Control of Hep G2-cell triacylglycerol and apolipoprotein B synthesis and secretion by polyunsaturated non-esterified fatty acids and insulin

Christopher D. BYRNE, Tim W. M. WANG and C. Nicholas HALES

University Department of Clinical Biochemistry, Addenbrookes Hospital, Hills Road, Cambridge CB2 2QQ, U.K.

Non-esterified fatty acids (NEFAs) and insulin are important factors in the control of lipoprotein secretion, but the mechanism of action is unclear. The present study was undertaken to determine whether insulin and NEFAs modulated hepatic secretion of triacylglycerol and apolipoprotein B (apo-B) by regulation of hepatic intracellular apo-B content. The experiments were performed with the human hepatoblastoma cell line Hep G2, for periods of up to 72 h in the presence and absence of NEFAs and insulin. Higher concentrations of eicosapentanoate (EPA) sustained for 72 h decreased cellular protein content (at 250 μM) or caused cell death (at 750 μM), and this effect was not observed with the other NEFAs studied, whereas 75 μM -EPA did not affect cell viability. Compared with the absence of NEFA, 75 μM -EPA did not alter the intracellular triacylglycerol content, but decreased the intracellular content of apo-B by 47 % ($P < 0.01$) and decreased secreted triacylglycerol and secreted apo-B by 13 % ($P < 0.05$) and 21 % ($P < 0.01$) respectively, after 72 h. However 250 μM -oleate increased the intracellular triacylglycerol by 36 % ($P < 0.01$), intracellular apo-B by 22 % ($P < 0.05$) and secreted triacylglycerol and apo-B by 20–30 % ($P < 0.05$ – 0.01). Insulin decreased secreted triacylglycerol and apo-B in the presence of each NEFA studied by 20–30 %. There was no correlation between the changes in intracellular triacylglycerol and the rate of secretion. However, when the secreted triacylglycerol or apo-B was plotted against intracellular apo-B content a significant correlation was observed ($r = 0.89$, $P < 0.001$ for both analyses). Apo-B mRNA levels did not change after 72 h incubation with oleate or EPA. These results demonstrate that EPA can be toxic to hepatocytes and that NEFAs and insulin control secretion of triacylglycerol and apo-B by regulation of the intracellular apo-B concentration, thus controlling assembly of apo-B with triacylglycerol to form lipoproteins.

INTRODUCTION

Increased concentrations of dietary saturated non-esterified fatty acids (NEFAs) have been positively correlated with the development of ischaemic heart disease (IHD) (Ahrens *et al.*, 1957; Keys *et al.*, 1965; Hegstead *et al.*, 1965). Dietary fat contains NEFAs of different acyl-chain length and degree of branching, which vary in the number and position of unsaturated double bonds and whose conformation may be *cis* or *trans*. Epidemiological studies of populations whose diet is rich in polyunsaturated fatty acid have revealed lower incidences of IHD (Sinclair, 1953; Dyerberg *et al.*, 1975; Bang *et al.*, 1976). The precise mechanism for these relationships remains unclear, but these populations have lower concentrations of LDL cholesterol. Increased concentrations of LDL cholesterol, and more recently triacylglycerol (Kannel & McGee, 1979; Abbott & Carroll, 1984; Cambien *et al.*, 1986) and lipoprotein (a) [Lp(a)] (Dahlen *et al.*, 1975; Kostner *et al.*, 1981; Rhoads *et al.*, 1986), have been recognized as independent cardiovascular risk factors in the development of IHD. In subjects with diabetes, the risk of IHD is increased 2–3-fold over age- and sex-matched controls (Assman & Shultz, 1988), and major contributors to this risk are the associated lipid and lipoprotein abnormalities (Uusitupa *et al.*, 1985). The most frequent lipid abnormality observed in subjects with diabetes mellitus is increased plasma triacylglycerol, often in association with increased plasma apo-B (for review see Howard, 1987). There is still controversy about the exact mechanism by which this occurs, but insulin and NEFAs are important regulatory factors (Reaven & Ida Chen, 1988).

Clinical studies of normal subjects whose diet has been

supplemented with *n*–3 NEFAs [e.g. eicosapentanoate (EPA) or docosahexanoate] have produced an improved lipid profile, with lowering of plasma triacylglycerol (Sanders *et al.*, 1985; Harris, 1989), LDL cholesterol and apolipoprotein B (apo-B) (Harris, 1989). *n*–3 NEFAs may also produce a decrease in the elevated plasma concentrations of Lp(a) (Herrmann *et al.*, 1989), and EPA is used therapeutically to decrease elevated plasma triacylglycerol and apo-B concentrations in subjects with diabetes (Schechtman *et al.*, 1988; Axelrod, 1989) and impaired glucose tolerance (Fasching *et al.*, 1991).

Studies *in vitro* to date are conflicting and have not clarified the exact mechanism by which EPA acts. EPA decreased triacylglycerol synthesis in perfused rat liver (Wong & Marsh, 1988) and rat hepatocytes (Wong *et al.*, 1985; Nossen *et al.*, 1986), and data from Hep G2 cells (Homan *et al.*, 1991) and rat hepatocytes (Lang & Davis, 1990) indicated that EPA and oleic acid were equipotent as substrates for triacylglycerol synthesis but, relative to oleate, EPA inhibited secretion.

