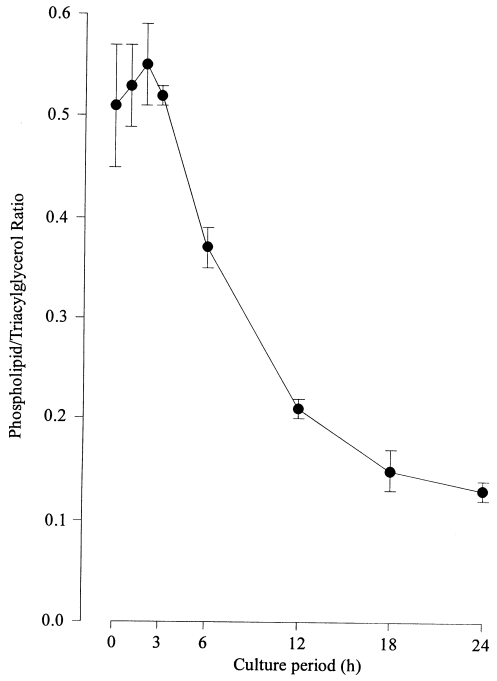

Figure 5 Relative changes in the labelling patterns of TAG and phospholipid

For details, see the legend to Figure 2. At each time point the ratio of radioactivity in TAG and phospholipid was calculated.

Table 5 Conversion of cellular phosphatidylcholine into VLDL TAG

Hepatocytes were cultured in the presence of 1,2-di[1-^{14}C]palmitoyl phosphatidylcholine (0.25 μCi; 111 μCi/μmol; added as a solution in 25 μl of ethanol) for 16 h. The medium was removed and the cells washed twice with phosphate-buffered saline. In each experiment, two dishes were removed at this stage and incorporation of label into cellular phospholipid and TAG fractions were determined. To the remaining dishes was added fresh medium without labelled substrate and the cells were cultured for a further 24 h. The secretion of labelled phospholipid and TAG into the VLDL fraction during this period was determined. Each value is the mean ± S.E.M. of duplicate determinations on each of four independent hepatocyte preparations.

	Phospholipid (10^{-3} × d.p.m./mg of cell protein)	TAG (10^{-3} × d.p.m./mg of cell protein)
Cell (initial)	62.9 ± 6.7	10.5 ± 1.7
Cell (final)	42.3 ± 5.2	5.6 ± 1.3
VLDL	4.7 ± 1.2	8.1 ± 0.8

with an increase in the output of both mass and label of TAG, probably as a result of the mobilization of the increased quantity of cytosolic TAG which accumulated during this time. This overall pattern is summarized in Figure 3, which shows a direct relationship between the concentration of intracellular TAG, most of which is present in the cell cytosol [22], and the rate of secretion of VLDL TAG mass.

Direct transfer of labelled fatty acid from cellular phospholipid to cellular and VLDL TAG

The nature of the relationship between changes in the labelling patterns of phospholipid and TAG shown in Figure 2 suggests that cellular phospholipid may represent the source of at least some of the additional TAG which cannot be accounted for by synthesis *de novo*. The experiment described by Figure 2 showed that the loss of phospholipid label from the cell was greater than could be accounted for by the appearance of phospholipid label in VLDL. Thus the changing balance of labelled glycerolipids in the cell and VLDL over a period of time was characterized by a decrease in the ratio of labelled phospholipid/TAG. The time course of changes in this ratio during the chase period of the experiment described in Figure 2 is shown in Figure 5. Although these results support the possibility of a metabolic transfer of phospholipid fatty acids to TAG, they are not conclusive. To investigate this aspect further, cellular phospholipids were pre-labelled by culturing hepatocytes overnight in the presence of 1,2-di[1-^{14}C]palmitoyl phosphatidylcholine. After removal of all unbound labelled phospholipid, the amounts of label incorporated into cellular phospholipid and TAG were measured (Table 5). Replicate dishes of cells (containing 62900 ± 6700 d.p.m. of cellular phospholipid) were cultured for a further period of 24 h and the secreted VLDL was isolated. During this period 8100 ± 820 d.p.m. (13.8 ± 2.2 % of the initial cellular phosphatidylcholine label) appeared as VLDL TAG. Not all of the label lost from phospholipids could be accounted for by labelled TAG in this experiment. It is possible that this label may have been associated with TAG precursors such as fatty acids and diacylglycerol. Since the main aim of this experiment was to investigate the capacity of the cell for phospholipid conversion, specifically to TAG, this aspect was not investigated further.

