

## Effects of Insulin and Glucose on Very Low Density Lipoprotein Triglyceride Secretion by Cultured Rat Hepatocytes

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### Research Article

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# Effects of Insulin and Glucose on Very Low Density Lipoprotein Triglyceride Secretion by Cultured Rat Hepatocytes

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**ABSTRACT** The effect of insulin on hepatic triglyceride synthesis and secretion is controversial. Previously, we have described a cell culture system of adult rat hepatocytes that synthesize and secrete very low density lipoprotein (VLDL) triglycerides with small and irreproducible effects of insulin on triglyceride metabolism. To study the primary effects of insulin on hepatic triglyceride metabolism a method was developed utilizing fibronectin-coated culture dishes that allowed adhesion, spreading, and maintenance of hepatocytes for 2–3 d in the absence of serum and insulin. This culture system allowed mass measurements of both cellular and secreted VLDL triglycerides for long time periods after the addition of physiological concentrations of insulin to hormone-free culture medium. In the absence of insulin and after an initial 4 h in culture, the medium was replenished and triglyceride mass was measured at the end of 18-h incubations. VLDL triglyceride accumulated in the culture medium at a linear rate over this time-course with increasing accumulation as the medium glucose concentration was raised from 2.5 to 25 mM glucose ( $1.77 \pm 0.24$  to  $3.09 \pm 0.76$   $\mu\text{g}$  triglyceride/mg cell protein per h). There was no apparent significant lipolysis or hepatocellular reuptake of secreted VLDL triglycerides. In the absence of insulin cellular triglyceride levels were unchanged between 3 and 24 h in culture

while insulin (50–500  $\mu\text{U}/\text{ml}$ ) significantly increased cellular triglyceride content at all glucose concentrations tested (0–25 mM). The addition of insulin to the culture medium progressively reduced the rate of VLDL triglyceride secretion accompanied by an increase in cellular triglyceride at insulin concentrations  $> 50$   $\mu\text{U}/\text{ml}$ . Most or all of the observed increase in cell triglyceride content could in all experiments be accounted for by the insulin-induced inhibition of VLDL secretion. Incorporation of  $[2\text{-}^3\text{H}]\text{glycerol}$  into cellular and VLDL triglycerides as a function of insulin concentration was also measured. Glycerol incorporation data at 20–22 h after plating of the cells closely paralleled the insulin-induced changes in cellular and VLDL triglyceride as determined by mass analysis. The observed effects of insulin occurred at concentrations close to the physiological range and suggest that the direct hepatic effect is to suppress VLDL secretion although the net effect in vivo will clearly reflect many additional accompanying changes.

## INTRODUCTION

Cell culture techniques have led to a considerable increase in knowledge concerning the mechanism by which low density lipoproteins (LDL)<sup>1</sup> are degraded and to the discovery of the defective high affinity cellular LDL receptor in familial hypercholesterolemia (1). By other techniques, defects in the degradation of chylomicrons have also been shown to be important in type I and type III hyperlipoproteinemia (2). However, in many other forms of hyperlipoproteinemia catabolic defects have not been clearly demonstrated and overproduction of lipoproteins may be important (2). In man, since LDL is substantially, if not exclu-

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<sup>1</sup> Abbreviations used in this paper: DME medium, Dulbecco's modified Eagle's medium; LDL, low density lipoproteins; VLDL, very low density lipoproteins.

sively, the product of the lipolysis of circulating very low density lipoproteins (VLDL) (3), the rate of VLDL secretion is likely to be an important determinant of the circulating levels of LDL. Many influences, in particular nutritional and endocrine factors, are believed to be important in regulating VLDL secretion (4). However, it has proved difficult to evaluate the individual importance of any particular hormone or substrate in man or whole animals because altering the concentration of one hormone or substrate inevitably modifies the secretion and the effects of other hormones and may affect the provision of other substrates. The methods for measurement of VLDL secretion in whole animals also do not allow short-term effects to be studied since they require measurements to be made over several hours or days and even then there is disagreement about the theoretical models used in the interpretation of results (5). Studies of isolated liver tissues have thus proved attractive since the environment may be precisely defined and much information has been gained from studies of isolated perfused liver (6–10), perfused liver slices (11), and isolated hepatocytes in suspension (12). These methods, however, have the limitation that the viability of the isolated liver or hepatocytes can only be maintained for a short time during which they may still be under the influence of conditions prevailing in the animal at the time of their isolation. Also, because of the short duration of the experiments and the small amounts of tissue studied, many investigators have measured the rates of VLDL triglyceride secretion by use of isotopically labeled precursors of triglycerides which, before their secretion in VLDL triglycerides, must be diluted in cellular triglyceride pools, the size of which may vary independently from VLDL secretory rates. We have developed a method that permits maintenance of isolated hepatocytes in culture for longer periods. This allows short-term experiments using isotopes but has the major advantage that in longer experiments mass measurements of secreted VLDL triglycerides can be made.

There has been disagreement about the role of insulin in the regulation of VLDL triglyceride secretion, some authors taking the view that it may stimulate secretion (8, 11, 13–15) and others that it may be either inhibitory (10, 16–18) or without effect (6). The present series of experiments was designed primarily to resolve this issue by observing the direct effect of insulin on VLDL triglyceride secretion. Glucose alone was supplied as substrate since there has been general agreement that this will stimulate triglyceride synthesis (6, 10–12, 18, 19). Fatty acids, which are also a major substrate for triglyceride synthesis in the whole animal (9, 19), were not studied in the present experiments.