We have found that oleate is utilized extremely rapidly by Hep G2 cells (Byrne *et al.*, 1991). Failure to take account of this in designing experiments to test the effects of NEFAs on this system can lead to a greatly underestimated effect of NEFAs to regulate triacylglycerol storage and secretion. Using regular NEFA supplementation of the medium, we found that sustained high concentrations of oleate decrease the action of insulin to inhibit triacylglycerol secretion. We now report a comparison of the metabolism by Hep G2 cells of NEFAs of different acyl chain length and degree of unsaturation. We have also studied the effects of the highly unsaturated fatty acid EPA and insulin in the regulation of intracellular and secreted triacylglycerol and

apo-B, over both short (< 24 h) and long (< 72 h) incubation periods.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle's Medium was purchased from Flow Laboratories, and fetal-calf serum from Advanced Protein Products (APP), Birmingham, U.K. Oleic acid, EPA, palmitic acid, stearic acid and fatty-acid-free BSA were from Sigma. [^{14}C]Oleic acid (54 mCi/mmol) [^{14}C]EPA (40 mCi/mmol), [^{14}C]palmitic acid (57 mCi/mmol), and [^{14}C]stearic acid (58 mCi/mmol) were purchased from Amersham.

Silica-coated t.l.c. plates and the solvents light petroleum, diethyl ether and acetic acid were purchased from BDH. The human hepatoma cell line Hep G2 was purchased from European Collection of Animal Cell Cultures, Porton Down, Wilts., U.K. and the insulin was a gift from the National Institute of Biological Standards, Potters Bar, Herts., U.K. Optiphase Hi-Safe II scintillant was obtained from LKB Scintillation Products.

Hydrazine, glycine, EDTA, MgCl_2 , NAD^+ , NADH, ATP and triolein standards were all purchased from Sigma. Tetraethylammonium hydroxide was from Kodak, and glycerol kinase and glycerol phosphate dehydrogenase were also from Sigma.

Both unconjugated and peroxidase-conjugated anti-(human apo-B) antibody were purchased from Binding Site (University of Birmingham, U.K.) and human apo-B reference standard was from Technicon. Sodium citrate, trisodium citrate, citric acid, H_2O_2 and 2,2-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (disodium salt) (ABTS) were purchased from Sigma.

[^{32}P]dCTP (3000 Ci/mmol) was purchased from Amersham. The apo-B cDNA was a gift from Professor J. Scott (RPMS, Hammersmith Hospital, London). The oligo(dT)-cellulose columns were purchased from Pharmacia, and all reagents used in the preparation of RNA and washing of the filters were of molecular-biology grade and purchased from Sigma.

Cell cultures

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

Incubation conditions

Medium for experiments involving radiolabelled NEFA was prepared by the following protocol. To form BSA-NEFA complexes, portions of [^{14}C]NEFA (oleate, palmitate, stearate and EPA, stored in either toluene or ethanol) were added to a solution of 0.1 M-KOH. The toluene and ethanol preservatives were removed by boiling, and the solution was added to medium supplemented with 3.6% BSA and 10% fetal-calf serum and stirred for 30 min to ensure adequate mixing of the solution. The medium then contained a known concentration of NEFA of known specific radioactivity, and was sterilized by passage through a $0.2\text{ }\mu\text{m}$ -pore cellulose acetate filter. The medium was removed from the confluent monolayer of cells, washed twice with phosphate-buffered saline (PBS; 0.15 M-NaCl, 1.8 mM- $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 8.2 mM- $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$), pH 7.4, and the prepared medium containing either labelled or unlabelled NEFAs was added to the cells. Cells which were to be exposed to insulin were preincubated with insulin (0.1 μM) for 16 h before commencement of the experiment.

Preliminary experiments were performed with [^{14}C]NEFAs

to document the rate of uptake of each NEFA by the hepatocytes. Consequently, during experiments where it was our intention to study the effects of prolonged incubation with known concentrations of NEFA, the medium was replaced every 8 h throughout the 72 h period with medium identical in composition with that at the beginning of the experiment.

Determination of triacylglycerol, phospholipid and NEFA concentrations

After a defined period of incubation (with or without insulin), the medium was removed and the cells were washed twice with PBS. The monolayers were removed from the flasks in 1.1 ml of 0.1 M-HCl with a cell scraper.

The lipid from the medium and cell suspensions were removed separately with a mixture of chloroform, methanol and water (Folch *et al.*, 1957), washed twice, dried down under nitrogen and applied to t.l.c. plates. The t.l.c. plates were placed in light petroleum (b.p. 40–60 °C)/diethyl ether/acetic acid (35:15:1, by vol.) in a solvent tank for 90 min. After staining with iodine, the triacylglycerol and fatty acid were removed, placed in tubes containing 10 ml of Optiphase Hi-Safe scintillant and counted for radioactivity in a Packard liquid-scintillation spectrometer. From the radioactivity (c.p.m.) recovered, the incorporation of [^{14}C]NEFA into intracellular and secreted triacylglycerol and phospholipid was then calculated, and the results were expressed as μmol of [^{14}C]NEFA incorporated into lipid/g of cell protein.

Enzymic determination of glycerol in acylglycerols

It is recognized that hepatocytes contain a large intracellular pool of triacylglycerol (Dashti & Wolfbauer, 1987; Wang *et al.*, 1988; Byrne *et al.*, 1991). Therefore, in order to study the effects of insulin and NEFA on newly formed intracellular and secreted triacylglycerol over short periods of time (< 5 h) [^{14}C]NEFA was used as a tracer with unlabelled NEFA, and [^{14}C]triacylglycerol was measured. When the prolonged effects of insulin and NEFA were studied (< 72 h), direct measurements of intracellular and secreted triacylglycerol were performed.

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

Determination of intracellular and secreted apo-B concentrations

The initial experiments were carried out by monitoring autoradiographically the incorporation of [^{35}S]methionine into apo-B immunoprecipitated from both medium and cell lysates. Apo-B100 was the major apo-B form in both situations, confirming the results of Thrift *et al.* (1986) (results not shown).

