Interaction of non-esterified fatty acid and insulin in control of triacylglycerol secretion by Hep G2 cells

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The role of insulin in the regulation of plasma triacylglycerol is poorly understood. Conflicting actions of insulin on rat liver cells have been reported, insulin inhibiting triacylglycerol secretion in short incubations (< 24 h) and stimulating triacylglycerol secretion in longer incubations (48–72 h). The present study was undertaken to examine regulation of triacylglycerol secretion by insulin and investigate the interaction between insulin and non-esterified fatty acid over 72 h in human hepatoblastoma (Hep G2) cells. Insulin inhibited triacylglycerol secretion throughout the 72 h period. The inhibition increased from 66 % in the first 24 h to 88 % in the final 24 h. Increasing the initial concentration of oleic acid from 200 μM to 1000 μM resulted in a 358 % increase in triacylglycerol secretion and a 712 % increase in accumulation over 24 h. Oleic acid uptake by the cells was rapid, with only 2.4 % of the initial concentration (500 μM) remaining after 24 h. Supplementation of the medium with oleic acid to maintain the concentration between 750 μM and 1000 μM throughout a 5 h period resulted in a 350 % increase in triacylglycerol secretion. Supplementation also decreased the insulin-induced inhibition of triacylglycerol secretion (18.2 to 7.8 %; $P < 0.001$). These results demonstrate that there is not a biphasic action of insulin on triacylglycerol secretion by Hep G2 cells. Experiments of this nature have not previously taken into account the rapid uptake of non-esterified fatty acid by hepatocytes and have consequently underestimated the effect of a sustained concentration on triacylglycerol metabolism. Oleic acid is therefore an even more potent stimulus to triacylglycerol synthesis and secretion than has previously been recognized. In addition, in the presence of a sustained increase in oleic acid concentration, there is a decrease in the action of insulin to inhibit triacylglycerol secretion.

INTRODUCTION

Prospective population studies in Framingham (Kannel & McGee, 1979; Abbot & Carroll, 1984) and Paris (Cambien *et al.*, 1986) have indicated that elevated triacylglycerol concentration is an independent risk factor for the development of cardiovascular disease. The concentration of triacylglycerol in plasma from fasted non-diabetic people is positively correlated with that of insulin (Olefsky *et al.*, 1974; Orchard *et al.*, 1983; Lääsko *et al.*, 1987; Juhan-Vague *et al.*, 1987). Insulin is a potent stimulator of both fatty acid (Salmon & Hems, 1973; Diamant & Shafir, 1975; Kirk *et al.*, 1976; Amatruda *et al.*, 1983) and triacylglycerol (Durrington *et al.*, 1982; Dich *et al.*, 1983; Wang *et al.*, 1988) synthesis. It might therefore be concluded that the observed close relationship between increases in insulin and triacylglycerol are explained by the former causing the latter.

The net effect of insulin on triacylglycerol appears to be governed by opposing forces. Insulin inhibits adipose-tissue lipolysis and decreases the non-esterified fatty acid (NEFA) flux into the plasma, and it also activates adipose-tissue lipoprotein lipase, both of these actions favouring a decrease in plasma triacylglycerol. Opposing this is the stimulatory effect of insulin on lipogenesis, esterification of fatty acid to form triacylglycerol and coupling of triacylglycerol to apolipoprotein-B (for review see Kissebah & Schectman, 1987). The situation is further confused by consideration of the relationships in diabetes mellitus. In diabetes the risk of ischaemic heart disease is increased 2–3-fold over age- and sex-matched controls (Assman & Shultz, 1988), and major contributions to this development are the associated lipid and lipoprotein abnormalities (Uusitupa *et al.*, 1985). The most frequent lipid abnormality in both insulin-dependent (IDDM) and non-insulin-dependent (NIDDM) diabetes is an increased plasma triacylglycerol (Howard, 1987).

IDDM is primarily a condition of insulin deficiency, whereas the insulin status of NIDDM remains controversial (Temple *et al.*, 1989; Banerji & Lebovitz, 1989). In NIDDM there are also increases in plasma glucose and NEFA (Liu *et al.*, 1983; Golay *et al.*, 1986; Ida Chen *et al.*, 1987), both of which are of great significance for triacylglycerol synthesis (Reaven & Ida Chen, 1988).