## METHODS

Arginine- and glucose-free Eagle's basal medium modified according to Dulbecco and Vogt (20) (DME medium), benzyl penicillin and streptomycin sulfate were bought from Gibco Laboratories Grand Island Biological Company (Grand Island, NY) and HEPES, L-ornithine, type 1 collagenase, pig skin gelatin, Tris-base, PPO, POPOP, and essentially fatty acid-free bovine serum albumin from Sigma Chemical Co. (St. Louis, MO). [ $^3\text{H}$ ]Glycerol and [ $^{14}\text{C}$ ]triolein were supplied by Amersham Corp. (Arlington Heights, IL). Crystalline porcine insulin was a generous gift from Dr. R. E. Chance (Eli Lilly Research Laboratories, Indianapolis, IN). The plastic culture dishes used were either 60-mm Diam (Lux Scientific Corp., Newbury Park, CA) or 100-mm Diam (Optilux, Oxnard, CA). Thin-layer chromatography was performed on 250- $\mu$  silica gel plates (Analtech, Inc., Newark, DE). For cell isolation, collagenase was dissolved in saline buffer modified from Hanks and Wallace (21) by the addition of 1 mM  $\text{Mg}^{++}$ , 15 mM glucose, 10 mM HEPES, and the exclusion of  $\text{Ca}^{++}$ . Saline for other purposes was buffered with phosphate (22). All solutions used for the isolation and culture of hepatocytes contained penicillin (500 U/ml) and streptomycin (500  $\mu\text{g}$ /ml). Fibronectin was prepared as described by Engvall and Ruoslahti (23). Citrated human plasma (300 ml) obtained from whole blood was run through a column (2.5  $\times$  60 cm) containing Sepharose 4 B (Pharmacia, Uppsala, Sweden) linked to gelatin (24). The column was successively eluted with 0.05 M Tris-HCl buffer pH 7.4, 0.1 M sodium chloride in Tris buffer, 1 M urea in Tris buffer, and finally with 5 M urea in Tris buffer. The protein peak obtained from the final elution was dialyzed repeatedly against 1 M urea in Tris buffer and stored at 4°C without further purification. Electrophoretic analysis indicated that ~90% of the protein in this fraction was fibronectin.

*Preparation of cultured hepatocytes.* Female Sprague-Dawley rats weighing 100–150 g were fed chow (Ralston Purina Co., St. Louis, MO) and water ad lib. and housed in a room with a 12-h light cycle (0700–1900 h). Hepatocytes were isolated from these animals by perfusion of the liver with collagenase solution by a previously described (26) modification of the method of Berry and Friend (27). Liver cells obtained by this method were washed initially in DME medium containing either 20% fetal calf serum or 30% neonatal calf serum at 4°C to inactivate collagenase and then two times in DME medium alone to remove serum proteins from cell surfaces. In our previous studies, isolated hepatocytes were plated in the presence of 10% fetal calf serum and 10  $\mu\text{g}$ /ml (240,000  $\mu\text{U}$ /ml) of insulin to promote cell viability and adhesion to the dishes. Under these conditions the hepatocytes could be shifted to serum-free medium within 24 h with good retention of metabolic function. However, in the 24-h period in serum-free medium enough insulin, as determined by radioimmunoassay, desorbed from cell surfaces, matrix proteins, and the plastic dish to mask potential effects of physiological levels of exogenous insulin on lipid and lipoprotein metabolism (data not shown).

In the present study the washed hepatocytes were plated onto fibronectin-coated dishes and neither insulin nor serum was required for adhesion and spreading of the cells. Under these conditions, effects of exogenous insulin on lipid and lipoprotein synthesis were consistent and reproducible. Culture dishes coated with fibronectin received either  $\sim 2.5\text{--}3 \times 10^6$  cells (60-mm dishes) in 3 ml or  $7\text{--}8 \times 10^6$  cells in 12 ml (100-mm dishes). Fibronectin-coated dishes were prepared by the addition of 20  $\mu\text{g}$  of fibronectin to 60-mm dishes containing 1.5 ml DME medium or 70  $\mu\text{g}$  of fibronectin to

100-mm dishes containing 5 ml of DME medium. Dishes were then maintained in a humidified atmosphere containing 95% O<sub>2</sub>/5% CO<sub>2</sub> at 37°C for 30 min, after which the DME medium was removed immediately before the introduction of the washed liver cells.

The time-course of the present studies also differs from that of our previous studies (25, 26). Hepatocytes were maintained on insulin- and serum-free DME medium containing 2.5 mM glucose for 4 h and then were washed with phosphate-buffered saline (22) to remove cellular debris and loosely adherent cell aggregates. The cells were incubated in DME medium containing the test concentrations of glucose and insulin for 18 h. This washing step results in the loss of 25–35% of the cellular protein mass plated at zero time. In experiments in which VLDL triglyceride mass was determined, the culture medium was changed and replaced with 2 ml (60-mm dishes) or 8 ml (100-mm dishes) of DME medium 4 h after plating of cells and then removed from the dishes for ultracentrifugation 18 h later. In experiments in which [2-<sup>3</sup>H]glycerol incorporation was measured the culture medium was also changed 4 h after plating and a pulse of [2-<sup>3</sup>H]glycerol was added to the medium 16 h later, the medium being removed for ultracentrifugation after a further 2-h incubation.

The protein content of the cultures after the 22-h incubation period was dependent upon both the collagenase and fibronectin preparations. In a series of 26 experiments utilizing three separate enzyme and fibronectin preparations, the average protein content per dish ranged between 0.7 and 1.8 mg protein. Insulin in the range 0–500 μU/ml did not affect cellular protein content when cells were plated onto the fibronectin-coated dishes. Cell number was not a useful criterion of cell viability or recovery since cells cannot be removed from the dishes as single cell suspensions after 22 h either on fibronectin-coated or serum-coated dishes. In a typical experiment the DNA and protein content of the original cell suspension (2.5 × 10<sup>6</sup> cells) was 37.8 ± 2.3 μg DNA and 2,680 ± 280 μg protein (n = 4); the dishes after the 4 h media change contained 28.0 ± 1.3 μg DNA and 1,795 ± 110 μg protein (n = 6); dishes at the termination of the 22-h incubation contained 25.7 ± 1.5 μg DNA and 1,584 ± 134 μg protein (n = 6). The DNA/protein ratios of the dishes after media changes at 4 h and at the end of the 22-h incubation were 64:1 and 62:1, respectively, suggesting that cell death and lysis was not a significant problem during the experiments. Less than 12% of the cells attached to the dish after the 4-h change were subsequently lost during the experimental test period.