The above results provide evidence that the cell contains the enzymic machinery for transferring phospholipid fatty acids into both cellular and VLDL TAG and suggest that the reciprocal relationship between changes in labelled phospholipid and TAG in the earlier pulse–chase experiment (Figure 2) may be one of cause and effect. If this is the case, neither the data shown in Table 5 nor those of Figure 2 provide information as to whether phospholipid fatty acids are transferred directly into VLDL TAG or whether they are initially transferred into the cytosolic TAG followed by recruitment for VLDL assembly.

DISCUSSION

There are two main questions relating to the provision of TAG for the synthesis of VLDL. These are, first, of the available primary sources of precursor fatty acids which is the most important? Secondly, what are the metabolic and cellular routes by which TAG is incorporated into VLDL? As regards the first question, extracellular fatty acids rather than fatty acids synthesized *de novo* appear to play a predominant role, but the metabolic pathways by which fatty acids from the extracellular source are incorporated into VLDL TAG are not clear. We have provided evidence that the major pathway follows an indirect route in which newly-synthesized TAG is transferred initially to the cell cytosol [13,14]. The second step involves transfer of the cytosolic TAG into the secretory lumen of the cell, a process which is achieved predominantly by lipolysis and re-esterification. It may be calculated that at least 70 % of the cytosolic TAG which eventually appears as VLDL has undergone this transformation [14,15]. The above calculation, however, does not provide any information relating to the immediate source of the cytosolic TAG, nor of the immediate fate of fatty acids, released by lipolysis, prior to their ultimate incorporation into VLDL TAG. The results of the TAG balance studies described above suggest that the cellular TAG pool(s) from which VLDL is derived is fed from sources in addition to those derived directly

from *de novo* synthesized or extracellular fatty acids. The pulse–chase studies (Figure 2) and the results of the phospholipid transfer study (Table 5) suggest that some of the TAG which ends up as VLDL is derived from a pool of intracellular phospholipid. In the presence of an extracellular supply of fatty acid, there is, however, no net loss of phospholipid, an observation which implies a rapid flux of extracellular fatty acid through the phospholipid pool. If this is the case, we are unable at the moment to determine whether this phospholipid pool is discharged into the cytosolic TAG pool prior to re-incorporation into VLDL on the one hand, or directly into VLDL TAG on the other.

Previous studies in cultured fibroblasts and smooth muscle cells have suggested the presence of the enzymic machinery required for the transfer of phospholipid fatty acids into TAG [33,34]. As far as we are aware, the present study provides the first evidence for the importance of this process in the provision of fatty acids for the synthesis of VLDL TAG in the liver. The nature of the enzymes involved, however, is obscure and both the present work and previous studies [33,34] imply the participation of phospholipases C and/or D to provide precursor diacylglycerol and/or phosphatidic acid respectively. Alternatively, phospholipases A_1 and/or A_2 may provide fatty acids for the acylation of glycerophosphate via the phosphatidic acid pathway of TAG synthesis. In the latter case, an opportunity for providing fatty acids from membrane phospholipids exists during the extensive lipolysis involved in phospholipid remodelling [35–38]. Enzymes responsible for these processes have been located in the Golgi [29] and in the ER [35]. It may be of some relevance that apoB itself has been reported to have intrinsic phospholipase A_1 and A_2 activity [39,40]. In the present work, the possible role of phospholipase A_2 in the assembly of VLDL was assessed by culturing cells in the presence of a phospholipase A_2 inhibitor, *p*-bromophenacyl bromide. At a concentration of 50 μM there was a decrease in the secretion of VLDL TAG by $40.0 \pm 16.2\%$ during a 24 h culture period. There was no change in cell viability during this time.