Studies *in vitro* to date have not greatly clarified the metabolic interactions by which insulin, glucose and NEFA control plasma triacylglycerol concentration. Data from short-term incubation (< 24 h) with rat hepatocytes and Hep G2 cells suggest that insulin inhibits triacylglycerol secretion (Durrington *et al.*, 1982; Patsch *et al.*, 1983; Pullinger & Gibbons, 1983; Mangiapane & Brindley, 1986; Dashti & Wolfbauer, 1987; Wang *et al.*, 1988) and also apolipoprotein-B secretion (Pullinger *et al.*, 1989). However, the effect of insulin on triacylglycerol secretion with rat hepatocytes has been thought to be biphasic during a 72 h incubation. Insulin inhibited triacylglycerol secretion during the first 48 h and stimulated during the remaining 24 h (Bartlett & Gibbons, 1988).

In an attempt to clarify these confusing observations *in vivo* and *in vitro*, we have undertaken systematic short (< 24 h)- and long- (72 h)-term studies of the interaction between glucose, NEFA and insulin on the control of triacylglycerol secretion by Hep G2 cells.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle's Medium was purchased from Flow Laboratories. Fetal-calf serum (FCS) was from Advanced Protein Products Ltd., Brierley Hill, West Midlands, U.K. Oleic acid and fatty-acid-free BSA were from Sigma. [$1\text{-}^{14}\text{C}$]Oleic acid

(54 mCi/mmol) was purchased from Amersham. Silica-coated t.l.c. plates and the solvents light petroleum, diethyl ether and acetic acid were from BDH. The human hepatoma cell line Hep G2 was purchased from Porton Down, and the insulin was a gift from the National Institute of Biological Standards, Potters Bar, Herts., U.K. Optiphase Hi-Safe II scintillant was obtained from LKB Scintillation Products. Hydrazine, glycine, EDTA, MgCl_2 , NAD^+ , NADH, ATP, triolein standards and glycerol kinase and glycerol phosphate dehydrogenase were all purchased from Sigma. Tetraethylammonium hydroxide was from Kodak.

Cell cultures

Cells were subcultured from large flasks (150 cm^2) into 25 cm^2 flasks with 9 ml of Dulbecco's Modified Eagle's Medium containing 10% (v/v) FCS, penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$). Every 3 days the medium was replaced with fresh medium of identical composition. After 5 days the flasks contained a confluent monolayer.

Incubation conditions

The concentration of fatty-acid-free BSA was chosen as 4% in an attempt to mimic closely the concentration of human albumin in serum, and the aim of the range of NEFA concentrations was an attempt to parallel the concentrations noted *in vivo* in both physiological and pathological conditions.

An insulin concentration of 0.1 μM was chosen as only 10% of this concentration remained after 8 h. Insulin was supplemented every 8 h in order to maintain the concentration between 0.1 and 0.01 μM . Our data on insulin dose-response curves verified those previously described by Podskalny *et al.* (1985).

Experiments were performed in the presence of 10% FCS. Initial short time-course experiments (< 24 h) were performed in both the absence and presence of FCS and the results were compared. There was no significant difference. However, since one objective was to investigate the effects of prolonged exposure of the hepatocyte to insulin, experiments were performed in the presence of serum. [Data from Bartlett & Gibbons (1988) have shown a 70% decrease in triacylglycerol secretion in rat hepatocytes during a 72 h incubation in serum-free medium.]

The presence of insulin in the 10% FCS was insignificant, as earlier dose-response experiments had revealed no inhibitory effect by insulin on triacylglycerol secretion at concentrations of 1 nM.

[1- ^{14}C]Oleic acid (54 mCi/mmol) in toluene was added to a solution of oleic acid in water at approx. 70°C . The toluene preservative was removed by boiling, and the residual volume (0.5 ml) was added to the medium supplemented with 4% BSA with or without FCS, to give a known concentration of oleic acid with known specific radioactivity.

The confluent monolayer of cells was washed twice with phosphate-buffered saline (0.15 M-NaCl, 1.8 mM- $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 8.2 mM- $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$), pH 7.4, and the prepared filtered sterilized labelled medium was added to the cells.

