Storage, mobilization and secretion of cytosolic triacylglycerol in hepatocyte cultures

The role of insulin

Julia M. DUERDEN and Geoffrey F. GIBBONS*

Metabolic Research Laboratory, Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE, U.K.

Cytosolic triacylglycerol labelled from [^3H]oleate accounted for almost 50% (57 ± 22 nmol/mg of protein) of the total cellular triacylglycerol which was newly synthesized by cultured hepatocytes during a 24 h incubation. Insulin decreased the export of triacylglycerol as very-low-density lipoprotein (VLDL) during this period. This resulted in a sequestration of newly synthesized triacylglycerol in the cytosol, rather than in the particulate fraction of the cell. Longer periods of incubation with [^3H]oleate resulted in increased concentrations of newly synthesized triacylglycerol within the cell, most of which ($78 \pm 3\%$ after 48 h; $80 \pm 3\%$ after 72 h) was located within the cytosolic fraction. The quantity of newly synthesized triacylglycerol in the cell cytosol was further increased by insulin. During these periods there were decreases in the amounts of triacylglycerol associated with the particulate fraction of the cell, irrespective of the presence or absence of insulin. In no case was a decrease in VLDL triacylglycerol secretion in response to insulin accompanied by an increased triacylglycerol content in the particulate fraction of the cell. In some experiments, the fate of the cytosolic triacylglycerol was studied by pulse labelling with [^3H]oleate. In these cases, when insulin was removed from the medium of cells to which they had previously been exposed, more newly synthesized triacylglycerol was secreted compared with cells which had not been exposed to insulin. This extra triacylglycerol was mobilized from the cytosolic rather than from the particulate fraction of the cell. Subsequent addition of insulin to the medium prevented the mobilization of cytosolic triacylglycerol. These results suggest that insulin enhances the storage of hepatocellular triacylglycerol in a cytosolic pool. Deficiency of insulin in the medium stimulates the mobilization of this pool which is channelled into the secretory pathway, entering the extracellular medium as VLDL.

INTRODUCTION

Exposure of cultured hepatocytes to insulin for periods of up to 24–48 h leads to a decrease in the rate of secretion of lipids and apoproteins associated with very-low-density lipoprotein (VLDL) [1–7]. This occurs despite an increase in the rate of triacylglycerol synthesis and results in an increase in the amount of triacylglycerol which accumulates within the cell [1,8]. The mechanism by which insulin exerts this inhibitory effect is not known. The intracellular site which accommodates the triacylglycerol which accumulates in the presence of insulin has also not yet been investigated. Prolonged treatment of rat hepatocytes and human Hep-G2 cells with insulin diminishes the inhibitory effect on the secretion of VLDL [6,8–10] and, in rat hepatocytes at least, if insulin is present for a sufficiently long period of time, this secretion process is stimulated [8,10]. There were two main objectives of the present work. The first objective was to establish whether insulin leads to the sequestration of newly synthesized triacylglycerol within the cytosolic pool or within the secretory apparatus represented by the membrane-bound fraction of the cell. Secondly, we attempted to determine whether triacylglycerol could be mobilized from this site when insulin was removed from the culture medium. This would explain a previous observation that removal of insulin from cells which had previously been exposed to the hormone gave rise to an increase in the secretion of VLDL triacylglycerol [8].

The effects of insulin on hepatic lipid synthesis, storage and secretion are complex and poorly understood [11]. Answers to the above questions may provide information about the means

by which insulin regulates the storage and secretion of hepatic triacylglycerol in the pre- and post-prandial states and why these processes are impaired in diabetes.

MATERIALS AND METHODS

Maintenance of animals and preparation of hepatocyte cultures

Animals were fed and housed as described previously [10,12]. Hepatocytes were prepared under sterile conditions [10] and were plated out (3.0 ml per dish) after suspension [$(0.5\text{--}0.75) \times 10^6$ cells/ml] in Waymouth's medium MB 752/1 containing added amino acids, antibiotics and serum [10]. After transfer to serum-free Waymouth's medium [10] the medium was supplemented with dexamethasone (1 μM), lactate (10 mM), pyruvate (1 mM) and [^3H]oleate (0.75 mM, 1 $\mu\text{Ci}/2.25$ μmol) (supplemented medium) with or without the addition of bovine insulin (78 nM) for 20 h or 24 h (day 1).

