Short- and longer-term regulation of very-low-density lipoprotein secretion by insulin, dexamethasone and lipogenic substrates in cultured hepatocytes

A biphasic effect of insulin

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1. The precise effects of insulin, dexamethasone and lipogenic precursors on the secretion of very-low-density lipoprotein (VLDL) cholesterol and triacylglycerol were dependent on the age of the culture and the duration of treatment. 2. The rates of secretion of triacylglycerol and cholesterol gradually declined with the age of the culture, although there was no detectable decrease within a given 24 h period. 3. Between 4 h and 24 h after cell preparation, insulin inhibited VLDL secretion. Inhibition was maximal between 6 and 12 h after addition of insulin. Longer-term treatment (24–48 h) with insulin resulted in a stimulation of VLDL secretion. This effect was less apparent when dexamethasone was simultaneously present. The secretion of triacylglycerol and cholesteryl ester was more sensitive to insulin than was that of non-esterified cholesterol. 4. Dexamethasone alone stimulated the secretion of VLDL to an extent which increased with the age of the culture. In young cultures (up to 24 h old) dexamethasone protected against inhibition by insulin, but was ineffective in older cultures. 5. In young cultures the stimulatory effect of lipogenic precursors (lactate and pyruvate) on the secretion of triacylglycerol and cholesterol was more pronounced in the presence of dexamethasone. In cultures older than 24 h, the secretion of these components was less sensitive to short-term stimulation by lactate and pyruvate.

INTRODUCTION

The utilization of endogenously synthesized triacylglycerol by extrahepatic tissues is facilitated by the assembly and secretion of VLDL by the liver. An important precursor of hepatic triacylglycerol is fatty acid synthesized *de novo* by the liver (Boogaerts *et al.*, 1984; Azain *et al.*, 1985). However, the relationship between the regulation of fatty acid synthesis and that of VLDL triacylglycerol secretion is obscure, and the rates at which these two processes operate do not always change in parallel with each other. For instance, insulin, which is generally considered to stimulate fatty acid synthesis *de novo* (Geelen *et al.*, 1980), has a direct inhibitory effect on hepatic VLDL secretion, at least in the short term (Durrington *et al.*, 1982; Patsch *et al.*, 1983; Pullinger & Gibbons, 1985; Mangiapane & Brindley, 1986). The inhibitory effect becomes less pronounced after longer periods of insulin treatment, and this has been ascribed to insulin resistance resulting from down-regulation of the insulin receptor (Patsch *et al.*, 1986). The role of glucocorticoids in potentiating the effect of insulin on hepatic lipid metabolism (Amatruda *et al.*, 1983) and in promoting insulin resistance (Caro & Amatruda, 1982) suggests that this type of hormone may be involved in the detailed regulation of VLDL secretion. Support for this concept has subsequently been forthcoming (Mangiapane & Brindley, 1986). One of the objectives of the present work was therefore to define the precise effects of insulin on VLDL triacylglycerol secretion in terms of the

duration of treatment, age of the culture and the role of the synthetic glucocorticoid dexamethasone.

In contrast with the opposing short-term effects of insulin on fatty acid synthesis and VLDL secretion, short-term (0–3 h) stimulation *in vitro* of fatty acid synthesis *de novo* by increasing the availability of intermediary metabolites such as lactate and pyruvate (Salmon *et al.*, 1974; Boyd *et al.*, 1981; Gibbons *et al.*, 1984) increased the secretion of VLDL triacylglycerol by isolated hepatocytes (Pullinger & Gibbons, 1985; Gibbons & Pullinger, 1987). A second objective of the present work was to examine the longer-term effects of lactate and pyruvate and to determine whether, and to what extent, these effects were modified by insulin and dexamethasone.

Finally, little is known of the extent to which changes in the secretion of VLDL triacylglycerol are accompanied by changes in the secretion of esterified and non-esterified cholesterol. Differential secretion of the lipid components of VLDL may affect its metabolism by lipoprotein lipase (Demel & Jackson, 1985) and its subsequent clearance from the plasma as intermediate- and low-density lipoprotein by cell-surface receptors (Goldstein & Brown, 1984; Lopes-Virella *et al.*, 1982; Witztum *et al.*, 1985). In view of the potential importance of these structural changes in determining the steady-state plasma lipoprotein concentration, we wished to investigate the effects of the above hormones and lipogenic precursors on the secretion of VLDL esterified and non-esterified cholesterol.

