Long-term maintenance of high rates of very-low-density-lipoprotein secretion in hepatocyte cultures

A model for studying the direct effects of insulin and insulin deficiency *in vitro*

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High rates of hepatic cellular triacylglycerol synthesis and very-low-density-lipoprotein (VLDL) triacylglycerol output were maintained *in vitro* for at least 3 days when hepatocytes were cultured in a medium lacking insulin but supplemented with 1 μ M-dexamethasone, 10 mM-lactate, 1 mM-pyruvate and 0.75 mM-oleate (supplemented medium). Under these conditions VLDL output remained constant, whereas cell triacylglycerol content increased 10-fold over 3 days, suggesting that the secretory process was saturated. Insulin, present during the first 24 h period, enhanced the storage of cellular triacylglycerol by inhibiting the secretion of VLDL. This stored triacylglycerol was subsequently released into the medium as VLDL if insulin was removed. With the supplemented medium the increased rate of VLDL secretion after insulin removal exceeded that observed under 'saturating' conditions, suggesting that pre-treatment with insulin enhanced the capacity for VLDL secretion. In contrast with the short-term (24 h) effects of insulin, longer-term exposure (> 48 h) to insulin enhanced the secretion of VLDL compared with insulin-untreated cultures. Under these conditions, insulin increased the net rates of triacylglycerol synthesis. The results suggest that insulin affects the secretion of VLDL triacylglycerol by two distinct and opposing mechanisms: first, by direct inhibition of secretion; second by increasing triacylglycerol synthesis, which stimulates secretion. The net effect at any time depends upon the relative importance of each of these processes.

INTRODUCTION

In view of the lipogenic role of insulin, until quite recently it was thought that insulin acted directly on the liver to promote the secretion of very-low-density lipoprotein (VLDL). This view was supported by observations in human subjects *in vivo* (Olefsky *et al.*, 1974; Reaven & Greenfield, 1981; Reaven & Mondon, 1984; Howard, 1987; Kostner & Karádi, 1988) and by the low rates of secretion of VLDL from the livers of diabetic animals *in vitro* (Woodside & Heimberg, 1976; Weiland *et al.*, 1980; Berry *et al.*, 1981). In contrast, there have been several reports that insulin administration *in vivo* suppresses hepatic VLDL secretion (Alcindor *et al.*, 1973; Vogelberg *et al.*, 1980; Pietri *et al.*, 1983), an effect which was not, at least in one case, due to a decreased uptake of plasma non-esterified fatty acids by the liver (Vogelberg *et al.*, 1980).

Studies on the direct effects of insulin on isolated liver preparations *in vitro* have also produced conflicting results (Nikkila, 1974; Topping & Mayes, 1982; Laker & Mayes, 1984; Topping *et al.*, 1988). Other studies, using hepatocytes in chemically defined media, have consistently shown that, during periods up to 24 h, insulin inhibits the secretion of VLDL lipids and apoproteins (Durrington *et al.*, 1982; Patsch *et al.*, 1983; Pullinger & Gibbons, 1985; Mangiapane & Brindley, 1986; Sparks *et al.*, 1986). This inhibitory effect became less pronounced after longer periods of exposure (Patsch *et al.*,

1986; Bartlett & Gibbons, 1988) and was reversed if insulin was present for 48 h (Bartlett & Gibbons, 1988; Gibbons, 1989). However, rates of VLDL secretion declined rapidly in culture media, and this made it difficult to assess the real significance of the later effects. One of the aims of the present work, therefore, was to establish conditions in which rates of triacylglycerol synthesis and secretion were maintained for several days in culture. Secondly, in view of the biphasic effect of insulin described above, we wished to study the direct consequences of varying periods of insulin deficiency *in vitro* and to compare these with the known effects of insulin deficiency on hepatic VLDL secretion *in vivo*. Finally, in other aspects of lipid metabolism, insulin has been shown to have different effects, depending upon the metabolic patterns imprinted on the liver by previous exposure to a given hormone or substrate mixture (Amatruda & Chang, 1983; Amatruda *et al.*, 1983). We considered it possible that similar effects of insulin on VLDL secretion may help to explain some of the conflicting observations described above.

MATERIALS AND METHODS

Maintenance of animals and preparation of hepatocyte cultures

Animals were housed as described previously (Bartlett & Gibbons, 1988) and were maintained on a diet containing (by wt.) 46.1% carbohydrate (mainly starch),

Abbreviation used: VLDL, very-low-density lipoprotein.

