Effects of oleic acid on the biosynthesis of lipoprotein apoproteins and distribution into the very-low-density lipoprotein by the isolated perfused rat liver

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The effects of oleic acid on the biosynthesis and secretion of VLDL (very-low-density-lipoprotein) apoproteins and lipids were investigated in isolated perfused rat liver. Protein synthesis was measured by the incorporation of L-[4,5-³H]leucine into the VLDL apoproteins ($d < 1.006$) and into apolipoproteins of the whole perfusate ($d < 1.21$). Oleate did not affect incorporation of [³H]leucine into total-perfusate or hepatic protein. The infusion of oleate, however, increased the mass and radioactivity of the VLDL apoprotein in proportion to the concentration of oleate infused. Uptake of oleate was similar with livers from fed or fasted animals. Fasting itself (24 h) decreased the net secretion and incorporation of [³H]leucine into total VLDL apoprotein and decreased the output of VLDL protein by the liver. A linear relationship existed between the output of VLDL triacylglycerol ($\mu\text{mol/h per g of liver}$) and secretion and/or synthesis of VLDL protein. Net output of VLDL cholesterol and phospholipid also increased linearly with VLDL-triacylglycerol output. Oleate stimulated incorporation of [³H]leucine into VLDL apo (apolipoprotein) E and apo C by livers from fed animals, and into VLDL apo B_h, B_i, E and C by livers from fasted rats. The incorporation of [³H]leucine into individual apolipoproteins of the total perfusate lipoprotein ($d < 1.210$ ultracentrifugal fraction) was not changed significantly by oleate during perfusion of livers from fed rats, suggesting that the synthesis *de novo* of each apolipoprotein was not stimulated by oleate. This is in contrast with that observed with livers from fasted rats, in which the synthesis of the total-perfusate lipoprotein ($d < 1.210$ fraction) apo B, E and C was apparently stimulated by oleate. The observations with livers from fed rats suggest redistribution of radioactive apolipoproteins to the VLDL during or after the process of secretion, rather than an increase of apoprotein synthesis *de novo*. It appears, however, that the biosynthesis of apo B_i, B_h, E and C was stimulated by oleic acid in livers from fasted rats. Since the incorporations of [³H]leucine into the VLDL and total-perfusate apolipoproteins were increased in fasted-rat liver when the fatty acid was infused, part of the apparent stimulated synthesis of the VLDL apoprotein may be in response to the increased formation and secretion of VLDL lipid.

INTRODUCTION

Non-esterified fatty acids (NEFA) taken up by the liver are converted into fatty acyl-CoA and subsequently esterified or oxidized [1–3]. Esterified fatty acid is released by the liver primarily in the form of triacylglycerol (TG) as a major constituent of the very-low-density lipoprotein (VLDL). In the normal fed state, the amount of TG and VLDL secreted by the liver depends on the rate of hepatic uptake of NEFA, which is proportional to the concentration of NEFA in the plasma *in vivo*, or in the medium perfusing the isolated liver *in vitro*. Concomitantly, NEFA also stimulate hepatic synthesis and secretion of other VLDL constituents, phospholipid and cholesterol [4–7], and increase the activity of hydroxymethylglutaryl-CoA reductase [8,9]. For transport of TG, the VLDL contains specific apolipoproteins that apparently are required for the stability of the particle and/or participate in its metabolism [10].

It is reasonable to assume that the synthesis and release of VLDL apoproteins are co-ordinately regulated

with that of VLDL lipids. Perfused livers from normal fed rats secrete TG at a rate proportional to NEFA uptake until a maximal rate of secretion is attained, after which TG accumulates intracellularly [11]. Some process involved in VLDL formation and release other than the synthesis of TG, such as the synthesis and/or secretion of surface lipids and apolipoproteins, must limit TG transport from the liver as VLDL. For example, it is possible that the synthesis and secretion of cholesterol, which is stimulated by NEFA [8,9], may be rate-limiting for secretion of the VLDL. Interestingly, Kosykh *et al.* [12] observed that secretion and synthesis of apolipoprotein (apo) B by human liver cells was stimulated by cholesterol, present in the incubation medium. In a comprehensive review, Vance & Vance [13] discussed the possible role which phospholipids, whose synthesis is increased by several NEFA [5], may play in the synthesis and secretion of lipoproteins by the liver. Nevertheless, specific information about how phospholipids might influence synthesis and secretion of VLDL is lacking. It is therefore logical to hypothesize that VLDL-protein

Abbreviations used: NEFA, non-esterified fatty acids; TG, triacylglycerol; VLDL, very-low-density lipoprotein; apo, apolipoprotein; BSA, bovine serum albumin.

