Extracellular fatty acids are not utilized directly for the synthesis of very-low-density lipoprotein in primary cultures of rat hepatocytes

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In hepatocytes cultured in the presence of oleate (initial concn. 0.75 mM), the secretion of very-low-density lipoprotein (VLDL) triacylglycerol and, to a lesser extent, apoprotein B (apoB) increased with time, whereas there was a large decline in the extracellular concentration of fatty acid. There was thus no synchronous relationship between the extracellular fatty acid concentration and the secretion of VLDL. Rather, the appearance of VLDL in the medium was dependent on the intracellular triacylglycerol concentration. At a given concentration of extracellular fatty acid, cells depleted of triacylglycerol secreted less VLDL triacylglycerol and apoB than did control cells. A similar pattern was observed for triacylglycerol newly synthesized from extracellular [^3H]oleate. By contrast, the synthesis and output of ketone bodies were directly dependent on the fatty acid concentration of the medium. These results suggest that, at least for oleic acid, extracellular fatty acids are not utilized directly for VLDL assembly, but first enter a temporary intracellular storage pool of triacylglycerol, which is the immediate precursor of secreted triacylglycerol. The size of this pool then determines the rate of secretion of VLDL triacylglycerol and apoB. Ketogenesis, on the other hand, relies mainly on the direct utilization of extracellular fatty acids.

INTRODUCTION

In vivo, there are several sources of fatty acids which are potentially available for the synthesis of hepatic very-low-density lipoprotein (VLDL) triacylglycerol, and the relative importance of each may depend on the nutritional state of the animal [1–4]. Nevertheless, it is well established that isolated liver preparations respond to an increased availability of extracellular fatty acid by increasing triacylglycerol secretion (see ref. [1] for a review). There is, however, little information as to the intracellular route by which these fatty acids are made available specifically for incorporation into VLDL triacylglycerol. Recent evidence has emphasized the importance of the intracellular pool of hepatic triacylglycerol as a source of substrate for VLDL synthesis [2,3,5,6], even in the presence of extracellular fatty acids [2,3]. In the absence of extracellular fatty acids, the size of the intracellular triacylglycerol pool is related to the rate of VLDL triacylglycerol secretion [7]. The exact intracellular route by which extracellular fatty acids enter VLDL as triacylglycerol may have important implications for the control of VLDL assembly, since prior incorporation into a storage pool rather than direct entry into the secretory apparatus imposes additional steps which may be subject to regulation. To investigate the biosynthetic relationship between extracellular fatty acids, intracellular and VLDL triacylglycerol, we have studied the time-dependent changes in these parameters in cultured hepatocytes over a period of 24 h. The effects of these changes on the output of apoprotein B (apoB) have also been determined.

MATERIALS AND METHODS

Cell cultures: harvesting of cells and preparation of VLDL

Rats were maintained as described previously [8] and used for hepatocyte preparation 2 h into the light phase of their diurnal

cycle. Hepatocytes prepared under sterile conditions were suspended in Waymouth's medium MB 752/1 containing fetal-calf serum, penicillin, streptomycin and added amino acids [8]. At 3–4 h after transfer of the suspension to culture dishes, the medium was removed, the cell monolayer was washed and serum-free Waymouth's medium containing added amino acids, antibiotics, dexamethasone, lactate, pyruvate and oleate (initial concns. 0.75 mM) was added [8]. This is subsequently referred to as the supplemented medium. In some experiments, oleate was not present in the medium at this stage. The cells were cultured overnight (i.e. for 18 h) under these conditions. The medium was removed, replaced with fresh supplemented medium and the cells were cultured for periods of 6, 12, 18 and 24 h. In some experiments, [^3H]oleate (1 μCi , 0.75 mM) was used at this stage in place of the unlabelled material. At the end of each period the cells were cooled to 4 °C and the medium was removed. The cells were washed and removed from the plate [5] and VLDL was isolated from the cell medium as described previously [5,9]. For storage of VLDL samples, a portion (0.2 ml) was added to a solution of BSA (1 %) in phosphate-buffered saline (0.2 ml) and kept at –20 °C or –80 °C before assay of apoB.

