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STIMULATION BY OLEIC ACID OF INCORPORATION OF L-[4,5-^{3}H]LEUCINE INTO VERY LOW DENSITY LIPOPROTEIN APOPROTEIN BY THE ISOLATED PERFUSED RAT LIVER[1]

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SUMMARY: Livers from normal fed or fasted (24h) rats were perfused in vitro to determine whether fatty acid affects the biosynthesis of very low density lipoprotein (VLDL) apoprotein. Oleate stimulated VLDL triacylglycerol output and increased incorporation of L-[4,5-^{3}H]leucine into VLDL apoprotein in both the fed and fasted groups. The increased incorporation of [^{3}H]leucine was mainly into VLDL-apoprotein E. The total mass of VLDL apoprotein secreted was also stimulated by oleate proportionately. These data suggest that fatty acids may stimulate hepatic synthesis and/or secretion of the VLDL apoproteins and that apo E, may be required for the formation and secretion of triacylglycerol in the VLDL.

Hepatic synthesis and secretion of the very low density lipoprotein (VLDL) proceeds through several major biosynthetic pathways for the synthesis of the lipid and apolipoprotein constituents. Hepatic secretion of VLDL, which involves proportional secretion of triacylglycerol, phospholipid, and cholesterol (1) is subject to nutritional and hormonal factors. These factors stimulate or reduce TG output, and have similar effects on other VLDL lipids and total apoprotein (2). The major VLDL apoproteins are produced by the liver or intestine. Virtually all the apoE (3) and nearly all the apo C are produced by the liver, while the liver and intestine both produce apo B (3).

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Abbreviations used:

VLDL, very low density lipoprotein; Apo, apolipoprotein, FFA, free fatty acid; TG, triacylglycerol; PL, phospholipid; C, cholesterol; CE, cholesteryl esters; TCA, trichloracetic acid; HMG-CoA, β-hydroxy β-methyl glutaryl coenzyme A.

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Fatty acids stimulate synthesis of TG and are also known to stimulate secretion of VLDL in perfused rat liver (4-9), synthesis of PL and C and activity of hepatic HMG-CoA reductase (2,10,11). In livers from normal fed rats, as the FFA uptake increases, triacylglycerol secretory rates reach a maximum, and triacylglycerol accumulates intracellularly (12,13,14). This observation implies that some process other than synthesis of TG limits lipid transport out of the liver. One possibility is the availability of VLDL apoproteins. It is clear that FFA stimulate hepatic cholesterol secretion in the VLDL, and also its biosynthesis. It is therefore logical to hypothesize that FFA might also stimulate VLDL protein synthesis. Since this particular issue (e.g., VLDL apoprotein biosynthesis) is of fundamental importance, and may be a critical rate-limiting factor in VLDL secretion by the liver, we carried out the experiments reported here using the isolated perfused rat liver preparation.

METHODS

Livers from male Sprague-Dawley rats (175-200g.), obtained from Harlan Industries, Indianapolis, IN), fed standard laboratory chow ad libitum or deprived of food 24h prior to the experiment, were perfused in vitro under the same procedures that have been used extensively in this laboratory (8). The perfusion medium consisted of 25% bovine erythrocytes, 6 g. bovine albumin/dl and 100 mg glucose/dl in Krebs-Henseleit bicarbonate buffer (pH 7.4). The medium also contained L-[4,5-^{3}H]leucine (3.6 µCi/ml), non radioactive leucine (8mM), and an amino acid mixture, except leucine, at the physiological concentrations in rat plasma (15). The initial volume of the perfusate was 80 ml. Bovine serum albumin, used for preparation of the fatty acid-albumin complex, was purified by a modification (16) of the Goodman procedure (17). The medium was gassed continuously with 95% O_2-5% CO_2. An infusion was started immediately and was continued at a constant rate (11.7 ml/hr) during the experiment (4 hr). For the experiments without oleate, the infusate contained 6g bovine albumin/dl in Krebs-Henseleit bicarbonate buffer, pH 7.4, L-[4,5-^{3}H]leucine (3.6 µCi/ml), non radioactive leucine (8 mM) and the amino acid mixture (17). For the experiments with oleate, the infusate contained, in addition to the above, a complex of oleic acid (166 or 332 µmol/hr,final conc. ≈ 0.7mM or 1.2 mM respectively) with 6g bovine albumin/dl (pH 7.8).