After 24 h of culture, the medium was removed and VLDL was isolated (see below). The cells were cooled to 4 $^{\circ}\text{C}$, washed with ice-cold phosphate-buffered saline [PBS: CaCl_2 (0.1 g/l), KCl (0.2 g/l), KH_2PO_4 (0.2 g/l), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.1 g/l), NaCl (8.0 g/l) and $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (2.16 g/l)] and scraped from the plate using 3.0 ml of ice-cold PBS. For longer-term studies, cells treated as above for 20 h had the medium removed and replaced with fresh supplemented medium with or without insulin. These dishes were incubated for a further 24 h (day 2) and the cells and VLDL were isolated (see below). In the remaining dishes, the supplemented medium was removed and replaced with the same

Abbreviations used: VLDL, very-low-density lipoprotein; ER, endoplasmic reticulum; PBS, phosphate-buffered saline.

* To whom correspondence should be addressed.

medium with or without insulin. The dishes were incubated for a further 24 h (day 3), after which the cell pellet and the VLDL fraction were isolated. In some experiments, cellular triacylglycerol was prelabelled with [^3H]oleate by incubating the cells during day 1 or during days 1 and 2 in the presence of supplemented medium (with or without insulin). The supplemented medium was then removed and the cells were washed with PBS. Basal medium lacking serum (with or without insulin) was added, and after 24 h secreted VLDL and the cell pellet were isolated. Incubation of cells in a medium free of [^3H]oleate such as this allowed the fate of pre-labelled cellular triacylglycerol to be determined during a period of 24 h in the presence or absence of insulin.

Isolation of VLDL

The medium from two dishes (each treated in the same way) was combined. VLDL was isolated as described previously [10], except that the final centrifugation was carried out at 40000 rev./min ($r_{av} = 81.1$ mm) for 16 h in a Beckman L8-70 ultracentrifuge with a 44 place 50.4 rotor.

Cell fractionation

Homogenization buffer (1.0 ml) containing mannitol (0.22 M), sucrose (0.07 M), Hepes (5 mM) and EDTA (2.17 mM) was added to the cell pellet and the cells were homogenized by 20 strokes in a 3 ml Dounce homogenizer. Cell disruption was confirmed by examination of a small portion of the homogenate under a light phase microscope. The homogenate was centrifuged at 3000 rev./min (800 g) to pellet the nuclei and cell debris. The supernatant from the latter spin was centrifuged at 40000 rev./min (149600 g) for 1 h in a Beckman ultracentrifuge SW 50.1 rotor. The floating cytosolic lipid was removed by slicing and retained for triacylglycerol analysis. The pellet containing the particulate material (mitochondria and microsomes) was sonicated in distilled water at 4 °C.

Lipid analysis

Total lipid was extracted from the VLDL, cytosolic and particulate fractions by the method of Folch *et al.* [13]. To adjust for losses of triacylglycerol during the extraction procedure, an internal standard of [^{14}C]triacylglycerol was added. Triacylglycerol mass was measured in a portion of the total lipid fraction by the glycerophosphate oxidase method [14] using a kit supplied by Boehringer Mannheim. Incorporation of [^3H]oleate

into the triacylglycerol was measured by t.l.c. of the remainder of the lipid extract on silica gel G, as previously described [12]. The triacylglycerol band was scraped into a scintillation vial for dual-label counting (^{14}C and ^3H).

Other methods

Cellular protein was measured by the method of Lowry *et al.* [15]. [^3H]Oleate bound to bovine serum albumin (essentially fatty-acid free) was prepared by the method of Van Harken *et al.* [16]. The initial concentration of [^3H]oleate in the medium was 0.75 mM. Its specific radioactivity varied from experiment to experiment, but was usually about 0.4 $\mu\text{Ci}/\mu\text{mol}$ (about 1 $\mu\text{Ci}/\text{dish}$). The albumin concentration in the culture medium was 0.5 %.