Measurement of cellular lipids and VLDL triacylglycerol and apoB

The total lipid fractions of the cell pellet and of the VLDL were isolated as described previously [10]. Portions of the lipid extracts were used for measurement of the mass of triacylglycerol [11] with a kit obtained from Boehringer-Mannheim ('Tri-glycerides GPO-PAP'). In those cases in which [^3H]oleate was used in the extracellular medium, the labelled triacylglycerol was purified from the total lipid extract by t.l.c. [12]. The radioactivity of this fraction was then used to calculate the rate of triacylglycerol synthesis. In all experiments, manipulative losses of triacylglycerol were accounted for by addition of glycerol

Abbreviations used: VLDL, very-low-density lipoprotein; apoB, apoprotein B.

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Fig. 1. Time-dependent changes in the concentration of non-esterified fatty acids and cellular and VLDL triacylglycerol

Cells were cultured overnight in either the presence (●, ▲) or the absence (○, △) of extracellular oleate (initial concn. 0.75 mM). In each case, the medium was removed and replaced with supplemented medium (containing 0.75 mM-oleate). At various times thereafter, the concentrations of secreted VLDL triacylglycerol (▲, △), cellular triacylglycerol (●, ○) and medium non-esterified fatty acids (■) were determined (this latter measurement was made only in the cells incubated overnight with oleate). Each point represents the mean $\pm$ S.E.M. of between three and seven individual experiments. Values marked * and † are significantly different from those values obtained in cells incubated overnight with oleate ($P < 0.05$ and $P < 0.01$ respectively).

Fig. 2. Effect of time of culture on the secretion of apoB

Cells were cultured overnight in the presence of oleate and then transferred to fresh medium containing oleate (0.75 mM) as described in the legend to Fig. 1. At various times thereafter, the amounts of VLDL apoB secreted into the medium were determined. Each point represents the mean $\pm$ S.E.M. of four individual experiments.

[14 C]trioleate as internal standard. The apoB content of VLDL was assayed as described previously [13], with VLDL apoB as a standard. Control experiments showed that the immunoreactivity of the apoB epitope recognized by the monoclonal antibody used in the assay was not adversely affected by freezing or storage at -20°C or -80°C of VLDL samples after dilution in 1% BSA.

Other methods

The concentrations of β -hydroxybutyrate and acetoacetate in the $d > 1.006$ infranantant (obtained as the bottom fraction after slicing the tube for VLDL) were measured by the method of Williamson *et al.* [14]. The results are expressed as the sum of these ketone bodies in the original culture medium ($\mu\text{mol/ml}$). Oleate bound to BSA (essentially fatty-acid-free) was prepared by the method of Van Harken *et al.* [15] to give a stock solution of 15 mM. This was sterilized by ultrafiltration and added to the culture dishes to give an initial concentration of 0.75 mM. The BSA concentration in the culture medium was 0.5%. The non-esterified fatty acid concentration of the medium was measured by the method of Shimizu *et al.* [16].

Statistical methods

All values are presented as means $\pm$ S.E.M. of several independent experiments. Significant differences were calculated by a paired or unpaired Student's t test.

Materials

All cell-culture media components were obtained from Gibco (Uxbridge, Middx, U.K.). Dexamethasone, oleate, lactate, pyruvate and fatty-acid-free BSA were obtained from Sigma (Poole, Dorset, U.K.). [^3H]Oleic acid was obtained from Amersham International (Little Chalfont, Bucks., U.K.) and was converted into the sodium salt before use.

