Very-low-density-lipoprotein secretion by isolated hepatocytes of fat-fed rats

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The very-low-density-lipoprotein secretion rate of isolated hepatocytes obtained from rats fed a high-fat diet was half that of cells from control animals. In fat-fed rats, the initial cellular uptake of [$1\text{-}^{14}\text{C}$]oleate *in vitro* was decreased by 25%, its esterification to triacylglycerols and phospholipids by 50% and its incorporation into very-low-density-lipoprotein triacylglycerols by 70%. Exogenous oleate was not the main precursor of very-low-density lipoproteins in these animals. Lipogenesis, a minor source of very-low-density lipoproteins with the control diet in our experimental conditions, was inhibited by 84% after fat-feeding. A short-term inhibition of lipogenesis *in vitro* did not result in a decrease in very-low-density-lipoprotein secretion rate. The results suggest that fat-feeding decreased availability of exogenous as well as endogenous fatty acids for synthesis of very-low-density lipoproteins.

The stimulatory effect of sucrose-containing diets on the secretion rate of VLD lipoproteins is well established (Schonfeld & Pfeiffer, 1971; Davis *et al.*, 1979), but the effects of high-fat diets have been studied less extensively (Reaven & Reaven, 1974). Fat-feeding in rats results in elevated plasma concentrations of ketone bodies, chylomicrons and non-esterified fatty acids (de Gasquet *et al.*, 1977; Kalopissis *et al.*, 1979), whereas VLD lipoprotein secretion rate is decreased by 40% (Kalopissis *et al.*, 1980). The reasons for this decrease in VLD-lipoprotein secretion *in vivo* are not clear since non-esterified fatty acids are known to enhance VLD-lipoprotein secretion rates by the perfused liver (Heimberg *et al.*, 1969) and by cultured hepatocytes (Dashti *et al.*, 1980). Furthermore, the role of the intestine, which normally contributes 10–20% to total plasma VLD lipoproteins (Windmueller & Levy, 1968; Holt & Dominguez, 1980), is unknown in the fat-fed rats, thus making the exact contribution of the liver in the process more difficult to assess. Therefore, in the present study, VLD-lipoprotein secretion rates of isolated hepatocytes from control and fat-fed rats were measured to ascertain the extent to which the liver of fat-fed rats is responsible for the decreased rates of VLD-lipoprotein secretion observed *in vivo*.

Abbreviation used: VLD lipoproteins, very-low-density lipoproteins ($d \leq 1.006 \text{ g/ml}$).

Experimental

Animals, diets and chemicals

Male Wistar rats (EVIC CEBA, Blanquefort, France) weighing 100 g were fed either a control or a high-fat diet for 3 weeks before study. The control diet contained (w/w): 24% casein, 63% starch, 6% salt mixture, 1% vitamins, 4% cellulose, 1% corn oil and 1% peanut oil. The high-fat diet contained (w/w): 31% casein, 26% starch, 7% salt mixture, 1.5% vitamins, 4.5% cellulose, 1.5% corn oil, 1.5% peanut oil and 27% pig lard. Rats fed on both diets had the same caloric intake but their weight gain was greater with the high-fat diet [body weights $\pm$ S.E.M. were: 224.8 ± 6.33 ($n = 9$) g and 248.6 ± 6.21 ($n = 11$) g, at $P < 0.05$, for the control and the fat-fed rats respectively]. $^3\text{H}_2\text{O}$ and [$1\text{-}^{14}\text{C}$]oleic acid (sp. radioactivity 52.3 Ci/mol) were obtained from C.E.A., Gif-sur-Yvette, France. Defatted bovine serum albumin and sodium oleate were from Sigma. Collagenase and triacylglycerol kit no. 124966 were from Boehringer.

Preparation of hepatocytes

After anaesthesia of the rats with sodium pentobarbital (4.8 mg/100 g rat) hepatocytes of control and fat-fed animals were prepared between 10.30 and 12.30 h by the method of Seglen (1972) modified as follows. The liver was perfused *in situ* for 2–3 min with a medium gassed with O_2 and containing

140 mM-NaCl/6.7 mM-KCl/5.5 mM-glucose/20 mM-Tricine {*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine} (pH 7.7), at a flow rate of 70 ml/min. Then 30 mg of collagenase and 74 mg of CaCl₂, in a re-circulating perfusion volume of 100 ml, were perfused for 6 min at the same flow rate. The cells thus obtained were washed three times with the initial medium and centrifuged (70 g for 45 s). Hepatocytes were suspended in 15 ml of Krebs-Henseleit (1932) saline containing 2% (w/v) dialysed serum albumin, pH 7.4, and kept for 15–20 min under O₂/CO₂ (19:1) at 37°C in a shaking water bath. Routine tests indicated that 80% of the hepatocytes excluded Trypan Blue. The wet weight of 1 × 10⁶ cells was approx. 7.7 mg and the dry weight 1.9 mg.