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15.4% protein, 3.1% fat and 4.4% crude fibre (Diet 41B; E. Dixon and sons, Ware, Herts., U.K.). Food and water were available *ad libitum*. Hepatocytes were prepared under sterile conditions (Bartlett & Gibbons, 1988) in a yield of approx. 10^8 cells/g of liver. The cells were suspended in Waymouth's medium MB752/1 containing added glutamine (3.4 mM), serine (0.5 mM), alanine (0.4 mM), penicillin (200 units/ml), streptomycin (200 μ g/ml) and foetal-calf serum (10%, v/v) at a concentration of $(0.50\text{--}0.75) \times 10^6$ cells/ml (3.0 ml/dish). After the cells had attached to the plates, the monolayer was washed several times with serum-free Waymouth's medium. To one batch of cells was then added serum-free Waymouth's medium containing the above amino acids and antibiotics (referred to hereafter as the basal medium). To another batch of cells was added the basal medium supplemented with dexamethasone (1 μ M), lactate (10 mM), pyruvate (1 mM) and oleate (0.75 mM). This is subsequently referred to as the supplemented medium.

After addition of the basal or supplemented medium, cells were incubated in the absence or presence of bovine insulin (78 nM) for either 20 h or 24 h (Day 1). After 24 h of culture, the medium was removed and the VLDL isolated (see below). The cells were washed, scraped from the plate and pelleted by centrifugation (Bartlett & Gibbons, 1988). After 20 h in culture, the medium was removed and replaced by the same medium in which insulin was either absent or present. Cells were cultured under these conditions for a further 24 h (Day 2), after which some dishes were used for isolation of VLDL and to obtain the cellular pellet. In the remaining dishes, the medium was removed and replaced with the same medium with or without insulin. The dishes were incubated for a further 24 h (Day 3), after which the cell pellet and the VLDL were isolated. In some experiments, insulin was added to or withdrawn from the culture medium at different times during the 3-day culture period. In other experiments, the basal medium was replaced by the supplemented medium (or vice versa) at different times. These refinements are described in the legends to the appropriate Figures and Tables.

Isolation and measurement of cellular and VLDL triacylglycerol

The medium from two dishes (each treated in the same way) was combined. VLDL was isolated as described previously (Bartlett & Gibbons, 1988), except that the final centrifugation was carried out at 40000 rev./min for 16 h in a Beckman L8-70 ultracentrifuge with a 44-place 50.4 rotor. Lipids were extracted from the VLDL and from the cellular pellet by the method of Folch *et al.* (1957). In each case the lipid residue was dissolved in 0.3 ml of ethanol, and the triacylglycerol content was determined by the method of Trinder (1969) with a kit supplied by Boehringer Corp. (London).

Other methods

Cellular protein was measured by the method of Lowry *et al.* (1951). Rates of protein synthesis were determined by measuring the incorporation of [3 H]leucine into cellular protein as described previously (Bartlett & Gibbons, 1988). Oleate bound to bovine serum albumin (essentially fatty acid-free) was prepared by the method of Van Harken *et al.* (1969). Albumin concentration in the culture medium was 1.0%.

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 unpaired Student's *t* test as specified in the text or in the Figure legends.

RESULTS

Long-term maintenance of high rates of hepatocellular VLDL triacylglycerol output *in vitro*

Hepatocytes incubated in the basal medium showed a decline in the rate of VLDL triacylglycerol secretion over the 3-day culture period (Fig. 1). This decrease occurred despite a significant increase in the cellular triacylglycerol content ($40.0 \pm 16.3\%$; $P < 0.05$) on Day 2 compared with Day 1. Supplementation of the serum-free medium from the outset with a mixture of 1 μ M-dexamethasone, 10 mM-lactate, 1 mM-pyruvate and 0.75 mM-oleate (supplemented medium) stimulated VLDL output on Day 1, and, in contrast with the basal medium, this elevated rate was sustained over the next 48 h in culture (Fig. 2). Preliminary experiments have shown that similar secretion rates could be maintained for at least 7 days under these conditions. Although the supplements

Fig. 1. Rates of VLDL triacylglycerol output in the basal medium: effects of insulin

Hepatocytes were cultured in the basal medium with (○—○) or without (●—●) 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 insulin that was present during Day 1 was removed during Day 2 (○---○). In other experiments insulin that was present during Days 1 and 2 was removed during Day 3 (○---□). VLDL triacylglycerol output was measured during each 24 h period (Day 1, Day 2, Day 3). Each value is the mean $\pm$ S.E.M. for at least six independent hepatocyte preparations.