Statistical methods

All values are expressed as means $\pm$ S.E.M. of several independent observations. The degree of significance of observed differences was tested by either a paired or an unpaired Student's *t* test. The mass of triacylglycerol synthesized from [^3H]oleate was calculated from the specific radioactivity of the [^3H]oleate present in the supplemented medium.

Materials

All culture-media components were obtained from Gibco (Uxbridge, Middlesex, U.K.). Bovine insulin, dexamethasone, oleate, pyruvate and lactate were obtained from Sigma (Poole, Dorset, U.K.). [^3H]Oleic acid was obtained from Amersham International (Little Chalfont, Bucks., U.K.) and was converted to the sodium salt before use.

RESULTS

Effect of insulin on the intracellular and extracellular distribution of newly synthesized triacylglycerol

When hepatocytes were cultured for 24 h with supplemented medium, intracellular triacylglycerol, newly synthesized from [^3H]oleate, was distributed almost equally between the cytoplasmic and particulate fractions of the cell (Table 1). Addition of insulin during this period suppressed the secretion of labelled triacylglycerol (Table 1) and there was a concomitant increase in the amount of triacylglycerol associated with the cytosolic fraction of the cell (Fig. 1). This accounted for a significantly

Table 1. Effect of insulin on the synthesis of cytosolic and VLDL triacylglycerol

Hepatocytes were cultured in the presence or absence of insulin for 1–3 days. At the end of each 24 h period the labelled VLDL triacylglycerol was isolated and its radioactivity was determined. This was equivalent to the mass of [^3H]triacylglycerol synthesized exclusively from [^3H]oleate and which had been secreted during each 24 h period. The cell pellet was harvested at the end of each period and the cytosolic and particulate fractions were isolated by centrifugation. The labelled triacylglycerol was isolated from each fraction and its radioactivity was determined. Values for cytosolic [^3H]triacylglycerol are presented as a percentage of the cellular cytosolic plus particulate triacylglycerol radioactivity. Each value is the mean $\pm$ S.E.M. for the number of independent determinations shown in parentheses. Values labelled * and ** are significantly different ($P < 0.05$ and $P < 0.01$ respectively) from the corresponding values in the absence of insulin. Key: M, supplemented medium only; M + I, supplemented medium plus insulin.

Incubation conditions			VLDL [^3H]triacylglycerol (nmol/mg of protein)	Cytosolic [^3H]triacylglycerol (% of total)
Day 1	Day 2	Day 3		
M			166.84 $\pm$ 18.70 (4)	49.91 $\pm$ 1.93 (4)
M + I			100.93 $\pm$ 9.10 (4)*	66.31 $\pm$ 5.68 (3)*
M	M		213.45 $\pm$ 23.28 (6)	78.01 $\pm$ 2.94 (5)
M + I	M + I		124.99 $\pm$ 24.53 (4)**	86.90 $\pm$ 2.13 (4)*
M	M	M	293.41 $\pm$ 37.76 (6)	80.32 $\pm$ 3.36 (4)
M + I	M + I	M + I	339.33 $\pm$ 40.90 (6)	84.04 $\pm$ 1.50 (3)

Fig. 1. Effect of different culture conditions on the accumulation of $[^3\text{H}]$ triacylglycerol in the cell cytosol

Hepatocytes were cultured in supplemented medium with (Δ) or without ($\bullet$) insulin for a total of 3 days. At the end of each day the medium was removed and replaced by medium of identical composition, again with or without insulin. In some experiments, at the end of day 1 the supplemented medium was replaced by the basal medium (without serum) either with (Δ) or without ($\circ$) insulin. In other experiments, at the end of day 2, the supplemented medium was replaced by the basal medium (without serum) either with ($\blacksquare$) or without ($\square$) insulin. At the end of each day, cells were harvested and the cytosolic triacylglycerol content was determined. Values marked * or ** are significantly different ($P < 0.05$ and $P < 0.01$ respectively) from the corresponding values in the presence of insulin. Each point represents the mean $\pm$ S.E.M. from at least four independent hepatocyte preparations.

increased proportion ($P < 0.05$) of the total intracellular triacylglycerol synthesized under these conditions (Table 1).