In order to measure the intracellular and secreted apo-B content, an immunoassay was developed. The measurements of total immunoreactive intracellular apo-B were made on cell lysates. The cells were lysed in a buffer containing Tris/HCl, Tris base (pH 7.4) and proteinase inhibitors [phenylmethanesulphonyl fluoride (1 mM in propan-2-ol), tosyl-phenylalanyl chloromethane (0.1 mM in methanol), *trans*-epoxysuccinyl-leucylamido-4-guanidinobutane (E64) 10 μM , pepstatin A 10 μM , and 5 mM-EDTA] and 1% Triton X-100. The cell lysates were passed three times through a 25-gauge needle and then sonicated at 4 μM amplitude for 10 s (MSE Soniprep 150). Samples were then applied to the microtitre plates and assayed. Secreted apo-B was determined by assaying samples of medium after periods of incubation with the cells.

Microtitre plates (96 well) were coated with polyclonal anti-(human apo-B) antibody (1:100 dilution in 0.1 M- NaHCO_3 ,

pH 9.2) at 4 °C for 16 h. After 16 h the antibody-containing solution was discarded and the wells were aspirated and then blocked with PBS containing 1 % fat-free BSA for 1 h at room temperature. The wells were washed with PBS containing 0.1 % Tween 20, aspirated and the samples to be assayed were applied to the wells and incubated for 16 h at 4 °C. The wells were washed again with PBS/0.1 % Tween, aspirated, and polyclonal anti-(human apo-B) peroxidase-conjugated antibody (1:150 dilution in PBS/0.1 % Tween) was added for 2 h incubation at room temperature. The wells were washed again with PBS/0.1 % Tween and then with PBS alone before applying the substrate 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) containing a 1:65000 dilution of 30 % H₂O₂. After adequate mixing and 2 h at room temperature, the absorbances were read at 405 nm on a microplate reader.

Preparation of total RNA and mRNA

Total RNA was prepared by the method of Chomczynski & Sacchi (1987). The RNA content was estimated by measuring the A_{260} . mRNA was prepared by passage of 1.2 mg of total RNA through an oligo(dT)-cellulose column, followed by elution of poly(A)⁺ RNA.

Northern blotting

Poly(A)⁺ RNA (10 µg) was denatured and analysed by electrophoresis on 1%-agarose denaturing gels containing formaldehyde (2.2 M) and then transferred to nylon filters by capillary action. Filters were baked at 80 °C for 2 h. The filters were prehybridized for 2 h at 65 °C in a solution containing 1 % BSA, 1 mM-EDTA, pH 8, 7 % SDS and 0.5 M-Na₂HPO₄, and then hybridized overnight at 65 °C with either a ³²P-labelled human β-actin cDNA probe or a 1.7 kb human apo-B cDNA probe. The specific radioactivity of the probes were 10⁹ c.p.m./µg. Filters were washed twice in a solution containing 0.5 % BSA, 0.25 M-EDTA, 5 % SDS and 1 M-Na₂HPO₄ (pH 7.2) (30 min each, 65 °C) and twice in a solution containing 0.25 M-EDTA, 1 M-Na₂HPO₄, pH 7.2, and 1 % SDS (30 min each, 65 °C). The relative quantities of mRNA were determined by scanning the density of the band obtained on the autoradiograph.

Lactate dehydrogenase (LDH) activity and protein measurements

LDH activity was measured by the method of Wroblewski & LaDue (1955), and actinomycin A (a respiratory-chain inhibitor) was added at a concentration of 5 µg/ml to cell lysates. Total cell protein content was measured by the method of Lowry *et al.* (1951).

Statistical analysis

Paired or unpaired Student's *t* tests were used; values of *P* < 0.05 were considered significant.

RESULTS

Time course of insulin effect and preincubation with insulin

There was no significant effect of insulin on triacylglycerol or apo-B secretion until approx. 12 hours of incubation with the hormone (results not shown). Therefore cells which were to be exposed to insulin were pre-treated with the hormone for 16 h before addition of fatty acids to the medium.

Direct and [¹⁴C]triacylglycerol measurements

Direct measurement of secreted and intracellular triacylglycerol gave parallel results to those obtained from incorporation of [¹⁴C]NEFA. Re-uptake and lipolysis of

labelled triacylglycerol were not significant, and there was no significant increase in either re-uptake or lipolysis in the presence of insulin (Byrne *et al.*, 1991).

[1-¹⁴C]NEFA uptake

The uptake of each NEFA was studied during the periods of time up to 24 h. With an initial concentration of 500 µM, recoveries of ¹⁴C-labelled fatty acid ranged from 75 % (EPA) to 80 % (oleate, palmitate and stearate), and this difference was not significant. As observed with oleate (Byrne *et al.*, 1991) NEFAs were rapidly taken up by the cells (Table 1). EPA was the NEFA most avidly taken up, and, when individual NEFAs were compared there was no difference in the insulin-stimulated uptake.

Between 5 h and 24 h incubation, the concentration of NEFA in the medium fell from 10–30 % of the initial concentration remaining at 5 h to approx. 2 %. Therefore in experiments of 72 h duration the medium was replaced with medium of identical composition every 8 h throughout the 3 days.

Effect of insulin and NEFA on intracellular [¹⁴C]triacylglycerol accumulation, [¹⁴C]triacylglycerol secretion, intracellular [¹⁴C]phospholipid accumulation and [¹⁴C]phospholipid secretion

We have previously demonstrated that oleate is rapidly taken up by Hep G2 cells (Byrne *et al.*, 1991). Therefore, the effect of a given concentration of NEFA cannot be studied unless an attempt is made to compensate for the rapid uptake.

In short-term experiments (< 5 h, initial NEFA concn. 500 µM) 29 % more intracellular triacylglycerol accumulated and 22 % more triacylglycerol was secreted with EPA than with oleate (Table 2). There was a corresponding 28 % increase in uptake of EPA compared with oleate during this incubation (Table 1).

Table 1. Effects of insulin and NEFA on [1-¹⁴C]NEFA uptake over 5 h

Initially 1000 nmol of NEFA was added to each flask (500 µM). Results are expressed as nmol of NEFA/mg of cell protein taken up by the cells (means ± S.E.M. of triplicate flasks from four independent experiments): **P* < 0.01 for EPA versus oleate, palmitate or stearate.