RESULTS

Fatty acid esterification

When hepatocytes were cultured with oleate at an initial concentration of 0.75 mM, there was a rapid clearance of the extracellular fatty acid from the culture medium. After 6 h, the concentration was decreased by 50% (Fig. 1). However, during this period, the output of VLDL triacylglycerol and apoB remained relatively low ($29.7 \pm 3.9 \mu\text{g/6 h}$ per mg of protein and $116.8 \pm 34 \text{ ng/6 h}$ per mg of protein respectively; see Figs. 1 and 2). By contrast, most of the newly esterified triacylglycerol remained within the cell during this period, and its intracellular concentration increased by about 2-fold (Fig. 1). This was also reflected by the distribution of label when the extracellular oleate was labelled with ^3H . In this case only 12.9% of the total newly synthesized triacylglycerol was secreted as VLDL, the rest remaining within the cell (Fig. 3). Between 12 and 18 h of culture, the average concentration of extracellular oleate decreased to between 0.13 and 0.22 mM. At the end of this period the concentration of intracellular triacylglycerol reached a peak at $450 \mu\text{g/mg}$ of protein, a 3-fold increase over the initial value. This was associated with a significant increase in the rate of secretion of VLDL triacylglycerol and apoB ($412 \pm 98\%$ and $273 \pm 69\%$ respectively; $P < 0.05$) compared with the initial rates when the extracellular oleate concentration was relatively high. This pattern was again paralleled by the changes in the secretion and cellular accumulation of newly synthesized triacylglycerol (Fig. 3). During the period 12–18 h, the proportion of the total newly synthesized triacylglycerol which was secreted as VLDL increased to $30.6 \pm 5.0\%$ ($P < 0.05$ compared with the proportion secreted between 0 and 6 h). Between 18 and 24 h

Fig. 3. Effect of time on the extracellular and intracellular distribution of newly synthesized triacylglycerol

Cells were cultured overnight in the presence of oleate (initial concn. 0.75 mM). The medium was removed and replaced with fresh supplemented medium containing [^3H]oleate (1 μCi , 0.75 mM). At various times thereafter, the amounts of labelled triacylglycerol in the secreted VLDL and that remaining within the cells were determined. Each point represents the mean $\pm$ S.E.M. of three independent experiments. Key: $\blacksquare$, cellular triacylglycerol; $\blacktriangle$, VLDL triacylglycerol; $\bullet$, total (cell+VLDL) triacylglycerol; $\triangle$, VLDL triacylglycerol as a percentage of the total.

there was little or no uptake of extracellular oleate. During this period the maximal rate of VLDL triacylglycerol secretion was maintained, although at the expense of intracellular triacylglycerol, net mobilization of which resulted in a decrease in its concentration (Fig. 1).

In the same series of experiments hepatocytes were depleted of intracellular triacylglycerol by overnight culture in the absence of oleate. This resulted in an intracellular triacylglycerol concentration of only 45 $\mu\text{g}/\text{mg}$ of protein, compared with 180 $\mu\text{g}/\text{mg}$ of protein in cells cultured overnight in the presence of oleate (Fig. 1). Subsequent culture of the triacylglycerol-depleted cells with oleate at the same initial concentration (0.75 mM) as that used with the high-triacylglycerol cells resulted in a decreased rate of VLDL triacylglycerol secretion. In this case, although the increase in cell triacylglycerol between 0 and 18 h was of the same order as previously, the maximum concentration of intracellular triacylglycerol was significantly lower ($P < 0.05$; Fig. 1). Under these conditions, there was a significant decrease in the secretion of VLDL triacylglycerol (Fig. 1) and in the secretion of apoB (79.9 $\pm$ 9.9% and 75.5 $\pm$ 9.8% of control, after 6 h and 24 h respectively; $P < 0.05$).

Fatty acid oxidation

The rate of ketogenesis (i.e. total production of acetoacetate and β -hydroxybutyrate) appeared to be directly dependent on the concentration of extracellular fatty acids in the culture medium. For instance, the relatively high residual concentration of oleate after the first 6 h of culture was associated with a high initial rate of ketogenesis (Fig. 4). By contrast, when there was

Fig. 4. Rate of ketogenesis

Cells were cultured according to the legend to Fig. 2. At various times thereafter, portions of the medium were removed for measurement of acetoacetate and β -hydroxybutyrate concentrations. The values presented are the sum of these ketone bodies. Each point represents the mean $\pm$ S.E.M. of four independent experiments.

little or no further uptake of oleate from the medium during the 18–24 h period, the rate of ketogenesis was relatively low (Fig. 4).