Viability of hepatocytes exposed to DME medium containing either 0 or 200 μU/ml of insulin was determined by measuring the incorporation of a mixture of <sup>3</sup>H-amino acids into cellular and secreted proteins at 18–22 h after plating of the cells. The difference in protein synthesis rates in these two groups was never >15%.

**Other methods.** VLDL were isolated from the culture medium by tube-slicing after ultracentrifugation at 100,000 g for 24 h (L-2-65B ultracentrifuge, Spinco Division of Beckman Instruments Inc., Palo Alto, CA) in either an SW-41 or SW-27 rotor depending on the volume of medium. The recovery of [2-<sup>3</sup>H]glycerol-labeled VLDL added to 60-mm dishes in 2 ml of the DME medium containing VLDL triglyceride (16 μg/ml) was 77% in the absence of cells and fibronectin, 88% when fibronectin was present, and 82 ± 8% when both cells and fibronectin were present.

After removal of medium from the culture dishes phosphate-buffered saline was introduced and cells were obtained by scraping the dishes with a rubber policeman. Cells

from each dish were pelleted by low-speed centrifugation and frozen. They were later sonicated in 0.5 ml of water. Cell protein was determined by the method of Lowry (31) and was in the range of 0.7–1.8 mg/60-mm dish. Lipids from both cells and VLDL were extracted by the method of Folch (32). The lipid extract was fractionated into lipid classes by thin-layer chromatography on silica gel (33). In experiments in which [2-<sup>3</sup>H]glycerol was used, the triglyceride-containing silica gel was scraped into vials containing 10 ml of toluene containing PPO (4 g/liter) and POPOP (0.054 g/liter) and its radioactivity measured. In experiments in which triglyceride mass was to be measured, the triglyceride-bearing silica gel was scraped into glass test tubes and analyzed by a charring method using concentrated sulphuric acid (34). Free fatty acids in the medium and cells were isolated by the method of Pittman et al. (35). Rat serum albumin appearing in the medium was measured directly in aliquots of medium by immunoelectrophoresis (36) and urea by the arsenic pentoxide method (37).

**Preparation of [2-<sup>3</sup>H]glycerol-labeled VLDL.** [2-<sup>3</sup>H]-Glycerol (20 μCi) was added to each of 50 dishes of hepatocytes when the initial culture medium was replenished with fresh DME medium. Medium was collected after a further incubation for 18 h and VLDL isolated by ultracentrifugation followed by dialysis against phosphate-buffered saline. Lipids in an aliquot of VLDL were extracted and fractionated by thin-layer chromatography. The mass of triglycerides and their radioactivity were then determined in order to obtain the specific activity of the triglycerides in the [2-<sup>3</sup>H]glycerol-labeled VLDL.

## RESULTS

**Characteristics of cultured hepatocyte system.** Typical rates of production of VLDL triglycerides, albumin, and urea by hepatocytes cultured on fibronectin in the absence of insulin are shown in Table I. The observed rate of VLDL-triglyceride secretion by hepatocytes on fibronectin-coated dishes (in the absence of insulin) is 5- to 15-fold greater than that of cells previously described in this laboratory (25, 26). The absence of insulin or reduction of the insulin concentration during cell maintenance and changes in physical state of the cells when stretched out on a fibronectin matrix both may be determinants of this increased VLDL output. The albumin secretion rate

TABLE I  
Rate of Accumulation in Culture Medium

VLDL triglycerides, μg/h/mg cell protein (n = 11)	3.09 ± 0.76
Albumin, μg/h/mg cell protein (n = 4)	2.7
Urea, μmol/h/mg cell protein (n = 4)	0.7–1.0

The rate at which VLDL triglyceride, albumin, and urea produced by hepatocytes cultured in DME medium containing 25.0 mM glucose accumulated in culture medium (mean ± SEM). The duration of experiments was 18 h in all experiments except those in which urea production was measured when it was 4 h. All hepatocyte cultures were plated in insulin- and serum-free medium 4 h before the start of the experiments.

of hepatocytes on fibronectin-coated dishes is only slightly greater (2.7 vs. 2.1  $\mu\text{g}/\text{mg}$  cell protein per h) than that previously observed (25, 26). The urea production rate is  $\sim 10$ -fold greater than that reported by Dashti et al. (28) for rat hepatocyte cultures and for perfused rat liver (29); however, Briggs and Freedland (30) have shown that several components present in our culture medium, particularly ornithine, greatly enhance urea production from ammonia. The mass of VLDL triglycerides (Fig. 1) and albumin (data not shown) in the culture medium increased linearly with respect to time for at least 24 h after the first 4 h of culture. Changes in the cell triglyceride content with respect to time in culture are shown in Table II. The triglyceride content of freshly isolated hepatocytes at the time of plating is  $20.7 \pm 5.2 \mu\text{g}/\text{mg}$  cell protein ( $n = 6$ ). During the first 3 h, when VLDL-triglyceride secretion is low, the cells accumulate triglycerides in the high glucose (25 mM) medium whether insulin is present or not. In the absence of insulin the cellular triglyceride levels are essentially unchanged between 3 and 24 h after plating, while 500  $\mu\text{g}/\text{ml}$  of insulin significantly increased cellular triglyceride content between 3 and 24 h.