When cells were cultured with supplemented medium containing $[^3\text{H}]$ oleate for 48 h, there was an increase in the amount of newly synthesized triacylglycerol within the cell, and this increment was located almost exclusively in the cytosolic fraction. This compartment now contained the bulk of the newly synthesized hepatocellular triacylglycerol (Table 1). The presence of insulin during this period again led to a decrease in the amount of triacylglycerol secreted (Table 1), which was accompanied by an increase in the cytosolic triacylglycerol content of the cell (Fig. 1). Increasing the culture period for a further 24 h (total 72 h) in medium supplemented with $[^3\text{H}]$ oleate led to a further accumulation of labelled triacylglycerol in the cytosol of the cell. The continued presence of insulin during this 24 h period again increased the amount of labelled triacylglycerol associated with the cytosolic fraction of the cell. However, in this case, the

Table 2. Effect of insulin on the proportion of $[^3\text{H}]$ triacylglycerol which is secreted as VLDL

Hepatocytes were cultured under conditions identical with those described in the legend to Table 1. The total amount of $[^3\text{H}]$ triacylglycerol at the end of each 24 h period was determined by summation of the amount of $[^3\text{H}]$ triacylglycerol secreted during that period and the amount of $[^3\text{H}]$ triacylglycerol remaining within the cell at the end of the period. Key: M, supplemented medium; M+I, supplemented medium plus insulin.

Incubation conditions			VLDL $[^3\text{H}]$ triacylglycerol secreted (% of total $[^3\text{H}]$ triacylglycerol available)
Day 1	Day 2	Day 3	
M			45.4 $\pm$ 7.3 (3)
M+I			20.4 $\pm$ 6.5 (3)
M	M		40.4 $\pm$ 8.3 (4)
M+I	M+I		17.1 $\pm$ 5.1 (3)
M	M	M	43.6 $\pm$ 7.2 (4)
M+I	M+I	M+I	29.5 $\pm$ 5.8 (4)

Table 3. Effect of insulin on the mobilization of intracellular triacylglycerol and the secretion of VLDL triacylglycerol

Hepatocytes were cultured in the supplemented medium with or without insulin for 24 h or 48 h (day 1 or day 1 + day 2). At the end of each period, some dishes were used for determination of the $[^3\text{H}]$ triacylglycerol content of the cellular cytosolic and particulate fractions. In the remaining dishes, the supplemented medium was replaced by serum-free basal medium with or without insulin and the cells were incubated for a further 24 h. At the end of this time the amount of $[^3\text{H}]$ triacylglycerol secreted into the medium as VLDL was determined. The amounts of $[^3\text{H}]$ triacylglycerol associated with the particulate and cytosolic fractions of the cell at the end of this period were also determined. These amounts were subtracted from the corresponding amounts of cytosolic and particulate $[^3\text{H}]$ triacylglycerol present within the cell at the start of the incubation in basal medium (i.e. 24 h earlier). Each value represents the mean $\pm$ S.E.M. of the number of independent determinations shown in parentheses. Values marked * and ** are significantly different ($P < 0.05$ and $P < 0.01$ respectively) from the corresponding values in the presence of insulin. Key: M, supplemented medium; M+I, supplemented medium plus insulin; -, basal medium only; I, basal medium plus insulin.

