Stimulation of Triacylglycerol Synthesis in Rat Adipocytes by Plasma Very-Low-Density Lipoproteins

By PIERRE THOMOPOULOS, MARTINE BERTHELIER, DOMINIQUE LAGRANGE,
M. JOHN CHAPMAN and MARIE-HÉLÈNE LAUDAT
I.N.S.E.R.M. U-35, Hôpital Henri Mondor, 94010 Creteil, France

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The effect of human plasma lipoproteins on lipogenesis from glucose has been studied in isolated rat adipocytes. The very-low-density lipoproteins increased lipogenesis specifically, whereas low-density lipoproteins and high-density lipoproteins were without effect. Such stimulation could be reproduced with partially delipidated very-low-density lipoproteins. Non-esterified fatty acids and glycerol were also without effect. Pretreatment of the adipocytes with trypsin did not alter the effect of very-low-density lipoprotein. The presence of Ca^{2+} was required for the full activation of lipogenesis. The synthesis of acylglycerol fatty acids and of acylglycerol glycerol were equally increased. The effect of very-low-density lipoprotein was not additive to that of insulin. It is suggested that very-low-density lipoprotein may directly stimulate lipogenesis in fat-cells, particularly in states when the lipoproteins are present at high concentration in the circulation.

Serum lipoproteins have important regulatory functions in lipid metabolism in addition to their role as lipid-transporting agents in blood. Thus extensive work with low-density lipoproteins has demonstrated their mechanism of action in the regulation of cholesterol synthesis by cultured cells (Brown & Goldstein, 1976). These effects are specific to low-density lipoproteins, the other lipoprotein classes appearing to be inactive. Very-low-density lipoproteins may, however, bind to pig tissue-membrane preparations (Bachoric *et al.*, 1976) and are capable of regulating membrane enzymes, such as the erythrocyte Mg^{2+} -ATPase (Shore & Shore, 1975) and the adipocyte adenylate cyclase (Pairault *et al.*, 1977). It is well known that the synthesis of very-low-density lipoprotein is closely related to nutritional status. Thus high-carbohydrate diets increase its production in normal and may induce hyperlipidaemia in susceptible humans (Nestel, 1973).

The aim of the present studies was to investigate the possible direct role of very-low-density lipoproteins on lipid metabolism in adipocytes. Evidence is presented that this class of lipoproteins stimulates lipogenesis from glucose in these cells.

Materials and Methods

Collagenase (from *Clostridium histolyticum*) type I (EC 3.4.24.3) (140 units/mg), trypsin (EC 3.4.21.4) (209 units/mg), soya-bean trypsin inhibitor and

Abbreviations used: Mg^{2+} -ATPase, Mg^{2+} -dependent adenosine triphosphatase; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

catalase (EC 1.11.1.6) (13 000 units/mg) were obtained from Worthington, Freehold, NJ, U.S.A. Bovine serum albumin (fraction V), fatty acids, glycerol, glucose and Hepes were purchased from Sigma, St. Louis, MO, U.S.A. Dinonyl phthalate (for g.l.c.) was from Merck, Darmstadt, Germany. Pig insulin monocomponent from Novo Research Institute, Copenhagen, Denmark, was dissolved in 10 mM-HCl and diluted appropriately in incubation medium. D-[3- ^3H]Glucose (2–5 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. PPO (2,5-diphenyloxazole) and dimethyl-POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene] were from Packard Instruments, Downers Grove, IL, U.S.A. All other chemicals and solvents were from Merck, Darmstadt, Germany, and were used without further purification.

Calcium-free bovine serum albumin was prepared by chromatography on cation-exchange resin (Chelex 100; Bio-Rad Laboratories, Richmond, CA, U.S.A.). Bovine serum albumin (15 g in 75 ml of water) was applied to a column (15 cm $\times$ 0.9 cm) of the resin equilibrated and eluted with water. The protein peak was collected and its calcium concentration measured by flame spectrophotometry.