*Uptake of VLDL triglycerides by cultured hepatocytes.* To test the possibility that significant quantities of VLDL triglycerides secreted into the culture

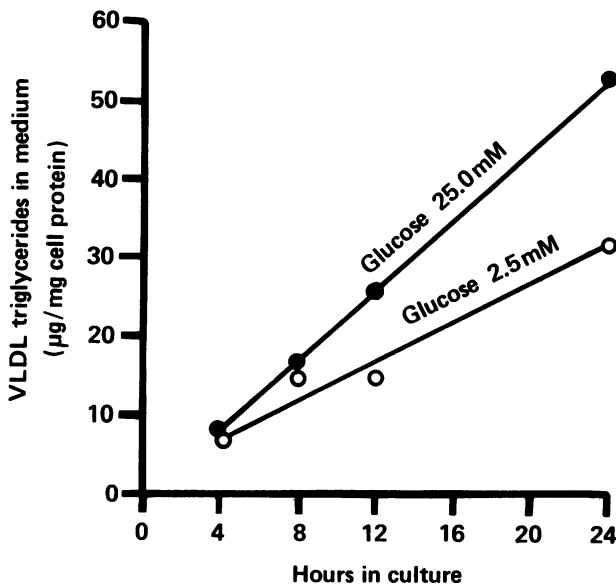


FIGURE 1 The concentration of VLDL triglycerides accumulating in culture medium containing 25.0 mM (●) or 2.5 mM (○) glucose at different times following the plating of hepatocytes into 60-mm culture dishes coated with fibronectin. Each point represents the mean of five plates. VLDL secretion rates for cells incubated in 2.5 and 25 mM glucose were significantly different at 12 and 24 h ( $P < 0.05$ ).

TABLE II  
*Changes in Hepatocyte Triglyceride Content*

Time in culture	Hepatocyte triglyceride content	
	No insulin	Insulin
<i>h</i>	$\mu\text{g}/\text{mg}$ cell protein	
1	$25.6 \pm 3.2$	$18.6 \pm 1.2$
2	$30.6 \pm 3.0$	$31.0 \pm 1.2$
3	$63.4 \pm 1.0$	$60.0 \pm 1.8$
24	$63.8 \pm 4.2$	$81.0 \pm 2.4$

The triglyceride content (mean  $\pm$  SEM) of hepatocytes cultured for different time intervals after initial plating in medium containing glucose (25.0 mM) either with or without insulin (500  $\mu\text{U}/\text{ml}$ ). At each time interval four culture dishes were harvested and analyzed for triglyceride and protein content.

medium might reenter the hepatocytes, experiments were carried out in which we measured the rate at which VLDL triglyceride labeled with  $[2\text{-}^3\text{H}]\text{glycerol}$  (Methods) became associated with cellular triglycerides of unlabeled cultured hepatocytes. The possible effects of different VLDL triglyceride concentrations and of insulin were examined. Results are shown in Table III. At the levels of VLDL triglycerides tested, which were similar to those present in hepatocyte cultures during the experiments reported here, uptake of labeled VLDL triglycerides by the cells was low. After

TABLE III  
*Uptake of VLDL Triglycerides into Cultured Hepatocytes*

Initial VLDL triglyceride concentration	VLDL triglyceride radioactivity associated with cell triglycerides per microgram cell protein at different incubation times (min)*						
	Insulin	0	15	30	60	120	240
$\mu\text{g}/\text{ml}$	$\mu\text{U}/\text{ml}$	%					
A. 16	0	0.8	0.8	1.0	1.2	1.7	1.2
B. 64	0	0.8	1.0	ND†	1.3	ND	1.1
C. 16	1,000	0.8	1.1	1.4	1.8	1.9	2.2

\* Each result is the mean of four dishes.

† Not determined.

The uptake of  $[2\text{-}^3\text{H}]\text{glycerol}$ -labeled VLDL prepared in hepatocyte culture (sp act 1,466 cpm/ $\mu\text{g}$  triglycerides) into cellular triglycerides when incubated with unlabeled cultured hepatocytes. Labeled VLDL containing 32  $\mu\text{g}$  of triglycerides was added to dishes containing 2 ml of DME culture medium. In experiment B culture medium had been preincubated with hepatocytes and contained 48  $\mu\text{g}/\text{ml}$  of triglycerides so that the final triglyceride concentration was 64  $\mu\text{g}/\text{ml}$ . In experiments A and C, DME medium had not been preincubated with cells so that the final triglyceride concentration was 16  $\mu\text{g}/\text{ml}$ . In experiment C insulin (1,000  $\mu\text{U}/\text{ml}$ ) was added to the culture. See text for full explanation.

an initial uptake of 0.8% of the radioactivity added, uptake proceeded at rates of  $\sim 0.1\%/h$  in the absence of insulin and  $0.4\%/h$  in the presence of insulin. No radioactive lipolysis products (mono- or diglycerides) were detected either in the cells or culture media. In other experiments in which smaller quantities of labeled VLDL were added to cultures ( $1\text{--}2\ \mu\text{g}/\text{ml}$  triglycerides), absolute rates of cellular uptake were similar but, of course, the fractional uptake was much larger ( $\sim 50\%$  in 24 h). It appears, then, that the reuptake mechanism was effectively saturated at the higher levels of VLDL triglycerides accumulating in the medium in our experiments of 18-h duration.