It has previously been assumed that intracellular membrane phospholipids play merely a structural role in the assembly of VLDL by providing a suitable framework to facilitate the proper folding of newly translated apoB during the subsequent construction of VLDL [41,42]. Although we still know next to nothing about the complex enzymology of bulk lipid recruitment for this process, the active metabolic participation of membrane phospholipids should be considered. This is not a new idea, but no direct experimental evidence for it has emerged since it was first proposed in 1971 [17]. Subsequent studies, although supportive of the concept, provided only indirect evidence. These later studies raised several questions which might possibly be answered in terms of a metabolic, as well as a structural, role for hepatic phospholipids. Why, for instance, does hepatic phospholipid turnover occur at such a high rate (15% of total phospholipid turnover per h; [35])? Why is the chemical structure of the phospholipids in the ER so important in permitting the efficient assembly of VLDL [43]? Why is a decreased production of phosphatidylcholine associated with a decline in the output of VLDL [44,45]? And, finally, as mentioned above, why does apoB contain intrinsic phospholipase A activity [39,40]?

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REFERENCES

1 Dixon, J. L. and Ginsberg, H. N. (1993) J. Lipid Res. **34**, 167–179
2 Sparks, J. D. and Sparks, C. E. (1994) Biochim. Biophys. Acta **1215**, 9–32
3 Borén, J., Wettesten, M., Rustaeus, S., Andersson, M. and Olofsson, S.-O. (1993) Biochem. Soc. Trans. **21**, 487–493
4 Sharp, D., Blinderman, L., Combs, K. A., Kienzle, B., Ricci, B., Wager-Smith, K., Gil, C. M., Turck, C. W., Bouma, M. E., Rader, D. J., Aggerbeck, L. P., Gregg, R. E., Gordon, D. A. and Wetterau, J. R. (1993) Nature (London) **365**, 65–69
5 Shoulders, C. C., Brett, D. J., Bayliss, J. D., Narcisi, T. M., Jarmuz, A., Grantham, T. T., Leoni, P. R., Bhattacharya, S., Pease, R. J. and Cullen, P. M. (1993) Hum. Mol. Genet. **2**, 2109–2116
6 Leiper, J. M., Bayliss, J. D., Pease, R. J., Brett, D. J., Scott, J. and Shoulders, C. C. (1994) J. Biol. Chem. **269**, 21951–21954
7 Gretch, D. G., Sturley, S. L., Wang, L., Lipton, B. A., Dunning, A., Grunwald, K. A. A., Wetterau, J. R., Yao, Z., Talmud, P. and Attie, A. A. (1996) J. Biol. Chem. **271**, 8682–8691
8 Du, E. Z., Wang, S.-L., Kayden, H. J., Sokol, R., Curtiss, L. K. and Davis, R. A. (1996) J. Lipid Res. **37**, 1309–1315
9 Borchardt, R. A. and Davis, R. A. (1987) J. Biol. Chem. **262**, 16394–16402
10 Rusinol, A., Verkade, H. and Vance, J. E. (1993) J. Biol. Chem. **268**, 3555–3562
11 Cartwright, I. J., Hebbachi, A.-M. and Higgins, J. A. (1993) J. Biol. Chem. **268**, 20937–20952
12 Swift, L. L. (1995) J. Lipid Res. **36**, 395–406
13 Gibbons, G. F., Bartlett, S. M., Sparks, C. E. and Sparks, J. D. (1992) Biochem. J. **287**, 749–753
