Decreased secretion of very-low-density lipoprotein triacylglycerol and apolipoprotein B is associated with decreased intracellular triacylglycerol lipolysis in hepatocytes derived from rats fed orotic acid or *n*–3 fatty acids

Abdel-Malek HEBBACHI, Marilia C. L. SEELAENDER, Paul W. BAKER and Geoffrey F. GIBBONS*

Oxford Lipid Metabolism Group, Metabolic Research Laboratory, Nuffield Department of Clinical Medicine, University of Oxford, Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE, U.K.

Hepatocytes from rats fed a chow (control) diet or from rats fed a chow diet supplemented with either orotic acid (OA; 1 %, w/w) or fish oil (FO; 20 %, v/w) were maintained in culture for periods up to 48 h. During the first 24 h period, the low rates of output of very-low-density lipoprotein (VLDL)-associated triacylglycerol (TAG) and apolipoprotein B (apoB) in hepatocytes from the FO- and OA-fed animals were associated with significantly lower rates of intracellular TAG lipolysis and re-esterification. Most of the VLDL TAG secreted was mobilized via lipolysis of the intracellular TAG pool, but the proportion of VLDL TAG secreted via this route in cells from the FO-fed and OA-fed animals was decreased compared with that in the control-fed animals' cells. In the presence of exogenous oleate the

inhibitory effect of OA feeding on VLDL apoB and TAG secretion persisted in the derived hepatocytes for up to 48 h following isolation. However, when oleate was absent no inhibitory effect on the secretion of TAG and apoB was observed between 24 and 48 h. Under these conditions the rate of intracellular TAG turnover returned to normal. The initial inhibitory effect of FO feeding on VLDL TAG and apoB secretion did not persist in the derived hepatocytes between 24 h and 48 h of culture in the presence of exogenous oleate. Although intracellular TAG lipolysis and VLDL TAG and apoB secretion rates appear to be positively correlated, a causal relationship has not been conclusively established.

INTRODUCTION

Very-low-density lipoprotein (VLDL), synthesized and secreted by the liver, is the body's most important source of endogenously synthesized triacylglycerol (TAG). The assembly of VLDL is a complex process that occurs in the secretory apparatus of the hepatocyte and involves the transfer of TAG to apolipoprotein B (apoB) (for reviews, see [1–4]). The question as to whether this process is completed in a single step [5,6] or is achieved by a sequential addition of TAG [7–10] has not yet been resolved. Nor has the major intracellular site of bulk TAG transfer to apoB yet been unequivocally identified, and, in rat liver, apoB₄₈ may differ from apoB₁₀₀ in this regard [11,12]. What is clear, however, is that TAG transfer shows an obligatory requirement for a microsomal TAG transfer protein (MTP), which consists of a heterodimer of protein disulphide isomerase and a specific 98 kDa subunit [13–15]. Although many dyslipidaemias are characterized by an excessive rate of production and secretion of hepatic VLDL [1,16,17], little is known about how these processes are regulated at the molecular level. For instance, short-term studies in HepG2 appeared to rule out transcriptional regulation of apoB [18–20]. Nor does MTP appear to be a regulatory target in the short-to-medium term [21], although long-term dietary studies have revealed changes in MTP mRNA levels [22,23]. In contrast, studies of post-translational events in the intracellular metabolism of apoB have suggested that variations in the

secretion of apoB-containing lipoproteins are associated with changes in pre-secretory degradation of apoB [5,24] and that this may result from changes in the availability of neutral lipid for lipoprotein assembly [25,26]. Other studies *in vitro*, however, using animal models of VLDL assembly and secretion have failed to establish a direct relationship between rates of TAG synthesis and VLDL apoB secretion [27–29]. TAG newly synthesized from exogenous fatty acids is a poor direct precursor of VLDL, and the most important immediate pool of TAG for VLDL assembly is that found in hepatocellular cytosolic storage droplets [30,31], from which it is mobilized via a process of lipolysis and re-esterification [31–34]. It is possible that the rate at which this process operates may determine the effective availability of TAG at the site(s) of VLDL assembly in the lumen of the secretory apparatus of the cell. Some evidence for a correlation between TAG-rich lipoprotein secretion and intra-hepatic TAG lipolysis has been obtained in rat hepatocytes [32] and in HepG2 cells [35], although such a relationship is not observed in the presence of insulin [32].

Certain dietary treatments are known to reduce the rate of hepatic VLDL secretion, and the primary purpose of the present work was to determine whether these treatments also affect hepatocellular TAG lipolysis. For instance, it has been known for many years that dietary orotic acid (OA) results in an impaired secretion of VLDL [36,37]. More recently, it has been conclusively established that the hypolipidaemic effect of dietary

Abbreviations used: VLDL, very-low-density lipoprotein; apoB, apolipoprotein B; TAG, triacylglycerol; PL, phospholipid; ER, endoplasmic reticulum; FO, fish oil; OA, orotic acid; LF, low fat; MTP, microsomal TAG transfer protein.

* To whom correspondence should be addressed.

n-3 fatty acid consumption is due primarily to a decreased secretion of hepatic VLDL [38–40]. In neither of these cases does the abnormally low secretion of VLDL result from a shortage of hepatic TAG, since TAG accumulates in the liver under these circumstances [37,41–43]. Rather, it appears that there may be an impediment in the mobilization and transport of TAG to the intracellular site(s) of VLDL assembly. The inhibitory effect of dietary OA and *n*-3 fatty acids on the secretion of VLDL is maintained, at least for short periods, in isolated liver preparations derived from donor animals. In view of the possible utility of these *in vitro* models for studying the regulation of VLDL assembly, a second objective of the present work was to establish the period of time over which the above inhibitory effects were retained in cultured hepatocytes derived from the donor animals. As far as we are aware, this is the first such longer-term study in cultured hepatocytes.

MATERIALS AND METHODS

Reagents

Waymouth's medium and Dulbecco's PBS were purchased from Gibco (Paisley, Scotland, U.K.). [9,10(*n*)-³H]Oleate, [1-¹⁴C]oleate, [2-³H]glycerol and glycerol [¹⁴C]trioleate were obtained from Amersham International (Aylesbury, Bucks., U.K.). BSA (fatty acid-free), oleate, glycerol, dexamethasone, anti-sheep IgG (whole molecule)-alkaline phosphatase conjugate and OA were obtained from Sigma (Poole, Dorset, U.K.). 3-Hydroxybutyrate dehydrogenase (*Rhodobacter spheroides*), TAG assay kit (GPO-PAP kit) and anti-(human apoB) serum were obtained from Boehringer-Mannheim (Lewes, Sussex, U.K.). Fish oil (FO; MaxEPA) was obtained from Seven Seas (Marfleet, Hull, U.K.).