Fig. 2. Rates of VLDL triacylglycerol output in the supplemented medium; effects of insulin

Hepatocytes were cultured in the supplemented medium with ($\triangle$ — $\triangle$) or without ($\blacktriangle$ — $\blacktriangle$) 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 insulin that was present during Day 1 was removed during Day 2 ($\triangle$ --- $\triangle$). In other experiments insulin that was present during Days 1 and 2 was removed during Day 3 ($\triangle$ --- $\square$). VLDL triacylglycerol output was measured during each 24 h period (Day 1, Day 2, Day 3). Each value is the mean $\pm$ S.E.M. for at least six independent hepatocyte preparations.

stimulated VLDL output by $64 \pm 24.5\%$ ($n = 7$; $P < 0.02$) on Day 1, cellular triacylglycerol content increased 3–4-fold (Fig. 3). During the next 2 days cellular triacylglycerol content increased by a further 2.8-fold (Fig. 3), whereas the rates of VLDL secretion remained constant (Fig. 2).

In some cases, the supplements were added at various times to cells which had been previously exposed only to the basal medium. If the medium was supplemented at the beginning of Day 2, the rate of VLDL secretion was increased to a level close to that observed when the supplements had been continuously present (Table 1). At the beginning of Day 3, although addition of the supplements led to a 5-fold stimulation of the basal rate of secretion, this elevated rate was lower (although not significantly so) than that observed when the supplements had been present continuously during the whole 3-day period (Table 1).

In other experiments, the supplements were withdrawn at various times from cultures in which they had previously been present. Removal of the supplements at the beginning of Day 2 or at the beginning of Day 3 resulted in small (16.4% and 25% respectively) and non-

Fig. 3. Effect of insulin on cellular triacylglycerol

Hepatocytes were cultured in either the basal or supplemented medium for 3 days. In each case insulin was either present or absent for the whole 3-day period. At the end of each day the medium was removed and replaced with corresponding medium of identical composition. $\bullet$, Basal medium, insulin absent; $\circ$, basal medium, insulin present; $\blacktriangle$, supplemented medium, insulin absent; $\triangle$, supplemented medium, insulin present. Each value is the mean $\pm$ S.E.M. for four independent hepatocyte preparations.

Table 1. Effects of addition and withdrawal of the substrate/dexamethasone mixture on VLDL triacylglycerol output

Cells were cultured for the whole 3-day period either in the basal medium or in the basal medium supplemented with the dexamethasone/substrate mixture (supplemented medium). The amounts of VLDL triacylglycerol secreted into the medium during the next 24 h period were determined. In some experiments, the supplemented medium was replaced by the basal medium at the end of Day 1 or Day 2, and the secretion of VLDL was determined during each subsequent 24 h period. In other experiments, the basal medium was replaced by the supplemented medium at the end of Day 1 or Day 2, and the subsequent rate of VLDL secretion was again determined over the next 24 h period. Key: —, basal medium; +, supplemented medium.

Culture conditions			VLDL triacylglycerol output ($\mu\text{g}/\text{mg}$ of protein)	
Day 1	Day 2	Day 3	Day 2	Day 3
—	—	—	39.2 ± 6.5 (6)	13.1 ± 2.5 (6)
+	+	+	90.9 ± 13.7 (6)	114.6 ± 23.0 (6)
+	—	—	76.0 ± 8.4 (5)	
—	+	—	73.8 ± 19.0 (5)	
—	—	+		62.7 ± 14.7 (5)
+	+	—		86.0 ± 17.1 (5)

Table 2. Pre-exposure of cells to different environments affects the subsequent response to insulin

Cells were cultured for either 24 h (Day 1, lines 1–4) or 48 h (Days 1 and 2, lines 5–8) in the presence of the supplemented or basal media with or without insulin. VLDL triacylglycerol output was measured during the next 24 h (Day 2, lines 1–4 or Day 3, lines 5–8) in the presence of the basal medium plus insulin. Cellular triacylglycerol contents were also measured at the corresponding times. Key: S, supplemented medium; B, basal medium; I, insulin. Values marked *, **, *** are significantly different from their corresponding controls (100%) at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively.