Preparation of lipoproteins

Lipoproteins were isolated at 5°C from the sera of normolipidaemic adult donors by sequential preparative ultracentrifugation (Havel *et al.*, 1955; Chapman & Goldstein, 1976). The density intervals used for the various fractions were: very-low-density lipoproteins $d < 1.006$; low-density-lipoproteins

$d = 1.024\text{--}1.045$ and high-density lipoproteins $d = 1.080\text{--}1.210$. All fractions were dialysed for 48 h at 5°C against a solution containing 150 mM-NaCl, 0.5 mM-EDTA (sodium salt) and 5 mM-Tris/HCl at pH 7.5. They were kept at 5°C and were used within 7 days of their isolation. Lipoproteins were partially delipidated by the method of Shore & Shore (1969). After delipidation, the very-low-density lipoproteins were dialysed for 18 h at 5°C against the NaCl/EDTA/Tris buffer described above. It has been confirmed (results not shown) that this procedure extracted 90% (by weight) of the triacylglycerols and 98% (by weight) of the total cholesterol, without altering the content of phospholipid in the lipoproteins.

Techniques with fat-cells

Male Wistar rats of 90–110 g body wt. were used, and food and water were supplied *ad libitum*.

Isolated fat-cells were prepared as described by Rodbell (1964), with the following modifications: the collagenase concentration was 70 units/ml (0.5 mg/ml), whereas the buffer was supplemented with 0.5 mM-glucose and 25 mM-Hepes (a unit of collagenase is the amount required to liberate amino acids from collagen equivalent in ninhydrin colour to 1.0 μ mol of L-leucine in 18 h at pH 7.4 at 37°C). After their preparation, the cells were washed three times in the incubation buffer (see below).

To measure the incorporation of radioactivity into triacylglycerols, the fat-cells were incubated at 37°C in a final volume of 0.2 ml in stoppered polypropylene tubes, each containing 5 mg/ml of fat-cell triacylglycerol and 0.3–0.5 μ Ci of D-[3-³H]-glucose. The incubation buffer was Krebs–Ringer bicarbonate (DeLuca & Cohen, 1964) containing one-half the suggested concentration of calcium, 0.5 mM-glucose, 25 mM-Hepes and 10 mg/ml of bovine serum albumin at pH 7.4. At the end of incubation, the total cell suspension was transferred to 500 μ l polyethylene tubes containing 150 μ l of dinonyl phthalate and centrifuged immediately for 30 s in Beckman microfuge at 5234 g_{av} . (Gliemann *et al.*, 1972). After centrifugation the tubes were cut transversely at the oil layer and the cells transferred to conical glass tubes for extraction. Water (0.45 ml), n-heptane (2 ml), 2-methylpropan-1-ol (3.5 ml) and 40 mM-H₂SO₄ (1 ml) were then added and fat-cell triacylglycerol was extracted by mixing on a vortex-type mixer for 20 s. The mixture was allowed to stand for 5 min to facilitate phase separation (Biggs *et al.*, 1975). A portion (1.5 ml) of the upper (n-heptane) phase was transferred to 7 ml of scintillation fluid (PPO, 5 g; POPOP, 0.3 g; toluene, 1 litre) and the radioactivity measured in an Intertechnique liquid-scintillation counter.

Characterization of the radioactive compounds in adipocytes

In the present experiments, centrifugation on dinonyl phthalate was omitted and the cell suspension was extracted in chloroform/methanol (2:1, v/v.). Portions of the chloroform phase were examined by t.l.c. on fluorescein-containing silica gel plates (Merck), in the presence of unlabelled lipid controls, in light petroleum (b.p. 40–65°C)/diethyl ether/16 M-acetic acid (75:24:1, by vol.). After drying in air, the lipid spots were made visible under u.v. light [360 nm (3600 Å)] and scraped directly into 7 ml of scintillation fluid. Thus the distribution of the radioactivity in triacylglycerol, phospholipid and cholesterol was measured.

Additional portions of the chloroform phase were saponified by the method of Jacobs *et al.* (1973). The effectiveness of the saponification was verified in experiments run in parallel with known amounts of unlabelled trioleoylglycerol. The products of saponification were examined by t.l.c. in the system described above. The radioactivity of the non-esterified fatty acids was measured directly in the organic phase after saponification (Jacobs *et al.*, 1973). The radioactivity in acylglycerol glycerol was obtained by subtraction of the above value from the total triacylglycerol radioactivity. The accuracy of this calculation was verified by measuring the radioactivity in acylglycerol glycerol in the aqueous phase of the saponification, after the appropriate correction for quenching.