Preparation of OA- and FO-containing diets

The control animals were fed on a commercially available pelleted low-fat (LF) chow diet (rat and mouse Breeding diet No 3) from Special Diet Service (Witham, Essex, U.K.), which contained 51.2% (w/w) carbohydrate, 4.3% (w/w) fat, 22.3% (w/w) protein, 4.5% (w/w) fibre and 7.7% (w/w) ash. To prepare OA- and FO- (MaxEPA) containing diets, chow diet (800 g) was powdered and mixed with de-ionized water (400 ml). OA (8 g) or MaxEPA (200 ml) was then added. After thorough mixing the diets were re-pelleted. The diet containing OA was dried at 40 °C in the dark. That containing FO was dried at room temperature in a well-ventilated area. The diets were kept frozen under an atmosphere of nitrogen at -20 °C before use. All diets contained the following antioxidants: α -tocopherol (110 mg/kg), vitamin E (120 mg/kg), vitamin C (10 mg/kg) and β -carotene (200 μ g/kg). After preparation of the diets, extraction of lipids (see below) followed by *trans*-methylation of the fatty acids and quantitative analysis by gas-chromatography indicated that these antioxidants were sufficient to prevent oxidation of the *n*-3 fatty acids during preparation. There was no decrease in the peak heights for eicosapentaenoic acid or docosahexaenoic acid relative to those for other fatty acids after preparation of the diet containing MaxEPA.

Maintenance of animals and preparation of hepatocyte cultures

In studies with the FO-containing diet, rats were fed either the control (chow) or the FO diet for 14 days. Studies with the OA diet used rats of the same age and initial body weights as those used above, but feeding was begun one week later and lasted for 7 days only. The temperature was kept constant at 20 $\pm$ 2 °C, and

Table 1 Characteristics of rats fed chow alone or chow supplemented with OA or FO

Rats were randomly allocated into four groups. One group was fed the LF (chow) diet for 2 weeks, and one group was fed the FO-supplemented diet for 2 weeks. The remaining two groups were fed the LF diet for the first week. One of these groups was then fed the OA-supplemented diet for the second week. The remaining group continued on the LF diet. The initial body weights of the latter groups correspond to the weights recorded at the beginning of the final week, during which the OA diet or the LF diet was consumed. The initial body weights of the FO-supplemented group and those of the corresponding chow-fed group were recorded at the start of the 2-week feeding period, i.e. 1 week before the initial weights of the OA-fed and the corresponding chow (LF)-fed groups were recorded. There were five animals in each group and the values represent the means $\pm$ S.E.M. Values marked *, ** and *** are significantly different ($P < 0.05$, $P < 0.01$, $P < 0.001$ respectively; paired *t* test) from those of the corresponding LF-fed controls.

Diet ...	Chow	Supplemented
OA-supplemented diet		
Initial body weight (g)	215.60 $\pm$ 9.5	207.20 $\pm$ 7.2
Final body weight (g)	258.70 $\pm$ 8.6	250.00 $\pm$ 5.2
Weight gain (g/day)	6.12 $\pm$ 0.3	6.10 $\pm$ 0.3
Liver weight (g)	11.44 $\pm$ 0.6	14.75 $\pm$ 1.1*
Food intake (g/day)	29.21 $\pm$ 0.7	31.40 $\pm$ 0.9
Hepatic cellular TAG (μ g/mg of protein)	14.47 $\pm$ 1.0	41.90 $\pm$ 7.9***
Plasma TAG (mg/dl)	42.80 $\pm$ 1.0	10.90 $\pm$ 2.1**
Plasma ketone bodies (μ mol/dl)	245.00 $\pm$ 2.0	213.00 $\pm$ 27.0
FO-supplemented diet		
Initial body weight (g)	164.5 $\pm$ 9.5	145.50 $\pm$ 7.2
Final body weight (g)	244.8 $\pm$ 8.2	232.80 $\pm$ 8.9
Weight gain (g/day)	6.04 $\pm$ 0.2	6.63 $\pm$ 0.2
Liver weight (g)	10.40 $\pm$ 0.6	13.75 $\pm$ 1.3**
Food intake (g/day)	26.00 $\pm$ 0.5	25.00 $\pm$ 1.4
Hepatic cellular TAG (μ g/mg of protein)	16.10 $\pm$ 2.7	48.00 $\pm$ 5.8***
Plasma TAG (mg/dl)	38.00 $\pm$ 1.2	40.90 $\pm$ 7.4
Plasma ketone bodies (μ mol/dl)	255.00 $\pm$ 20	558 $\pm$ 116*

the room was artificially lighted with a 10 h light/14 h dark cycle. Food and water were given *ad libitum*. Food was replaced daily, with any uneaten portions discarded. Food intake and body weights were recorded daily (Table 1). Hepatocytes were prepared from all groups at 10:00 h. At any one time, two rats were used simultaneously for hepatocyte preparation: one control and one either OA fed or FO fed. Hepatocytes from all groups were prepared under sterile conditions and plated out (at a density of 0.65×10^6 cells/ml, 3 ml/dish) into collagen-coated dishes [44] as a suspension in Waymouth's medium containing 10% (v/v) fetal calf serum and supplements (3.6 mM glutamine, 0.36 mM alanine, 0.45 mM serine, 90 000 units/l penicillin and 90 000 units/l streptomycin) [45]. The viability of isolated hepatocytes, as assessed by Trypan Blue exclusion, varied from 89 to 94%. At this stage, hepatocytes were treated in one of two ways, depending upon the experimental objectives. In experiments designed to determine cellular TAG lipolysis and re-esterification, cellular TAG was pre-labelled, during the plating period, by the addition of [³H]glycerol (0.25 mM; 2.93×10^4 d.p.m./nmol) and [¹⁴C]oleate (0.375 mM; 1.96×10^3 d.p.m./nmol, bound to BSA). After 4 h, the medium containing the labelled substrates was removed and the cell monolayer was washed twice with PBS. Two dishes of cells from each experimental group were harvested for measurement of the ³H:¹⁴C ratio of the cellular TAG. This was the 'initial' cell TAG ratio. To the remaining dishes in each group was added Waymouth's medium (3 ml) with the above supplements, dexamethasone (1 μ M), lactate (10 mM) and pyruvate (1 mM), but lacking serum and labelled substrates. Cells were cultured under these conditions for 6, 12, 24 or 48 h, after which the medium was removed (for isolation of VLDL) and the cells were harvested. Cells cultured for 48 h had the

medium replaced after 24 h and were cultured under identical conditions for a further 24 h. The $^3\text{H}:^{14}\text{C}$ ratios of the VLDL and cellular TAG were determined after each period of culture (see below).

In all remaining experiments, after plating, hepatocytes from each of the dietary groups were cultured in the serum-containing Waymouth's medium as above but without the labelled TAG precursors. After 4 h, the medium was removed and replaced with the serum-free medium as described above. Cells were cultured for 6, 12, 24 or 48 h in the absence or presence of [^3H]oleate (initial concentration 0.75 mM; 9.78×10^3 d.p.m./nmol). Cells cultured for 48 h had the medium removed after 24 h and replaced with medium of identical composition (i.e. with or without labelled oleate). In some instances, cells were cultured in the absence of oleate for the first 24 h and in the presence of labelled oleate (0.75 mM) for the next 24 h. The medium of cells cultured in the absence of labelled oleate contained albumin only at an equivalent concentration (0.5%).

Harvesting of cells and isolation of VLDL particles

At the end of each culture period, the medium was removed from the dishes and the VLDL-containing fraction was isolated by ultracentrifugation as described previously [45]. The cell monolayer was washed, scraped from the dishes and pelleted by centrifugation [45].