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synthesis might also be stimulated by NEFA and may become rate-limiting for formation and secretion of the VLDL. We therefore investigated the effects of oleate on VLDL-apoprotein biosynthesis, using the isolated rat liver model. A preliminary report of this work has appeared [14].

EXPERIMENTAL

Chemicals

All chemicals used were reagent grade. Oleic acid (99% purity) was obtained from Applied Science Inc., State College, PA, U.S.A. BSA (fraction V; powder), obtained from Pentex, Kankakee, IL, U.S.A., was purified before use as described previously [5]. Silica-gel G plates (250 mm thick) were purchased from Analtec, Newark, DE, U.S.A. L-[4,5-³H]Leucine was obtained from New England Nuclear. Tissue solubilizer (TS-1) and scintillation fluid (Biocount) were obtained from Research Products International Corp., Mount Prospect, IL, U.S.A.

Animals

Male Sprague-Dawley rats (175–200 g), obtained from Harlan Industries, Indianapolis, IN, U.S.A., were fed on standard Purina laboratory chow and water *ad libitum* until the time of the experiment. Fasted animals were deprived of food 24 h before the experiment. The housing facility maintained a 12 h-light/12 h-dark cycle (dark 18:00 to 06:00 h).

Perfusion of livers

Rats were generally killed between 08:00 and 10:00 h. Livers were perfused *in vitro* for 4 h by procedures similar to those reported previously from this laboratory [14,15]. Livers were surgically removed from rats anaesthetized with pentobarbital (60 mg/kg). The basal perfusion medium consisted of 30% (v/v) bovine erythrocytes, 6 g of BSA/dl and 100 mg of glucose/dl in Krebs-Henseleit bicarbonate buffer [4] (pH 7.4), L-[4,5-³H]leucine (3.6 μ Ci/ml), non-radioactive leucine (8 mM), and amino acids at concentrations similar to those in rat plasma [16]. BSA used in the perfusion medium was delipidated and purified as described previously [5,17]. The liver was placed in the perfusion apparatus containing the basal medium, and an infusion was started immediately, which was continued at a constant rate (11.7 ml/h) during the experiment (4 h). For the experiments without oleate, the infusate consisted of Krebs-Henseleit bicarbonate buffer (lacking Ca^{2+} or Mg^{2+}), containing L-[4,5-³H]leucine (3.6 μ Ci/ml), 8 mM-leucine, and other amino acids at physiological concentrations [16] and BSA (6 g/dl). Thus leucine of constant specific radioactivity was provided to the livers throughout the entire experiment by adding the [³H]-leucine to the original medium as well as infusing it with the BSA in the presence or absence of added oleate. For the experiments with oleate, the perfusate was similar to that described above, but contained, in addition, oleic acid at concentrations sufficient to supply the fatty acid at rates of 55, 110, 166 or 332 μ mol/h. These procedures permitted us to maintain a relatively constant concentration of NEFA in the perfusate for the duration of the experiment [11] (Table 1), along with a constant concentration of leucine (8 mM) and other

amino acids at physiological concentrations. Although a priming dose of NEFA was not added in these experiments, a steady-state concentration was generally attained within 15–30 min. A sample of the perfusate was removed for analysis every hour during the 4 h experimental period. After the termination of perfusion, the liver was rinsed with ice-cold 0.9% NaCl, weighed, and 1 g samples were homogenized in chloroform/methanol (2:1, v/v) or buffer for analysis of lipid and radioactivity respectively.

Chemical analysis

After sedimentation of the erythrocytes from the perfusate, the VLDL was isolated by ultracentrifugation in a Beckman 50.2 rotor [14] at the density of the protein-free perfusion medium ($d = 1.006$). A sample of perfusate plasma, or isolated lipoprotein, was extracted with chloroform/methanol (2:1, v/v) [18]. Individual lipid classes were separated by t.l.c. on silica gel G plates [5] and appropriate bands were analysed for TG [19], cholesterol [20] and phospholipid [21]. Analysis of NEFA in lipid extracts was done with the extraction mixture of Stajner & Suva [22]. The VLDL protein was measured by the method of Lowry *et al.* [23], modified to eliminate turbidity by the addition of SDS [24].