NEFA	Control	Insulin
Palmitate	110 ± 2.3	115 ± 3.1
Oleate	100 ± 2.6	109 ± 3.1
Stearate	100 ± 1.3	104 ± 1.8
EPA	129 ± 2.3*	133 ± 1.8

Table 2. Effect of insulin and [1-¹⁴C]NEFA on intracellular and secreted [¹⁴C]triacylglycerol over 5 h

The initial concentration of all NEFA was 500 µM. Results are expressed as µmol of NEFA incorporated into triacylglycerol/g of cell protein (means ± S.E.M. of triplicate flasks from four independent experiments): **P* < 0.01 for EPA versus oleate.

NEFA		Control	Insulin
Palmitate	Intracellular	28.4 ± 1.8	33.1 ± 0.42
	Secreted	0.57 ± 0.05	0.39 ± 0.46
Oleate	Intracellular	26.4 ± 1.3	32.5 ± 0.78
	Secreted	0.57 ± 0.05	0.39 ± 0.06
Stearate	Intracellular	23.4 ± 1.9	26.9 ± 0.93
	Secreted	0.48 ± 0.03	0.36 ± 0.03
EPA	Intracellular	34.1 ± 2.34*	38.6 ± 1.02
	Secreted	0.70 ± 0.05*	0.45 ± 0.06

After 5 h the concentration of NEFAs remaining in the medium was 20–30 % of the initial concentration. When the same initial concentration of NEFA was used without supplementation for longer periods of time (24 h), less triacylglycerol was synthesized and secreted with EPA than with oleate ($31 \pm 0.3 \mu\text{mol}$ of $[1\text{-}^{14}\text{C}]\text{EPA}$ incorporated into cellular triacylglycerol/g of cell protein, compared with $48.0 \pm 0.2 \mu\text{mol}$ of $[1\text{-}^{14}\text{C}]\text{oleate}$ incorporated/g of cell protein; $P < 0.01$; and $1.15 \pm 0.01 \mu\text{mol}$ of $[1\text{-}^{14}\text{C}]\text{EPA}$ incorporated into secreted triacylglycerol/g of cell protein, compared with $1.30 \pm 0.01 \mu\text{mol}$ of $[1\text{-}^{14}\text{C}]\text{oleate}$ incorporated/g of cell protein; $P < 0.05$). Therefore during a 24 h period, depending on the time points chosen, it is possible to show both a stimulatory and an inhibitory effect of EPA on triacylglycerol synthesis and secretion compared with oleate. Insulin inhibited triacylglycerol secretion with all NEFAs during both 5 h and 24 h incubations.

Incorporation of $[1\text{-}^{14}\text{C}]\text{NEFA}$ into cellular and secreted phospholipid was also studied. EPA was preferentially incorporated into cellular phospholipid compared with other NEFAs. During a 5 h incubation there was $17.4 \pm 1.5 \mu\text{mol}$ of $[1\text{-}^{14}\text{C}]\text{-oleate/g}$ of cell protein incorporated into phospholipid, compared with $22.5 \pm 1.8 \mu\text{mol}$ of $[1\text{-}^{14}\text{C}]\text{EPA/g}$ of cell protein incorporated into phospholipid ($P < 0.01$). There was no significant difference between $[1\text{-}^{14}\text{C}]\text{oleate}$ and $[1\text{-}^{14}\text{C}]\text{EPA}$ incorporated into secreted $[^{14}\text{C}]\text{phospholipid}$.

Effect of insulin and NEFA on intracellular and secreted triacylglycerol over 72 h

Neither $75 \mu\text{M}$ -EPA nor $75 \mu\text{M}$ -oleate significantly altered the intracellular concentration of triacylglycerol compared with the absence of NEFA from the medium (Table 3). At $250 \mu\text{M}$ -oleate the intracellular triacylglycerol content was increased compared with zero NEFA. At $75 \mu\text{M}$ EPA did not decrease the effect of oleate to increase intracellular triacylglycerol. The intracellular triacylglycerol content further increased with increasing concentrations of exogenous oleate. Oleate maintained at $750 \mu\text{M}$ throughout a 72 h period doubled the intracellular triacylglycerol content (Table 3).

Increasing concentrations of exogenous oleate increased the concentration of secreted triacylglycerol, but this increase was not as marked as that observed in intracellular triacylglycerol. Oleate maintained at $750 \mu\text{M}$ throughout the 72 h increased secreted triacylglycerol by 30 %. In the presence of $75 \mu\text{M}$ -EPA there was a 13 % decrease in secreted triacylglycerol compared with the absence of NEFA from the medium ($P < 0.01$), and a 32 % decrease compared with $250 \mu\text{M}$ -oleate ($P < 0.01$) (Table 3).

Insulin inhibited triacylglycerol secretion without significantly affecting intracellular triacylglycerol.

Effect of insulin and NEFA on apo-B secretion over 5 h

With an initial NEFA concentration of $500 \mu\text{M}$ the results obtained for apo-B secretion were qualitatively similar to those for $[^{14}\text{C}]\text{triacylglycerol}$ secretion, with the most apo-B secreted in the presence of EPA (Table 4). EPA increased apo-B secretion by 46 % ($P < 0.01$) compared with the absence of exogenous NEFA, and increased apo-B secretion by 17 % compared with an identical concentration of oleate ($P < 0.05$). The percentage increase in $[^{14}\text{C}]\text{triacylglycerol}$ secretion attributable to EPA as compared with oleate was 22 %, which was not significantly different from the 17 % increase in apo-B attributable to EPA compared with oleate. There was no difference in apo-B secretion when palmitate and stearate were compared with oleate during this period. Insulin inhibited apo-B secretion by 28–40 %, a range comparable with the insulin-induced inhibition of $[^{14}\text{C}]\text{triacylglycerol}$ during the same period.