Incubation conditions

For the measurement of VLD-lipoprotein secretion, cellular uptake and esterification of [1-¹⁴C]-oleate, 20 × 10⁶ viable cells were incubated in Krebs medium containing 20 mM-glucose, 0.7 mM-oleate and 1 μCi of [1-¹⁴C]oleic acid, in a final volume of 4 ml; incubation times were 0, 5, 10, 15, 20, 30, 45, 60 and 120 min. Total lipogenesis (from all sources of C₂ units) was measured after 60 and 120 min incubations of 20 × 10⁶ viable cells/flask with 0.5 mCi of ³H₂O and 20 mM-glucose in a final volume of 3 ml of Krebs medium with or without oleate (1 mM).

Chemical determinations

The content of the incubation flasks was centrifuged at 4°C (1000 g·min). The lipids in the cell-free supernatant were extracted by the method of Dole (1956) and separated by t.l.c. on silica-gel plastic plates. The solvent system consisted of light petroleum (b.p. 35–60°C)/diethyl ether/acetic acid (170:30:1, by vol.). The spots were revealed by I₂ vapour, cut out and their radioactivity measured. Non-esterified fatty acids were present at all time points, whereas triacylglycerols only from 20 min incubations onwards. These medium triacylglycerols probably corresponded to secreted VLD lipoproteins. VLD lipoproteins were also obtained by ultracentrifugation of the supernatant at 1.4 × 10⁸ g·min after 0, 60 and 120 min incubations in duplicate. After lipid extraction (Folch *et al.*, 1957), the VLD-lipoprotein triacylglycerols were determined enzymically and their radioactivity measured.

The sedimented hepatocytes were washed extensively with cold NaCl (0.9%); cell lipids were extracted (Folch *et al.*, 1957), washed twice to remove aqueous contamination (Denton & Randle, 1967) and analysed by t.l.c. as above.

Fatty acid synthesis was measured by the method of Brunengraber *et al.* (1973) after extensive washing of the lipidic phase with methanol/water

(1:1, v/v) to remove all traces of ³H₂O. It was expressed as nmol of synthesized palmitate using the calculation proposed by Windmueller & Spaeth (1966, 1967):

$$\frac{(\text{d.p.m. of } ^3\text{H in fatty acids})}{(\text{d.p.m./nmol of } ^3\text{H}_2\text{O}) \times 13.3}$$

Results were analysed with the Student's *t* test and expressed as means ± s.e.m. for the numbers of animals in parentheses.

Results and discussion

VLD-lipoprotein secretion

After incubating the hepatocytes from control and fat-fed rats with oleate (0.7 mM), secretion of VLD-lipoprotein triacylglycerols (nmol/10⁶ cells) was decreased by 50% with the high-fat diet (Table 1). [1-¹⁴C]Oleate incorporation in the VLD-lipoprotein triacylglycerols was even more decreased, by 70%, in these animals (Table 1). In our experimental conditions this incorporation represents VLD-lipoprotein precursor fatty acids of exogenous origin. The labelled triacylglycerols appeared in the incubation medium from 20 min onwards and then increased linearly until the end of the experiment (Fig. 1*b*). That these triacylglycerols were secreted as VLD lipoproteins is supported by two observations. (a) The time of appearance in the medium (20 min) corresponds to the time needed for VLD-lipoprotein synthesis and secretion (Glaumann *et al.*, 1975). (b) Their secretion was inhibited when colchicine (0.1 mM) was added to the incubation medium (A.-D. Kalopissis, S. Criglio & X. Le Liepvre, unpublished work).

The large decrease of VLD-lipoprotein secretion rate (50%) and exogenous oleate incorporation in the VLD-lipoprotein triacylglycerols strongly suggests that the decreased rate of VLD-lipoprotein secretion reported *in vivo* (Kalopissis *et al.*, 1980) is attributable to a decreased synthesis by the hepatocyte.