14 Wiggins, D. and Gibbons, G. F. (1995) Adv. Enzyme Regul. **35**, 179–198
15 Wiggins, D. and Gibbons, G. F. (1992) Biochem. J. **284**, 457–462
16 Yang, L.-Y., Kuksis, A., Myher, J. J. and Steiner, G. (1995) J. Lipid Res. **36**, 125–136
17 Bar-on, H., Roheim, P. S., Stein, O. and Stein, Y. (1971) Biochim. Biophys. Acta **248**, 1–11
18 Kondrup, J., Damgaard, S. E. and Fleron, P. (1979) Biochem. J. **184**, 73–81
19 Duerden, J. M., Bartlett, S. M. and Gibbons, G. F. (1989) Biochem. J. **263**, 937–943
20 Bartlett, S. M. and Gibbons, G. F. (1988) Biochem. J. **249**, 37–43
21 Edelstein, C. and Scanu, A. M. (1986) Methods Enzymol. **128**, 151–155
22 Duerden, J. M. and Gibbons, G. F. (1990) Biochem. J. **272**, 583–587
23 Folch, J., Lees, M. and Sloane-Stanley, G. H. (1957) J. Biol. Chem. **226**, 497–509
24 Trinder, P. (1969) Ann. Clin. Biochem. **6**, 24–30
25 Takayama, M., Itoh, S., Nagasaki, T. and Tanimizu, I. (1977) Clin. Chim. Acta **79**, 93–98
26 Goldfarb, S., Barber, T. A., Pariza, M. A. and Pugh, T. D. (1978) Exp. Cell Res. **117**, 39–46
27 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
28 van Harken, D. R., Dixon, C. W. and Heimberg, M. (1969) J. Biol. Chem. **244**, 2278–2285
29 Gibbons, G. F., Khurana, R., Odwell, A. and Seelaender, M. C. L. (1994) J. Lipid Res. **35**, 1801–1808
30 Duerden, J. M., Marsh, B., Burnham, F. J. and Gibbons, G. F. (1990) Biochem. J. **271**, 761–766
31 Gibbons, G. F. and Burnham, F. J. (1991) Biochem. J. **275**, 87–92
32 Chao, F.-F., Stiers, D. L. and Ontko, J. A. (1986) J. Lipid Res. **27**, 1174–1181
33 Gavino, V. C., Miller, J. S., Dillman, J. M., Milo, G. E. and Cornwell, D. G. (1981) J. Lipid Res. **22**, 57–62
34 Rosenthal, M. D. (1980) Lipids **15**, 838–848
35 Schmid, P. C., Johnson, S. B. and Schmidt, H. H. O. (1991) J. Biol. Chem. **266**, 13690–13697
36 Patton, G. M., Fasulo, J. M. and Robins, S. J. (1994) J. Lipid Res. **35**, 1211–1221
37 Lawrence, J. B., Moreau, P., Cassagne, C. and Morre, D. J. (1994) Biochim. Biophys. Acta **1210**, 146–150
38 Wilson, H.-M. P., Neumuller, W., Eibl, H., Welch, Jr., W. H. and Reitz, R. C. (1995) J. Lipid Res. **36**, 429–439
39 Reisfeld, N., Lichtenberg, D., Dagan, A. and Yedgar, S. (1993) FEBS Lett. **315**, 267–270
40 Parthasarathy, S. and Barnett, J. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 9741–9745
41 Spring, D. J., Chen-Liu, L. W., Chatterton, J. E., Elovson, J. and Schumaker, V. N. (1992) J. Biol. Chem. **267**, 14839–14845
42 Spring, D. J., Lee, S.-M., Puppione, D. L., Phillips, M., Elovson, J. and Schumaker, V. N. (1992) J. Lipid Res. **33**, 233–240
43 Rusinol, A. E., Chan, E. Y. W. and Vance, J. E. (1993) J. Biol. Chem. **268**, 25168–25175
44 Verkade, H. J., Fast, D. G., Rusinol, A. E., Scraba, D. G. and Vance, D. E. (1993) J. Biol. Chem. **268**, 24990–24996
45 Yao, Z. and Vance, D. E. (1988) J. Biol. Chem. **263**, 2998–3004