For characterization of VLDL apoproteins, the VLDL was dialysed exhaustively against 1 mM-EDTA/0.02% NaN_3 , pH 7.0, freeze-dried, and then delipidated with chloroform/methanol (2:1, v/v) at 4 °C [25]. The solubilized apoproteins [in 0.2 M-Tris (pH 8.6)/0.2 M-SDS] were examined routinely by vertical-slab SDS-gradient polyacrylamide-gel electrophoresis [26]. Polyacrylamide gels were stained with Coomassie Blue and scanned with an LKB laser scanner. The gels were analysed with an LKB recording integrator. Percentages relative to the total are reported without correction for possible differences among the apolipoproteins in chromogenicity.

Measurement of incorporation of [³H]leucine into protein

Incorporation of radioactive leucine into protein of the VLDL, total perfusate and liver was estimated after precipitation with 10% (v/v) trichloroacetic acid in the presence of excess unlabelled leucine. Additionally, the trichloroacetic acid precipitate of the VLDL was extracted with chloroform/methanol (2:1, v/v) to allow correction for any conversion of radioactive leucine into lipid-soluble metabolites. The precipitated protein was solubilized in Hyamine hydroxide, and, after neutralization with acetic acid, radioactivity was determined by liquid-scintillation spectrometry (Beckman LS3801). After scanning, stained bands were sliced from gels and solubilized with H_2O_2 and TS-1 for determination of radioactivity.

Isolation of total perfusate lipoprotein apoprotein

Fresh rat plasma (0.3 ml) was added as carrier in a manner similar to that described by Windmueller & Spaeth [27] to a sample (4.5 ml) of the 4 h perfusate plasma, the density was adjusted to $d = 1.21$, and the sample was ultracentrifuged for 22 h at 60000 rev./min in a Beckman-Ti 80 rotor. The $d < 1.21$ lipoprotein fraction was recovered in the top 1 ml after the single ultracentrifugation. After dialysis and delipidation, the lipoprotein fraction was subjected to SDS/poly-

Table 1. Effect of oleic acid on secretion of VLDL TG and protein, and on incorporation of [³H]leucine into VLDL protein

Livers from rats fed on standard chow diet, or fasted for 24 h, were perfused for 4 h with L-[4,5-³H]leucine, in the presence or the absence of oleate. Synthesis of VLDL apoprotein was measured by incorporation of [³H]leucine. The steady-state concentration of NEFA ($\mu\text{mol/ml}$ of perfusate) during perfusion is also indicated. Numbers of observations are given in parentheses; ND, not determined. * $2P < 0.05$ compared with appropriate group (fed or fasted state) when oleate was not infused (A.1. or B.1.). † $2P < 0.05$ compared to fed state when oleate was not infused (A.1.).

Group	Rate of infusion of oleate	Incorporation of [³ H]leucine (d.p.m./h per g)	Output of VLDL protein mass ($\mu\text{g/h}$ per g)	Sp. radioactivity (d.p.m./ μg)	Output of VLDL TG ($\mu\text{mol/h}$ per g)	Perfusate plasma NEFA concn. (mm)†	Hepatic uptake of NEFA ($\mu\text{mol/h}$ per g)
A. Fed	1. None (20)	18 530 $\pm$ 1680	55 $\pm$ 5	395 $\pm$ 47	0.21 $\pm$ 0.01	0.12 $\pm$ 0.01 (16)	-0.5 $\pm$ 0.1
	2. 55 $\mu\text{mol/h}$ (4)	20 240 $\pm$ 4070	66 $\pm$ 7	269 $\pm$ 14	0.32 $\pm$ 0.08	0.44 $\pm$ 0.06	3.6 $\pm$ 0.1
	3. 110 $\mu\text{mol/h}$ (6)	27 200 $\pm$ 2580*	90 $\pm$ 2*	401 $\pm$ 69	0.70 $\pm$ 0.02*	0.47 $\pm$ 0.07	8.3 $\pm$ 0.7
	4. 166 $\mu\text{mol/h}$ (14)	33 100 $\pm$ 4700*	96 $\pm$ 10*	378 $\pm$ 35	0.77 $\pm$ 0.09*	0.61 $\pm$ 0.05 (13)	12.6 $\pm$ 0.5
B. Fasted (24 h)	5. 332 $\mu\text{mol/h}$ (3)	35 000 $\pm$ 4700*	ND	ND	1.00 $\pm$ 0.15*	1.29 $\pm$ 0.15	30.1 $\pm$ 1.7
	1. None (14)	5050 $\pm$ 100†	31 $\pm$ 6†	188 $\pm$ 29†	0.14 $\pm$ 0.04†	0.09 $\pm$ 0.01 (14)	-0.6 $\pm$ 0.1
	2. 166 $\mu\text{mol/h}$ (13)	22 700 $\pm$ 3600*	65 $\pm$ 6*	326 $\pm$ 32*	0.38 $\pm$ 0.12*	0.54 $\pm$ 0.03 (13)	14.7 $\pm$ 1.0