Culture conditions			Cytosolic loss (nmol of $[^3\text{H}]$ triacylglycerol/ mg of protein)	Particulate loss (nmol of $[^3\text{H}]$ triacylglycerol/ mg of protein)	VLDL secreted (nmol of $[^3\text{H}]$ triacylglycerol/ mg of protein)
Day 1	Day 2	Day 3			
			day 1-day 2	day 1-day 2	
1	M	-	52.26 $\pm$ 21.06 (3)	46.2 $\pm$ 13.69 (3)	85.50 $\pm$ 18.93 (5)
2	M	I	35.38 $\pm$ 17.81 (3)	40.63 $\pm$ 19.21 (3)	54.38 $\pm$ 12.98 (3)
3	M+I	-	101.46 $\pm$ 30.09 (3)	36.0 $\pm$ 8.68 (3)	171.74 $\pm$ 20.39 (5)**
4	M+I	I	54.89 $\pm$ 30.87 (3)	20.68 $\pm$ 13.59 (3)	97.67 $\pm$ 20.48 (3)
			day 2-day 3	day 2-day 3	
5	M	M	123.54 $\pm$ 27.15 (4)	31.66 $\pm$ 13.22 (4)	131.76 $\pm$ 14.41 (3)**
6	M	M	46.03 $\pm$ 12.54 (3)	9.12 $\pm$ 7.43 (3)	48.33 $\pm$ 13.47 (5)
7	M+I	M+I	264.12 $\pm$ 55.58 (4)	18.91 $\pm$ 6.77 (4)	257.24 $\pm$ 28.58 (3)*
8	M+I	M+I	189.13 $\pm$ 30.49 (4)	21.26 $\pm$ 10.61 (4)	188.01 $\pm$ 25.21 (3)

amount of labelled triacylglycerol secreted increased compared with that in cells not exposed to insulin (Table 1).

In contrast with the accumulation of labelled triacylglycerol in the cytosol of the cell, that associated with the particulate fraction remained constant or declined with increasing time of culture. This was the case irrespective of the presence or absence of insulin in the medium. At no time did the presence of insulin give rise to a significant increase in the amount of labelled triacylglycerol associated with the particulate fraction of the cell. Amounts observed in the presence and absence of insulin respectively were (means $\pm$ S.E.M.): day 1, 74.2 ± 25.6 and 62.8 ± 26.1 ; day 2, 54.8 ± 15.9 and 47.7 ± 10.5 ; day 3, 55.5 ± 9.9 and 50.5 ± 14.1 nmol/mg of cell protein.

If the amount of newly synthesized triacylglycerol secreted as VLDL was expressed as a proportion of the total (cellular + VLDL) [^3H]triacylglycerol available, then this quantity appeared to be constant at around 40% during each successive 24 h period. During each period, the presence of insulin decreased this proportion. However, in this case, during the final 24 h the proportion of labelled triacylglycerol secreted increased compared with that secreted in the previous 24 h period (Table 2).

Mobilization of cellular triacylglycerol for VLDL secretion

The effect of insulin on the rate of transfer of pre-existing cellular triacylglycerol into VLDL triacylglycerol was determined by pre-labelling cellular cytosolic and particulate triacylglycerol with [^3H]oleate for 24 or 48 h. After pre-labelling for 24 h in the presence of insulin, subsequent removal of [^3H]oleate led, during the next 24 h, to a higher secretion of labelled triacylglycerol compared with that observed when pre-labelling was carried out in the absence of insulin (Table 3, line 3 versus line 1, $P < 0.01$). This was accompanied by a greater loss of cytosolic [^3H]triacylglycerol from the cells previously exposed to insulin (Fig. 1 and Table 2, line 3 versus line 1). There was little or no difference in the amounts of [^3H]triacylglycerol lost from the particulate fraction of the cell (Table 3).