Experiments performed with cells which had been preincubated with insulin were previously exposed to insulin (0.1 μM) for 16 h before commencement. Cells which were continuously exposed to a high concentration of NEFA were supplemented hourly with a known concentration of oleic acid of specific radioactivity identical with that introduced at the beginning of the experiment.

Determination of triacylglycerol and NEFA concentrations

After a defined period of incubation (with or without insulin), the medium was removed and the cells were washed twice with

phosphate-buffered saline. The monolayers were removed from the flasks in 1.1 ml of 0.1 M-HCl with a cell scraper.

The lipid from the medium and cell suspensions were removed separately with a mixture of chloroform/methanol/water (Folch *et al.*, 1957), washed twice, dried down under nitrogen and applied to t.l.c. plates. The t.l.c. plates were placed in light petroleum (b.p. $40\text{--}60^\circ\text{C}$)/diethyl ether/acetic acid (35:15:1, by vol.) in the solvent tank for 90 min. After staining with iodine, the triacylglycerol and fatty acid were removed, placed in tubes containing 10 ml of Optiphase Hi-Safe scintillant and counted for radioactivity in a Packard liquid-scintillation spectrometer.

Enzymic determination of glycerol in acylglycerols

Acyl esters of glycerol can be hydrolysed by tetraethylammonium hydroxide under conditions that do not cleave phosphate esters (Brockerhoff, 1963). Thus the acylglycerol may be determined without prior removal of the phospholipids. The glycerol formed was measured by a coupled enzymic assay and the fluorescence of NADH was measured (Cherniek, 1969).

Protein measurements

Total cell protein content was measured by the method of Lowry *et al.* (1951).

Statistical analysis

Paired or unpaired Student's *t* tests were used where applicable.

RESULTS

Direct and [^{14}C]triacylglycerol measurements

Changes of triacylglycerol metabolism estimated by enzymic determination of glycerol paralleled data obtained with [1- ^{14}C]oleic acid. There was no significant increase in re-uptake or lipolysis of secreted triacylglycerol attributable to insulin (results not shown), corroborating the data of Durrington *et al.* (1982).

An initial oleic acid concentration of 500 μM resulted in 3 ± 0.1 nmol of triacylglycerol/mg of protein being secreted compared with 0.6 ± 0.01 nmol of [^{14}C]triacylglycerol/mg of protein in 24 h; 14 ± 1 nmol of intracellular triacylglycerol/mg of protein accumulated, compared with 15 ± 1 nmol of [^{14}C]triacylglycerol/mg of protein, during this period. The percentage inhibition of the secreted triacylglycerol and the percentage increase in intracellular triacylglycerol accumulation attributable to insulin were the same for the two methods. The decreased specific radioactivity of secreted triacylglycerol presumably reflected dilution in a pool of unlabelled intracellular precursor. In contrast, no dilution of accumulated triacylglycerol was ob-

Table 1. Effects of insulin on [^{14}C]triacylglycerol secretion with increasing concentrations of glucose

Glucose (mM)...	5	12.5	25
Control	299 ± 15	335 ± 6	410 ± 30
Insulin	137 ± 5	171 ± 5	215 ± 15
Inhibition (%)	54	49	48

Results are expressed as μg of triacylglycerol/24 h per g of cell protein (means $\pm$ s.d. of triplicate flasks). Initial concentration of oleic acid was 500 μM . There was a 37% increase in triacylglycerol secretion with a glucose concentration of 25 mM compared with 5 mM. Insulin (0.1 μM) produced approx. 50% inhibition of triacylglycerol secretion at all three glucose concentrations. There was no significant difference in the percentage inhibition by insulin at all three concentrations.

Fig. 1. (a) Time course of the effect of insulin on triacylglycerol secretion over 24 h (no preincubation), and (b) effects of 16 h preincubation with insulin on triacylglycerol secretion over the subsequent 24 h

Results are expressed as μg of triacylglycerol/g of cell protein, and are means $\pm$ S.E.M. of three experiments performed with triplicate flasks. (a) Initial concentration of oleic acid during all experiments was $500 \mu\text{M}$. $\square$, No preincubation, control; $\blacklozenge$, no preincubation, +insulin. (b) Duration of preincubation was 16 h before the onset of the experiment. Initial concentration of oleic acid during all experiments was $500 \mu\text{M}$. $\square$, Preincubated, control; $\blacklozenge$, preincubated, +insulin.