**Lipolysis of triglycerides secreted into culture medium.**  $20\ \mu\text{l}$  of [ $^{14}\text{C}$ ]triolein ( $5\ \text{mCi}/\text{mol}$ ) equivalent to  $600\ \text{nmol}$  of fatty acid was added to hepatocytes cultured in the absence or in the presence of insulin ( $1,000\ \mu\text{U}/\text{ml}$ ). The combined radioactivity of free fatty acids in medium and cells was determined after 1 h, yielding lipolytic activities of  $0.52\ \text{nmol}$  of fatty acid/h per mg cell protein in the absence of insulin and  $0.38\ \text{nmol}$  of fatty acid/h per mg cell protein in the presence of insulin. Triglyceride lipolysis was also studied using native VLDL as substrate. Medium from 24-h incubations with hepatocytes was collected with or without the addition of heparin ( $0.1$  and  $1.0\ \text{mg}/\text{dl}$ ) 2 min before removal of the medium. This was incubated with [ $2\text{-}^3\text{H}$ ]glycerol-labeled VLDL at pH 7.4 and 8.5 for 3 h. There was no evidence of any decrease of the radioactivity recovered in the medium triglycerides during the incubation. Thus, under the culture conditions described above no significant lipolytic activities were detectable.

**Effect of glucose concentration on secretion of VLDL triglycerides and on cellular triglyceride content.** The rate of secretion of VLDL triglycerides was related to the glucose concentration of the medium (Fig. 2), increasing from  $1.77\pm 0.24\ \mu\text{g}/h$  per mg cell protein (mean $\pm$ SEM) at  $2.5\ \text{mM}$  glucose to  $3.09\pm 0.76\ \mu\text{g}/h$  per mg cell protein at  $25.0\ \text{mM}$  glucose. This effect was consistently observed in all experiments.

The total triglycerides in the system (cellular and VLDL triglycerides) thus increased by  $>50\%$  suggesting that triglyceride synthesis was stimulated to that extent (assuming no change in triglyceride breakdown). The cell triglyceride content also increased in all experiments with increasing glucose concentration (Fig. 2), from a mean of  $40.5\pm 4.2\ \mu\text{g}/\text{mg}$  cell protein at  $2.5\ \text{mM}$  to  $63.0\pm 8.6\ \mu\text{g}/\text{mg}$  at  $25.0\ \text{mM}$  glucose.

**Effect of insulin on secretion of VLDL triglycerides and on cellular triglyceride content.** The rate of secretion of VLDL triglycerides was progressively reduced with increasing concentrations of insulin in the medium (Fig. 3 and Table IV, line 1). At concentrations of insulin  $>100\ \mu\text{U}/\text{ml}$ , the reduction in secretory

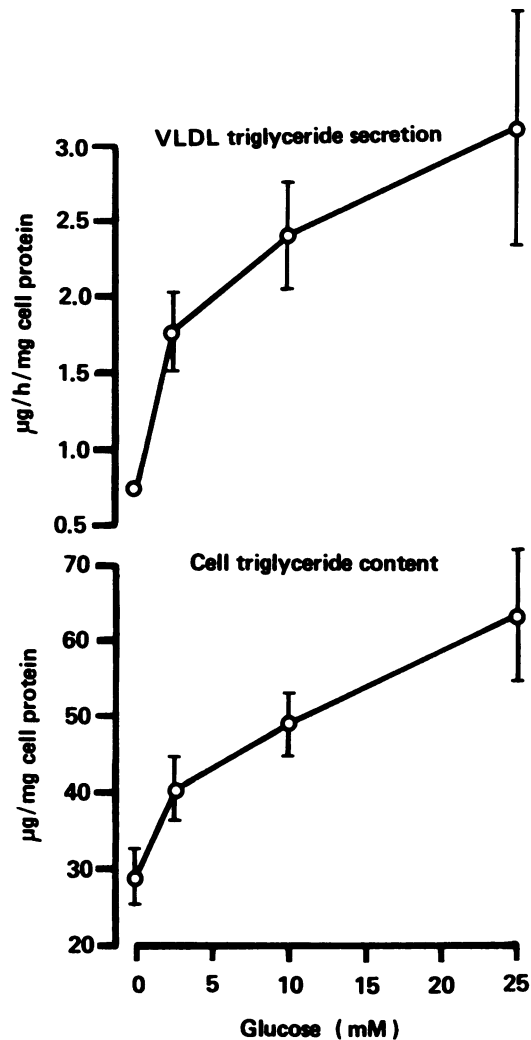


FIGURE 2 The rate of secretion of VLDL triglycerides and the cell triglyceride content of cultured hepatocytes as a function of the glucose concentration of the culture medium. Each point is the mean $\pm$ SEM derived from six experiments with the exception of VLDL triglyceride secretion in the absence of glucose that was the mean from a single experiment in which it was measured. VLDL triglyceride secretion and cell triglyceride content at 10 and 25 mM glucose were significantly different from that at 5 mM glucose using Willcoxon's paired sign test.

rate was observed in all experiments. This effect of insulin was apparent throughout the range of glucose concentrations tested ( $0\text{--}25\ \text{mM}$ ) (Fig. 4). In all experiments,  $>90\%$  of the secreted triglycerides were recovered as VLDL. Cellular triglyceride content increased in the presence of insulin in the culture medium (Fig. 3 and Table IV, line 2), consistently so at concentrations  $>50\ \mu\text{U}/\text{ml}$ . Similar absolute increments in cellular triglyceride content were produced by insulin regardless of glucose concentration (Fig. 4).