[1-¹⁴C]Oleate uptake

Oleate disappearance from the incubation medium obeyed first-order kinetics. Initial oleate uptake (up to 45 min) by the hepatocytes was lower with the high-fat diet (Fig. 1*a*) as revealed by the different slopes of the regression lines [−0.0409 ± 0.00189 (*n* = 3) for the control rats and −0.0303 ± 0.00124 (*n* = 3) for the fat-fed rats; *P* < 0.001]. In the first 45 min, hepatocytes from control rats took up approx. 25% (i.e. 13 nmol/10⁶ cells) more oleate than hepatocytes from fat-fed rats. Afterwards, this difference was gradually abolished as oleate concentration fell; at 120 min both hepatocyte preparations had taken up similar amounts of

Table 1. Effects of the high-fat diet on VLD-lipoprotein secretion, [$1\text{-}^{14}\text{C}$]oleate incorporation in VLD-lipoprotein triacylglycerols and lipogenesis of isolated rat hepatocytes

For experimental details and calculations, see the Experimental section. All results are means $\pm$ s.e.m. for the numbers of animals in parentheses. VLD-lipoprotein secretion is expressed as nmol of VLD-lipoprotein triacylglycerol/ 10^6 cells, oleate incorporation in VLD-lipoprotein triacylglycerols as nmol of [$1\text{-}^{14}\text{C}$]oleate/ 10^6 cells and lipogenesis as nmol of synthesized palmitate/ 10^6 cells. Statistically significant differences between fat-fed and control rats are indicated by * $P < 0.05$; ** $P < 0.01$ (no oleate added) and † $P < 0.001$ (0.7 mM-oleate).

Type of diet	[Oleate] (mM)	VLD-lipoprotein secretion			Oleate incorporation		Lipogenesis	
		Incubation time (min)	60	120	60	120	60	120
Control	None	8.76 $\pm$ 0.04 (3)	22.3 $\pm$ 1.7 (3)	—	—	—	5.1 $\pm$ 0.9 (3)	12.5 $\pm$ 2.6 (3)
Control	0.7	19.4 $\pm$ 2.8 (4)	33.4 $\pm$ 3.0 (4)	—	15.2 $\pm$ 2.0 (5)	—	—	—
Control	-1	9.9 $\pm$ 1.6 (3)	23.0 $\pm$ 3.9 (3)	—	—	—	1.27; 1.46	5.33; 7.01
High-fat	None	—	—	—	—	—	0.85 $\pm$ 0.13 (3)**	2.02 $\pm$ 0.31 (3)*
High-fat	0.7	8.3 $\pm$ 2.3 (4)†	19.1 $\pm$ 3.4 (4)†	4.95 $\pm$ 0.66 (6)†††	10.3 $\pm$ 0.9 (6)†††	—	—	—

oleate (95 and 90 nmol/ 10^6 cells for the control and the fat-fed rats respectively).

It seems unlikely that the 25% lower initial oleate uptake with the high-fat diet is responsible for the 50% decreased VLD-lipoprotein secretion rate. Besides, this exogenous fatty acid was not the major precursor of VLD-lipoprotein triacylglycerols in our experimental conditions. This point is discussed below.

Hepatic esterification of [$1\text{-}^{14}\text{C}$]oleate

In the hepatocytes, [$1\text{-}^{14}\text{C}$]oleate was rapidly esterified to triacylglycerols and phospholipids (Figs. 1c and 1d). At no time point could we detect intracellular non-esterified fatty acids by t.l.c. The label increased until 45 min in both fractions, the triacylglycerols being quantitatively the major esterified product of oleate. Then a plateau was attained, apparently because oleate concentration was greatly diminished in the incubation medium; also part of the triacylglycerols (and presumably of the phospholipids) was secreted as VLD lipoproteins from 20 min onwards.

With the high-fat diet, oleate esterification to triacylglycerols and phospholipids was decreased by 50%. As this decrease was measured at all incubation times, it cannot be only due to the lower initial oleate uptake; a greater oxidation of oleate in the fat-fed rats seems a more probable explanation. With the control diet, approx. 30% of the oleate taken up by the cells is estimated to be oxidized, as the sum of the label recovered in the cell plus medium triacylglycerols and phospholipids amounts to 70% of oleate uptake at all incubation times between 20 and 120 min. By contrast, with the high-fat diet the sum of the recovered label at the same time points corresponds only to 40% of oleate uptake, the remaining 60% being oxidized. This rough calculation indicates a 2-fold stimulation of fatty acid oxidation in the fat-fed rats, as expected (Krebs & Hems, 1970).

Lipogenesis and VLD-lipoprotein secretion

Lipogenesis was strongly inhibited by the high-fat diet (Table 1), as expected (Hill *et al.*, 1960). To establish an eventual effect of lipogenesis on VLD-lipoprotein secretion, lipogenesis was inhibited in hepatocytes from control rats *in vitro* by addition of oleate (1 mM) to the incubation medium. Lipogenesis was inhibited by 75% at 60 min and only by 50% at 120 min (Table 1). However, this inhibition of fatty acid synthesis achieved *in vitro*, unlike in the fat-fed rats, was transient and already partly relieved at the second incubation hour when medium oleate concentration was very low. VLD-lipoprotein secretion was similar in incubations with or without oleate (1 mM), indicating that it was not influenced directly by the transient inhibition of lipogenesis. One cannot