EXPERIMENTAL

Maintenance of animals

Male Wistar rats were housed in a windowless room artificially lit between 08:00 h and 20:00 h. The animals were fed on a commercially available pelleted diet (Diet 41B; E. Dixon and Sons, Ware, Herts., U.K.). This contained (w/w) 46.7% carbohydrate (mainly starch), 15.4% protein, 3.1% fat and 4.4% crude fibre. Food and water were available *ad libitum*. The animals were used for hepatocyte preparation at 11:00 h and weighed between 230 g and 300 g.

Preparation of hepatocyte cultures and incubation procedures

Hepatocytes were prepared by the two-step collagenase-perfusion technique (Seglen, 1976) as described previously (Pullinger & Gibbons, 1983, 1985). In the present experiments, however, the Krebs-Henseleit (1932) perfusion buffer and the collagenase were sterilized by passage through Millex-GS Filters (Millipore S. A., Molsheim, France). The hepatocytes were finally suspended in Waymouth's medium MB752/1 (1.0×10^6 cells/ml) containing glutamine (3.4 mM), serine (0.5 mM), alanine (0.4 mM), penicillin (200 units/ml), streptomycin (200 µg/ml) and newborn-calf serum (10%). The cell suspension was plated out (3.0 ml per dish) on to Falcon 'Primaria' culture dishes (Becton Dickinson Labware, Oxnard, CA, U.S.A.). The dishes were placed in an incubator under an atmosphere of air/CO₂ (19:1) at 37 °C. After 4 h most of the cells had attached to the base of the plate, and the medium containing unattached cells was removed. The monolayer was washed three times with the above medium lacking serum. Finally, serum-free medium (3.0 ml) was added to each dish and the dishes were returned to the incubator. Where appropriate, insulin, dexamethasone, lactate and pyruvate were added, to give initial concentrations of 78 nM, 1 µM, 10 mM and 1 mM respectively. The high initial insulin concentration was used to counteract the possible effects of insulin depletion, owing to its high rate of degradation by hepatocytes (Juul & Jones, 1982). Similar concentrations of insulin to that used here provoked maximal inhibition of labelled VLDL-triacylglycerol secretion (Durrington *et al.*, 1982).

After addition of the serum-free Waymouth's medium (i.e. 4 h after plating), batches of cells were treated by one of three different protocols, depending on the time of addition of hormones and substrates and the duration of treatment, as follows. Protocol A: hormones and/or substrates were added immediately and incubated for 24 h (i.e. between 4 and 28 h after isolation of the cells). Protocol B: hormones and/or substrates were added 20 h after transfer to the serum-free medium and incubated for a further 24 h (i.e. between 24 and 48 h after isolation of the cells). Protocol C: as for Protocol B, except that at the end of the 24 h treatment period the medium was removed and replaced with medium containing the appropriate hormone and/or substrate. The cells were then incubated for a further 24 h (i.e. between 48 h and 72 h after isolation of the cells). These cells had thus been exposed to the hormones and/or substrates for a total of 48 h.

Isolation of VLDL and measurement of cholesterol (esterified and non-esterified) and triacylglycerol

At the end of each 24 h period, the medium from two identically treated dishes was combined, the density was adjusted to 1.006 g/ml, and NaN₃ was added to give a concentration of 0.1 g/litre. The supernatant was centrifuged at 20000 g for 20 min, followed by centrifugation at 40000 rev./min in a Beckman SW-50.1 rotor for 18 h. The floating fraction, of $d < 1.006$, was obtained by tube slicing. A portion of the VLDL-containing fraction was used for extraction of the total lipids (Folch *et al.*, 1957). The total lipid fraction was dissolved in ethanol (0.3 ml) and the triacylglycerol content was determined by the glycerol-3-phosphate oxidase method (Trinder, 1969). The materials for this assay were obtained as a kit supplied by Boehringer Corp. (London). Total (esterified and non-esterified) cholesterol and non-esterified cholesterol were determined by a modification of a method described previously (Pullinger & Gibbons, 1985). In the present work the derived cholest-4-en-3-one was isolated directly by chromatography of the enzyme-treated VLDL fraction on Whatman (Maidstone, Kent, U.K.) LK5D thin-layer silica-gel plates. The chromatoplate was developed with chloroform, and the heptafluorobutyryl enol ester of cholest-4-en-3-one was prepared and assayed by gas chromatography (Pullinger & Gibbons, 1985).