Potential differences in size of the precursor pool of leucine (e.g., fed vs. fasted state) were minimized by expanding the intracellular hepatic pool by including a high concentration of leucine (8 mM), a protocol characterized by Feldhoff et al (15) in examining albumin synthesis.

The VLDL was isolated from the final perfusate (45-50 ml) by ultracentrifugation (8) and dialysed against 0.15 M NaCl-0.002M EDTA, pH 7.4, and 0.01% NaN_3 for 48 h. For characterization of the VLDL apoproteins, the dialysed VLDL was delipidated with $CHCl_3/CH_3OH$ (2:1, v/v) at 4°C (18). The solubilized apoproteins (0.2M-TRIS, pH 8.6, and 0.2M sodium dodecylsulfate) were then examined by vertical slab SDS gradient polyacrylamide gel electrophoresis (19). Gels were stained with coomassie blue. Bands were cut from gels, destained, and counted by liquid scintillation spectrometry (18).

Incorporation of [^{3}H]leucine into VLDL protein was estimated by precipitation with 10% TCA in the presence of excess cold leucine (1%). The total precipitated protein was solubilized in hydroxide of hyamine and radioactivity was determined by liquid scintillation spectrometry using a Beckman LS-3801 counter programmed for quench count to correct to dpm. Duplicate aliquots of VLDL were precipitated with TCA as above and extracted with $CHCl_3$:CH_3OH (2:1,v/v) to correct for incorporation of [^{3}H]leucine into lipid.

Triacylglycerol was estimated by the method of Van Handel and Zilversmit (20) as modified by Newman (21). Protein was estimated by the method of Lowry et al. (22) modified to eliminate turbidity by addition of sodium dodecyl sulfate (23).

RESULTS

When oleic acid was added to the medium perfusing livers from fed rats, the incorporation of [^{3}H]leucine into VLDL apoproteins was two fold greater than that of livers perfused in the absence of oleic acid (37242 ± 2715 vs 17200 ± 2052/dpm/g/hr), whereas in the fasted state the stimulation was five fold (Fig. 1). When oleic acid was not infused, incorporation of [^{3}H]leucine into VLDL apoproteins was lower with livers from fasting rats compared to fed animals (7163 ± 1510 vs 17200 ± 2052 dpm/g/hr). However, when oleic acid was

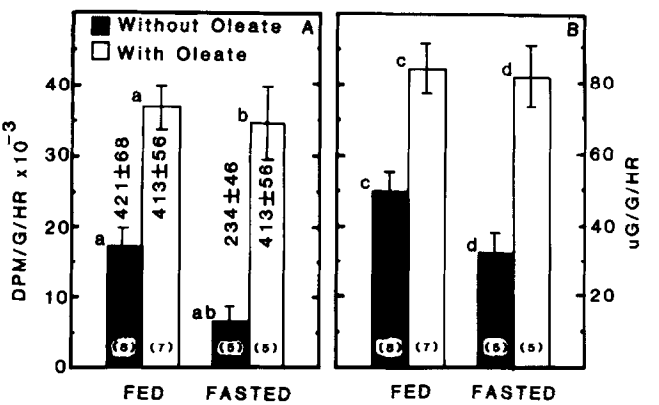

Figure 1: Effects of Oleate on incorporation of [^{3}H]leucine into total VLDL apoprotein.
Livers of rats fed standard chow diet or fasted 24h were perfused for 4h with L-[4,5-^{3}H]leucine, in the presence or absence of oleate (166 µmol/hr). VLDL apoprotein synthesis was measured by incorporation of [^{3}H]leucine into protein. The dpm in the TCA precipitates were corrected for incorporation of [^{3}H]leucine into lipid by extraction with $CHCl_3$:CH_3OH (2:1; v/v). Total VLDL apoprotein mass secretion was determined by a modification of the Lowry method. Figures in parentheses indicate number of perfusion experiments in each group. Panel A shows incorporation of [^{3}H]leucine into VLDL protein, while panel B depicts mass release of protein, measured chemically. The figures above the bars in panel A indicate the specific activity of the isolated protein (dpm/µg protein). Bars with identical letters are significantly different from one another, with $2P < 0.05$. In panel B, the output of VLDL protein mass by livers from fasted rats when fatty acid was not infused was less than that for fed livers, but was only of borderline significance ($2P < 0.10$).