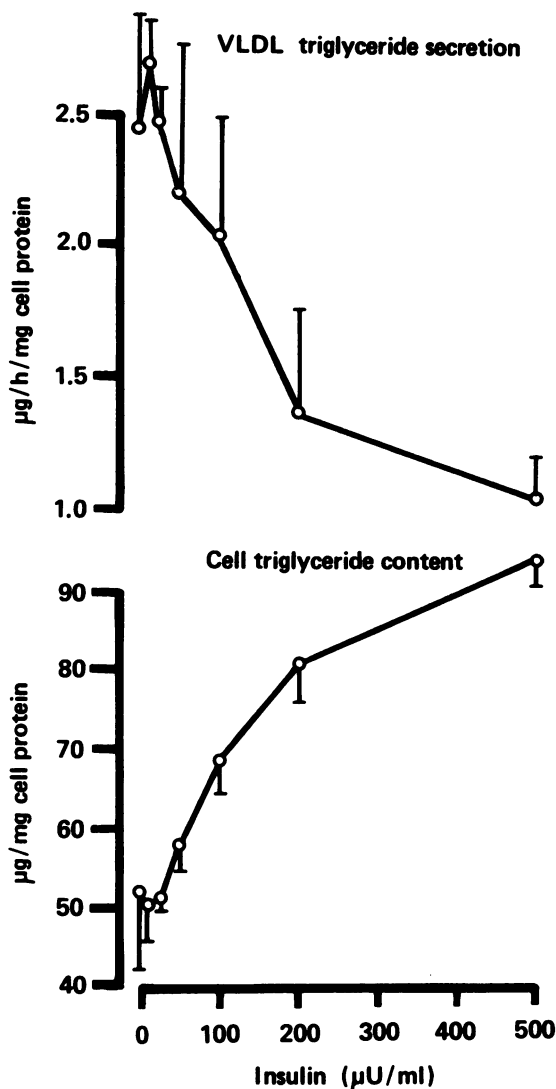


FIGURE 3 The rate of secretion of VLDL triglycerides and the cell triglyceride content of hepatocytes cultured in medium containing 25.0 mM glucose as a function of the insulin concentration of the medium. Each point represents the mean  $\pm$  SEM derived from seven experiments.

The increments in cellular triglyceride content as a function of increasing insulin concentrations are calculated in Table IV (line 4). These increments in cellular triglycerides reflected primarily or exclusively the block in VLDL-triglyceride secretion in response to insulin. The total triglyceride in the system only increased by  $\sim$ 10–15% even at the highest insulin level (Table IV, line 3) and these did not achieve statistical significance.

*Effect of insulin on the incorporation of [ $^3$ H]glycerol into cellular triglycerides and secreted VLDL triglycerides.* The radioactivity from [ $^3$ H]glycerol incorpo-

rated into cellular triglycerides increased with increasing concentrations of insulin in the culture medium up to a concentration of 1,000  $\mu$ U/ml (Fig. 5). Despite this consistent increase in cellular triglycerides labeled with  $^3$ H, there was no corresponding increase in radioactivity in VLDL triglycerides, which was overall reduced with increasing insulin concentration. There was a marked reduction in extracellular labeled VLDL triglycerides with increasing insulin concentration expressed as the fractional secretion of hepatic triglycerides in VLDL (Fig. 6). The glycerol incorporation data should not be taken to reflect the absolute rates of triglyceride synthesis in hepatocytes since the specific activity of the three-carbon pools and cell diglyceride (the precursor of triglyceride) is unknown. [ $^3$ H]Glycerol incorporation was used only as a relative index of alterations in the distribution of newly synthesized triglycerides between intracellular and extracellular pools in response to insulin.

## DISCUSSION

In the present study, it was found that glucose stimulated both triglyceride synthesis and VLDL triglyceride secretion by cultured hepatocytes. Insulin, on the other hand, inhibited VLDL triglyceride secretion and favored the accumulation of triglycerides within the cells. The accumulation of VLDL triglycerides was linear during the course of the experiments and lipolysis and cellular reuptake of VLDL triglycerides were shown to be minimal. Assuming that 15% of liver weight is protein and that the rat liver represents 5% of the total body wt, then the rates of VLDL triglyceride secretion in the present experiments (266–464  $\mu$ g/h per g wet liver depending on the concentration of glucose) were higher than in liver perfusion studies and similar to the highest reported for rat hepatocytes in suspension (38). Our reported triglyceride secretory rate is 10% higher than that reported by Patsch et al. (39) for triglyceride secretion from perfused livers of carbohydrate-induced female rats. The rate of albumin secretion (405  $\mu$ g/h per g wet liver weight) was similar to that in the intact rat (40). The use of fibronectin-coated dishes allowed hepatocytes to remain viable in culture without the requirement for insulin in the method as previously reported from this laboratory (25, 26).

The stimulatory effects of glucose on triglyceride synthesis was likely to be due to increased provision of substrate for glycerol and fatty acid synthesis. The insulin-induced increases in cellular triglyceride content appeared to be principally or exclusively due to inhibition of the secretion of VLDL triglycerides. The effect was present at both high and low concentrations of glucose, supporting the view that it was not exclu-

TABLE IV  
Effect of Increasing Insulin Concentrations on Triglyceride Synthesis and Secretion

Triglycerides ( $\mu\text{g}/\text{mg}$ cell protein)	Insulin ( $\mu\text{U}/\text{ml}$ )						
	0	10	25	50	100	200	500
1. VLDL TG accumulating in medium in 18 h	43.7	48.1	44.1	39.4	36.4	24.5	18.7
2. Cellular TG	51.9	51	50.9	58.0	68.6	80.5	93.2
3. Total (1 + 2)	95.6	99.1	95.0	97.4	105.0	105.0	111.9
4. Increment in cellular TG with insulin*	—	-0.9	-1.0	+6.1	+16.7	+28.6	+41.3

TG, triglycerides.

\* Value in line 2 less the cellular TG content in the absence of insulin, i.e., 51.9. The data are taken from the seven experiments described in Fig. 3.