Determination of cellular protein concentration and rates of protein synthesis

At the beginning of each 24 h incubation period, [³H]-leucine (5 µCi) was added to the cells to give a final specific radioactivity of 0.67 µCi/µmol. After incubation, the medium was removed for isolation of the VLDL (see above) and ice-cold Dulbecco's phosphate-buffered saline (140 mM-NaCl/2.7 mM-KCl/8.0 mM-Na₂HPO₄/0.9 mM-CaCl₂/1.5 mM-KH₂PO₄/0.6 mM-MgCl₂) was added to the cells. The cells were detached from the plate with a rubber 'policeman' and transferred to a centrifuge tube. The dish was washed with a further 2.0 ml of phosphate-buffered saline, and the cells were obtained by centrifugation at 500 g for 5 min. The supernatant was removed, and the cells were sonicated (MSE M-126 sonicator, 10 s at 6 µm) after suspension in 0.5 ml of phosphate-buffered saline. A sample of the sonicated material was removed for determination of protein mass (Lowry *et al.*, 1951). Another sample was transferred to a filter paper soaked with a solution of trichloroacetic acid (5%, v/v). The filter paper was washed with 5% trichloroacetic acid (1.0 ml), followed by ethanol (1.0 ml), and the precipitated protein was assayed for radioactivity by scintillation counting.

Materials

All culture-media components were obtained from Gibco (Paisley, Scotland, U.K.). Bovine insulin, dexamethasone, pyruvate and lactate were obtained from Sigma (Poole, Dorset, U.K.).

RESULTS

Effect of age of the culture on the secretion of VLDL

In the absence of any hormone or substrate supplement, the rates of secretion of lipids associated with VLDL declined after each successive 24 h incubation period (Fig. 1) but there was no change in the rate at

Fig. 1. Effect of the age of the hepatocyte culture on the rate of VLDL lipid secretion

Hepatocytes were plated into dishes in Waymouth's medium supplemented with serum (10%) at a concentration of 1.0×10^6 cells/ml. After 4 h, the medium was removed and the monolayer was washed three times with 3.0 ml of serum-free Waymouth's medium; 3.0 ml of the same medium was added to all dishes. The dishes were then divided into three batches, A, B and C, and treated according to Protocols A, B and C respectively, as described in the Experimental section. All values represent means $\pm$ S.E.M. for three independent hepatocyte preparations. ●, Triacylglycerol; ▲, total cholesterol; ■, non-esterified cholesterol.

which [3 H]leucine was incorporated into cellular protein (55.5, 59.0, 63.1 nmol/mg of cell protein over each 24 h period). However, during the period 24–48 h after cell preparation, the cells secreted $46.3 \pm 2.3 \mu\text{g}$ of triacylglycerol/mg of cell protein, and the rate at which this triacylglycerol appeared in the medium was linear with time during this period (Fig. 2), as was the rate of cellular protein synthesis (results not shown).

Short-term effects of dexamethasone, insulin and lipogenic substrates on VLDL secretion in young cultures (Protocol A)

Insulin significantly inhibited the secretion of VLDL triacylglycerol and total (esterified plus non-esterified) cholesterol to approximately the same extent. However, there was a tendency towards a greater inhibition of the secretion of total cholesterol compared with that of non-esterified cholesterol (Table 1). Although dexamethasone and the substrate mixture, when added singly, had little stimulatory effect on lipid secretion, when added together they produced significant increases in the secretion of triacylglycerol, total and non-esterified cholesterol (Table 1). When the lactate plus pyruvate mixture was added to cultures containing dexamethasone alone, there was a $140.8 \pm 14.4\%$ ($P < 0.05$) increase in the rate of triacylglycerol secretion (Table 1, line 7 versus line 3). This was in contrast with the insignificant effects observed on triacylglycerol secretion when dexamethasone or lactate plus pyruvate were added singly to cultures containing no other addition (Table 1, lines 3 and 4).