DISCUSSION

There are two major routes by which extracellular fatty acids are metabolized by the liver. These are (a) oxidation to ketone bodies and CO_2 , and (b) esterification to glycerolipids followed by secretion as VLDL [17]. The control of fatty acid flux into the esterification or oxidative pathways is well established and is determined by the catalytic activity of carnitine palmitoyl-transferase, which is dependent on the concentration of malonyl-CoA [18–21]. In the absence of extracellular fatty acids, the hepatic pool of stored triacylglycerol is capable of providing substrate for both ketogenesis [22,23] and VLDL [3,5,6,23]. However, the relative importance of this pool in the simultaneous presence of extracellular fatty acids is not precisely known. In particular, a question remains as to the extent to which extracellular fatty acids first enter the bulk intracellular pool of glycerolipid before mobilization for ketogenesis and VLDL triacylglycerol secretion. The studies *in vivo* by Chao *et al.* [24] investigated the channelling of triacylglycerol, newly synthesized on the microsomal membrane from exogenous [^3H]glycerol, into the cytosolic pool on the one hand and into nascent VLDL on the other. However, their experimental protocol precluded calculation of the contribution of cytosolic triacylglycerol to VLDL. This is a difficult task, in view of the fact that a large proportion of cytosolic triacylglycerol utilized for VLDL assembly is mobilized via lipolysis followed by re-esterification [25]. Thus, with labelled glycerol as the triacylglycerol precursor, there is considerable uncertainty as to its effective specific radioactivity during the assembly of VLDL.

The present results provide evidence, for the first time, that a major proportion of the extracellular fatty acid is esterified and temporarily stored intracellularly before mobilization for VLDL assembly. This is indicated by several observations. First, after the addition of oleate, there was a lag phase of approx. 12 h before the secretion of triacylglycerol became maximal. Fatty

acids esterified during this period were utilized to increase the net triacylglycerol content of the cell, rather than for secretion. Second, maximal rates of VLDL secretion occurred when there was no net extraction of oleate from the culture medium (between 18 and 24 h). Third, at equivalent initial concentrations of extracellular oleate, cells with a high intracellular triacylglycerol concentration secreted more VLDL triacylglycerol (Fig. 1). An alternative explanation for our results is that at lower concentrations of extracellular fatty acids there is normally direct incorporation into VLDL, but at the higher concentration used in the present work this pathway becomes saturated and a storage pool of triacylglycerol is temporarily formed. Two observations militate against this argument. First, the rate of VLDL secretion increased after fatty acid depletion in the medium suggesting that some factor other than extracellular fatty acid concentration was initially rate-limiting for VLDL secretion. Second, our previous studies with increasing concentrations of fatty acid in freshly prepared hepatocytes have shown that, despite an increase in the rate of VLDL secretion, even at the highest fatty acid concentration, most of the VLDL triacylglycerol was derived from the cytosolic pool [2].

It is apparent from Fig. 1 that, at zero time, cells which had been pretreated with oleate overnight for 18 h contained less triacylglycerol than the non-pretreated cells at the 18 h time point. In other words, cells that had been treated with oleate for exactly the same time contained different amounts of triacylglycerol. However, after 18 h in the presence of oleate, the non-pretreated cells were older by 18 h than those pretreated with oleate overnight (i.e. at zero time in Fig. 1). We have shown previously [26] that the rate of triacylglycerol synthesis increases with the age of the culture, and this may explain the difference in cellular triacylglycerol content. It is also of interest that this higher triacylglycerol content at 18 h in the non-pretreated cells (compared with zero time in the oleate-treated cells) was accompanied by an increased rate of VLDL triacylglycerol secretion during the next 6 h period (again compared with 0–6 h in the oleate-treated cells). We do not wish to imply, however, that the size of the intracellular triacylglycerol pool is the only factor determining VLDL output, and it is clear from Fig. 1 that the relationship between intracellular and VLDL triacylglycerol is not linear. Nevertheless, the present results suggest that the secretion of VLDL is related, in some as yet unknown manner, to the content of cellular triacylglycerol, rather than to the concentration of extracellular fatty acid. Under some conditions, intracellular cholesterol becomes rate-limiting for VLDL secretion [27]. We do not currently have any information concerning this potential regulatory factor under the culture conditions used.