Analytical methods

The protein content of cultured hepatocytes was determined using the method of Lowry and co-workers [46]. Lipids from cells and VLDL fractions were extracted by the method of Folch and co-workers [47]. In those experiments in which a [^{14}C]-labelled TAG precursor had not been used, glycerol [^{14}C]trioleate was added to samples as internal standard. This was used to calculate the loss of TAG after extraction. Cellular and VLDL TAG was determined enzymically using the GPO-PAP kit obtained from Boehringer-Mannheim. Different classes of labelled lipids [TAG, phospholipids (PL) and fatty acids] were separated by TLC using a solvent system (n-hexane/diethyl ether/glacial acetic acid, 70:30:1.6, by vol.), visualized using Rhodamine 6G and assayed for [^{14}C] and [^3H] radioactivity by scintillation counting. Ketone bodies in the rat plasma were determined enzymically [48]. Oleate bound to BSA (essentially fatty acid-free) was prepared by the method of Van Harken and co-workers [49] to give a stock solution of 15 mM oleate. Total apoB (apoB₁₀₀ and apoB₄₈) was assayed by an ELISA method developed in our laboratory, using rat VLDL apoB as a standard [50].

Calculations

The proportion of VLDL TAG secreted via lipolysis of intracellular TAG was calculated from the relative loss of [^3H]glycerol in the VLDL TAG (as determined by the $^3\text{H}:^{14}\text{C}$ ratio) secreted by cells in which the intracellular TAG had been pre-labelled with [^{14}C]oleate and [^3H]glycerol as described previously [32,35]. Calculations of the effects of dietary OA and $n-3$ fatty acids on the turnover (i.e. lipolysis and re-esterification) of intracellular TAG were also carried out as previously described [32]. It should be emphasized that after the initial pre-labelling of cellular TAG, during the subsequent periods over which measurements were made neither fatty acids (labelled or unlabelled) nor glycerol (labelled or unlabelled) were added to the medium. This procedure ensured that subsequent changes in the specific radioactivity of either the TAG glycerol or of the TAG fatty acid was

not due to new synthesis from exogenously added substrates. Thus any decline in the specific radioactivity of cellular or VLDL ^3H -labelled TAG after pre-labelling cellular TAG with [^3H]glycerol resulted either from the synthesis of unlabelled TAG *de novo*, via the acetyl-CoA carboxylase pathway, or from lipolysis followed by re-esterification with unlabelled glycerol (or, more precisely, unlabelled glycerophosphate) produced as a result of cellular metabolism. The contribution of unlabelled TAG synthesis *de novo* may be determined by the decrease in the specific radioactivity of the [^{14}C]oleate moiety of the pre-labelled TAG. Thus any excess decline in the specific radioactivity of [^3H]glycerol-labelled TAG compared with that of [^{14}C]oleate-labelled TAG will reflect, specifically, the amount of unlabelled glycerol that has entered the TAG pool via lipolysis of the original doubly labelled TAG followed by re-esterification of the product fatty acids. In practice this relative decrease in specific radioactivity of [^3H]TAG is equivalent to the decrease in the $^3\text{H}:^{14}\text{C}$ radioactivity ratio of cellular or VLDL TAG compared with the initial values immediately after pre-labelling. For instance, it may be calculated that the relative decline in the specific radioactivity of the [^3H]TAG (or the final $^3\text{H}:^{14}\text{C}$ ratio) is $\chi\%$ of the initial cell $^3\text{H}:^{14}\text{C}$ value. The amount of unlabelled glycerol that must have entered the TAG pool to achieve this decrease is $[(100/\chi) - 1]$ pools. This is equivalent to the fractional rate of lipolysis (or turnover). As the total mass of the cellular TAG pool is known, this calculation may be used to estimate the total mass of cellular TAG that has undergone lipolysis followed by re-esterification (i.e. fractional turnover multiplied by pool size). For example, in Figure 4A the initial mean $^3\text{H}:^{14}\text{C}$ ratio of the cellular TAG in the control cells was 3.90. After 6 h of culture, some of this material had been secreted as VLDL. At this time, the $^3\text{H}:^{14}\text{C}$ ratio of the sum of the remaining cellular TAG and the secreted VLDL TAG was 2.60, or 66.6% of the original value. Using the formula above, this suggested that 0.5 pools of unlabelled glycerol had entered the original TAG pool during this period. In other words, the fractional turnover (lipolysis/re-esterification) of the TAG pool was 0.50. Multiplication of this value by the pool size at this time (158.7 $\mu\text{g}/\text{mg}$ of protein) gives a value that represents the total TAG turnover.

Statistical analysis

All values are presented as the means $\pm$ S.E.M. of several independent experiments. Significant differences were obtained using a paired Student's *t* test.

RESULTS

VLDL lipid and apoB secretion in hepatocytes from donor animals fed OA

Groups of animals fed either the control or the OA-supplemented diets had similar daily food intake, and body weight gain during the feeding period (Table 1a). However, the hepatic TAG content of OA-fed rats after 7 days of feeding was about three times more than that of normal rats, whereas the plasma TAG levels in OA-fed rats decreased substantially compared with those of normal rats (Table 1a). The liver weight of OA-fed rats was about 22% higher than that of normal rats.

Secretion of VLDL-associated TAG, PL and total apoB (apoB₁₀₀ and apoB₄₈) by hepatocytes prepared from OA-fed rats was significantly inhibited compared with that observed in hepatocytes from the control animals (Figures 1 and 2). The extent of inhibition of each component of VLDL was similar (about 50%), and this difference persisted at each time point up to 48 h. There did not appear to be any diminution of the

Figure 1 The effect of OA feeding on VLDL TAG secretion

Hepatocytes from chow- and OA-fed rats were cultured for 6, 12, 24 and 48 h in the presence of [3 H]oleate, as described in the Materials and methods section. At the end of each culture period, the medium was removed and VLDL was isolated. Cells cultured for 48 h had the medium changed at 24 h. [3 H]Oleate was again added. Values for 48 h are the cumulative totals of TAG secreted during the periods 0–24 h and 24–48 h. TAG mass represents the total amount of TAG secreted (derived from endogenous and exogenous sources) (A). Newly synthesized TAG represents the amount of TAG derived from exogenous sources (B). Values are means $\pm$ S.E.M. of six independent experiments. Values marked * and ** are significantly different ($P < 0.05$ and $P < 0.01$ respectively) from those of the corresponding chow-fed animals. □, Chow-fed rats; ■, OA-fed rats.

inhibitory effect of OA feeding over this period of time. Both the total mass of VLDL TAG (which includes that derived from pre-existing intracellular TAG) and VLDL secretion of newly synthesized TAG (estimated from the incorporation of [3 H]oleate) were inhibited. The TAG:apoB ratio of VLDL particles secreted by treated cells was not significantly different from that of normal cells throughout the culture period, indicating that both groups of cells secreted VLDL particles of the same size. However, in each group the TAG:apoB ratio increased with time, suggesting that larger particles were secreted at longer time periods (Table 2).