Table 3. Effect of insulin, EPA and oleate on intracellular and secreted triacylglycerol over 72 h

Results are expressed as mg/triacylglycerol/g of cell protein (means $\pm$ S.E.M. of triplicate flasks from four independent experiments). Values marked with asterisks are significantly different from their corresponding zero controls (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). The secreted triacylglycerol is that secreted during the final 8 h of the 72 h incubation, whereas the intracellular triacylglycerol is the total extracted after 72 h.

NEFA (concn.)		Control	Insulin
Zero	Intracellular	41.9 ± 3.5	42.9 ± 3.5
	Secreted	1.30 ± 0.05	1.05 ± 0.06
EPA ($75 \mu\text{M}$)	Intracellular	40.5 ± 3.8	38.7 ± 4.0
	Secreted	$1.14 \pm 0.03^*$	1.06 ± 0.07
Oleate ($75 \mu\text{M}$)	Intracellular	48.0 ± 4.0	48.9 ± 5.0
	Secreted	1.52 ± 0.07	1.18 ± 0.05
Oleate ($250 \mu\text{M}$)	Intracellular	$56.8 \pm 5.3^{**}$	62.1 ± 6.0
	Secreted	$1.69 \pm 0.08^{**}$	1.14 ± 0.08
EPA ($75 \mu\text{M}$) + oleate ($250 \mu\text{M}$)	Intracellular	$57.0 \pm 5.0^{**}$	62.0 ± 6.0
	Secreted	$1.52 \pm 0.12^{**}$	1.20 ± 0.11
Oleate ($750 \mu\text{M}$)	Intracellular	$81.0 \pm 6.5^{***}$	93.9 ± 9.1
	Secreted	$1.70 \pm 0.08^{**}$	1.18 ± 0.05

Table 4. Effect of insulin and NEFA on apo-B secretion over 5 h

Cells treated with insulin were preincubated with insulin for 16 h before the experiment. The initial concentration of each NEFA was $500 \mu\text{M}$. Results are expressed as ng of apo-B/mg of cell protein (means $\pm$ S.E.M. of triplicate flasks from four independent experiments); * $P < 0.01$, EPA versus zero.

NEFA	Control	Insulin
Zero	20.0 ± 1.3	14.5 ± 1.5
Oleate	25.0 ± 1.5	17.6 ± 1.9
Palmitate	25.0 ± 1.8	17.7 ± 2.0
Stearate	24.8 ± 1.6	17.3 ± 2.1
EPA	$29.3 \pm 1.7^*$	18.1 ± 1.8

Table 5. Effect of insulin and NEFA on apo-B secretion over 72 h

The initial concentration of each NEFA was $750 \mu\text{M}$. Cells treated with insulin were preincubated with insulin for 16 h before the experiment. Results are expressed as ng of apo-B/h per mg of cell protein (means of triplicate flasks $\pm$ S.E.M. from four independent experiments): * $P < 0.05$ for palmitate/stearate versus zero; ** $P < 0.01$ for insulin versus controls; *** $P < 0.01$ for oleate versus zero.

NEFA	Control	Insulin
Zero	7.4 ± 0.5	$5.7 \pm 0.5^{**}$
Oleate	$9.4 \pm 0.6^{***}$	$7.5 \pm 0.6^{**}$
Palmitate	$9.1 \pm 0.9^*$	$7.3 \pm 0.7^{**}$
Stearate	$8.7 \pm 0.7^*$	$7.0 \pm 0.7^{**}$

Effect of insulin and NEFA on intracellular and secreted apo-B over 72 h

Oleate, palmitate and stearate increased apo-B secretion compared with zero NEFA but did not differ from each other (Table 5). The percentage inhibition of apo-B secretion attributable to insulin was similar to that demonstrated for triacylglycerol secretion (Table 3).

Table 6. Effect of insulin, EPA and oleate on intracellular and secreted apo-B over 72 h

Results are expressed as ng of apo-B/mg of cell protein (means $\pm$ S.E.M. of duplicate flasks from five independent experiments): * $P < 0.05$ for oleate versus zero; ** $P < 0.01$ for EPA versus zero; *** $P < 0.01$ for EPA + oleate versus 250 μ M-oleate. The secreted apo-B is that secreted during the final 8 h of the incubation, whereas the intracellular apo-B is that present after 72 h.

NEFA (concn.)		Control	Insulin
Zero	Intracellular	14.7 $\pm$ 1.4	11.0 $\pm$ 1.1
	Secreted	25.2 $\pm$ 1.1	20.4 $\pm$ 1.4
EPA (75 μ M)	Intracellular	7.7 $\pm$ 2.2**	7.2 $\pm$ 0.3
	Secreted	19.8 $\pm$ 1.4**	15.7 $\pm$ 1.5
Oleate (75 μ M)	Intracellular	16.1 $\pm$ 0.7	15.1 $\pm$ 0.4
	Secreted	27.2 $\pm$ 2.7	20.4 $\pm$ 2.6
Oleate (250 μ M)	Intracellular	18.0 $\pm$ 1.5*	15.5 $\pm$ 0.9
	Secreted	30.2 $\pm$ 1.5*	22.1 $\pm$ 1.5
EPA (75 μ M) + oleate (250 μ M)	Intracellular	16.0 $\pm$ 1.5	15.0 $\pm$ 1.0
	Secreted	23.0 $\pm$ 1.8***	17.0 $\pm$ 1.5

EPA and oleate had different effects on intracellular apo-B. Compared with the absence of exogenous NEFA from the medium, 75 μ M-oleate increased the intracellular apo-B content by 9.5% and 250 μ M-oleate increased the intracellular apo-B content by 22% (Table 6). In contrast with the results obtained for intracellular triacylglycerol, increasing the exogenous oleate concentration to 750 μ M throughout the 72 h did not produce a further increase in the intracellular apo-B content. EPA (75 μ M) lowered the intracellular apo-B content by 52% compared with 75 μ M-oleate ($P < 0.01$). Similarly, compared with the absence of exogenous NEFA from the medium, EPA produced a 47% decrease in intracellular apo-B ($P < 0.01$) (Table 6). Combining EPA and oleate resulted in a small but not significant decrease in intracellular apo-B, compared with the effect of oleate alone. The effects of EPA and oleate on apo-B secretion were qualitatively similar to those on its intracellular apo-B content: 250 μ M-oleate increased secretion by 20% ($P < 0.05$) and 75 μ M-EPA decreased secretion by 22% ($P < 0.01$). Combining EPA and oleate resulted in a 23% decrease in secretion compared with oleate alone ($P < 0.01$).