The concentration of triacylglycerol in the fat-cells was assayed by the method of Biggs *et al.* (1975). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Stimulation of lipogenesis by very-low-density lipoprotein

At 37°C, the incorporation of radioactive glucose into lipids was linear for a 75-min period in the absence or presence of very-low-density lipoproteins (results not shown); the incubation time used in the subsequent experiments varied from 30 to 60 min.

The effect of very-low-density lipoprotein reached a plateau at 200 μ g of protein/ml, the concentration required for half-maximal stimulation being 25 μ g of protein/ml (Fig. 1).

Low-density lipoprotein and high-density lipoprotein displayed a small effect without a concentration-dependence from 25 to 1000 μ g of protein/ml for the former and from 25 to 1500 μ g of protein/ml for the latter. Similar results were obtained with three different fresh preparations of low-density-lipo-

Fig. 1. *Effect of various concentrations of very-low-density, low-density and high-density lipoproteins on lipogenesis*
Lipogenesis from D-[3-³H]glucose was measured as described in the Materials and Methods section, in the presence of the indicated concentrations of very-low-density lipoproteins (●), low-density lipoproteins (▲), or high-density lipoproteins (△). Results are means of triplicate determinations. The results shown are taken from one experiment considered typical of a series of five.

Fig. 2. *Effect of native and partially delipidated very-low-density lipoproteins on lipogenesis*
Lipogenesis from D-[3-³H]glucose was measured as described in the Materials and Methods section, in the presence of the indicated concentrations of native (△) or partially delipidated very-low-density lipoprotein (▲). Results are means of triplicate determinations. The results shown are taken from one experiment considered typical of a series of four.

Table 1. *Conversion of glucose into the fatty acid and glycerol moieties of triacylglycerol*
Isolated rat adipocytes were incubated with D-[3-³H]glucose in the presence or absence of very-low-density lipoprotein (100 μg of protein/ml) and insulin (25 μunits/ml). After 60 min the triacylglycerols were extracted, saponified and radioactivity measured (see the Materials and Methods section). Results are expressed as nmol of glucose incorporated/h per mg of fat-cell triacylglycerol; they are means ± s.d. of quadruplicate determinations. The results presented are taken from one experiment considered typical of a series of three.

Additions	Glucose incorporated into:	
	Fatty acids	Glycerol
None	0.20 ± 0.02	0.80 ± 0.02
Very-low-density lipoprotein	0.42 ± 0.06	1.58 ± 0.06
Insulin	2.04 ± 0.12	1.96 ± 0.12

proteins and high-density lipoproteins and with several preparations of very-low-density lipoproteins.

Distribution of radioactivity after saponification of fat-cell triacylglycerols

To investigate the nature of the lipids containing the radioactivity, the chloroform/methanol extract of the fat-cells obtained at the end of a 60-min incubation was separated by t.l.c. (see the Materials and Methods section). More than 90 % of the radioactivity migrated with the triacylglycerols (results not shown), either in the basal or in the stimulated state. In the same experiments, the chloroform/methanol extract

was saponified (see the Materials and Methods section) and the radioactivity measured in the fatty acid and glycerol moieties of the triacylglycerols. Table 1 shows that the distribution of the radioactivity was not modified in the stimulated state by the very-low-density lipoprotein, whereas insulin clearly increased the incorporation of the radioactivity into fatty acids.

Evaluation of the components of the very-low-density lipoprotein molecule mediating the stimulation of lipogenesis

Formation of non-esterified fatty acids during the incubation of very-low-density lipoproteins with adipocytes could stimulate lipogenesis from glucose (Saggerson, 1972). To investigate this possibility, fat-cells were incubated in the absence and presence of non-esterified fatty acids, 0.05–1 mM (palmitate, oleate, stearate, arachidonate) or glycerol, 0.02–0.3 mM. No increase in the rate of incorporation of glucose into triacylglycerols was observed (results not shown). Furthermore, by using partially delipidated very-low-density lipoprotein, which lacked 90 % (by weight) of its triacylglycerol and 98 % (by weight) of its cholesterol (see the Materials and Methods section), the stimulation of lipogenesis was unaltered (Fig. 2).