† With the infusion of oleate as indicated, these values represent the concentration of the fatty acid maintained throughout the 4 h perfusion.

acrylamide-gel electrophoresis as described above for the VLDL. Densitometric scanning revealed only 10–15% contamination by rat or bovine albumin. Stained apolipoprotein bands were sliced from the gel and solubilized for determination of radioactivity as discussed above.

Statistics

Values in all Tables are reported as means $\pm$ S.E.M. Statistical comparisons were made by using Student's unpaired *t* test. Statistical significance was taken as $2P < 0.05$.

RESULTS

Effect of oleic acid on secretion of VLDL apoprotein and incorporation of [³H]leucine

The incorporation of [³H]leucine into total VLDL apoproteins by livers from fed rats was stimulated by oleate and increased with increasing concentration of oleate in the perfusate plasma (Table 1). This was accompanied by increased secretion of VLDL protein. As expected, the output of VLDL TG was also stimulated by infusion of oleate. Fasting itself decreased incorporation of [³H]leucine (5050 versus 18 530 d.p.m./h per g) and mass of VLDL apoprotein secreted (31 versus 55 $\mu\text{g/h}$ per g), when livers were perfused without oleate. Incorporation of [³H]leucine into VLDL apoproteins by livers from fasted rats increased about 4-fold in response to oleic acid, whereas the mass of protein secreted in VLDL doubled. The amount of oleic acid provided to the fasted liver did not stimulate the incorporation of [³H]leucine into VLDL protein, or net accumulation of VLDL protein in the medium to that value reached with livers from fed animals under similar conditions. The specific radioactivity (d.p.m./ μg) of the total VLDL apoprotein was not different with livers from fed animals when NEFA was infused in amounts of 55–166 $\mu\text{mol/h}$. Fasting decreased the specific radioactivity of total VLDL apoprotein in comparison with the fed control in experiments in which oleate was omitted (395 versus

Table 2. Incorporation of [³H]leucine into total perfusate and hepatic protein

Data presented are means $\pm$ S.E.M. The total incorporation of [³H]leucine into perfusate and hepatic protein was determined by trichloroacetic acid precipitation as described in the text. Oleate was infused at 166 $\mu\text{mol/h}$ as indicated. * $2P < 0.05$ compared with fed state when oleate was not infused (A.1.).

Group	Leucine incorporated (d.p.m./h per g)	
	Perfusate ($\times 10^{-6}$)	Liver ($\times 10^{-5}$)
A. Fed		
1. No oleate (12)	1.62 $\pm$ 0.10	0.82 $\pm$ 0.09
2. Oleate infused (12)	1.48 $\pm$ 0.09	0.94 $\pm$ 0.09
B. Fasted (24 h)		
1. No oleate (12)	1.34 $\pm$ 0.14	1.25 $\pm$ 0.10*
2. Oleate infused (11)	1.20 $\pm$ 0.07	1.16 $\pm$ 0.17

Table 3. Effect of oleate on incorporation of [³H]leucine into individual VLDL apoproteins

Data are means $\pm$ S.E.M. Where indicated, 166 μ mol of oleate/h was infused. Incorporation was calculated from the percentage distribution of radioactivity on the SDS/polyacrylamide gel used to separate the apolipoproteins and the incorporation rate of radioactivity in the total VLDL protein (Table 1). * $2P < 0.05$ compared with appropriate group (fed or fasted state) when oleate was not infused (A.1. or B.1.). † $2P < 0.05$ compared with fed state when oleate was not infused (A.1.).