Effect of NEFA on protein synthesis and cell viability

The concentrations of each NEFA examined were 75 μ M, 250 μ M, 500 μ M, 750 μ M during periods of time from 5 h to 72 h. There was no alteration in cellular protein content with palmitate, oleate and stearate at all the concentrations studied over the 72 h incubation, compared with incubation of cells with medium from which NEFAs were absent. However, incubation of cells with 250 μ M-EPA and 750 μ M-EPA decreased total cellular protein by 25% and 75% respectively after 72 h. Only when the EPA concentration was decreased to 75 μ M was there no alteration of cellular protein after 72 h incubation.

To assess cell viability further during incubation with EPA, LDH release into the medium was measured. The results were expressed as percentages $\pm$ S.D. and represented LDH released into the medium expressed as a percentage of the total LDH content of cells and medium. It was necessary to assess cell viability during short-term (< 5 h) incubations with 500 μ M-EPA to establish whether this concentration was toxic to cells in the short term, resulting in loss of cytosolic constituents, and also whether lower EPA concentrations were toxic during a 72 h incubation. After a 5 h incubation with 500 μ M-EPA, 4.6 $\pm$ 0.6% of LDH was released into the medium, compared with 4.0 $\pm$ 0.5%

Fig. 1. Regression analysis of (a) apo-B secretion and (b) triacylglycerol secretion versus intracellular apo-B content

of LDH released from control cells incubated in the absence of NEFA. However, after a 72 h incubation with 500 μ M-EPA 93.5 $\pm$ 5.9% of LDH was released, compared with 4.5 $\pm$ 0.8% with 75 μ M-EPA and 3.9 $\pm$ 0.6% from control cells incubated in the absence of NEFA. Therefore, since 75 μ M-EPA did not affect cell viability over 72 h, all the EPA data shown are with this concentration.

Effect of oleate and EPA on apo-B mRNA over 72 h

Cells were incubated in conditions similar to those described above, and triacylglycerol and apo-B were measured during and after a 72 h incubation with oleate and EPA. Cells were incubated in the absence of NEFA or with 750 μ M-oleate or 75 μ M-EPA for 72 h. To maintain conditions similar to those described for intracellular and secreted apo-B and triacylglycerol, the medium was replaced every 8 h with fresh medium of identical composition. Northern-blot analysis of apo-B mRNA revealed that there was no significant difference between cells incubated in the absence of NEFA and those incubated in 750 μ M-oleate or 75 μ M-EPA (results not shown).

Regression analysis of triacylglycerol and apo-B secretion with intracellular apo-B content

In order to investigate a possible relationship between triacylglycerol and apo-B secretion and intracellular apo-B content, the data obtained during 72 h incubations with insulin and various NEFAs were pooled and regression analysis was performed. There was a significant positive correlation between apo-B secretion and intracellular apo-B content ($r = 0.89$, $P < 0.001$) (Fig. 1a). Similarly there was an equally significant positive correlation between triacylglycerol secretion and intracellular apo-B content ($r = 0.89$, $P < 0.001$) (Fig. 1b).

DISCUSSION

These studies were designed to take account of the very rapid NEFA utilization by Hep G2 cells in tissue culture. The importance of considering this problem, which has not been

addressed in previous studies, is shown by our data. We have demonstrated that EPA may produce either a stimulatory or an inhibitory effect on triacylglycerol synthesis and secretion (relative to the effect of oleate), depending on the concentration and duration of the incubation. During short time-course experiments (< 5 h) high concentrations of EPA (500 μM) were more rapidly taken up by the cells, and during this time more triacylglycerol was synthesized and secreted than with oleate. The percentage increase in the rate of uptake was comparable with the percentage increase in triacylglycerol synthesis and secretion. There was also a similar increase in apo-B secretion with EPA compared with oleate during this period, suggesting that there is an increase in secretion of the assembled VLDL particle. It was important to assess whether the high EPA concentration (500 μM) used in the short-term experiments resulted in increased loss of cytosolic components, which might have contributed to the increased triacylglycerol and apo-B secretion observed during this period. Therefore LDH released into the medium was measured during short- (< 5 h) and long- (72 h) term incubations. Our data show that incubation of cells with 500 μM -EPA for 5 h did not alter LDH release, whereas incubation with 500 μM for 72 h resulted in a marked increase in LDH release, affecting cell viability. Incubation of cells with 75 μM -EPA did not affect LDH release during the 72 h incubation.

Lower concentrations of EPA were studied to determine whether there was an effect on lipid and lipoprotein metabolism in the absence of any effect on protein synthesis and cell viability. Lower concentrations of EPA did not alter triacylglycerol synthesis compared with the absence of NEFAs from the medium, and triacylglycerol secretion was inhibited. These results contrasted with those with oleate, which increased both triacylglycerol synthesis and secretion.