Line	Culture conditions		VLDL triacylglycerol output (Day 2) (%)	Cell triacylglycerol content (Day 2) (%)
	Day 1	Day 2		
1	S+I	B+I	100 (68.5 ± 11.3 µg/mg of protein)	100 (321.2 ± 64.0 µg/mg of protein)
2	S	B+I	31.1 ± 3.9**	68.8 ± 2.7*
3	B+I	B+I	100 (17.2 ± 2.1 µg/mg of protein)	100 (154.2 ± 22.1 µg/mg of protein)
4	B	B+I	90.3 ± 19.1	64.5 (2)

	Culture conditions			VLDL triacylglycerol output (Day 3) (%)	Cell triacylglycerol content (Day 3) (%)
	Day 1	Day 2	Day 3		
5	S+I	S+I	B+I	100 (148.3 ± 29.2 µg/mg of protein)	100 (501 ± 21.0 µg/mg of protein)
6	S	S	B+I	40.9 ± 14.5**	59.6 ± 4.8***
7	B+I	B+I	B+I	100 (33.7 ± 17.3 µg/mg of protein)	100 (203.6 ± 31.5 µg/mg of protein)
8	B	B	B+I	19.4 ± 8.4***	40.7 (2)

significant decreases in the rate of VLDL secretion (Table 1). Under these conditions, cellular triacylglycerol content declined to a much greater extent (63.1% and 52.3% respectively).

Effects of insulin on the basal and stimulated rates of VLDL secretion

Addition of insulin to the basal and supplemented media for the first 24 h after removal of serum (Day 1) suppressed the secretion of VLDL triacylglycerol to $46.7 \pm 8.9\%$ ($P < 0.001$) and $55.0 \pm 5.8\%$ ($P < 0.001$) of the respective control values (Figs. 1 and 2). The inhibitory effect of insulin therefore persisted in cells stimulated to secrete larger quantities of VLDL. This inhibitory effect was maintained during the next 24 h (Day 2) when exposure to insulin was continued over this period. However, in this case, the suppression was significantly more pronounced in the basal medium compared with that observed in the supplemented medium ($45.5 \pm 5.3\%$ compared with $68.9 \pm 3.0\%$ of the respective control values; $n = 6$; $P < 0.005$). Furthermore, when insulin was continuously present in the basal medium, the rate of secretion on Day 2 was significantly less ($63.3 \pm 12.8\%$; $n = 6$; $P < 0.01$) than on Day 1, although in the supplemented medium this was not the case ($130 \pm 24.1\%$; $n = 6$).

In contrast with the inhibitory effects of insulin described above, continued exposure of the hepatocytes to insulin for a further 24 h (Day 3) stimulated the secretion of VLDL triacylglycerol irrespective of whether or not the medium contained the supplements (Figs. 1 and 2). Despite these biphasic effects of insulin on the secretion of VLDL triacylglycerol, continuous exposure of the cells to insulin resulted in a sustained increase in the content of cellular triacylglycerol. This occurred even when the cellular triacylglycerol was already increased in the presence of the supplemented medium (Fig. 3).

The detailed effects of insulin on the secretion of VLDL triacylglycerol was dependent upon the environment to which the cells had previously been exposed. For instance, addition of insulin for 24 h to cells in the basal medium which had previously been cultured for 24 h or 48 h in the presence of the supplemented medium alone gave rise to a secretion of VLDL triacylglycerol which was only 31.1% and 40.9% respectively of that when the cells had previously been cultured for the corresponding periods with the supplemented medium plus insulin (Table 2). The cellular triacylglycerol also decreased under these conditions (Table 2). However, when insulin was added for 24 h to cells which had previously been cultured in the unsupplemented medium (as opposed to the supplemented medium) during the previous 24 h, the output of VLDL triacylglycerol was similar irrespective of whether insulin had previously been present or absent (Table 2). In contrast, addition of insulin to cultures which had been exposed for the previous 48 h to the basal medium only led to a 80.6% decline in VLDL output compared with those which had previously been exposed to the basal medium plus insulin during this 48 h period (Table 2).

Effects of insulin removal on the secretion and storage of triacylglycerol

In the basal medium during the first 24 h in serum-free medium (Day 1) insulin decreased the secretion of VLDL triacylglycerol (Fig. 1; $P < 0.001$). During this period, the cellular triacylglycerol content increased almost 2-fold (Fig. 3; $P < 0.05$). When insulin was removed from the medium of the cells, then during the next 24 h period (Day 2) VLDL triacylglycerol secretion increased ($P = 0.05$), whereas cells which had not received insulin on Day 1 showed a decline (Fig. 1; $P < 0.01$). The increase observed when insulin was removed appeared to occur at

Fig. 4. Effect of insulin on cellular plus VLDL triacylglycerol

Hepatocytes were cultured in the basal or supplemented media with or without insulin as described in the legends to Figs. 1, 2 and 3. In each case the amount of cellular triacylglycerol present at the end of each day was added to the amount of VLDL triacylglycerol secreted during the previous 24 h period. ●, Basal medium, insulin absent; ○, basal medium, insulin present; ▲, supplemented medium, insulin absent; △, supplemented medium, insulin present. Each value is the mean $\pm$ S.E.M. for four independent hepatocyte preparations. Values marked * and *** mean that insulin gave rise to significant increases ($P < 0.05$ and $P < 0.001$ respectively) compared with the non-insulin control on the same day.

the expense of the cellular triacylglycerol which accumulated the previous day when insulin was present.