Table 2. Time course of the effect of insulin and increasing concentrations of oleic acid on [^{14}C]triacylglycerol secretion

Results are expressed as μg of triacylglycerol/g of cell protein (means $\pm$ S.E.M. of triplicate flasks in three experiments). Insulin-treated cells were also preincubated with insulin ($0.1 \mu\text{M}$) for 16 h before the experiment.

[Oleic acid] (μM)	Time ...	2 h	4 h	8 h	24 h
50	Control	4.6 ± 0.2	14 ± 0.9	20 ± 2.3	81 ± 9
	Insulin	3.3 ± 0.6	11 ± 1.1	15 ± 1.6	21 ± 3.0
500	Control	34 ± 4.0	125 ± 13	256 ± 30	607 ± 70
	Insulin	31 ± 3.1	99 ± 10	183 ± 20	258 ± 31
1500	Control	127 ± 14	331 ± 40	628 ± 62	1199 ± 151
	Insulin	128 ± 3	300 ± 30	440 ± 50	417 ± 63

Table 3. Effect of insulin and $200 \mu\text{M}$, $500 \mu\text{M}$ and $1000 \mu\text{M}$ initial concentrations of oleic acid on the secretion and intracellular accumulation of [^{14}C]triacylglycerol over 24 h

Results are expressed as μg of triacylglycerol/24 h per g of cell protein, and are means $\pm$ S.E.M. of triplicate flasks in three experiments.

[Oleic acid] (μM)...		200	500	1000
Secreted	Control	341 $\pm$ 32	498 $\pm$ 59	865 $\pm$ 90
	Insulin	108 $\pm$ 15	306 $\pm$ 43	453 $\pm$ 61
Intracellular	Control	2665 $\pm$ 321	8130 $\pm$ 878	19200 $\pm$ 2450
	Insulin	2816 $\pm$ 333	9216 $\pm$ 892	20720 $\pm$ 2533

served. Therefore all of the increase in intracellular triacylglycerol can be attributed to the exogenous oleate substrate, whereas the triacylglycerol that is secreted throughout this period is derived from both a pre-existing unlabelled and a newly labelled pool. Consequently only a small percentage of the total secreted triacylglycerol is labelled. All the results shown in the Figures and Tables represent [^{14}C]triacylglycerol measurements.

Effects of glucose and insulin

The effects of glucose and insulin on triacylglycerol secretion at $500 \mu\text{M}$ -oleic acid were studied at glucose concentrations corresponding to normal, moderately and severely hyperglycaemic conditions (Table 1), assuming that Hep G2 cells represent the behaviour of perivenous hepatocytes. (This may not be true, as the exact derivation of this cell line is unknown.) There was a 37% increase in triacylglycerol secretion in control cells incubated in 25 mM-glucose compared with those incubated in 5 mM-glucose. Insulin inhibited triacylglycerol secretion by 54%, 49% and 48% at 5 mM, 12.5 mM- and 25 mM-glucose respectively. This difference was not statistically significant. Consequently, in order to maximize triacylglycerol secretion by the cells, all subsequent experiments were performed in media containing 25 mM-glucose.

Time course of insulin effect and preincubation with insulin

Experiments were performed to determine the time course of the insulin effect. There was a delay after the addition of insulin, during which time there was no significant difference in the concentration of triacylglycerol secreted in the control cells and those exposed to insulin (Fig. 1a). The duration of this delay

Table 4. Effects of insulin on oleic acid uptake

Initial concentration of oleic acid was 500 μM . Results are expressed as concentrations of oleic acid in μM , and are means $\pm$ S.E.M. of triplicate flasks in 4–6 experiments. *P* values shown demonstrate the significance of the insulin effect at each time point.