Group	Apoprotein ...	Leucine incorporated (d.p.m./h per g)			
		B _h	B _i	E	C
A. Fed					
	1. No oleate (11)	7775 ± 1103	3158 ± 384	4235 ± 1050	2978 ± 422
	2. Oleate infused (10)	6814 ± 950	5400 ± 635*	8720 ± 855*	5370 ± 950
B. Fasted					
	1. No oleate (11)	1680 ± 40†	730 ± 280†	245 ± 16†	830 ± 240
	2. Oleate infused (9)	8520 ± 1939*	3520 ± 490*	1885 ± 190*	3080 ± 775

Table 4. Percentage distribution of mass among VLDL apoproteins

Data are means $\pm$ S.E.M. Where indicated, 166 μ mol of oleate/h was infused. VLDL was treated as described in Table 3. The percentage relative area was calculated after scanning the VLDL SDS/polyacrylamide gel on a laser densitometer as described in the text. The distribution reported here is that for known apoproteins only (100 %); 11–16 % of the integrated stained gel occurred in areas of unknown composition, although the primary contaminant was albumin. * $2P < 0.05$ compared with appropriate group (fed or fasted) when oleate was not infused (A.1. or B.1.). † $2P < 0.05$ compared with fed state when oleate was not infused (A.1.).

Group	Apoprotein . . .	Distribution (%)					
		B _h	B _i	AIV	E	AI	C
A. Fed							
	1. No oleate (15)	22.0±3.6	21.9±1.9	0.4±0.1	11.1±2.1	0.2±0.1	44.3±2.3
	2. Oleate infused (14)	15.1±2.7	19.9±1.8	1.1±0.3	20.3±3.8*	4.4±0.9*	39.0±3.6
B. Fasted (24 h)							
	1. No oleate (12)	27.5±4.8	22.1±2.6	6.8±2.3†	0.7±1.4†	0.7±0.4	42.1±4.8
	2. Oleate infused (13)	30.9±3.9	17.6±2.1	2.1±1.3	9.2±2.8*	2.4±0.9	37.4±6.9

188). The specific radioactivity, however, increased approximately to normal fed values when oleate was infused. In agreement with previous studies, the hepatic uptake of NEFA was unaffected by nutritional state of the animal, and uptake was proportional to the amount of NEFA infused [11]. The molar ratios of fatty acid to albumin in the medium were maintained constant during the 4 h perfusion (0.51, 0.54, 0.76 and 1.48 from the lowest to the highest rate of oleate infused respectively, corresponding to final concentrations of NEFA in perfusate plasma as indicated in Table 1). Even at the highest ratio of 1.48, when 332 μ mol of oleate was infused, there was no appreciable haemolysis of erythrocytes.

Incorporation of [³H]leucine into total perfusate and hepatic protein

In agreement with earlier data [28], [³H]leucine was incorporated into total protein in a linear manner after a lag time of 30 min during the 4 h perfusion (results not shown). Fasting or availability of exogenous NEFA did not affect the lag period, linearity, or total incorporation of radioactivity. Incorporation of [³H]leucine into hepatic or total perfusate protein was unaffected by infusion of fatty acid with livers from either fed or fasted rats (Table

2). Incorporation of radioactivity tended to be greater into perfusate protein and lesser into hepatic protein by livers from animals in the fed than in the fasted state (Table 2); however, only the incorporation into hepatic protein was significantly different.

Effects of oleic acid on secretion and incorporation of [³H]leucine into specific VLDL apoproteins

To determine whether incorporation of [³H]leucine into specific VLDL apoproteins was stimulated by fatty acid, the distribution of [³H]leucine among individual apoproteins was determined after separation by SDS/polyacrylamide-gel electrophoresis. When oleate was added to the medium perfusing livers from fed rats, the incorporation of [³H]leucine into apo B_h (high-molecular-mass apolipoprotein B₁₀₀) was unaffected, whereas that into apo E, apo B_i (low-molecular-mass apolipoprotein B₄₈) and apo C of the VLDL was greater than that with livers perfused in the absence of oleate (Table 3). When oleate was infused in experiments with livers from fasted rats, an increase in the incorporation of radioactivity into all VLDL apoproteins was observed, in comparison with VLDL from experiments when oleate was not infused. The percentage of VLDL apo E, measured by densitometry, increased in response to infusion of oleate