In the presence of the supplemented medium, insulin decreased the secretion of VLDL triacylglycerol during the first 24 h in the serum-free medium ($P < 0.02$; Fig. 2), whereas cellular triacylglycerol increased ($P = 0.06$; Fig. 3). If insulin was then removed from the medium, during the next 24 h (Day 2) VLDL triacylglycerol secretion increased to $187.9 \pm 19.0 \mu\text{g/mg}$, compared with $90.9 \pm 13.7 \mu\text{g/mg}$ ($P < 0.01$) during the same period in cells not pre-treated with insulin.

Effect of insulin on the net synthesis of triacylglycerol

The effects of insulin on the net synthesis of triacylglycerol may be determined by comparing the sum of the cellular and secreted triacylglycerol in the presence and absence of insulin. This calculation involved, where appropriate, subtraction of the initial cellular triacylglycerol content from the final content. That rat hepatocyte cultures do not take up secreted VLDL has been shown previously (Durrington *et al.*, 1982). If this is done for each of the 3 days in culture, then on Day 1 insulin had no effect on net synthesis, in either the basal

or the supplemented medium (Fig. 4). However, the continued presence of insulin resulted in increased net synthesis on Day 2, and an even greater increase on Day 3. Again, these increases occurred irrespective of whether the medium was supplemented or not.

DISCUSSION

Capacity of hepatocytes for the secretion of VLDL triacylglycerol

One of the reasons for this work was the need to develop a model to study the long-term effects of insulin and insulin deficiency on hepatic VLDL output *in vitro*. Previous experiments had shown that, in cells cultured in unsupplemented Waymouth's medium, the rates of triacylglycerol secretion fell sharply over a 3-day period (Bartlett & Gibbons, 1988). In the present work, a continuously high rate of VLDL triacylglycerol secretion was maintained for at least 3 days, provided that the basal Waymouth's medium was supplemented with dexamethasone and the triacylglycerol precursors, lactate, pyruvate and oleate (Fig. 2). Addition of the supplements to cells which had previously been maintained in the basal medium for up to 48 h also stimulated the originally low rate to a level which was not significantly different from that which occurred if the supplements had been present throughout (Table 1). This suggests that, despite their low rate of VLDL output, cells maintained in the basal medium did not lose their capacity for secreting large quantities of VLDL when culture conditions became favourable.

The high but constant rate of VLDL output over the 3-day period in the presence of the supplements ($\sim 100 \mu\text{g}$ of triacylglycerol/24 h per mg of protein) occurred against a large increase in the content of cellular triacylglycerol during this time. This supports the suggestion that hepatic VLDL secretion is a saturable process and that any further uptake or synthesis of triacylglycerol results in increased cellular storage (Chao *et al.*, 1986). However, this high constant rate of triacylglycerol secretion was exceeded when cells pre-treated with insulin were subsequently cultured in an insulin-free medium. This suggests that insulin, although acutely inhibitory to VLDL secretion, had a latent effect by increasing the capacity for this process. This latency was realized only when insulin was removed from the medium. This delayed effect of insulin may explain the observation by Reaven & Mondon (1984) that the livers of animals exposed to high concentrations of plasma insulin *in vivo* secreted more VLDL than normal when perfused without insulin *in vitro*.