Fig. 2. Time course of VLDL-triacylglycerol secretion

Hepatocytes were plated out at a concentration of 1.0×10^6 cells/ml (3.0 ml per dish). The medium was removed 4 h later and the monolayer was washed three times with 3.0 ml of serum-free Waymouth's medium. The cells were incubated for a further 20 h, after which the medium was again replaced with 3.0 ml of fresh Waymouth's medium. The rates of secretion of VLDL triacylglycerol during the next 6 h, 12 h and 24 h were then determined. Values for each time point are means $\pm$ S.E.M. for three independent hepatocyte preparations.

Short-term effects of dexamethasone, insulin and lipogenic substrates on the secretion of VLDL in older cultures (Protocol B)

Insulin again gave rise to a decrease in the secretion of VLDL from these older cultures, and the extent of inhibition was similar to that observed in young cultures (Table 2). The stimulatory effects of dexamethasone, added alone, on the secretion of VLDL triacylglycerol was more pronounced in these cultures. However, the most important difference between these and the younger cultures was the inability of lactate plus pyruvate to sustain an increased rate of VLDL lipid secretion, in the presence of dexamethasone. In the presence of this glucocorticoid, the substrate mixture had no further stimulatory effect, and, in fact, under these conditions there was actually a significant and substantial decline in the rate of triacylglycerol secretion (Table 2, line 7). In the absence of hormones and substrates, the rate of protein synthesis corresponded to an incorporation of [3 H]leucine of 50.7 ± 6.5 nmol/24 h per mg of cell protein. None of the hormone or substrate additions had any effect on this rate.

Longer-term effects of hormones and lipogenic substrates on VLDL secretion (Protocol C)

In this series of experiments, cells which had been incubated for 20 h in the absence of serum were used. At this point, the medium was changed and the appropriate hormone and/or hormone/substrate mixture was added. The cells were incubated for a further 24 h, the medium was changed again, and the appropriate hormones and substrates were again added to corresponding dishes. VLDL secretion into the medium was then determined at the end of a further 24 h period. Thus VLDL secretion

Table 1. Effects of hormones and lipogenic substrates on the secretion of VLDL lipids in young hepatocyte cultures (i.e. 4–28 h after plating)

At 4 h after plating the hepatocytes, the medium was removed and the monolayer was washed three times with serum-free Waymouth's medium. Then 3.0 ml of the same medium was added, followed by additions of hormones and substrates as indicated. The cells were incubated for 24 h, after which the medium was removed and the VLDL-containing fraction was isolated. The mass of triacylglycerol and of total and non-esterified cholesterol associated with the VLDL was determined. The values represent means $\pm$ S.E.M. for at least three independent hepatocyte preparations. Values marked *, ** and *** are significantly different from the controls, at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively.

Addition(s)	Rate of secretion of VLDL lipids (% of control)		
	Triacylglycerol (control = 95.1 ± 5.3 μ g/mg of cell protein)	Total cholesterol (control = 4.38 ± 0.44 μ g/mg of cell protein)	Non-esterified cholesterol (control = 3.57 ± 0.76 μ g/mg of cell protein)
1. –	100	100	100
2. Insulin	$45.5 \pm 2.5^{***}$	$39.6 \pm 3.4^{**}$	$52.1 \pm 15.0^*$
3. Dexamethasone	107.3 ± 12.8	124.2 ± 12.5	$133.5 \pm 12.2^*$
4. Lactate/pyruvate	109.0 ± 13.0	$149.7 \pm 20.7^*$	128.9 ± 24.8
5. Insulin + dexamethasone	$55.1 \pm 2.7^{**}$	86.8 ± 41.4	102.9 ± 38.9
6. Insulin + lactate/pyruvate	$37.8 \pm 10.3^{**}$	$52.5 \pm 9.9^*$	$42.1 \pm 9.0^*$
7. Dexamethasone + lactate/pyruvate	$148.6 \pm 19.9^*$	$167.7 \pm 30.6^*$	$202.0 \pm 42.2^*$
8. Insulin + dexamethasone + lactate/pyruvate	$57.0 \pm 2.9^{**}$	$44.1 \pm 10.0^{**}$	$62.0 \pm 1.5^{***}$