Insulin also has differing effects on triacylglycerol synthesis and secretion. Insulin stimulates synthesis (Durrington *et al.*, 1982; Dich *et al.*, 1983; Wang *et al.*, 1988; Byrne *et al.*, 1991) and inhibits secretion (Durrington *et al.*, 1982; Patsch *et al.*, 1983; Pullinger & Gibbons, 1985; Mangiapane & Brindley, 1986; Dashti & Wolfbauer, 1987; Wang *et al.*, 1988; Byrne *et al.*, 1991). Several other workers have also observed the large increase in intracellular triacylglycerol when hepatocytes are incubated in the presence of exogenous oleate (Thrift *et al.*, 1986; Ellsworth *et al.*, 1986; Wang *et al.*, 1988; Pullinger *et al.*, 1989; Byrne *et al.*, 1991), but only a small percentage of this is secreted.

From these and other experiments it is clear that triacylglycerol secretion is not simply regulated by the availability of intracellular triacylglycerol. Another possibility is that it is regulation of the intracellular apo-B concentration which determines incorporation of triacylglycerol into the VLDL lipoprotein particle, and hence controls the rate of VLDL secretion. We therefore measured intracellular and secreted apo-B under a variety of conditions, using a sensitive immunoassay, to determine whether there was a relationship between secretion of apo-B and triacylglycerol and the intracellular apo-B content. Intracellular and secreted apo-B was decreased by EPA and increased by oleate over 72 h. Insulin decreased apo-B secretion throughout the 72 h and tended to decrease intracellular apo-B (although this difference did not reach statistical significance). When the intracellular apo-B content from all conditions studied was compared with secreted triacylglycerol and apo-B there were highly significant correlations between these parameters, suggesting that the intracellular apo-B content was regulating lipoprotein secretion, whereas the intracellular triacylglycerol content was not associated with secreted triacylglycerol or apo-B.

EPA is available on prescription as a constituent of a preparation manufactured for the treatment of subjects with

hypertriglyceridaemia. However, the mechanism of action of EPA is not clear from studies *in vitro*. The effects of high concentrations of EPA (500 μM) which we have demonstrated are probably not relevant *in vivo*, as it is unlikely that dietary manipulation with fish oils would ever produce a plasma concentration approaching this. Currently there is no information available indicating patients' plasma concentrations during treatment with EPA, and this was confirmed after discussion with the manufacturers of the pharmaceutical preparation. However, it is unlikely that plasma concentrations as high as 500 μM would be achieved with oral medication, and administration of conventional doses of EPA to subjects with hyperlipidaemia does not cause overt hepatic necrosis. Consequently, the effects of high concentrations of EPA on hepatic lipoprotein secretion are probably irrelevant *in vivo*. The decreases in triacylglycerol and apo-B secretion observed with low concentrations of EPA (75 μM) are probably more relevant *in vivo*, and these results are consistent with the results of clinical studies (Sanders *et al.*, 1985; Schectman *et al.*, 1988; Axelrod, 1989; Harris, 1989; Fasching *et al.*, 1991).

The mechanism by which insulin, oleate and EPA regulate the intracellular apo-B content is not clear. Apo-B is synthesized and then either assembled into lipoprotein particles and secreted, or degraded intracellularly (Davis *et al.*, 1990; Klausner & Sitia, 1990). Published data suggest that insulin increases degradation of intracellular apo-B (Jackson *et al.*, 1990) and oleate may increase secretion by protecting intracellular apo-B from degradation (Dixon *et al.*, 1991).

In short-term experiments (< 24 h), Pullinger *et al.* (1989) did not observe any effect of insulin or oleate to regulate transcription of the apo-B gene or stability of the apo-B mRNA. Likewise EPA did not affect gene transcription during the same period of incubation, although when the effects of both EPA and docosahexaenoic acid were combined a significant decrease in apo-B mRNA occurred (Wong *et al.*, 1989). We have also been unable to detect any difference in the levels of apo-B mRNA when oleate or EPA was separately applied to cells for 72 h. It is possible that even longer periods of incubation are necessary before changes in mRNA are detectable. Rats fed with cholesterol-rich diets had a several-fold increase in hepatic apo-B mRNA (Matsumoto *et al.*, 1987), and patients with the rare recessive disease abetalipoproteinaemia were found to have a 6-fold increase in hepatic mRNA compared with control subjects (Lackner *et al.*, 1986).

Our data shows that high concentrations of EPA affect cell viability and suggest that the toxic effects of EPA should perhaps be considered when large therapeutic doses are employed in the treatment of subjects with hyperlipidaemias. The availability of intracellular apo-B for the synthesis of the VLDL particle is a key factor in the control of triacylglycerol secretion from the liver. Both insulin and NEFAs regulate intracellular apo-B content, and this is a major contribution to their regulation of the secreted lipoprotein. Further research to unravel the mechanisms by which the availability of intracellular apo-B is controlled may lead to novel approaches to decreasing elevated plasma triacylglycerol concentrations.

We thank Professor K. Siddle for his helpful comments, Professor J. Scott for the use of his apo-B cDNA and Dr. R. Pease for his advice. This work was supported by grants from the British Diabetic Association, CORDA, Glaxo and Medical Research Council (M.R.C.). C.D.B. is an M.R.C. Training Fellow.

REFERENCES

- Abbott, R. D. & Carroll, R. J. (1984) *Am. J. Epidemiol.* **119**, 830–836
- Ahrens, E. H., Hirsch, J., Insull, W., Tsaltas, T. T., Blomstrand, R. & Peterson, M. L. (1957) *Lancet* **i**, 943–953