Table 5. Incorporation of L-[4,5-³H]leucine into *d* < 1.21 perfusate apolipoproteins

Values are means $\pm$ s.e.m., relative to the 'fed, no oleate' group set at 100%. The methodology may not have allowed identical recovery of each individual apoprotein. It is assumed that there is a constant recovery of each apoprotein among the four groups, since carrier plasma was added to provide excess apoproteins and allow dilutions of label for dissociated apoproteins (see the Experimental section). Apoproteins were separated by SDS/polyacrylamide-gel electrophoresis as described in the text. Where indicated, 166 μ mol of oleate/h was infused. * $2P < 0.05$ compared with appropriate group when oleate was not infused. † $2P < 0.05$ compared with fed state when oleate was not infused. ‡ $2P < 0.10$ compared with fed state when oleate was not infused.

Group	Apoprotein . . .	Incorporation (%)			
		B _h	B _i	E	C
A. Fed					
1. No oleate (6)		100 ± 17	100 ± 12	100 ± 20	100 ± 16
2. Oleate infused (7)		78 ± 10	83 ± 10	55 ± 9	89 ± 7
B. Fasted					
1. No oleate (7)		64 ± 8†	42 ± 5†	46 ± 5†	78 ± 20
2. Oleate infused (5)		130 ± 22*	103 ± 17*	76 ± 2*	60 ± 23

Fig. 1. Correlation between output of VLDL TG and VLDL cholesterol or phospholipid

Livers from male rats were perfused with increasing oleate concentrations. The output of VLDL TG (i.e. net accumulation in the VLDL) was plotted as a function of output of VLDL cholesterol (a) and phospholipid (c) by livers from fed animals, and that of VLDL cholesterol (b) and phospholipid (d) by livers from fasted rats. The data points are the experimental observations with individual livers. Livers were perfused with oleate as follows: □, none; ◆, 55 μ mol/h; ×, 110 μ mol/h; ■, 166 μ mol/h; ▲, 332 μ mol/h.

in experiments with livers from fed rats (Table 4). Fasting itself decreased the percentage of VLDL apo E compared with the fed state. Furthermore, in the fasted state, the percentage of apo E increased when oleic acid was infused.

Incorporation of radioactivity into VLDL protein, and the output of VLDL protein mass, were measured only at the termination of the perfusion experiment (4 h). The secretion of VLDL protein mass was observed, in a limited number of perfusions, to be linear during the

Fig. 2. Correlation between output of VLDL TG and total VLDL protein

Livers from male rats were perfused with increasing oleate concentrations. The output of VLDL TG (i.e. net accumulation in the VLDL) by livers from fed animals was plotted as a function of incorporation of [3 H]leucine into VLDL protein (d.p.m./h per g; a) and secretion (i.e. accumulation) of VLDL protein mass (μ g/h per g; c). The incorporation (d.p.m./h per g; b) and mass secretion (μ g/h per g; d) by livers from fasted animals are also shown. Livers were perfused with oleate as follows: $\square$, none; $\blacklozenge$, 55 μ mol/h; $\times$, 110 μ mol/h; $\blacksquare$, 166 μ mol/h; $\blacktriangle$, 332 μ mol/h.

4 h experimental period, in the presence or absence of added oleate. Incorporation of [3 H]leucine into the lipoprotein apoprotein was also observed to be linear with time. A lag period preceding incorporation of radioactivity was not observed, perhaps because of the low amounts of radioactivity during the early time periods, and the limited number of observations. However, output of TG, as shown previously by our laboratory [4], accumulated in a linear fashion (i.e. 49% accumulated during the first 2 h and 51% in the second 2 h) whether the livers were obtained from fed or fasted rats, or whether fatty acid was added to the medium. Presumably, secretion of total perfusate TG is a reflection of secretion of VLDL TG, since in the experiments reported here approx. 85% of the perfusate TG was recovered in the VLDL isolated at the termination of the experiment.

In contrast with the VLDL apoproteins, oleate did not stimulate the incorporation of [3 H]leucine into individual $d < 1.210$ apolipoproteins by livers from fed rats (Table 5). Fasting itself decreased the incorporation of [3 H]leucine into $d < 1.210$ apo B_{100} , B_1 and E; furthermore, this incorporation of [3 H]leucine by livers from fasted rats was stimulated when oleate was infused, just as for the VLDL.