The above results suggested that when exogenous oleate was present in the culture medium, VLDL secretion remained suppressed for up to 48 h in hepatocytes from OA-fed rats. By contrast, when oleate was absent, cells from the control-fed animals secreted very little VLDL TAG and apoB during the 24–48 h culture period (Table 3). Under these conditions, cells from the OA-fed animals secreted more VLDL TAG and apoB than those from the control-fed animals. These results may be explained by the more rapid initial depletion of the cellular pool

Figure 2 The effect of OA feeding on VLDL PL and total apoB (apoB₁₀₀ and apoB₄₈) secretion

Hepatocytes from chow- and OA-fed rats were cultured as described in the legend to Figure 1. At the end of each culture period, the medium was removed and VLDL was isolated. Newly synthesized PLs represent the amount of PLs derived from exogenous oleate (A), and total apoB represents the amount of apoB₁₀₀ and apoB₄₈ secreted (B). Values are means $\pm$ S.E.M. of six independent experiments. Values marked * and ** are significantly different ($P < 0.05$ and $P < 0.01$ respectively) from those of the corresponding chow-fed animals. □, Chow-fed rats; ■, OA-fed rats.

of TAG in the hepatocytes from the control-fed rats than from the OA-fed rats. In the absence of continued TAG synthesis from exogenous oleate, cellular TAG became rate limiting for VLDL assembly much earlier than that in the cells from the OA-fed rats.

Dietary OA effects on hepatocellular lipid metabolism and TAG lipolysis

Hepatocytes from the OA-fed animals synthesized rather less TAG and phospholipids from exogenous oleate than did those from the control-fed animals (Figure 3), and this difference became more pronounced with increasing time of culture. The increase in lipid synthesis after 24 h in both types of cell preparation resulted from the replenishment of the medium containing fresh [3 H]oleate at this time. The differences in lipid synthesis were not due to any differences in the rate of uptake of [3 H]oleate from the cell medium between the two cell types (results not shown). When the total mass (measured enzymically) of TAG synthesized during the 48 h culture period was determined by summation of the cellular and VLDL TAG (after subtraction of the endogenous TAG present in the cell at zero time), this amount was greater in the hepatocytes from the chow-

Table 2 Time-dependent changes in the TAG:apoB ratios of secreted VLDL

Hepatocytes from rats fed chow (LF) alone or chow supplemented with OA were cultured for periods of 6, 12, 24 or 48 h as described in the Materials and methods section. Cells cultured for 48 h had the medium changed at 24 h, and fresh oleate was added. The values represent the means $\pm$ S.E.M. of six independent hepatocyte preparations within each group. Values marked * and ** are significantly different ($P < 0.05$ and $P < 0.01$ respectively; paired *t* test) from the corresponding values at 6 h.

Dietary treatment	Time of culture (h)	TAG:apoB ratio
Chow (LF)	6	15.6 $\pm$ 2.7
	12	17.3 $\pm$ 1.9
	24	23.2 $\pm$ 3.5*
	48	28.4 $\pm$ 2.2**
OA-supplemented	6	16.9 $\pm$ 2.8
	12	19.4 $\pm$ 2.5
	24	20.7 $\pm$ 6.5
	48	26.2 $\pm$ 5.8*

Table 3 Effect of OA feeding on VLDL output during prolonged culture of hepatocytes

Hepatocytes from animals fed either a chow diet or a chow diet supplemented with 1% (w/w) OA were cultured either for 24 h or for 48 h in the presence or absence of oleic acid. After 24 h, the medium was replaced with fresh medium with or without oleic acid. Measurements of VLDL apoB and VLDL TAG output were made during the periods 0–24 h and 24–48 h. The values below represent the means $\pm$ S.E.M. of six hepatocyte preparations in each group. Values marked * and ** are significantly different ($P < 0.05$ and $P < 0.01$ respectively) from the corresponding values in the hepatocytes from the chow-fed animals.

Diet	Period of culture (h)	VLDL TAG (μ g/mg of protein)		VLDL apoB (μ g/mg of protein)	
		—Oleate	+ Oleate	—Oleate	+ Oleate
Chow	0–24	119.0 $\pm$ 18.0	148.3 $\pm$ 16.5	8.21 $\pm$ 0.53	6.48 $\pm$ 0.78
	24–48	32.1 $\pm$ 5.7	197.3 $\pm$ 14.3	1.12 $\pm$ 0.25	5.47 $\pm$ 0.48
Chow + OA	0–24	76.1 $\pm$ 15.0**	65.4 $\pm$ 11.8**	5.48 $\pm$ 0.79**	3.48 $\pm$ 0.30**
	24–48	65.5 $\pm$ 11.3**	104.8 $\pm$ 20.1*	2.36 $\pm$ 0.59**	3.06 $\pm$ 0.36**

fed compared with those from the OA-fed animals (425 ± 42 and 283 ± 29 μ g/mg protein respectively; $n = 5$, $P < 0.05$). Thus the decreased incorporation of [3 H]oleate was not an artefactual result of a lower specific radioactivity resulting from an increased pool size of unlabelled oleate in the hepatocytes from the OA-fed rats. It is, of course, possible that changes in the rate of degradation (e.g. oxidation) of newly synthesized lipids could affect the relative amounts of [3 H]oleate incorporated into TAG and phospholipids in the control and OA-fed animals. However, we found no evidence for any significant change in the rate of fatty acid oxidation as evidenced by ketogenesis. For instance, in the cells from the control animals, the amounts of ketone bodies (acetoacetate plus β -hydroxybutyrate) produced were 1.15 ± 0.21 , 2.06 ± 0.17 and 3.86 ± 0.59 μ mol/mg of cell protein at 6, 12 and 24 h respectively. The corresponding amounts produced in the cells from the OA-fed animals were 0.86 ± 0.19 , 1.79 ± 0.36 and 3.07 ± 0.29 μ mol/mg of cell protein respectively.

The effect of OA feeding on the proportion of the VLDL TAG secreted after lipolysis and re-esterification was determined by measurement of the relative decline in the 3 H specific radioactivity of the VLDL TAG, as reflected by the decline in 3 H: 14 C ratio after 24 h culture compared with that of the cellular TAG at the start of the culture period. These results are shown in Table 4, which suggests that, in the hepatocytes from the OA-fed rats, the

Figure 3 The effect of OA feeding on total newly synthesized TAG and PL (cellular + VLDL)

Hepatocytes from chow- and OA-fed rats were cultured as described in the legend to Figure 1. The medium was changed at 24 h in the cells cultured for 48 h, and fresh [3 H]oleate was added. The sum of the newly synthesized cellular and secreted TAG (A) and PL derived from exogenous oleate (B) were determined. Values are means $\pm$ S.E.M. of six independent experiments. Values marked * are significantly different ($P < 0.05$) from those of the corresponding chow-fed animals. □, Chow-fed rats; ■, OA-fed rats.

lower rate of VLDL TAG secretion was associated with a decrease in the proportion of this material that had been recruited via lipolysis and re-esterification of cellular TAG. Although this decrease was statistically significant by a Student's *t* test only after 6 h of culture, analysis of the complete data by repeated measures analysis of variance showed a significant overall effect ($P < 0.01$).