Table 2. Effects of hormones and lipogenic substrates on the secretion of VLDL lipids in older hepatocyte cultures (24–48 h after plating)

At 4 h after plating the hepatocytes, the medium was removed and the monolayer was washed three times with serum-free Waymouth's medium. After addition of 3.0 ml of the same medium, the cells were incubated for a further 20 h. The medium was replaced by 3.0 ml of fresh medium, and hormones and substrates were added as indicated. The amounts of VLDL triacylglycerol, total and non-esterified cholesterol secreted into the medium during the subsequent 24 h were determined. The values represent means $\pm$ S.E.M. for three independent hepatocyte preparations, except where otherwise indicated, in which two preparations only were used. Values marked † and †† are significantly different from the controls, at $P < 0.05$ and $P < 0.001$ respectively.

Addition(s)	Rate of secretion of VLDL lipids (% of control)		
	Triacylglycerol (control = 46.3 ± 2.3 μ g/mg of cell protein)	Total cholesterol (control = 3.91 ± 0.98 μ g/mg of cell protein)	Non-esterified cholesterol (control = 2.73 ± 0.11 μ g/mg of cell protein)
1. –	100	100	100
2. Insulin	$38.9 \pm 5.8^{\dagger\dagger}$	$42.8 \pm 5.2^{\dagger\dagger}$	36.2 (2)
3. Dexamethasone	$132.7 \pm 9.2^{\dagger}$	155.8 ± 27.5	$142.2 \pm 13.4^{\dagger}$
4. Lactate/pyruvate	113.6 ± 7.4	99.6 ± 12.5	124.0 ± 49.2
5. Insulin + dexamethasone	35.3 (2)	38.4 (2)	33.4 (2)
6. Insulin + lactate/pyruvate	28.6 (2)	26.5 (2)	63.3 (2)
7. Dexamethasone + lactate/pyruvate	$66.7 \pm 8.7^{\dagger}$	143.0 ± 43.0	149.8 ± 29.0
8. Insulin + dexamethasone + lactate/pyruvate	31.0 (2)	28.3 (2)	37.9 (2)

was measured during the last 24 h of a 48 h period in which the cells were treated with a particular hormone, either alone or in combination with another hormone or substrate.

The most striking difference between these cells and those treated for only 24 h was in their response to insulin. Thus, during the last 24 h of the 48 h treatment period, insulin stimulated the rate of secretion of each lipid component of VLDL. The increase was particularly marked in the case of triacylglycerol, and the increase in total cholesterol was more marked than that of non-

esterified cholesterol (Table 3). Although dexamethasone also tended to increase VLDL secretion, when the two hormones were present simultaneously the stimulatory effect was not additive. Indeed, under these conditions insulin, in contrast with its effects when present alone, failed to produce a significant stimulation of total and non-esterified cholesterol secretion.

Time course of the effect of insulin on VLDL secretion

In view of the biphasic effect of insulin on VLDL secretion, it was considered important to identify the

length of time required for the hormone to exert its maximum inhibitory effect. Thus, hepatocyte cultures were incubated for different times in the presence and the absence of insulin, and the amounts of triacylglycerol, total and non-esterified cholesterol secreted during each interval were measured (Fig. 3). In the case of triacylglycerol, the inhibitory effect of insulin increased to reach a maximum between 6 and 12 h after its addition to the medium. At this point the cells secreted only $7.9 \pm 4.1\%$ of the control value, compared with $57.6 \pm 13.6\%$ during the previous 6 h period. During the next 12 h period the inhibitory effect was less pronounced ($44.2 \pm 7.7\%$ of control). This represented the beginning of the reversal

of the insulin effect, which culminated in a stimulation of triacylglycerol secretion between 24 and 48 h (Table 3). The response of esterified and non-esterified cholesterol showed a similar pattern to that of triacylglycerol, with a maximal inhibitory effect occurring between 6 h and 12 h after addition of insulin (Fig. 3).