The above hypothesis assumes that the increase in intracellular triacylglycerol accumulation between 0 and 18 h of culture is not due to a storage of triacylglycerol as VLDL within the secretory apparatus. This possibility appears unlikely, in view of the short half-life of apoB in the secretory apparatus [28–30] and that only a small proportion of the total intracellular triacylglycerol is present in the secretory pool [31,32].

Previous reports have also shown that, in freshly isolated liver preparations, extracellular fatty acids make only a relatively small direct contribution to VLDL triacylglycerol, the bulk of the secreted lipid in the short-term being derived from intracellular glycerolipids [2,4]. This indirect route for the utilization of extracellular fatty acid is similar to that which operates in muscle tissue [33–35], in which extracellular fatty acids are first esterified to cellular triacylglycerol before utilization for energy production.

The rate of secretion of apoB also appeared to be dependent on the intracellular concentration of triacylglycerol over the 24 h

period during which oleate was present in the extracellular medium (Fig. 2), although this relationship was less pronounced than that observed for VLDL triacylglycerol. In four time-course experiments carried out, although the apoB/triacylglycerol ratios were somewhat variable, in each case there was a steady decline in the ratio between 12 and 24 h. Thus the amount of triacylglycerol per VLDL particle increased [36] and, because there is a finite amount of apoB per particle, implies an increase in particle size with increasing time of culture.

Previous reports have suggested that at least some of the intracellular triacylglycerol utilized for VLDL assembly in the absence of extracellular fatty acid is mobilized via lipolysis followed by re-esterification [6,23]. If intracellular glycerolipid is a major contributor to VLDL triacylglycerol, this may have important implications for the mechanism by which VLDL particles are assembled intracellularly. The present results would imply that extracellular fatty acid is initially esterified at the cytosolic face of the endoplasmic reticulum [37], but that this triacylglycerol does not enter the secretory apparatus. Instead, it enters the cytosolic pool. Fatty acids, subsequently released from this pool may, however, be re-esterified at some site on the membrane of the secretory apparatus which is in close proximity to functional apoB. Binding of this newly synthesized triacylglycerol to apoB at this site would then facilitate transfer of the latter from the membrane to the lumen of the secretory apparatus as an immature VLDL particle [38–40]. The delayed effect of oleate on the secretion of VLDL may contribute to the controversy surrounding the precise effects of extracellular fatty acids on apoB secretion in several models *in vitro* [39,41,42]. Compared with oleate, *n*–3 fatty acids have a decreased stimulatory effect on the secretion of VLDL [43]. This may result from their inability to sustain a suitably high level of intrahepatic triacylglycerol, since triacylglycerol synthesis is suppressed under these conditions [43].

In contrast with the lack of synchrony between fatty acid uptake and VLDL secretion, the rate of ketogenesis appeared to be directly dependent on the concentration of fatty acids in the external medium rather than on the concentration of intracellular triacylglycerol (Figs. 1 and 4). This implies that extracellular fatty acids are in the main utilized directly for ketogenesis without prior esterification and lipolysis. The initial rate of ketogenesis in the present work was $0.21 \pm 0.04 \mu\text{mol/h per mg}$ of protein. Assuming a protein content per whole liver of 160 mg/g, this is equivalent to $33.6 \mu\text{mol/h per g}$ of liver. This rate is considerably higher than values quoted for isolated perfused livers, i.e. $5\text{--}13.3 \mu\text{mol/h per g}$ [4,44,45] and similar to rates observed *in vivo* [46]. From this viewpoint, therefore, primary cultures of rat hepatocytes appear to be a useful model for studying the regulation of ketogenesis.

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