OA feeding also decreased the fractional turnover of TAG in the derived hepatocytes. Fractional turnover is a measure of the rate of operation of the lipolysis/re-esterification of cellular TAG [32,35] and was determined by measurement of the relative decrease in the specific radioactivity of the sum of VLDL and cellular TAG at the end of the culture period, as reflected by the decline in the 3 H: 14 C ratio (see Figure 4 and legend). The product of the fractional turnover and the total TAG pool size (μ g/mg of protein) is the total quantity of TAG that has undergone lipolysis and re-esterification. These values are also shown in Figure 4, which suggests that total TAG turnover was considerably decreased in the hepatocytes from the OA-treated rats.

VLDL secretion and intracellular TAG lipolysis in hepatocytes from rats fed *n*–3 fatty acids

In this experiment, animals were fed the FO diet (MaxEPA) for two weeks. Food intake was similar to that in the control animals

Table 4 Effect of feeding a diet supplemented with OA on the mode of secretion of VLDL TAG

Hepatocytes from animals fed a chow (LF) or a chow diet supplemented with OA were cultured for 4 h with [^3H]glycerol and [^{14}C]oleate. The labelled medium was removed and the cells were cultured for periods of 6, 12, 24 and 48 h as described in the Materials and methods section. After each time point, the secreted VLDL was isolated and the ^3H : ^{14}C ratio of its TAG was determined. The proportion of the VLDL secreted via lipolysis was determined as described in the Materials and methods section. The values represent the means $\pm$ S.E.M. of five independent experiments. Values marked *, ** and *** are significantly different ($P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively) from the corresponding initial value of the cellular ^3H : ^{14}C TAG ratio. Values marked † are significantly different ($P < 0.05$) from the corresponding values in the chow-fed animals.

Diet	Period of culture (h)	Initial cell TAG ^3H : ^{14}C ratio	VLDL TAG ^3H : ^{14}C ratio	VLDL TAG	
				Secreted after lipolysis (% of total)	Secreted without lipolysis (% of total)
Chow		3.90 $\pm$ 0.42			
	6		1.84 $\pm$ 0.25***	49 $\pm$ 3	51 $\pm$ 3
	12		1.75 $\pm$ 0.29***	56 $\pm$ 5	44 $\pm$ 5
	24		1.42 $\pm$ 0.19***	60 $\pm$ 3	40 $\pm$ 4
OA-supplemented	48		1.22 $\pm$ 0.24***	67 $\pm$ 3	33 $\pm$ 4
		3.65 $\pm$ 0.36			
	6		2.46 $\pm$ 0.40**	32 $\pm$ 5†	68 $\pm$ 5†
	12		2.01 $\pm$ 0.40**	43 $\pm$ 12	57 $\pm$ 12
	24		1.68 $\pm$ 0.30**	54 $\pm$ 78	46 $\pm$ 78
	48		1.35 $\pm$ 0.15**	61 $\pm$ 45	39 $\pm$ 5

(chow fed), as was body-mass gain. Both the liver weight and the hepatic TAG content in the FO-fed animals were higher than those of the controls (Table 1b).

Hepatocytes from the FO-fed and the chow-fed animals were cultured for 4 h in the presence of [^3H]glycerol and [^{14}C]oleate to label intracellular TAG. Following removal of the labelled substrates, during the next 24 h, cells from the FO-fed animals secreted significantly less VLDL TAG than those from the chow-fed rats (Table 5). This occurred despite the higher initial hepatic TAG content of the FO-fed animals (Table 1), suggesting a decreased mobilization of this pool for VLDL assembly. VLDL apoB secretion was also decreased in the cells from the FO-fed animals under similar conditions (Table 5). Of the decreased VLDL TAG secreted by the cells from the FO-fed animals, a significantly smaller proportion was secreted following lipolysis and re-esterification of cellular TAG. Furthermore, the fractional turnover of the cellular TAG in the cells from the FO-fed animals was also significantly decreased. As previously observed [32], a large proportion of the cellular TAG that undergoes lipolysis and re-esterification was not secreted as VLDL, but was retained by the cell, probably in the cytosol. The proportions of lipolysed cell TAG that were recycled in this way were similar in the cells from both the FO-fed and the chow-fed animals (Table 5). Compared with the LF-fed controls, in the FO-fed animals the decreased rate of VLDL apoB and TAG secretion observed during the first 24 h of culture in the absence of extracellular oleate did not persist for longer periods (Table 6). This is similar to that observed in hepatocytes from OA-fed rats cultured under similar conditions and may reflect a decreased availability of intracellular TAG in the control group at the end of the first 24 h culture period, during which oleate was not present. Such a decline in cellular TAG levels would result from a higher initial rate of VLDL TAG output during the earlier part of the culture period (i.e. 0–24 h) in the control group.

DISCUSSION

Although both apoB and MTP are obligatory requirements for the packaging and secretion of hepatic TAG as VLDL, there is little evidence that, in the short term at least, changes in the availability of these proteins at the hepatocellular site(s) of VLDL

assembly are primarily responsible for the regulation of VLDL output. For instance, in rats, changes in the quantity and activity of MTP did not vary during pathophysiological and developmental changes associated with decreased output of VLDL [21]. In HepG2, MTP fails to respond acutely to the inhibitory effect of insulin on lipoprotein output, although there was a decrease in its mRNA level [51]. Stimulation of lipoprotein output by oleate was not accompanied by changes either in MTP itself or its mRNA. However, longer-term dietary changes in hamster are associated with changes in hepatic MTP mRNA levels [22,23]. Changes in the rate of secretion of apoB-containing lipoproteins are not, in the short term at least, accompanied by changes in the rate of apoB transcription [18–20], and controversy exists as to whether such a correlation exists even after longer-term nutritional changes in the hamster in which dietary tri-palmitate increased hepatic apoB mRNA levels [52], whereas hydrogenated coconut oil did not [22]. On the other hand, it is generally agreed that intracellular apoB availability is limited by post-translational degradation, which is believed to be secondary to an effect on translocation across the endoplasmic reticulum (ER) membrane and assembly with lipid [24–26].

On the basis of the above results, it might appear reasonable to conclude that hepatic VLDL assembly is primarily dependent upon the rate of neutral lipid synthesis, which then determines the extent of apoB degradation, and, thus, the output of VLDL TAG and apoB. However, in rat hepatocytes, although changes in VLDL apoB output are positively correlated with *de novo* lipogenesis in the donor animals, apoB output is resistant to manipulations which increase TAG synthesis *in vitro* (for a review, see [1]). Furthermore, although increased hepatic TAG content is sometimes associated with an elevated VLDL output [53–55], this is not always the case, and several instances are known in which dietary and developmentally induced increases in hepatic TAG are associated with a decreased output of hepatic VLDL [37,38,56,57]. The latter include dietary supplementation with either OA [36,37] or with FOs containing *n*–3 polyunsaturated fatty acids [38–40,43]. The present work describes an investigation of lipid and apoB metabolism in hepatocytes derived from donor animals maintained under these conditions.