Differential effects of hormones on the secretion of VLDL lipids

Although, in general, changes in the rates of secretion of a particular lipid were accompanied by parallel changes in those of the other lipid fractions, this was not always the case. In this respect one of the most striking

Table 3. Longer-term effects of hormones and lipogenic substrates on the secretion of VLDL lipids

Monolayers were treated as described in the legend to Table 2. However, after incubation for 24 h in the presence of the appropriate hormone(s) and/or substrates, the medium was replaced with 3.0 ml of fresh medium and the appropriate additions were made to corresponding dishes. The secretion of VLDL triacylglycerol, total and non-esterified cholesterol into the medium during the next 24 h period was determined. The values represent means $\pm$ S.E.M. for three independent hepatocyte preparations. Values marked $\dagger$ and $\dagger\dagger$ are significantly different from the controls, at $P < 0.05$ and $P < 0.01$ respectively.

Addition(s)	Rate of secretion of VLDL lipids (% of control)		
	Triacylglycerol (control = 25.6 ± 3.1 $\mu\text{g}/\text{mg}$ of cell protein)	Total cholesterol (control = 2.51 ± 0.86 $\mu\text{g}/\text{mg}$ of cell protein)	Non-esterified cholesterol (control = 2.00 ± 0.95 $\mu\text{g}/\text{mg}$ of cell protein)
1. –	100	100	100
2. Insulin	$201.5 \pm 29.1\dagger$	$150.1 \pm 17.5\dagger$	$121.4 \pm 6.5\dagger$
3. Dexamethasone	230.6 ± 67.9	150.5 ± 26.4	$209.8 \pm 41.0\dagger$
4. Lactate/pyruvate	183.2 ± 60.0	$167.8 \pm 4.4\dagger\dagger$	174.3 ± 55.8
5. Insulin + dexamethasone	257.2 ± 88.1	100.6 ± 23.6	96.5 ± 26.0
6. Insulin + lactate/pyruvate	$235.2 \pm 37.3\dagger$	137.5 ± 29.0	107.9 ± 17.2
7. Dexamethasone + lactate/pyruvate	245.7 ± 128.8	133.9 ± 24.3	171.6 ± 35.2
8. Insulin + dexamethasone + lactate/pyruvate	$299.0 \pm 68.0\dagger$	99.9 ± 33.3	87.3 ± 13.7

Fig. 3. Effect of time on the extent of insulin inhibition of VLDL secretion

Hepatocytes were plated out and treated as described in the legend to Fig. 2. Insulin was added at the time of the second change of medium (i.e. 20 h after the change to serum-free medium). The rates of secretion of VLDL triacylglycerol (a), total cholesterol and non-esterified cholesterol (first and second columns of each pair respectively; b) were determined during the next successive 6 h, 6 h, 12 h periods. The total height of each column represents the control rate of secretion. The shaded areas represent the rates of secretion in the presence of insulin. Each value represents the means $\pm$ S.E.M. of at least three independent determinations.

examples was the differential effect of insulin on the secretion of esterified and non-esterified cholesterol. For instance, when the inhibitory effect of insulin on VLDL secretion was increasing (i.e. between 0 and 6 h after treatment), although the secretion of total cholesterol declined to $54.4 \pm 11.1\%$ of the control value ($P < 0.05$), there was no significant effect on the secretion of non-esterified cholesterol ($87.9 \pm 26.1\%$ of control). This discrepancy could be accounted for by a decline in the secretion of esterified cholesterol from $0.29 \pm 0.05 \mu\text{g}/\text{mg}$ of cell protein (control) to $0.06 \pm 0.06 \mu\text{g}/\text{mg}$ in the presence of insulin. There was no significant difference in the rates of secretion of non-esterified cholesterol (1.02 ± 0.09 and $0.90 \pm 0.26 \mu\text{g}/\text{mg}$ respectively). When insulin stimulated VLDL triacylglycerol and cholesterol secretion (i.e. during the last 24 h of the 48 h treatment period), the effect tended to be more pronounced on cholesteryl ester [$0.96 \pm 0.21 \mu\text{g}/\text{mg}$ of protein (insulin) versus $0.51 \pm 0.22 \mu\text{g}/\text{mg}$ of protein (control)] than on non-esterified cholesterol (1.70 ± 0.43 and $2.00 \pm 0.95 \mu\text{g}/\text{mg}$ of protein respectively).