The relationship between the secretion of the VLDL TG and the synthesis and secretion of the constituent

VLDL apoproteins was evaluated. The relationship of the output (i.e. net accumulation in the perfusate) of VLDL TG and total VLDL protein, in the ranges of output of VLDL TG studied, was linear, as was the relationship between output of VLDL TG and incorporation of [3 H]leucine into VLDL protein, with livers from fed rats (Fig. 1). Although the mass of secreted VLDL protein and radioactivity incorporated diminished with livers from fasted rats when VLDL-TG output was minimal, as the output of VLDL TG increased by infusion of oleate, synthesis and output of VLDL protein relative to TG output were elevated to values approaching those in the fed state. This linearity was also observed for VLDL cholesterol and for VLDL phospholipid when the net accumulation was compared with that of VLDL TG (Fig. 2). Similar relationships were reported previously between VLDL-TG secretion and that of VLDL cholesterol and phospholipid in livers from both male and female rats [7].

DISCUSSION

Our current and previous studies [14] demonstrated that, when exogenous oleate is available to livers from fed or fasted rats, VLDL-TG and apoprotein secretion are increased concomitantly. We reported on other occasions the apparent proportionality of output of

VLDL TG and VLDL protein [29]. Ruderman *et al.* [30] and Petersburg *et al.* [31] derived similar conclusions from their data with perfused rat livers. In contrast, Patsch *et al.* [32], Davis *et al.* [33] and Dashti & Wolfbauer [34] failed to observe a stimulation of secretion of VLDL protein by fatty acid, concomitant with an increased secretion of TG by hepatocytes or HepG2 cells in tissue culture. However, Bell-Quint *et al.* [35] and Rash *et al.* [36] reported that apo B synthesis was stimulated by oleate in hepatocytes or HepG2 cells, respectively. The reasons for discrepancies in response to fatty acid among the various studies with intact perfused livers or using isolated hepatocytes in tissue culture are not understood at present. As stated by Forte [37], however, conditions must be defined carefully if tissue-culture experiments are to be compared properly in evaluation of regulation of apolipoprotein synthesis.

Since incorporation of [^3H]leucine into VLDL apo B₁, E and C was increased by oleate, yet that into the total $d < 1.210$ apolipoproteins (apo B_n, B₁, E and C) was not affected in the experiments with fed rats, the additional protein associated with the VLDL probably was not a result of stimulated synthesis *de novo* of the apoproteins, but rather was derived from additional apolipoproteins independently secreted into the medium. Oleate, however, did stimulate the synthesis of apoproteins by livers from 24 h-fasted animals, as indicated by the increased incorporation of [^3H]leucine into both VLDL and $d < 1.210$ apoproteins. Even so, with the increased secretion of VLDL, just as with VLDL produced by livers from fed rats, some of the apoprotein of the VLDL may represent redistributed apoprotein. Without addition of exogenous fatty acid, the synthesis of all VLDL apoproteins by livers from fed animals exceeded that of fasted rats, which, except for apo C, was reflected in the incorporation of [^3H]leucine into apoprotein of the whole perfusate.

A major portion of the additional apoprotein associated with the VLDL secreted by livers from fed rats was apo E, as indicated by the increased proportion of mass (Table 4) and radioactivity (Table 3) along with the increased output of total VLDL protein (Table 1). Apo B₁ and apo C were also increased, but to a lesser extent. We reported previously that apo E (determined by radioimmunoassay) is shifted to VLDL when synthesis and output of TG are stimulated by oleate [38]. Our present radioactivity data support that observation. Although we do not have similar quantitative data (i.e. radioimmunoassay) also for apo B_n, B₁ and C, it can be estimated from the increase in VLDL protein mass (Table 1) and the relative mass percentages (Table 4) that the amount of apo B₁ associated with VLDL increased from 12 to 19 $\mu\text{g/h}$ per g, apo C from 24 to 37 $\mu\text{g/h}$ per g, and apo E from 6 to 20 $\mu\text{g/h}$ per g when fed livers were stimulated with infusion of 166 μmol of oleate/h. However, the amount of apo B_n in the VLDL was not changed significantly (12 to 14 $\mu\text{g/h}$ per g).