A similar pattern was observed in cells pre-incubated with [^3H]oleate for 48 h in the presence or absence of insulin. Again, removal of [^3H]oleate from the insulin-treated cells resulted, over the next 24 h, in a higher rate of transfer of labelled cellular triacylglycerol into VLDL triacylglycerol than in the cells not pre-treated with insulin (Table 3, line 7 versus line 5; $P < 0.05$). This was again due an increased mobilization of labelled cytosolic triacylglycerol in the insulin-treated cells (Fig. 1 and Table 3, line 7 versus line 5). If insulin was present in the medium during the 24 h period after removal of [^3H]oleate, then the mobilization of cytosolic triacylglycerol was decreased (Fig. 1), resulting in a decreased secretion of labelled VLDL triacylglycerol (Table 3). For instance, Fig. 1 shows that, with cells pre-incubated with [^3H]oleate alone for 48 h followed by removal of [^3H]oleate, the presence of insulin during the next 24 h resulted in a 10-fold increase in the amount of labelled triacylglycerol remaining in the cytoplasm of the cells compared with that observed when insulin was absent during this period. The secretion of labelled VLDL declined by 60–70% under these conditions (Table 3, lines 5 and 6). A similar pattern was observed when insulin had been present with [^3H]oleate during a pre-incubation period of 48 h (Fig. 1). However, in this case, when oleate was removed during the final 24 h of culture the presence of insulin during this period did not prevent the mobilization of cytosolic triacylglycerol to the same extent as that which occurred when insulin had been absent during the pre-incubation period. In other words, prior exposure to insulin for 48 h desensitized the cell to the normal inhibitory effect of insulin on the mobilization of cytosolic triacylglycerol. Neither was the inhibitory effect of

Table 4. Contribution of exogenous oleate to VLDL and cytosolic triacylglycerol

Hepatocytes were cultured for 1, 2 or 3 days in the supplemented medium in the presence or absence of insulin in exactly the same way as described in the legend to Table 1. At the end of each 24 h period, the VLDL and cytosolic triacylglycerol fractions were isolated and their total mass and radioactivities were determined. Each value is the mean $\pm$ S.E.M. of the number of independent determinations shown in parentheses. Values are marked * and ** are significantly different ($P < 0.05$ and $P < 0.01$ respectively) from the corresponding values observed in the absence of insulin. Key: M, supplemented medium only; M+I, supplemented medium plus insulin.

Incubation conditions				VLDL triacylglycerol			Cytosolic triacylglycerol		
Day 1	Day 2	Day 3		Total mass (nmol/mg of protein)	Amount due to [^3H]oleate (%)	Specific activity (nmol of [^3H]oleate/ nmol of triacylglycerol)	Total mass (nmol/mg of protein)	Amount due to [^3H]oleate (%)	Specific activity (nmol of [^3H]oleate/ nmol of triacylglycerol)
M				221.46 $\pm$ 12.99 (4)	74.71 $\pm$ 4.21 (4)	2.36 $\pm$ 0.15 (3)	160.35 $\pm$ 11.28 (3)	69.45 $\pm$ 10.95 (3)	2.23 $\pm$ 0.21 (3)
M+I				133.48 $\pm$ 18.87** (4)	77.72 $\pm$ 5.75 (4)	2.54 $\pm$ 0.15 (3)	229.46 $\pm$ 12.74* (3)	73.39 $\pm$ 20.38 (3)	2.13 $\pm$ 0.25 (3)
M				254.96 $\pm$ 28.05 (6)	89.55 $\pm$ 3.71 (5)	2.81 $\pm$ 0.11 (6)	220.42 $\pm$ 47.68 (5)	78.94 $\pm$ 9.11 (5)	2.61 $\pm$ 0.23 (5)
M+I				167.58 $\pm$ 33.45* (5)	86.68 $\pm$ 5.12 (4)	2.73 $\pm$ 0.16 (5)	417.16 $\pm$ 47.25* (4)	76.93 $\pm$ 8.42 (4)	2.93 $\pm$ 0.35 (3)
M				301.80 $\pm$ 51.24 (6)	96.12 $\pm$ 3.69 (5)	3.05 $\pm$ 0.12 (6)	299.88 $\pm$ 108.78 (4)	91.05 $\pm$ 4.56 (4)	2.89 $\pm$ 0.21 (4)
M+I				406.09 $\pm$ 48.00 (6)	81.84 $\pm$ 5.3 (5)	2.59 $\pm$ 0.15 (6)	545.2 $\pm$ 140* (4)	81.95 $\pm$ 6.74 (4)	2.58 $\pm$ 0.22 (4)
	M+I	M+I							

insulin on the secretion of VLDL triacylglycerol so pronounced under these conditions (Table 3, lines 7 and 8).