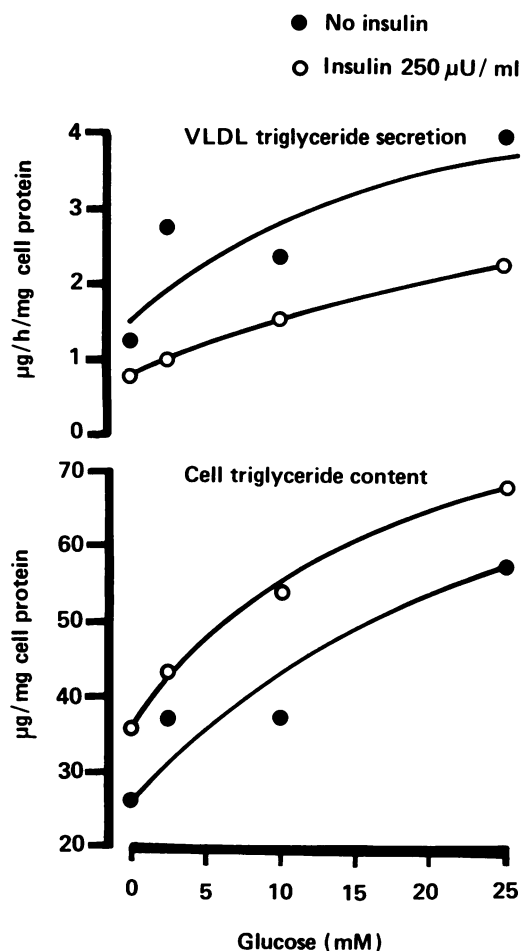


FIGURE 4 The rate of secretion of VLDL triglycerides and the cell triglyceride content of hepatocytes cultured in media containing either no insulin or 250  $\mu\text{U}/\text{ml}$  insulin as a function of the glucose concentration of the media. Each point is the mean derived from three experiments.

sively the result of increased synthesis due to enhanced glucose uptake by hepatocytes. A primary effect of insulin on hepatic triglyceride synthesis has been postulated by other workers using liver slices (18), perfused liver slices (11), isolated perfused liver (7, 8, 10), and hepatocytes in suspension (12, 19). Insulin may enhance the conversion of acetyl-CoA to fatty acids (41) and it has been suggested that it might reduce the entry of these into mitochondria for  $\beta$ -oxidation because of inhibition of acyl-carnitine transferase by malonyl-CoA produced as an intermediate in fatty acid synthesis (42). However, it has recently been reported that in hepatocytes isolated from fasted rats insulin has a minimal effect on either triglyceride synthesis or intracellular levels of malonyl-CoA and that its effect in the fed state may be due to antagonism of glucagon-induced suppression of triglyceride synthesis (43). In our experiments hepatocytes were prepared from fed rats, but since the experiments were performed between 4 and 22 h in culture glucagon effects were unlikely to have persisted. This may account for the statistically insignificant effect of insulin on triglyceride synthesis. It is also possible that the inhibition of triglyceride breakdown may have contributed in part to the insulin-induced increase in hepatocyte triglyceride content observed in our experiments.

We consistently observed an inhibitory effect of insulin on VLDL triglyceride secretion by liver cells. Our results are not in agreement with the recent report of Beynen et al. (44) who concluded that insulin increases secretion of newly synthesized VLDL from suspensions of freshly isolated hepatocytes and that this effect reflects the influence of insulin on triglyceride synthesis and not on the secretory process. This study is difficult to interpret since the isolated hepatocytes were incubated for only 2 h. As shown in Table



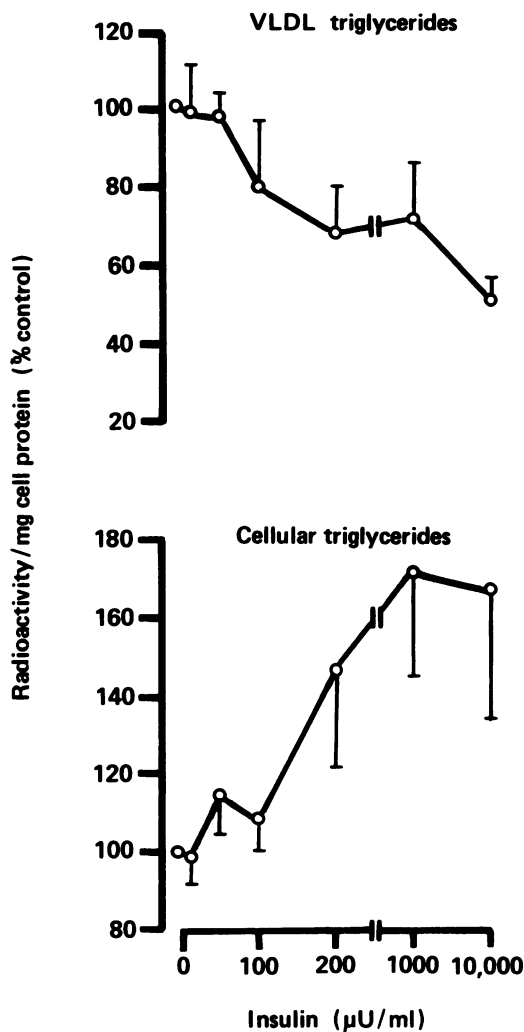


FIGURE 5 Incorporation of [<sup>3</sup>H]glycerol into cellular and VLDL triglyceride as a function of insulin concentration. Hepatocyte cultures were plated in insulin-free DME medium containing 25 mM glucose for 4 h and then incubated in the test media containing 0–10,000 μU/ml of insulin. After 16 h, 20 μCi of [2-<sup>3</sup>H]glycerol was added to each culture dish and the cells and media were harvested after an additional 2 h. The results are reported as mean±SEM for four experiments. Incorporation of [<sup>3</sup>H]glycerol into triglyceride is expressed as a percentage of that when no insulin was present (100% value).