There were two major reasons for carrying out this study. First, we wished to assess the value of these *in vitro* models for

Figure 4 Turnover of cellular TAG (labelled from [^3H]glycerol) during VLDL secretion in hepatocytes prepared from rats fed chow diet only or chow diet supplemented with OA

Hepatocytes from rats fed chow diet or chow diet supplemented with OA were plated in the presence of [^3H]glycerol and [^{14}C]oleate as described in the Materials and methods section. Changes in the $^3\text{H}:^{14}\text{C}$ ratios of the total (cellular + VLDL) TAG were determined (A). Calculations of fractional turnover (added pools) (B) and TAG hydrolysis (C) were carried out according to the procedures described in the Materials and methods section. Values marked * and ** are significantly different ($P < 0.05$ and $P < 0.01$ respectively) from those of the corresponding chow-fed animals. The values represent the means $\pm$ S.E.M. of five independent experiments. $\square$, Chow-fed rats; $\blacksquare$, OA-fed rats. Where no error bars are shown, the standard error falls within the size of the respective symbol.

the study of the molecular events involved in the assembly of VLDL. This involved a characterization of the time-frame over which diet-induced metabolic changes imprinted in the liver of donor animals *in vivo* were retained in isolated hepatocytes *in vitro*. Secondly, we [31,32] and others [33,34] have provided evidence that a large proportion of VLDL TAG is derived from the intracellular cytosolic TAG pool by a process that involves lipolysis followed by re-esterification. Whether this is an obligatory process in the assembly of VLDL is not known, although there are instances in HepG2 [35] and in rat liver [32] in which lipolysis and secretion appear to be correlated. It was of interest, therefore, to measure rates of intracellular TAG lipolysis under conditions in which VLDL output is curtailed following dietary consumption of OA or *n*-3 polyunsaturated fatty acids.

The present results suggest that signals resulting from the above dietary manipulations are retained within the liver cell for at least 24 h following removal from the body, during which time the secretion of VLDL apoB and TAG remains suppressed

Table 5 Relationship between VLDL secretion and cellular TAG turnover in hepatocytes from chow and FO-fed animals

Hepatocytes from animals fed a chow (LF) diet or chow diet supplemented with FO were cultured for 4 h in the presence of [^3H]glycerol and [^{14}C]oleate as described in the Materials and methods section. After the removal of labelled medium the cells were washed and cultured for a further 24 h. The VLDL was isolated. Calculation of the parameters was carried out according to the procedures described in the Materials and methods section. The values below represent the means $\pm$ S. E. M. of several hepatocyte cultures from the chow-fed and the FO-fed animals (numbers in parentheses). Values marked * and ** are significantly different ($P < 0.05$ and $P < 0.01$ respectively) from the corresponding values in the chow-fed animals.

Animals ...	Chow-fed	FO-fed
VLDL apoB secretion ($\mu\text{g}/\text{mg}$ of cell protein)	9.09 ± 0.51 (5)	$4.55 \pm 0.85^*$ (5)
VLDL TAG secretion ($\mu\text{g}/\text{mg}$ of cell protein)	132 ± 29 (5)	$53.10 \pm 11.20^*$ (5)
VLDL $^3\text{H}:^{14}\text{C}$ (% of the initial cell TAG $3\text{H}:^{14}\text{C}$ ratio)	36.30 ± 2.30 (8)	$45.60 \pm 1.60^*$ (7)
VLDL TAG secretion via lipolysis (% of total secreted)	64.70 ± 2.30 (8)	$54.40 \pm 1.60^*$ (7)
(Cell + VLDL) TAG $^3\text{H}:^{14}\text{C}$ (% of initial cell $^3\text{H}:^{14}\text{C}$)	37.10 ± 2.50 (8)	$49.70 \pm 1.80^*$ (7)
Fractional turnover (added pools)	1.79 ± 0.20 (8)	$1.00 \pm 0.07^{**}$ (7)
VLDL TAG secretion (% of total TAG hydrolysed)	47.50 ± 5.00 (8)	59.10 ± 3.80 (7)

Table 6 Effect of FO feeding on VLDL output during prolonged culture of hepatocytes

Hepatocytes from rats fed either a LF- or a FO-enriched diet were cultured for either 24 h or 48 h. During the period 24–48 h the media were supplemented with oleate. Measurements of VLDL apoB and VLDL TAG output were made during 0–24 h and 24–48 h of culture. The values below represent the means $\pm$ S.E.M. of five hepatocyte preparations in each group. Values marked ** and *** are significantly different ($P < 0.01$ and $P < 0.001$ respectively) from the corresponding values in the hepatocytes from the LF-fed animals.

Diet	Period of culture (h)	VLDL secretion ($\mu\text{g}/\text{mg}$ of cell protein)	
		TAG	ApoB
Chow (LF)	0–24	95 ± 13.5	8.51 ± 0.71
	24–48	69 ± 13.5	1.46 ± 0.45
FO	0–24	$34.2 \pm 5.4^{**}$	$3.11 \pm 0.42^{***}$
	24–48	57.6 ± 9.9	2.76 ± 0.59

(Figures 1 and 2, Table 5). However, surprisingly, the decreased output of VLDL was not accompanied by any decrease in the plasma TAG concentration of the rats fed FO compared with those fed the low-fat diet (Table 1b). Separation of the plasma into fractions containing chylomicrons and VLDL, however, showed a higher proportion of chylomicron TAG and a lower proportion of VLDL TAG in the plasma from the animals fed the FO diet. This plasma contained 24.5 ± 3.0 and 7.5 ± 1.6 mg/dl of chylomicron and VLDL TAG respectively. The corresponding values for the control-fed animals were 13.1 ± 3.1 and 23.0 ± 6.0 mg/dl of plasma respectively. In addition, many reports of the hypotriglyceridaemic effects of FO feeding have used human subjects or animals fed other types of fatty acid, rather than a low-fat diet, as a control. However, two recent reports [58,59] suggest that, compared with a low-fat diet, diets rich in FO are not hypotriglyceridaemic. In studies conducted in parallel with the present work, we found that after feeding a diet supplemented with 20% (v/w) olive oil, the plasma TAG concentration rose to 88.5 ± 12.9 mg/dl, compared with 40.9 ± 7.4 mg/dl when 20% (v/w) FO was used in the diet. Cells from the former animals secreted significantly more VLDL than

did those from the latter [60]. We did not measure rates of lipolysis and re-esterification of TAG in hepatocytes from these animals.

The underlying mechanisms involved in the inhibitory effects of OA and *n*-3 fatty acids are not well understood, and, in the case of suppression by *n*-3 fatty acids, several explanations have been proposed, including an increase in the intracellular degradation of apoB [40,61]. Our recent studies suggest, however, that decreased synthesis of apoB, particularly of apoB₄₈, makes an important contribution to the decreased secretion of VLDL that occurs during consumption of a diet rich in *n*-3 fatty acids [60]. In hepatocytes from OA-fed rats, despite their large TAG pool size, apoB is packaged with very small quantities of TAG in the lumen of the endoplasmic reticulum compared with that in normal hepatocytes. There is no further addition of lipid and these small particles are not secreted [8]. This inhibitory effect on VLDL assembly may be due, at least partly, to impaired TAG recruitment resulting from the inhibition of intracellular lipolysis and re-esterification in these cells. Our current data do not allow us to speculate as to whether there is any causal relationship between impaired lipolysis and decreased synthesis of apoB₄₈ [60]. In the present work, the similarity in the TAG/apoB ratios (Table 2) in the secreted VLDL from the control-fed and the OA-fed hepatocytes does not contradict these previous findings [8]. What the present results suggest is that, in the hepatocytes from the OA-fed rats, although many of the incipient lipoprotein particles formed contain small quantities of TAG and are not secreted, the relatively smaller number of particles that manage to acquire a normal amount of TAG are secreted in the usual way. Thus the hepatocytes from the OA-treated rats secrete a smaller number of normal-size particles.