- Assman, G. & Shultz, H. (1988) *Am. Heart J.* **116**, 1713–1724
- Axelrod, L. (1989) *Diabetes* **38**, 539–543
- Bang, H. O., Dyerberg, J. & Hjorne, N. (1976) *Acta Med. Scand.* **200**, 69–73
- Brockerhoff, H. (1963) *J. Lipid Res.* **4**, 96–100
- Byrne, C. D., Brindle, N. P. J., Wang, T. W. M. & Hales, C. N. (1991) *Biochem. J.* **280**, 99–104
- Cambien, F., Jacqueson, A., Richard, J. L., Warnet, J. M., Ducimetiere, P. & Claude, J. R. (1986) *Am. J. Epidemiol.* **124**, 624–632
- Chernick, S. S. (1969) *Methods Enzymol.* **14**, 627–630
- Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- Dahlen, G., Berg, K., Gillnas, T. & Ericson, C. (1975) *Clin. Genet.* **7**, 334–341
- Dashti, N. & Wolfbauer, D. (1987) *J. Lipid Res.* **28**, 423–437
- Davis, R. A., Thrift, R. N., Wu, C. C. & Howell, K. E. (1990) *J. Biol. Chem.* **265**, 10005–10011
- Dich, J., Bro, B., Grunnet, N., Jensen, F. & Kondrup, J. (1983) *Biochem. J.* **212**, 617–623
- Dixon, J. L., Furukawa, S. & Ginsberg, H. N. (1991) *J. Biol. Chem.* **266**, 5080–5086
- Durrington, P. N., Newton, S., Weinstein, D. B. & Steinberg, D. (1982) *J. Clin. Invest.* **70**, 63–73
- Dyerberg, J., Bang, H. O. & Hjorne, N. (1975) *Am. J. Clin. Nutr.* **28**, 958–966
- Ellsworth, J. L., Erickson, S. K. & Cooper, A. D. (1986) *J. Lipid Res.* **27**, 858–874
- Fasching, P., Ratheiser, K., Waldhausl, W., Rohac, M., Osterrode, W., Nowotny, P. & Vierhapper, H. (1991) *Diabetes* **40**, 583–589
- Folch, J. M., Lees, M. & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509
- Harris, W. S. (1989) *J. Lipid Res.* **30**, 785–807
- Hegstead, D. M., McGandy, R. B., Myers, M. L. & Stare, F. J. (1965) *Am. J. Clin. Nutr.* **17**, 281–95
- Herrmann, W., Biermann, J., Lindhofer, H. G. & Kostner, G. (1989) *Med. Klin.* **84**, 429–433
- Homan, R., Grossman, J. E. & Pownall, H. J. (1991) *J. Lipid Res.* **32**, 231–241
- Howard, B. V. (1987) *J. Lipid Res.* **28**, 613–628
- Jackson, T. K., Salhanick, A. I., Elovson, J., Deichman, M. L. & Amatruda, J. M. (1990) *J. Clin. Invest.* **86**, 1746–1751
- Kannel, W. B. & McGee, D. L. (1979) *J. Am. Med. Soc.* **241**, 2035–2038
- Keys, A., Anderson, J. T. & Grande, F. (1965) *Metab. Clin. Exp.* **14**, 776–787
- Klausner, R. D. & Sitia, R. (1990) *Cell* **62**, 611–614
- Kostner, G., Avogaro, P., Cazzolato, G., Marth, E., Bittolo-Bon, G. & Quinci, G. B. (1981) *Atherosclerosis* **38**, 51–61
- Lackner, K. J., Monge, J. C., Gregg, R. E., Hoeg, J. M., Triche, T. J., Law, S. W. & Brewer, H. B. (1986) *J. Clin. Invest.* **78**, 1707–1712
- Lang, C. A. & Davis, R. A. (1990) *J. Lipid Res.* **31**, 2079–2085
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mangiapane, E. H. & Brindley, D. N. (1986) *Biochem. J.* **233**, 151–160
- Matsumoto, A. H., Aburatani, H., Shibasaki, T., Kodama, T., Takaku, F. & Itakura, H. (1987) *Biochem. Biophys. Res. Commun.* **142**, 92–99
- Nossen, J. O., Rustan, A. C., Gløppstad, S. H., Malbakken, S. & Drevon, C. A. (1986) *Biochim. Biophys. Acta* **879**, 56–65
- Patsch, W., Franz, S. & Schonfeld, G. (1983) *J. Clin. Invest.* **71**, 1161–1174
- Pullinger, C. R. & Gibbons, G. F. (1985) *Biochim. Biophys. Acta* **833**, 44–51
- Pullinger, C. R., North, J. D., Teng, B. B., Rifci, V. A., Ronhild de Brito, A. E. & Scott, J. (1989) *J. Lipid Res.* **30**, 1065–1077
- Reaven, G. M. & Ida Chen, Y. D. (1988) *Diabetes Metab. Rev.* **4**, 639–652
- Rhoads, G. G., Dahlen, G., Berg, K., Morton, N. E. & Danneberg, A. L. (1986) *J. Am. Med. Soc.* **256**, 2540–2544
- Sanders, T. A. B., Sullivan, D. R., Reeve, J. & Thompson, G. R. (1985) *Arteriosclerosis* **5**, 459–465
- Schechtman, G., Kaul, S. & Kissebah, A. H. (1988) *Diabetes* **37**, 1567–1573
- Sinclair, H. M. (1953) *Proc. Nutr. Soc.* **12**, 69–82
- Thrift, R. N., Forte, T. M., Cahson, B. E. & Shore, V. G. (1986) *J. Lipid Res.* **27**, 236–250
- Uusitupa, M., Siitonen, O., Pyörälä, K., Aro, K., Herzio, K., Penttilä, J. & Voutilainen, E. (1985) *Diabetologia* **28**, 653–665
- Wang, S. R., Pessah, M., Infante, J., Catala, D., Salvat, C. & Infante, R. (1988) *Biochim. Biophys. Acta* **961**, 351–363
- Wong, S. & Marsh, J. B. (1988) *Metab. Clin. Exp.* **37**, 1177–1181
- Wong, S., Reardon, M. & Nestel, P. (1985) *Metab. Clin. Exp.* **34**, 900–905
- Wong, S. H., Fisher, E. A. & Marsh, J. B. (1989) *Arteriosclerosis* **9**, 836–841
- Wroblewski, F. & LaDue, J. S. (1955) *Proc. Soc. Exp. Biol. Med.* **90**, 210–213

Received 6 February 1992/14 April 1992; accepted 14 May 1992