Studies on the mechanism of action of very-low-density lipoprotein

Comparison of the effects of maximally active concentrations of insulin and very-low-density lipoproteins evaluated either separately or together showed that their actions were not additive (Table 2).

The stimulation of glucose metabolism in fat-cells

Table 2. *Lack of additivity of the effect of very-low-density lipoprotein and insulin*

Isolated rat adipocytes were incubated with D-[3-³H]glucose in the presence or absence of maximally active concentrations of very-low-density lipoproteins (200 µg of protein/ml) or insulin (50 µunits/ml) or both. The incorporation of radioactivity into fat-cell triacylglycerol was measured as described in the Materials and Methods section. Results are expressed as nmol of glucose incorporated/h per mg of fat-cell triacylglycerol; they are means ± s.d. of quadruplicate determinations. The results shown are taken from one experiment considered typical of a series of four.

Additions	Incorporation of D-[3- ³ H]glucose into triacylglycerols
None	1.02 ± 0.04
Very-low-density lipoprotein	2.12 ± 0.07
Insulin	4.62 ± 0.22
Insulin + very-low-density lipoprotein	4.53 ± 0.21

Table 3. *Effect of very-low-density lipoproteins on lipogenesis of trypsin-treated adipocytes*

Isolated rat adipocytes (15 mg of fat-cell triacylglycerol/ml) were incubated for 15 min at 37°C in the presence of trypsin (1 mg/ml). Enzyme activity was terminated by the addition of soya-bean trypsin inhibitor (1 mg/ml). In the controls, soya-bean inhibitor was added before trypsin. Lipogenesis from D-[3-³H]glucose was subsequently measured as described in the Materials and Methods section, and, in addition, in the presence or absence of insulin (25 µunits/ml) or very-low-density lipoproteins (100 µg of protein/ml). Results are expressed as nmol of glucose incorporated/h per mg of fat-cell triacylglycerol; they are means ± s.d. of quadruplicate determinations. The results shown are taken from one experiment considered typical of a series of three.

Additions	Glucose incorporated	
	Controls	Trypsin-treated cells
None	1.15 ± 0.06	1.09 ± 0.05
Insulin	4.37 ± 0.23	1.07 ± 0.05
Very-low-density lipoprotein	2.30 ± 0.12	2.18 ± 0.09

by insulin may be abolished by treatment of the cells with trypsin (Kono, 1969; El-Allawy & Gliemann, 1972) and is strongly dependent on the presence of Ca²⁺ [see Kissebah *et al.* (1975) for a review].

Trypsin treatment of the adipocytes did not abolish the effect of very-low-density lipoprotein on lipogenesis (Table 3), whereas incubation in Ca²⁺-free medium was clearly inhibitory (Table 4).

Finally, Livingston *et al.* (1977) provided evidence

Table 4. *Effect of Ca²⁺ on the stimulatory action of very low-density lipoprotein*

Lipogenesis from D-[3-³H]glucose was studied as described in the Materials and Methods section in either a Ca²⁺-free or a normal medium and in the presence or absence of insulin (25 µunits/ml) or very-low-density lipoproteins (100 µg of protein/ml). Results are expressed as nmol of glucose incorporated/h per mg of fat-cell triacylglycerol; they are means ± s.d. of quadruplicate determinations. The results shown are taken from one experiment considered typical of a series of three.

Additions	Glucose incorporation	
	Normal medium (1.7 mM-Ca ²⁺)	Ca ²⁺ -free medium
None	1.25 ± 0.06	1.18 ± 0.07
Insulin	4.00 ± 0.24	2.36 ± 0.18
Very-low-density lipoprotein	2.50 ± 0.16	1.65 ± 0.10

Table 5. *Effect of catalase on the stimulatory action of very-low-density lipoprotein*

The stimulatory effect of insulin (25 µunits/ml), very-low-density lipoprotein (100 µg of protein/ml) and H₂O₂ (10 µM) on lipogenesis from D-[3-³H]glucose was measured in the absence or presence of catalase (20 µg/ml). Results are expressed as nmol/h of glucose incorporated per mg of fat-cell triacylglycerol; they are means ± s.d. of quadruplicate determinations. The results shown are taken from one experiment considered typical of a series of three.