Following OA feeding, the period over which the inhibitory effect was retained in hepatocytes could be extended to 48 h if TAG synthesis did not become rate limiting (Table 3). This could be achieved by culturing cells in the presence of oleic acid. In the absence of oleate, however, the greater decrease in the secretion of VLDL in the chow-fed cells compared with the OA-fed cells after prolonged culture resulted in the latter cell type secreting more VLDL than the former. A similar pattern was also observed after prolonged culture of cells from the FO-fed group of animals (Table 6). Again, the decreased initial output of VLDL TAG and apoB from these cells compared with those from the chow-fed animals was not maintained after 24 h in culture. The decline in the secretion of apoB was most particularly marked in the latter case. This pattern may have been related to a combination of two effects. First, to a loss of the 'imprint' on hepatic metabolism caused by the FO diet *in vivo*, and, secondly, to an increased availability of intracellular TAG for VLDL assembly in the FO-fed cells at the end of the first 24 h culture period [36.9 ± 6.3 (FO) compared with 23.4 ± 0.9 $\mu\text{g}/\text{dish}$ (control)]. The larger quantity of cellular TAG at this time resulted from a decreased initial removal of TAG as VLDL in the FO-fed animals. It would appear that this extra TAG was sufficient to maintain a substantial rate of apoB secretion compared with that observed in the chow-fed cells. We believe that this is the first such detailed study of the effects of dietary imprinting on longer-term hepatocellular lipid metabolism *in vitro*. The results suggest that, particularly with OA feeding, the inhibitory effect is observed for a sufficiently long period to permit studies of its possible *in vitro* reversal.

In hepatocytes from both OA-fed and FO-fed animals, the decreased secretion of VLDL was associated with a decline in the rate of intracellular TAG lipolysis and re-esterification compared with that observed in hepatocytes from the chow-fed controls (Figure 4, Table 5). In both cases there was also a decline in the

proportion of the secreted VLDL TAG that was recruited via the lipolytic pathway (Tables 4 and 5). Thus these results would suggest that there has been a net loss of labelled glycerol during the recruitment of TAG for VLDL assembly. Superficially at least, this observation might imply complete hydrolysis of cellular TAG to [¹⁴C]fatty acids and [³H]glycerol followed by dilution of the labelled glycerol prior to re-esterification to labelled fatty acids with a relatively constant specific radioactivity. Other workers have, however, provided evidence that the pool of cellular TAG recruited for VLDL production undergoes only limited hydrolysis to diacylglycerol or monoacylglycerol [33,34]. These results are not incompatible with our own observations, which represent, simply, the empirical outcome of what is probably a complex series of enzymic steps involving lipases and acyltransferases, the overall result of which appears as a net removal of labelled glycerol. This net loss was greater in the cells from the control animals than in those from the OA-fed or FO-fed animals.

Irrespective of the exact degree of lipolysis of intracellular TAG, our present and previous [31,32,62] results and those of others [34,35] suggest that cytosolic TAG is not predominantly transported *en bloc* (i.e. as intact molecules) across the membrane bilayer of the ER or the Golgi into the secretory lumen where VLDL assembly occurs. Instead, transport across the membrane probably occurs as fatty acids and/or diacylglycerol, monoacylglycerol or a combination of these lipolytic products of cytosolic TAG. To account for the kinetics of intracellular and VLDL TAG fatty acid metabolism, we have proposed that there are at least two sites of TAG synthesis in the ER membrane, one of which is concerned exclusively with the provision of TAG for VLDL assembly [31,62]. In addition, the transfer of TAG lipolysis products across the membrane implies the presence of a TAG-synthesizing site on the luminal surface of the microsomal membrane. Evidence for the presence of a luminal form of diacylglycerol acyl transferase has recently been presented [63]. Furthermore, it has also been suggested that the presence of both malonyl-CoA-sensitive and malonyl-CoA-insensitive carnitine acyltransferases in the microsomal fraction of the cell may serve as a means by which cytosolic TAG fatty acids produced by lipolysis on the cytosolic surface of the microsomal membrane are shuttled across the membrane for esterification in the lumen [64].

It would appear, therefore, that the enzymology involved in the recruitment and transport of lipid for VLDL assembly is more complex than originally thought. As discussed above, some details of the process are now beginning to emerge. If lipolysis turns out to be an obligatory requirement for VLDL assembly, it is possible that this step might be a regulatory target. The present results and those of our previous work have shown that, under some conditions, intracellular TAG lipolysis and lipoprotein secretion rates are positively correlated. However, it must be emphasized that, at the present time, there is no conclusive evidence for a causal relationship.

We thank Reginald Hems for help in the preparation of hepatocyte cultures and Madge Barber for typing the manuscript. FO (MaxEPA) was the generous gift of Dr. F.-W. Vas Dias (Seven Seas, Marfleet, Hull, U.K.). P.W.B. thanks the Australian Heart Foundation for a travel grant. M.C.L.S. thanks FAPESP (Brazil) for a personal support grant. This work was funded by a Programme Grant from the Medical Research Council (M.R.C.) to G.F.G., and we thank the Council for its support. G.F.G. is a member of the External Scientific Staff of the M.R.C. and Research Lecturer at the University of Oxford.

REFERENCES

- Gibbons, G. F. (1990) *Biochem. J.* **268**, 1–13
- Sparks, J. D. and Sparks, C. E. (1994) *Biochim. Biophys. Acta* **1215**, 9–32