Fig. 1. Effect of the high-fat diet on $[1\text{-}^{14}\text{C}]$ oleate uptake and incorporation in cell plus medium triacylglycerols and phospholipids

Isolated hepatocytes from control (●) and fat-fed (■) rats were incubated with $[1\text{-}^{14}\text{C}]$ oleate (0.7 mM) up to 120 min. For experimental details see the Experimental section. Each point is the average for three experiments. (a) shows the percentage of $[1\text{-}^{14}\text{C}]$ oleate remaining in the incubation medium; equations of the regression lines of medium oleate (y) on time (x) (from 0 to 45 min) were: $\log y = (4.66 \pm 0.05) - (0.0409 \pm 0.0019)x$ for the control rats and $\log y = (4.69 \pm 0.03) - (0.0303 \pm 0.0012)x$ for the fat-fed rats; the slopes were significantly different from each other ($P < 0.001$). (b) shows $[1\text{-}^{14}\text{C}]$ oleate incorporation in medium triacylglycerols (y), secreted as VLD lipoproteins; the equations of the regression lines on time (x) were: $y = (-5.60 \pm 1.25) + (0.27 \pm 0.02)x$ for the control rats and $y = (-0.95 \pm 0.81) + (0.08 \pm 0.01)x$ for the fat-fed rats; the slopes were significantly different from each other ($P < 0.01$). (c) and (d) show hepatic $[1\text{-}^{14}\text{C}]$ oleate esterification to phospholipids and triacylglycerols respectively.

exclude that a greater and longer inhibition of fatty acid synthesis, as after fat-feeding, could have had an effect on VLD-lipoprotein secretion.

However, the lack of stimulation of the VLD-lipoprotein secretion rate of control hepatocytes by 1 mM-oleate is surprising in itself, this secretion being even lower (by 30%) than that measured in the presence of 0.7 mM-oleate. Recently Åkesson (1980) also found no stimulation of VLD-lipoprotein secretion by 1 mM-oleate, whereas he measured near-maximal stimulations with 0.25, 0.50 and 0.75 mM-oleate in the incubation medium of hepatocytes from rats fed a stock diet. Apparently, the fatty acid concentration influences the subsequent VLD-lipoprotein secretion.

Origin of the VLD-lipoprotein-triacylglycerol fatty acids

With the control diet, a total of 100 nmol of fatty acids was secreted at 120 min as VLD-lipoproteins (calculated by the triacylglycerol content of VLD lipoproteins multiplied by a factor of 3, as 1 nmol of

sn-glycerol esterifies 3 nmol of fatty acid). At this time, 33 nmol of exogenous oleate was incorporated in the VLD lipoproteins and a maximum of 12.5 nmol could be provided by lipogenesis (i.e. without oleate in the medium). Apparently, half of the VLD-lipoprotein-triacylglycerol fatty acids ($100 - 33 - 12.5 = 54.5$ nmol) originates from hydrolysis of the endogenous pre-existing pool of liver triacylglycerols. With the high-fat diet, the same calculation yields a total of 57 nmol of fatty acids secreted at 120 min as VLD lipoproteins, 10 nmol of which was the exogenous oleate and 2 nmol could be provided by lipogenesis. The remaining 45 nmol of fatty acids ($57 - 10 - 2 = 45$ nmol) originates also from hydrolysis of the endogenous triacylglycerol pool. These calculations suggest that, in fat-fed rats, endogenous triacylglycerols were less utilized, resulting eventually in their accumulation in the liver. In fact, these animals had a 2.5-fold greater hepatocyte triacylglycerol content than the control rats [77.8 ± 12.10 ($n = 6$) and 33.1 ± 5.20 ($n = 6$) nmol/ 10^6 cells respectively; $P < 0.01$].

In conclusion, incorporation of exogenous fatty acids into hepatic VLD lipoproteins is greatly decreased in fat-fed rats and so is their hepatic esterification to triacylglycerols and phospholipids and, to a lesser extent, their cellular uptake. Furthermore, lipogenesis is strongly inhibited after fat-feeding and the endogenous triacylglycerol pool contributes less to the VLD-lipoprotein triacylglycerols than in the control animals. Thus it appears that fatty acids from exogenous as well as from endogenous sources are not readily available as substrates for VLD-lipoprotein triacylglycerols in the fat-fed rats, suggesting that fatty acid availability at the assembly point could be a limiting factor of VLD-lipoprotein secretion. These metabolic changes, together with the known stimulation of fatty acid oxidation in these animals, lead to a decrease of re-exportation of fatty acids in the plasma as hepatic VLD lipoproteins, and to an acceleration of their catabolism in the liver. Such adaptative processes subsequent to fat-feeding are probably of prime importance in controlling plasma lipid concentrations.

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