Additions	Glucose incorporated	
	Controls	Catalase
None	1.04 ± 0.05	1.02 ± 0.05
Insulin	4.57 ± 0.24	4.39 ± 0.21
Very-low-density lipoprotein	2.08 ± 0.13	2.04 ± 0.14
H ₂ O ₂	4.16 ± 0.21	1.03 ± 0.04

that the insulin-like effects of polyamines on fat-cells result from their oxidative deamination, with formation of H₂O₂, a compound that has been shown to stimulate glucose uptake. A similar mechanism, which might be possible with highly complex macromolecules such as very-low-density lipoproteins, has been eliminated by incubating the fat-cells in the absence and presence of catalase and by comparing the results with those obtained with H₂O₂ (Table 5). Catalase did not alter the effect of very-low-density lipoproteins on lipogenesis.

Discussion

The present studies provide evidence for the increased incorporation of glucose into triacylglycerol in fat-cells by serum very-low-density

lipoproteins. This effect is specific to very-low-density lipoproteins and is not induced by low-density or high-density lipoproteins. Partially delipidated very-low-density lipoproteins, which mainly consist of phospholipid-apolipoprotein complexes, stimulate lipogenesis in a similar way. Before considering the mechanism and possible consequences of such a phenomenon, it is important to demonstrate that the stimulatory effect is directly related to very-low-density lipoprotein. Thus such large and complex molecules might generate metabolites that could mediate the observed effects. Vernon (1977) observed an inhibition by fatty acids of lipogenesis from [$1\text{-}^{14}\text{C}$]acetate in adipose tissue. Conversely, Saggerson (1972) has demonstrated that palmitate may stimulate incorporation of glucose into acylglycerol glycerol and fatty acids in normal fat-cells. This effect was dependent on the palmitate and glucose concentrations. Thus one could hypothesize that, in our experiments, the very-low-density lipoprotein might be degraded by the adipocyte lipoprotein lipase and that non-esterified fatty acids might be formed, which could then account for the stimulated lipogenesis. However, under our experimental conditions, a wide range of concentration of various fatty acids could not reproduce this stimulation. Besides, our results with partially delipidated lipoproteins are not consistent with this hypothesis.

H_2O_2 (and other oxidants) can stimulate glucose uptake in isolated fat-cells (Czech *et al.*, 1974). Production of H_2O_2 may mediate the insulin-like effect of thiols (Czech *et al.*, 1974) and polyamines on fat-cells (Livingston *et al.*, 1977). Our data with catalase demonstrate that such a phenomenon does not take place during the stimulation of lipogenesis from glucose by very-low-density lipoprotein. Finally, mild proteolysis (Kono & Barham, 1971) has been reported to stimulate glucose utilization in fat-cells and Krishnaiah & Weigandt (1974) have proposed that lipoproteins may display trypsin-like activity. Trypsin treatment of the adipocytes, sufficient to inhibit the action of insulin, did not interfere with the stimulation of lipogenesis by very-low-density lipoprotein (Table 3). This is not consistent with a proteolysis-mediated mechanism of lipoprotein action. Furthermore, it suggests that their effect is independent of insulin and of non-suppressible insulin-like activities of serum (El-Allawy & Gliemann, 1972).