- 3 Yao, Z. and McLeod, R. S. (1994) *Biochim. Biophys. Acta* **1212**, 152–166
- 4 Innerarity, T. L., Borén, J., Yamanaka, S. and Olofsson, S.-O. (1996) *J. Biol. Chem.* **271**, 2353–2356
- 5 Borchardt, R. A. and Davis, R. A. (1987) *J. Biol. Chem.* **262**, 16394–16402
- 6 Rusiñol, A., Verkade, H. and Vance, J. E. (1993) *J. Biol. Chem.* **268**, 3555–3562
- 7 Alexander, C. A., Hamilton, R. L. and Havel, R. J. (1976) *J. Cell. Biol.* **69**, 241–263
- 8 Cartwright, I. J., Hebbachi, A.-M. and Higgins, J. A. (1993) *J. Biol. Chem.* **268**, 20937–20952
- 9 Cartwright, I. J. and Higgins, J. A. (1995) *Biochem. J.* **310**, 897–907
- 10 Bamberger, M. J. and Lane, M. D. (1988) *J. Biol. Chem.* **263**, 11868–11878
- 11 Borén, J., Rustaeus, S. and Olofsson, S.-O. (1994) *J. Biol. Chem.* **269**, 25879–25888
- 12 Swift, L. L. (1995) *J. Lipid Res.* **36**, 395–406
- 13 Gordon, D. A., Jamil, H., Sharp, D., Mullaney, D., Yao, Z., Gregg, R. E. and Wetterau, J. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 7628–7632
- 14 Leiper, J. M., Bayliss, J. D., Pease, R. J., Brett, D. J., Scott, J. and Shoulders, C. C. (1994) *J. Biol. Chem.* **269**, 21951–21954
- 15 Pease, R. J. and Leiper, J. M. (1996) *Curr. Opin. Lipidol.* **7**, 132–138
- 16 Howard, B. V. (1987) *J. Lipid Res.* **28**, 613–628
- 17 Laws, A. (1996) *Curr. Opin. Lipidol.* **7**, 172–177
- 18 Pullinger, C. R., North, J. D., Teng, B.-B., Rifici, V. A., Ronhild de Brito, A. E. and Scott, J. (1989) *J. Lipid Res.* **30**, 1065–1077
- 19 Moberly, J. B., Cole, T. G., Alpers, D. H. and Schonfeld, G. (1990) *Biochim. Biophys. Acta* **1042**, 70–80
- 20 Dashti, N. (1992) *J. Biol. Chem.* **267**, 7160–7169
- 21 Brett, D. J., Pease, R. J., Scott, J. and Gibbons, G. F. (1995) *Biochem. J.* **310**, 11–14
- 22 Lin, M. C. M., Arbeen, C., Bergquist, K., Kienzie, B., Gordon, D. A. and Wetterau, J. R. (1994) *J. Biol. Chem.* **269**, 29138–29145
- 23 Bennett, A. J., Billett, M. A., Salter, A. M. and White, D. A. (1995) *Biochim. Biophys. Res. Commun.* **212**, 473–478
- 24 Bonnardel, J. A. and Davis, R. A. (1995) *J. Biol. Chem.* **270**, 28892–28896
- 25 Rusiñol, A. E., Chan, E. Y. W. and Vance, J. E. (1993) *J. Biol. Chem.* **268**, 25168–25175
- 26 Wu, X., Shang, A., Jiang, H. and Ginsberg, H. N. (1996) *J. Lipid Res.* **37**, 1198–1206
- 27 Davis, R. A. and Boogaerts, J. R. (1982) *J. Biol. Chem.* **257**, 10908–10913
- 28 Gibbons, G. F. (1994) *Curr. Opin. Lipidol.* **5**, 191–199
- 29 Coussons, P. J., Bourgeois, C. S., Wiggins, D. and Gibbons, G. F. (1996) *Arterioscler. Thromb. Vasc. Biol.* **16**, 889–897
- 30 Gibbons, G. F., Bartlett, S. M., Sparks, C. E. and Sparks, J. D. (1992) *Biochem. J.* **287**, 749–753
- 31 Gibbons, G. F. and Wiggins, D. (1995) *Adv. Enzyme Regul.* **35**, 179–198
- 32 Wiggins, D. and Gibbons, G. F. (1992) *Biochem. J.* **284**, 457–462
- 33 Yang, L.-Y., Kuksis, A., Myher, J. J. and Steiner, G. (1995) *J. Lipid Res.* **36**, 125–136
- 34 Yang, L.-Y., Kuksis, A., Myher, J. J. and Steiner, G. (1996) *J. Lipid Res.* **37**, 262–274
- 35 Gibbons, G. F., Khurana, R., Odwell, A. and Seelaender, M. C. L. (1994) *J. Lipid Res.* **35**, 1801–1808
- 36 Windmueller, H. G. (1964) *J. Biol. Chem.* **239**, 530–537
- 37 Windmueller, H. G. and Spaeth, A. E. (1966) *J. Biol. Chem.* **241**, 2891–2899
- 38 Harris, W. S., Connor, W. E., Illingworth, D. R., Rothrock, D. W. and Foster, D. M. (1990) *J. Lipid Res.* **31**, 1549–1558
- 39 Parks, J. S., Johnson, F. L., Wilson, M. D. and Rudel, L. L. (1990) *J. Lipid Res.* **31**, 455–466
- 40 Wang, H., Chen, X. and Fisher, E. A. (1993) *J. Clin. Invest.* **91**, 1380–1389
- 41 Pottenger, L. A. and Getz, G. S. (1971) *J. Lipid Res.* **12**, 450–459
- 42 Otto, D. A., Tsai, C. E., Baltzell, J. K. and Wooten, J. T. (1991) *Biochim. Biophys. Acta* **1082**, 37–48
- 43 Wong, S. H., Nestel, P. J., Trimble, R. P., Storer, G. B., Illman, R. J. and Topping, D. L. (1984) *Biochim. Biophys. Acta* **792**, 103–109
- 44 Michalopoulos, G. and Pitot, H. (1975) *Exp. Cell Res.* **94**, 70–78
- 45 Duerden, J. M., Bartlett, S. M. and Gibbons, G. F. (1989) *Biochem. J.* **263**, 937–943
- 46 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- 47 Folch, J., Lees, M. and Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509
- 48 Williamson, D. H., Mellanby, J. and Krebs, H. A. (1962) *Biochem. J.* **82**, 90–96
- 49 Van Harken, D. R., Dixon, C. W. and Heimberg, M. (1969) *J. Biol. Chem.* **244**, 2278–2285
- 50 Duerden, J. M. and Gibbons, G. F. (1993) *Biochem. J.* **294**, 167–171
- 51 Lin, M. C. M., Gordon, D. and Wetterau, J. R. (1995) *J. Lipid Res.* **36**, 1073–1081
- 52 Bennett, A. J., Billett, M. A., Salter, A. M., Mangiapane, E. H., Bruce, J. S., Anderton, K. L., Marenah, C. B., Lawson, N. and White, D. A. (1995) *Biochem. J.* **311**, 167–173
- 53 Yamamoto, M., Yamamoto, I., Tanaka, Y. and Ontko, J. A. (1987) *J. Lipid Res.* **28**, 1156–1165
- 54 Wiggins, D., Hems, R. and Gibbons, G. F. (1995) *Metabolism* **44**, 841–847
- 55 Bourgeois, C. S., Wiggins, D., Hems, R. and Gibbons, G. F. (1995) *Am. J. Physiol.* **269**, E208–E215
- 56 Duerden, J. M., Bartlett, S. M. and Gibbons, G. F. (1989) *Biochem. J.* **262**, 313–319
- 57 Coleman, R. A., Haynes, E. B., Sand, T. M. and Davis, R. A. (1988) *J. Lipid Res.* **29**, 33–42
- 58 Yaqoob, P., Sherrington, E. J., Jeffery, N. M., Sanderson, P., Harvey, D. J., Newsholme, E. A. and Calder, P. C. (1995) *Int. J. Biochem. Cell Biol.* **27**, 297–310
- 59 Lottenberg, A. M. P., Oliveira, H. C. F., Nakandakare, E. R. and Quintão, E. C. R. (1992) *Lipids* **27**, 326–330
- 60 Brown, A.-M., Baker, P. W. and Gibbons, G. F. (1997) *J. Lipid Res.* **38**, 469–481
- 61 Lang, C. A. and Davis, R. A. (1990) *J. Lipid Res.* **31**, 2079–2086
- 62 Gibbons, G. F. and Wiggins, D. (1995) *Biochem. Soc. Trans.* **23**, 495–500
- 63 Owen, M. R., Corstorphine, C. G. and Zammit, V. A. (1997) *Biochem. J.* **323**, 17–21
- 64 Broadway, N. M. and Saggerson, E. D. (1995) *Biochem. Soc. Trans.* **23**, 490–494