II hepatocytes require 2–3-h incubations in nutrient-rich media before a steady-state concentration of cellular triglyceride is established. Our findings appear to be at variance with the conclusions reached by other workers using other experimental systems (7, 8, 11). However, in many of those experiments there were increases in the cellular pool of labeled triglyceride in response to insulin and this was not fully taken into

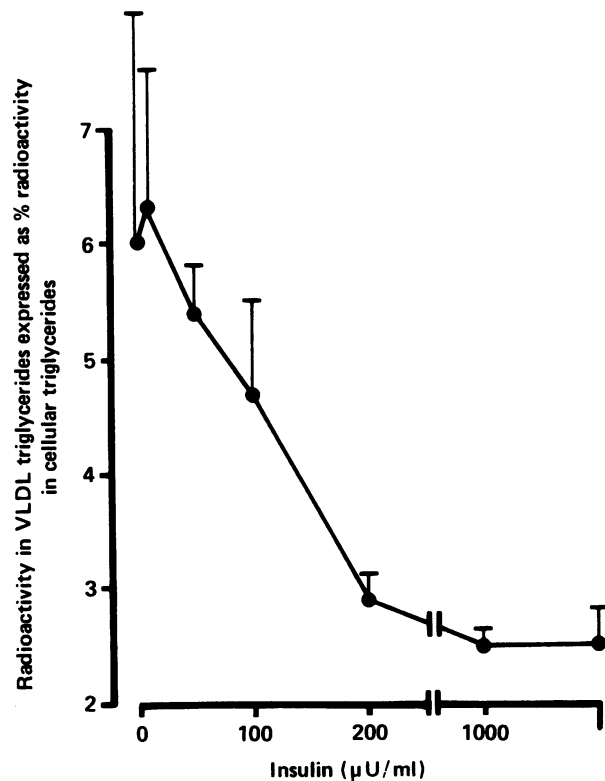


FIGURE 6 Incorporation of [<sup>3</sup>H]glycerol into VLDL triglycerides secreted into the culture medium as a percentage of that in cellular triglyceride at the end of the 2-h radioactive pulse-labeling period. The experimental conditions are described in the legend to Fig. 5. The data represents the mean±SEM of four experiments. The ratio of radioactivity in VLDL and cells at insulin concentrations at and >200 μU/ml differs significantly from that at zero insulin concentration (Student's *t* test).

account when interpreting the data. An increase in the amount of labeled triglyceride released by the cells would be expected even with no change in the true net rate of triglyceride secretion and possibly even if the mass of triglycerides released was reduced in response to insulin. In the present experiments, our finding of an inhibitory effect of insulin on VLDL triglyceride secretion was based on direct mass measurements. Heimberg and co-workers (8) found no significant change in the secretion of triglycerides by perfused livers isolated from rats treated with insulin; Nikkilä (10) reported that insulin abolished the increased incorporation of [<sup>14</sup>C]acetate into triglycerides secreted by livers isolated from rats receiving glucose either shortly before or for several days before liver perfusion.

The hypothesis that insulin stimulates VLDL triglyceride secretion has been advanced on the basis of

a number of clinical studies and experiments in whole animals. Much of the case for this hypothesis rests on a positive correlation between serum insulin concentration or integrated serum insulin response to glucose, on the one hand, and the fasting serum VLDL triglyceride concentration, on the other. The correlation is seen in such diverse circumstances as carbohydrate induction, type IV hyperlipoproteinemia, type II diabetes mellitus, and obesity (13–15). Such observations, however, do not establish that insulin is itself the cause of the increased serum triglyceride secretion. In particular, the increased flux of free fatty acids in many hypertriglyceridemic states may both increase hepatic triglyceride secretion and increase insulin secretion either directly (45) or indirectly as a consequence of fatty acid-induced insulin resistance (46). In keeping with the present finding of an inhibitory effect of insulin on VLDL triglyceride secretion there is evidence in animals (47) and man that insulin infusion can reduce serum triglyceride levels (17, 18) as do insulin-secreting tumors (10). An inverse relationship between mean daily integrated serum triglyceride concentrations and mean daily integrated serum insulin concentrations has been reported in men receiving 45% of their calories as sucrose or corn syrup and after carbohydrate induction with a diet containing 65% dietary calories as sucrose or corn syrup (16).

The suggestion that insulin may be responsible for the hypertriglyceridemia of type IV hyperlipoproteinemia (15) is unconvincing since only one-quarter of patients with this condition have an increased insulin response to glucose whereas in more than one-third the insulin response is low (48). Similarly the contention that hyperinsulinemia is responsible for increased serum triglyceride in obesity is put in question by the finding that the triglyceride secretory rate is increased not only in obese patients with high serum insulin responses to glucose but also in those with lower than normal responses (10). In diabetes mellitus increased secretion of VLDL is in part, at least, responsible for the hypertriglyceridemia (49). In dogs given insulin antiserum the most immediate effect observed in liver perfusion studies, in which radiolabeled palmitate was provided as substrate, was an increase in the secretion of labeled triglycerides (50) consistent with the present findings that insulin has an inhibitory effect on secretion. In similar experiments, in which insulin replacement therapy had been withdrawn for 2 d from pancreatectomized dogs, triglyceride secretion was found to be low (51). However, reduced triglyceride synthesis in more chronic insulin deficiency, whether a primary or secondary effect, must inevitably reduce VLDL triglyceride secretion. The reduction in serum triglyceride when insulin is administered to patients with

either type I or type II diabetes (52) and primary hypertriglyceridemia (53, 54) is consistent with an inhibitory effect of insulin on triglyceride secretion, although a major contribution to this effect may be the reduction in serum free fatty acid levels or effects on lipoprotein lipase levels.

The differential effect of insulin on hepatic triglyceride synthesis and secretion could function to limit hypertriglyceridemia postprandially, when high concentrations of both insulin and glucose reach the liver by the portal circulation and increase the synthesis of triglycerides. The inhibitory effect of insulin on VLDL secretion during absorption of meals could serve a useful function by directing triglycerides into storage pools to be secreted later when the insulin levels have fallen and triglycerides are no longer being supplied to peripheral tissues as chylomicrons secreted from the gut. Whether insulin acts directly on the mechanism by which the lipid and protein components of VLDL are assembled or on the secretory mechanism itself remains to be determined. This could be established by studying the effects of insulin on apolipoprotein B synthesis and on the composition of secreted VLDL.

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