Time ...	2 h	4 h	8 h	12 h	24 h
Control	206 $\pm$ 10.2	151 $\pm$ 9	74 $\pm$ 6	37 $\pm$ 3.1	12 $\pm$ 2
Insulin	196 $\pm$ 10	121 $\pm$ 7.2	55 $\pm$ 5.5	27 $\pm$ 3.2	7 $\pm$ 1.8
<i>P</i>	< 0.01	< 0.01	< 0.001	< 0.001	< 0.001

Table 5. Effects of a sustained high concentration of oleic acid on the inhibition of [^{14}C]triacylglycerol secretion by insulin over 5 h

Results are expressed as μg of triacylglycerol secreted/g of cell protein during a 5 h incubation. Cells were preincubated for 16 h before the start of the experiment. The initial concentration of oleic acid was 750 μM . The concentration of oleic acid was maintained between 750 μM and 1000 μM by hourly addition of oleic acid (of identical specific radioactivity) to the supplemented group. The above data represent a typical experiment of three similar ones performed with the results expressed as means $\pm$ S.D. of triplicate flasks. The inhibition of triacylglycerol secretion is expressed as a percentage for the two groups. A sustained oleic acid concentration decreased the inhibition of triacylglycerol secretion by insulin (**P* < 0.001).

	Triacylglycerol secretion	Inhibition (%)
Control	377 $\pm$ 35	
Insulin	308 $\pm$ 21	18.2
Supplemented control	1236 $\pm$ 108	
Supplemented insulin	1140 $\pm$ 38	7.8*

could be decreased by preincubating the cells before the beginning of the experiment with insulin (Fig. 1*b*). The percentage inhibition of triacylglycerol secretion attributable to insulin increased with preincubation, and therefore, in order to maximize the effects due to insulin, all subsequent experiments were performed after a 16 h preincubation. In addition, the insulin-mediated per-

centage inhibition of triacylglycerol increased with time as the concentration of NEFA fell throughout the 24 h period.

In the presence of a falling oleic acid concentration, intracellular triacylglycerol did not increase after 8 h (results not shown). Similarly, the effect of insulin on inhibition of triacylglycerol secretion increased markedly after 8 h (Fig. 1*b*).

Effects of NEFA concentration

Experiments to determine the effect of increasing concentrations of oleic acid on triacylglycerol production were performed (Table 2). There was a tendency toward an earlier and more marked insulin-induced inhibition of triacylglycerol secretion the lower the initial oleic acid concentration. NEFA was found to be a potent stimulus of triacylglycerol production (Table 3). There was a 358 % increase in triacylglycerol secretion and a 712 % increase in intracellular triacylglycerol when the initial concentration of oleic acid was raised from 200 μM to 1000 μM .

Effect of insulin on NEFA uptake

There was a rapid uptake of oleic acid by the cells (Table 4). An initial concentration of 500 μM -oleic acid in the medium had fallen by approx. 60 % during the first 2 h of incubation. Insulin significantly increased the uptake of oleic acid at all the time points examined.

Effect of sustained NEFA concentration on insulin-induced inhibition of triacylglycerol secretion

The rapid fall in oleic acid concentration made it difficult to define the effect of a given concentration during experiments performed for times in excess of 2 h. Therefore, by using repeated additions of oleic acid, the oleic acid concentration was maintained between 750 μM and 1000 μM for 5 h (Table 5). Triacylglycerol secretion increased by 350 % when the concentration of oleic acid was maintained. Also, a sustained oleic acid concentration decreased the percentage inhibition of triacylglycerol secretion by insulin (from 18.2 % to 7.8 %, *P* < 0.001).

Time course of prolonged insulin effect on NEFA uptake, intracellular triacylglycerol accumulation and secretion

The effects of prolonged exposure (72 h) to insulin in the presence of an initial concentration of 500 μM -oleic acid was then examined. There was a successive decrease in NEFA uptake with each 24 h period throughout the 72 h (*P* < 0.001). Insulin produced a small but successive increase in NEFA uptake during

Table 6. Effect of prolonged incubation with insulin over 72 h on [^{14}C]oleic acid uptake, intracellular [^{14}C]triacylglycerol accumulation and [^{14}C]triacylglycerol secretion