Thus our data demonstrate that the stimulation of the incorporation of glucose into fat-cell triacylglycerol by very-low-density lipoprotein is directly related to the native lipoprotein molecule. It may be asked which part of the lipoprotein molecule is responsible for this effect; our results can reply at least in part to this question. Thus the cholesterol and triacylglycerol components are unlikely candidates, since partially delipidated very-low-density lipo-

proteins, which were deficient in these lipids, were as active as the native molecules. Furthermore, apolipoproteins A and B would not appear to be involved in the stimulatory phenomenon, since the native lipoproteins in which they predominate, i.e. high-density and low-density lipoproteins respectively, similarly lacked activity. The phospholipid and apolipoprotein components (primarily apolipoproteins C), either alone or in an associated form, therefore appear to be responsible for the observed effect. Future studies, with purified preparations of apolipoproteins C, phospholipids or both may clarify this problem. The final answer to this question thus necessitates reconstitution of the molecule from its components (for a review for the apolipoprotein components of very-low-density lipoproteins, see Morriset *et al.*, 1975).

The exact mechanism of the stimulation of lipogenesis by very-low-density lipoproteins remains unknown. It does require the presence of Ca^{2+} , as does the stimulatory action of insulin (Table 4). However, although insulin stimulates preferentially the synthesis of the fatty acid moiety of triacylglycerol, as previously reported (Moody *et al.*, 1974), very-low-density lipoprotein equally increases the incorporation of glucose into fatty acids and glycerol (Table 1). The lack of additivity of the effects of maximal concentrations of insulin and very-low-density lipoproteins thus demonstrates that some of the steps involved in their action are shared.

The net effect of very-low-density lipoprotein on lipogenesis has not been studied in our experiments. However, Sutherland *et al.* (1976) observed that basal lipolysis is not modified by these lipoproteins. This suggests that the observed stimulation of the incorporation of glucose into adipocyte triacylglycerol reflects a real accumulation of triacylglycerol in the cell.

The regulation of fatty acid metabolism in adipocytes differs from that in other tissues. Thus free fatty acids inhibit their own biosynthesis in fibroblasts (Jacobs & Majerus, 1973) and in liver (Mayes & Topping, 1974). Recently, Lakshmanan *et al.* (1977) demonstrated that triacylglycerol-rich lipoproteins also inhibit fatty acid synthesis in liver cells. On the contrary, in adipocytes, non-esterified fatty acids may stimulate their own synthesis (Saggerson, 1972) and inhibit lipolysis (Fain & Shepherd, 1975). Our studies show that very-low-density lipoproteins may also stimulate lipogenesis from glucose in these cells.

The significance *in vivo* of this finding remains to be evaluated. In our model, rat adipocytes were incubated with human very-low-density lipoproteins; although this is a heterologous system, it is noteworthy that rat fat tissue behaves in most situations like that of man. Some investigators have been unable to demonstrate fatty acid synthesis from glucose in human fat-cells (Galton, 1968). However, subsequent

work clearly showed that, depending on the cell size and the experimental conditions, conversion of glucose into acylglycerol fatty acids takes place *in vitro* in human adipose tissue (Goldrich *et al.*, 1969; Smith, 1971). In addition, some similarity exists in the chemical composition (Mills & Taylaur, 1971) and in the nature of the protein components of rat and human very-low-density lipoproteins (Bersot *et al.*, 1970; Swaney *et al.*, 1977). Our results should however be confirmed in an homologous system.

The concentration of very-low-density lipoprotein that elicited a half-maximal response in our experiments was 25 µg/ml, but a significant effect was observed at 10 µg/ml. The protein concentration of very-low-density lipoprotein in normal human plasma is about 100 µg/ml (Mills & Taylaur, 1971). The concentration of lipoproteins in extravascular fluids is not known with accuracy. Studies of lymph fluid and of pleural effusion (Reichl *et al.*, 1973; Rerabek, 1977) suggest that the extravascular very-low-density lipoprotein concentration is 1.3 % of that of serum. Thus the stimulation of lipogenesis that we report is observed with concentrations of very-low-density lipoproteins that are above the calculated physiological range. However, their concentration in pathological states may often overlap with those used in our studies.

Insulin is the main and most potent stimulatory agent of lipid synthesis in adipocytes. Its postprandial concentration in plasma increases and allows for the storage of part of the nutrients as fat-cell triacylglycerol. The very-low-density lipoprotein concentration, like that of insulin, tends to reflect the nutritional status of the body, being increased during high carbohydrate intake and obesity. In such situations, these macromolecules might contribute to the stimulation of lipogenesis in a less acute way than does insulin.

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