Studies *in vivo* have shown that the turnover of hepatic triacylglycerol is very rapid. For instance, assuming a constant rate of secretion, and that the liver is the major source of plasma VLDL, it may be calculated that a secretion rate of 29–38 mg of triacylglycerol/g of liver over a 24 h period (Goh & Heimberg, 1979; Marrino *et al.*, 1987; Duerden & Gibbons, 1988) was achieved with a steady-state hepatic triacylglycerol content of only 7 mg/g. Most of this secreted triacylglycerol does not equilibrate with the bulk of the hepatic triacylglycerol (Higgins & Hutson, 1984; Chao *et al.*, 1986; Duerden & Gibbons, 1988). In the present model *in vitro*, the maximum rate of VLDL triacylglycerol output was $188 \mu\text{g}/24 \text{ h}$ per mg of protein which approximates to $30 \text{ mg}/24 \text{ h}$ per g of liver. However, cellular tri-

Fig. 5. Relationship between the change in VLDL output and the change in net triacylglycerol synthesis when insulin was added to the basal medium

Changes in net triacylglycerol synthesis caused by insulin were calculated from the difference between the sum of the cellular and VLDL triacylglycerol over a 24 h period in the presence and absence of insulin. All other parameters were identical. Changes in VLDL triacylglycerol output in the presence, compared with the absence, of insulin was calculated by using the same dishes as those used for triacylglycerol synthesis over the same 24 h period. Each point represents a single measurement. Key: ○, effect of insulin on Day 1; △, effect of insulin on Day 2; □, effect of insulin on Day 3; ●, insulin present on Day 1, absent on Day 2; ▲, insulin present on Days 1 and 2 but absent on Day 3.

acylglycerol content was 468 ± 50 $\mu\text{g}/\text{mg}$ of protein (74.8 mg/g of liver), a value which is considerably higher than that which occurs under normal conditions *in vivo*. The reason for this discrepancy between hepatocellular and VLDL triacylglycerol *in vivo* and *in vitro* is not clear, but it may be related to the availability of other factors such as lipoproteins, cholesterol and phospholipid *in vitro*.

Comparison of the effects of insulin deficiency *in vitro* and *in vivo*

The present results confirm that short-term (< 24 h) insulin deficiency *in vitro* stimulates the secretion of VLDL triacylglycerol. However, the effects of short-term insulin deficiency *in vivo* are contradictory. For instance, administration of anti-insulin serum to rats decreased VLDL secretion within 4 h in livers derived from them (Woodside & Heimberg, 1976), whereas similar treatment in dogs had the opposite effect (Balasse *et al.*, 1972). Despite this, the well-known inhibitory effect of chronic

Fig. 6. Relationship between the change in VLDL output and the change in net triacylglycerol synthesis when insulin was added to the supplemented medium

Conditions were identical with those described in the legend to Fig. 5, except that the cells were cultured in the supplemented medium rather than the basal medium. Key: see legend to Fig. 5.

insulin deficiency *in vivo* on hepatic VLDL secretion (Woodside & Heimberg, 1976; Berry *et al.*, 1981; Reaven & Mondon, 1984) may be simulated by removing insulin from the medium for longer than 48 h (Figs. 1 and 2).

Relationships between triacylglycerol synthesis and VLDL output: the effect of insulin

When insulin was added to the medium of cells, VLDL triacylglycerol output increased or decreased according to whether the cells had previously been exposed to insulin. If, in each case, this net change was compared with the corresponding effects of insulin on net triacylglycerol synthesis in the same cells, then a consistent relationship emerged (Figs. 5 and 6). In both the basal and supplemented media, when the effect of insulin on net triacylglycerol synthesis was small, then insulin inhibited VLDL output. Conversely, when the net increase in triacylglycerol synthesis was large, insulin stimulated the secretion of VLDL. The increased net synthesis threshold that was required for a stimulation of VLDL output was between 100 and 125 μg of triacylglycerol/24 h per mg of protein. Interestingly, this

threshold value of 100–125 μg was obtained independently in cells cultured in both basal (Fig. 5) and supplemented (Fig. 6) media, and was thus independent of the absolute rates of triacylglycerol synthesis and VLDL output. These results suggest that there are at least two effects of insulin, which oppose each other in determining the precise rate of VLDL output. One is a direct inhibitory effect. The other is an indirect stimulatory effect which results from the increase in triacylglycerol synthesis. These observations may be relevant to the controversy surrounding the effects of insulin on hepatic VLDL secretion *in vitro*. The present results would suggest that, if experimental conditions were such that insulin was able to maintain an increase in net triacylglycerol synthesis greater than a certain amount (in the present case 100–125 $\mu\text{g}/24\text{ h}$ per mg of protein), then VLDL secretion would increase (Topping & Mayes, 1982; Topping *et al.*, 1988). If not, then the direct inhibitory effect becomes the over-riding factor and VLDL secretion declines. *In vivo* the magnitude of the increase in triacylglycerol synthesis in response to insulin, and thus the effect on VLDL secretion, may depend upon the hormone balance of the plasma to which the liver had previously been exposed.

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