The [^{14}C]oleic acid uptake results are expressed as concentration of oleic acid (μM) present in the medium at the end of each 24 h period. The initial concentration of oleic acid was 500 μM at the beginning of each 24 h, and the medium was exchanged for fresh medium of identical composition after each 24 h period throughout the 72 h. All results are the means $\pm$ S.E.M. of triplicate flasks (*n* = 3). The concentration of oleic acid in the medium of the insulin-treated cells, expressed as a percentage of the controls, was 48 % at the end of day 1, 33 % at the end of day 2 and 28 % at the end of day 3. The insulin-induced increase in uptake of oleic acid was significant (**P* < 0.001). There was a successive decrease in uptake of oleic acid in the control cells with time (20 μM at the end of day 3 compared with 12.8 μM at the end of day 1; ***P* < 0.001). The results for intracellular and secreted [^{14}C]triacylglycerol are expressed as μg of triacylglycerol/g of cell protein, and the increase in triacylglycerol secretion in the control group of cells was significant when days 2 and 3 were compared with day 1 (****P* < 0.001).

	Day 1		Day 2		Day 3	
	Control	Insulin	Control	Insulin	Control	Insulin
[^{14}C]Oleate uptake	12.8 $\pm$ 2.4	6.2 $\pm$ 1.2*	18.1 $\pm$ 1.3	6.1 $\pm$ 0.8*	20.0 $\pm$ 2.2**	5.6 $\pm$ 1.3*
[^{14}C]Triacylglycerol (intracellular)	11271 $\pm$ 552	13113 $\pm$ 458	9956 $\pm$ 348	15297 $\pm$ 1336	7349 $\pm$ 389	12229 $\pm$ 904
[^{14}C]Triacylglycerol (secretion)	560 $\pm$ 56	192 $\pm$ 8	1029 $\pm$ 133***	170 $\pm$ 11	1804 $\pm$ 351***	208 $\pm$ 11

each 24 h period and, unlike the control cells, there was no successive decrease in NEFA uptake with time (Table 6).

There was an increase in intracellular triacylglycerol accumulation throughout the 72 h. However, in the absence of insulin there was a decrease in intracellular triacylglycerol accumulation in each successive 24 h period (Table 6). In the presence of insulin the accumulation of intracellular triacylglycerol was constant for each successive 24 h period. Therefore the incremental increase in triacylglycerol accumulation attributable to insulin increased from 16 % in the first 24 h to 66 % in the last 24 h. There was an increase in the rate of triacylglycerol secretion with each successive 24 h period (Table 6). Insulin inhibited triacylglycerol secretion throughout the 72 h, and the absolute concentration of triacylglycerol secreted during each 24 h period remained the same. Therefore the insulin-induced percentage inhibition of triacylglycerol secretion increased from 66 % in the first 24 h to 88 % in the final 24 h.

DISCUSSION

The present data differ from previously published studies of the long-term effect of insulin on triacylglycerol secretion by liver cells. The additional insights into the interaction between insulin and NEFA in the control of this process may help to reconcile the apparent anomaly that appears to exist between the role of insulin *in vivo* and *in vitro*. Studies which have examined the effect of insulin and NEFA on triacylglycerol metabolism have not recognized the rapid fall in NEFA concentration that occurs during studies of hepatocytes. To the best of our knowledge there is no published information on the effects of prolonged incubation with insulin on triacylglycerol metabolism in Hep G2 cells. Nevertheless, several authors have concluded that they are a useful model for examining lipid and lipoprotein metabolism (Thrift *et al.*, 1986; Ellsworth *et al.*, 1986; Wang *et al.*, 1988).