We reported previously that apo E has a unique affinity for VLDL, which may be related to the TG content of the VLDL [38]. Dolphin *et al.* [39] have also observed this association of apo E with the VLDL, which may represent a necessary condition for subsequent metabolism of the VLDL. Our previous report suggested that apo E is necessary for hepatic uptake of nascent VLDL [38], as has also been inferred by others [40], and has been shown for remnant particles [41]. Whether

apo E becomes associated with the VLDL before or after secretion remains to be determined. Apo E has been observed in VLDL isolated directly from Golgi apparatus [42]. Apo E is thought to be secreted with a nascent high-density-lipoprotein fraction by perfused livers [43], as well as in a lipid-free form [44], either of which may provide additional apoprotein available for association with the VLDL.

It has been generally accepted that synthesis of apo B is required for structure and for the formation of the VLDL [45], even though evidence is not yet completely conclusive as to individual roles for apo B_n and B₁. Our present data suggest that apo B_n is synthesized by the liver at a constant rate in the formation of VLDL in the fed state. That apo B_n synthesis was not increased by oleate with livers from fed animals, but was increased in those of fasted rats, suggests the essential role of this apo B subclass in VLDL formation, and that its synthesis alone may be required to form a basic structure to carry lipid. Swift *et al.* [42] presented evidence for either an independent regulation or a precursor-product relationship of apo B₁ and B_n. Our data indicate indirectly that synthesis of apo B (apo B_n, in particular) was required for VLDL formation and subsequent secretion. However, the data also suggest that apo B synthesis may become maximal without curtailing additional TG transport, as noted in the experiments with fed rats. Windmueller & Spaeth [27] concluded that the synthesis and secretion of the apo B forms were physiologically regulated. They suggested that the secretion of B₄₈ (B₁) was directly related to secretion of TG. Although it is probable that most apo B_n is secreted by the liver as a component of the VLDL, as our data would indicate, some apo B₁ may be secreted in other fractions, and possibly may associate with VLDL after secretion. For example, it is possible that some apo B₁ may be derived from nascent HDL. Indeed, Fainaru *et al.* [43] described the presence of 'apo B' in nascent HDL of liver perfusates. We have also noted the presence of radioactive apo B (apo B₁ primarily) in the high-density-lipoprotein fraction ($d = 1.063\text{--}1.210$) of liver perfusates (H. G. Wilcox & M. Heimberg, unpublished work).

Apo C (particularly apo C III) has been shown by Nestruck & Rubenstein [46] to be acquired by VLDL after hepatic secretion. Their data suggested independence of apo C synthesis and VLDL formation and secretion. Our data support their report, since synthesis of apo C was not affected by fasting or by oleate. The association of apo C (apo C III), like apo E, and perhaps apo B₁, may be related to the size and/or lipid composition [38] of the VLDL particle, which may then result in the purported modulation of VLDL catabolism by these apoproteins.

The specific radioactivity (d.p.m./ μg) of total VLDL apoprotein produced by livers from fed rats was unaffected by oleate. With livers from 24 h-fasted rats, the specific radioactivity of VLDL apoprotein was lower than that observed in livers from fed rats, but was increased by the addition of oleate. This difference between the fed and the fasting state could reflect variations in leucine pool size. However, this should have been equalized in our experiments by the constant infusion into the medium of a large excess of leucine (8 mM) with constant specific radioactivity [16]. Such differences may also reflect possible differences in rate of protein turnover, in the size of the existing unlabelled

pool of the specific apoprotein, and/or, less likely, differences in the leucine content of the apoprotein. However, it more probably represents the stimulated synthesis of the apolipoproteins by oleic acid by livers from rats in the fasted versus the fed state.

The data presented here suggest that, in the fed state, the extent of apoprotein synthesis by the livers is maximal, and that apo B_h may be the primary apoprotein required for VLDL formation. Furthermore, in the fed state TG transport can be increased in response to increased synthesis of TG from exogenous fatty acid (oleate), without synthesis of additional apolipoprotein. However, as TG output increases, additional apolipoprotein becomes associated with the VLDL, particularly apo E, but also B_i and C. In livers from 24 h-fasted rats, however, apolipoprotein synthesis is stimulated by increased TG synthesis and transport. The mechanism of this stimulation is at present unclear.

We express our appreciation to Karen White, Marie Hurt and David Young for their excellent technical assistance. This research was supported by grants HL-27850 and BRSG-84-09 from the National Institutes of Health, U.S. Public Health Service. W.H.S. is a postdoctoral trainee.

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