Source of fatty acid for triacylglycerol synthesis

Comparison of the total mass of VLDL triacylglycerol secreted with that derived exclusively from extracellular oleate suggested that the latter contributed by far the largest proportion (Table 4). Neither the length of the culture period nor the presence of insulin had much effect on this contribution. Furthermore, the specific radioactivity of the cytosolic triacylglycerol was very similar to that of the secreted VLDL triacylglycerol, suggesting that the incubation period (24 h) was sufficiently long to enable the establishment of equilibrium between the extracellular fatty acid and the cytosolic and VLDL triacylglycerol pools.

DISCUSSION

The present results show that when the secretion of VLDL triacylglycerol from hepatocytes is impaired by insulin, triacylglycerol accumulates in the cytosolic fraction of the cell rather than as a membrane-bound form. This implies that, under these conditions, triacylglycerol does not accumulate in any part of the secretory apparatus as part of a developing or mature VLDL particle. For instance, during the first 24 h period (day 1) in the absence of insulin, newly synthesized triacylglycerol was almost equally distributed between the cytosolic and membrane-bound (containing the secretory apparatus) compartments of the cell (Table 1). This is similar to a previous observation [17], which suggested that 50% of the newly synthesized hepatic triacylglycerol *in vivo* was contained within a secretory pool. However, in the presence of insulin, during this period there was a significant increase in the mass and proportion of newly synthesized triacylglycerol associated with the cytosolic fraction of the cell (Table 1, Fig. 1). In this respect, insulin behaves differently from orotic acid, which prevents the secretion of VLDL by sequestering triacylglycerol within a specific compartment of the secretory apparatus [18]. The current observations suggest that insulin directs a greater proportion of the triacylglycerol, newly synthesized at the cytosolic face of the endoplasmic reticulum (ER) [19], into the cell cytosol rather than into the ER lumen where it is destined for secretion as VLDL [20–23]. This is an important branchpoint in hepatic triacylglycerol metabolism [11], but the factors which regulate channelling of the newly synthesized triacylglycerol into the cytosol on the one hand, or into the ER lumen on the other, remain obscure [24].

An interesting observation which emerged from this work was that insulin prevented the mobilization or disappearance of pre-labelled triacylglycerol from the cytosol of the cell (Fig. 1), and this was accompanied by a decline in the rate of secretion of VLDL triacylglycerol (Table 3). This suggests that cytosolic triacylglycerol is an important source of the triacylglycerol required for VLDL assembly and that its recruitment for this process is impaired by insulin. Previous studies have highlighted the importance of cytosolic triacylglycerol for VLDL synthesis [11,25]. There is also evidence that cytosolic triacylglycerol undergoes hydrolysis and re-esterification before it can be made available for incorporation into nascent VLDL [25,26]. The lipolytic step is probably mediated by a lysosomal acid lipase [25,27] (EC 3.1.1.3) which may be inhibited by insulin, in common with the hormone-sensitive lipase of adipose tissue [27]. This would explain, at least in part, the observed effect of insulin on cytosolic triacylglycerol mobilization and VLDL triacylglycerol secretion. Further evidence for a precursor–product relationship

between cytosolic and VLDL triacylglycerol comes from the observation that, under conditions where the inhibitory effect of insulin on VLDL secretion was small (Table 3, line 8 versus line 7), the inhibitory effect on the mobilization of cytosolic triacylglycerol was also decreased (Fig. 1, day 3). Previous studies such as this have also shown that prolonged exposure of hepatocytes to insulin either diminishes [6,9] or reverses [8,10] the relatively short-term inhibitory effect of insulin on the secretion of VLDL. The mechanism by which chronic exposure to insulin releases the suppression of cytosolic triacylglycerol mobilization and enhances the secretion of VLDL triacylglycerol is obscure, but may be related to the acquisition of insulin-resistance by the cell [9].