The results presented here confirm the short-term (< 24 h) inhibition by insulin of hepatic triacylglycerol secretion. The results also show that the inhibitory effect is time-dependent, with a delay in onset that is much decreased by a 16 h pre-incubation (Figs. 1a and 1b). In confirmation of Wang *et al.* (1988), a very potent effect of NEFA to increase triacylglycerol secretion was found. In experiments longer than 2 h there was a marked decrease in the ambient concentration of oleic acid, falling to as low as 2.4 % after 24 h. Consequently the true effect of raised NEFA concentration to increase triacylglycerol secretion was underestimated by 350 % over only 5 h, if the medium was not regularly replenished (Table 5). There was an interaction between the effects of NEFA to increase and the effects of insulin to decrease triacylglycerol secretion, such that at sustained higher NEFA concentration the effect of insulin was decreased (Table 5). On the other hand, NEFAs alone led to a successive decrease in their own uptake (Table 6), but an increasing rate of triacylglycerol secretion (Table 6). Insulin, on the other hand, produced a small but significant stimulation of NEFA uptake over the initial 24 h period, and the rate of uptake in the presence of insulin did not decline with time (Table 6). As a result of these two opposing effects of NEFA and insulin, there was a significant increase in the insulin-induced stimulation of NEFA uptake from the first to the last 24 h period of a 72 h incubation. Similarly the rate of triacylglycerol secretion in the presence of insulin remained constant in each 24 h period, despite the rise seen in the presence of NEFA alone (Table 6). Hence the insulin inhibition increased from 66 % to 88 % between the beginning and the end of the 72 h period.

NEFA increased the intracellular deposition of triacylglycerol. Insulin further increased this by 16 % over 24 h and by 42 % over 72 h (Table 6). Over a longer period the increments in tri-

acylglycerol accumulation tended to decrease in the presence of NEFA alone, but to be maintained when insulin was present. Thus the proportional effect of insulin rose from 16 % in the first 24 h period to 66 % in the last, over 72 h. There was no biphasic effect of insulin on triacylglycerol secretion. These results differ from those of Bartlett & Gibbons (1988). A possible explanation of this may be the different species of hepatocyte and experimental conditions examined. Our data were obtained in the presence of serum with an immortalized cell line. The data of Bartlett & Gibbons (1988) were from rat hepatocytes in the absence of serum. In order to compensate for the absence of serum, they used a supplemented medium containing dexamethasone (1 μ M), lactate (10 mM), pyruvate (1 mM) and oleate (0.75 mM). Using a supplemented medium Bartlett & Gibbons (1988) and Duerden *et al.* (1989) have observed a constant secretion of triacylglycerol over 72 h in the absence of insulin. However, the present results show an increased secretion of triacylglycerol with time. In the presence of insulin the absolute amount of triacylglycerol remained constant in each 24 h period of the 72 h, and the percentage of insulin-induced triacylglycerol inhibition increased with time.

Triacylglycerol secretion increased in the control cells during each 24 h period throughout the 3 days. There is increasing evidence that the rate of triacylglycerol synthesis is controlled by co-ordinate regulation of the activities of phosphatidate phosphohydrolase and diacylglycerol acyltransferase and by the availability of substrates. These two enzymes are key regulatory enzymes in triacylglycerol production, and the activity of both enzymes has been shown to increase in the presence of NEFA [Cascales *et al.* (1984) and Haagsman & Van Golde (1981) respectively]. Furthermore, the activity of phosphatidate phosphohydrolase is increased in diabetes (Pittner *et al.*, 1985).

If the present data may be extrapolated to the situation *in vivo*, they imply a consistent and clear view of the role of insulin in the regulation of triacylglycerol metabolism. The normal action of insulin after nutrition would be to increase triacylglycerol storage in liver and adipose tissue. It would do this by inhibiting hepatic export and stimulating hepatic storage of triacylglycerol. This effect is enhanced by the action of insulin to lower plasma NEFA concentrations which otherwise act powerfully to increase hepatic triacylglycerol secretion. Insulin would enhance adipose-tissue triacylglycerol storage by activating lipoprotein lipase, inhibiting hormone-sensitive lipase and accelerating fatty acid synthesis and esterification. Under abnormal conditions, increased plasma triacylglycerol concentrations may arise through insulin deficiency, which reverses the above effects, or through resistance to the action of insulin to inhibit NEFA release from adipocytes. In the latter situation, as we have shown, NEFAs are potent antagonists of the hepatic action of insulin to decrease triacylglycerol secretion, and hence increased output of triacylglycerol results. Thus the situation in which both plasma triacylglycerol and plasma insulin are increased is a result of insulin resistance and another example of the ability of NEFAs to act as insulin antagonists, described several years ago as a feature of 'the glucose-fatty acid cycle' (Randle *et al.*, 1963). The most frequently observed condition in which this type of insulin resistance occurs is obesity.

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