In other cases, a particular treatment produced changes in the rate of secretion of VLDL triacylglycerol, but not of esterified or non-esterified cholesterol, e.g. during the final 24 h of the 48 h treatment with dexamethasone plus insulin plus lactate and pyruvate (Table 3).

DISCUSSION

Rates of VLDL secretion by cultured hepatocytes: comparison with isolated perfused liver and rates *in vivo*

During the first 24 h after removal of serum from the medium (i.e. 4–28 h after plating), in the absence of added hormones and substrates the rate of VLDL triacylglycerol secretion was $95.1 \pm 5.3 \mu\text{g}/\text{mg}$ of cell protein. This rate was similar to, or somewhat higher than, those quoted by other authors under similar conditions [e.g. $72 \mu\text{g}/\text{mg}$ of cell protein (Durrington *et al.*, 1982); $60 \mu\text{g}/\text{mg}$ of protein (Patsch *et al.*, 1983)]. Between 24 h and 48 h in culture, the rate of triacylglycerol secretion declined to $46.3 \pm 2.3 \mu\text{g}/\text{mg}$ of protein, a value which compared well with that reported previously for cultures of a similar age [i.e. $27.1 \mu\text{g}/\text{mg}$ of cell protein (Davis & Boogaerts, 1982)]. In the present work, the rates of secretion were considerably higher than any of five previously quoted values for isolated perfused livers (Marsh, 1986), but were lower than the rate *in vivo* determined after treatment of intact animals with Triton WR-1339 (Otway & Robinson, 1967; Goh & Heimberg, 1979). Although the rates of VLDL secretion declined in each subsequent 24 h period (Fig. 1), the rate of triacylglycerol secretion remained linear during the period 24–48 h after preparation of the cells (Fig. 2). The decrease in the rate of VLDL secretion as the cells got older was not accompanied by a decline in the rate of protein synthesis from [^3H]leucine. Unlike the cells which had been cultured for a total of 72 h and which had undergone three changes of medium, during the 24 h period over which linearity of VLDL secretion was maintained, the medium was not changed and the cells remained undisturbed. It therefore appears likely that, as regards VLDL secretion, the cells remain stable for at least 24 h, provided that the medium remains unchanged.

Effects of lipogenic precursors on VLDL secretion

It is becoming increasingly clear that C_3 acids such as lactate and pyruvate are the preferred substrate for anabolic processes, including lipogenesis, in the liver (Salmon *et al.*, 1974; Boyd *et al.*, 1981; Katz & McGarry, 1984; Gibbons *et al.*, 1984). In freshly isolated hepatocytes, the increase in the rate of fatty acid synthesis *de novo* which occurs in the presence of these substrates is accompanied by an increase in the rates of secretion of VLDL triacylglycerol and cholesterol (Pullinger & Gibbons, 1985; Gibbons & Pullinger, 1987). However, in the cultured cells, during the period 4–28 h after plating, the stimulatory effect of these lipogenic precursors on the secretion of triacylglycerol and non-esterified cholesterol only became marked when dexamethasone was simultaneously present. If glucocorticoids are a prerequisite for a positive response to lipogenic precursors, it is possible that, in freshly prepared cells, the intracellular concentration of hormone remains sufficiently high to permit a positive response to lactate plus pyruvate during a 3 h incubation period. In cells cultured for a longer period, the intracellular glucocorticoid concentration probably declines and, under these conditions, lipogenic substrates alone are incapable of stimulating VLDL secretion. The lack of a significant stimulatory effect of the glucocorticoid/substrate combination on cultures older than 28 h possibly reflects the loss of some other mediating factor during this period.