We thank Mrs. M. Barber for typing the manuscript. This work was supported by a grant from the Medical Research Council (M.R.C.). G.F.G. is a member of the External Scientific Staff of the M.R.C. at the University of Oxford.

REFERENCES

- Durrington, P. N., Newton, R. S., Weinstein, D. B. & Steinberg, D. (1982) *J. Clin. Invest.* **70**, 63–73
- Patsch, W., Franz, S. & Schonfeld, G. (1983) *J. Clin. Invest.* **71**, 1161–1174
- Pullinger, C. R. & Gibbons, G. F. (1985) *Biochim. Biophys. Acta* **833**, 44–51
- Sparks, C. E., Sparks, J. D., Bolognino, M., Salhanick, A., Strumph, P. S. & Amatruda, J. M. (1986) *Metab. Clin. Exp.* **35**, 1128–1136
- Mangiapan, E. H. & Brindley, D. N. (1986) *Biochem. J.* **233**, 151–160
- Dashti, N., Williams, D. L. & Alaupovic, P. (1989) *J. Lipid Res.* **30**, 1365–1373
- Pullinger, C. R., North, J. D., Teng, B.-B., Rifci, V. A., Ronhild de Brito, A. E. & Scott, J. (1989) *J. Lipid Res.* **30**, 1065–1078
- Duerden, J. M., Bartlett, S. M. & Gibbons, G. F. (1989) *Biochem. J.* **263**, 973–943
- Patsch, W., Gotto, A. M., Jr. & Patsch, J. R. (1986) *J. Biol. Chem.* **261**, 9603–9606
- Bartlett, S. M. & Gibbons, G. F. (1988) *Biochem. J.* **249**, 37–43
- Gibbons, G. F. (1990) *Biochem. J.* **268**, 1–13
- Duerden, J. M. & Gibbons, G. F. (1988) *Biochem. J.* **255**, 929–935
- Folch, J., Lees, M. & Sloane-Stanley, G. N. (1957) *J. Biol. Chem.* **226**, 497–509
- Trinder, P. (1969) *Ann. Clin. Biochem.* **6**, 24–30
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Van Harken, D. R., Dixon, C. W. & Heimberg, M. (1969) *J. Biol. Chem.* **244**, 2278–2285
- Palmer, J. F., Cooper, C. & Shipley, R. A. (1978) *Biochem. J.* **172**, 219–226
- Hay R., Fleming, R., O'Connell, W., Kirschner, J. & Oppliger, W. (1988) *J. Lipid Res.* **29**, 981–995
- Coleman, R. & Bell, R. M. (1978) *J. Cell Biol.* **76**, 245–253
- Boström, K., Borén, J., Wettsten, M., Sjöberg, A., Bondjers, G., Wiklund, O., Carlsson, P. & Olofsson, S.-O. (1988) *J. Biol. Chem.* **263**, 4434–4442
- Borchardt, R. A. & Davis, R. A. (1987) *J. Biol. Chem.* **262**, 16394–16402
- Bamberger, M. J. & Lane, M. D. (1988) *J. Biol. Chem.* **263**, 11868–11878
- Higgins, J. A. (1988) *FEBS Lett.* **232**, 405–408
- Olofsson, S.-O., Bjursell, G., Boström, K., Carlsson, P., Elovson, J., Protter, A. A., Reuben, M. A. & Bondjers, G. (1987) *Atherosclerosis* **68**, 1–17
- Francone, O. L., Kalopissis, A.-D. & Griffaton, G. (1989) *Biochim. Biophys. Acta* **1002**, 28–36
- Bar-On, H., Roheim, P. S., Stein, O. & Stein, Y. (1971) *Biochim. Biophys. Acta* **248**, 1–11
- Debeer, L. J., Beynen, A. C., Mannaerts, G. P. & Geelen, M. J. H. (1982) *FEBS Lett.* **140**, 159–164