Biphasic effect of insulin on VLDL secretion

The inhibitory effect of insulin on VLDL triacylglycerol and cholesterol secretion increased with time of exposure to reach a maximum between 6 h and 12 h, during which the secretion of VLDL decreased to only 8% of the control value. Between 12 h and 24 h inhibition became less pronounced (Fig. 3), and after 24 h of insulin treatment VLDL secretion increased (Table 3). Direct short-term inhibitory effects of insulin on hepatic VLDL secretion have been observed previously (Durrington *et al.*, 1982; Patsch *et al.*, 1983; Pullinger & Gibbons, 1985; Mangiapane & Brindley, 1986), and are in apparent contrast with the increased hepatic VLDL secretion associated with high plasma insulin concentrations *in vivo* (Olefsky *et al.*, 1974; Reaven & Greenfield, 1981; Steiner *et al.*, 1984). The reasons for this discrepancy have been discussed previously (Gibbons, 1986), and may relate to indirect effects of insulin on extrahepatic metabolism *in vivo* or to hepatic insulin resistance. A diminution of the inhibitory effect of insulin after 15 h pretreatment with the hormone has been reported, and this has been ascribed to insulin resistance resulting from a down-regulation of the insulin receptor (Patsch *et al.*, 1986). However, the present results show that this mitigation of the inhibitory response is merely the prelude to an eventual stimulation of VLDL secretion if insulin treatment was continued for a sufficient length of time (i.e. > 24 h). Whether this effect of chronic insulin exposure has any physiological relevance remains to be determined. However, it should be noted that *in vivo* the liver is normally exposed to a rhythmic fluctuation in the concentration of plasma insulin (Schlierf & Dorow, 1973; Knox *et al.*, 1979; Teidgen & Seitz, 1980), and this is determined by the cyclical pattern of food intake. Under these conditions the liver would never be exposed to a continuously high concentration of insulin for a

sufficiently lengthy period to promote stimulation of VLDL secretion. However, in conditions characterized by chronic hyperinsulinaemia, such as obesity and non-insulin-dependent diabetes, the normal control of VLDL secretion may be over-ridden, resulting in a hypersecretion of hepatic triacylglycerol and cholesterol.

Modification of the insulin effect by dexamethasone

The short-term inhibitory effect of insulin on VLDL lipid secretion was mitigated to some extent when dexamethasone was present simultaneously (Table 1). The ability of dexamethasone to over-ride the inhibitory effect of insulin has also been noted by other authors (Mangiapane & Brindley, 1986). The stimulation of VLDL secretion by chronic insulin treatment was also modified by dexamethasone (Table 3). In view of the apparent importance of insulin resistance in determining the exact response of VLDL secretion, it is possible that glucocorticoids in some way increase insulin resistance, a possibility for which there is some experimental evidence (Caro & Amatruda, 1982).

Differential effect of insulin on the secretion of VLDL lipids

Between 0 and 6 h after addition of insulin, although there was a significant decline in the secretion of triacylglycerol and cholesteryl ester, there was no significant change in the secretion of unesterified cholesterol. Non-esterified cholesterol forms part of the polar sheath of the VLDL particle, in contrast with esterified cholesterol and triacylglycerol, which are located in the hydrophobic core. In this respect, Patsch *et al.* (1983) have shown that the secretion of phospholipid, which also forms part of the outer envelope, is also less sensitive to inhibition by insulin than is that of triacylglycerol. The differential inhibition of the secretion of esterified and non-esterified cholesterol gave rise to a change in the relative proportion of these lipids in the newly secreted VLDL particle. Changes of this type give rise to differences in the rates of transfer of cholesterol between different types of lipoprotein, and may ultimately affect the rate at which lipoprotein cholesterol is cleared from the plasma (Fielding *et al.*, 1982, 1984).

We thank Professor D. N. Brindley and his colleagues (University of Nottingham, U.K.) for help and advice in the preparation of hepatocyte cultures. We thank Mrs. M. Barber for typing the manuscript. This work was supported by a grant from the Medical Research Council (U.K.).

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