infused, a similar level of incorporation was attained in both the fed and fasted state. This increased incorporation of [^{3}H]leucine into VLDL apoprotein was accompanied by stimulation of secretion of VLDL apoprotein mass (Figure 1). The total mass of VLDL apoprotein secreted increased from 51 ± 6 to 84 ± 8 µg/g/hr in the fed state when the livers were perfused with oleic acid, and from 33 ± 5 to 82 ± 8 µg/g/hr in the fasting state. Fasting itself decreased the total apoprotein mass secreted from 51 to 33 µg/g/hr, when livers were perfused in the absence of oleate. Similar data were reported earlier by Marsh and co-workers (24) who showed that the total apoprotein mass secreted decreased from 75 to 53 µg/g/hr in livers from rats fasted for 24h compared to the fed control. Infusion of oleic acid stimulated secretion of VLDL-TG from 0.24 ± 0.04 to 0.84 ± 0.10 µmol/g/hr in the fed state, and from 0.15 ± 0.02 to 0.62 ± 0.14 µmol/g/hr in the fasted group. The addition of oleate to the medium did not alter the specific activity (dpm/µg)of the VLDL protein secreted in the fed group, but increased it in the fasted group (Fig. 1). Incorporation of [^{3}H]leucine into VLDL apoproteins was not further stimulated by infusing 332 µmol/h oleate (final conc. ≈ 1.2 mM).

To determine whether synthesis of any specific apoprotein subclass of the VLDL was stimulated preferentially, incorporation of [^{3}H]leucine into the apoproteins was determined after separation by polyacrylamide gel electrophoresis. Incorporation into apo E was increased from 36 ± 3.5 to 49.7 ± 3.1 % of the total apoprotein. This represents approximately a 2.5 fold stimulation of incorporation of [^{3}H]leucine into apo E by livers from fed animals (6932 ± 674 vs 17367 ± 1083 dpm/hr/g liver, for experiments without and with oleic acid respectively). Incorporation into apo AI and into a protein with M_r of about 45000 (apo AIV?) were also stimulated by oleate, but incorporation into the two isoforms of apo B and C group proteins were unaffected (Table 1).

DISCUSSION

It was reported earlier from our laboratory that free fatty acids stimulate the synthesis and secretion by the isolated perfused rat liver, not only of triacylglycerol, but of all lipid components of VLDL (7,8,9). It was of

TABLE I

INCORPORATION OF L[4,5-^{3}H]LEUCINE INTO VLDL APOLIPOPROTEINS SEPARATED BY SDS-PAGE

FATTY ACID INFUSED	B_h	B_l	A-IV	E	A-I	C Group
None (n=3)						
% of total counts	21.4 ±3.5	17.2 ±3.0	3.7 ±0.9	36.0 ±3.5	1.9 ±1.0	17.7 ±4.4
DPM/g/hr	4121 ±676	3312 ±578	713 ±173	6932 ±674	366 ±192	3408 ±847
Oleate (n=3)						
% of total counts	13.4 ±4.2	15.2 ±2.1	7.6 ±1.4	49.7* ±3.1	4.1 ±0.9	8.1 ±0.9
DPM/g/hr	4682 ±1210	2743 ±807	3832† ±645	22832† ±1170	2703† ±565	4155 ±161

+ Data presented are Means ± SEM. VLDL was isolated by ultracentrifugation, washed, delipidated, and separated by SDS-PAGE as described in the text. These data represent percent distribution of radioactivity in separated apolipoproteins and the rate of incorporation of the radiolabelled amino acid precursor. Experimental conditions were as described in the legend to figure (1), with the exception of oleate infused at 332 µmol/h (final conc. ≈ 1.2 mM). Two percent of total counts were albumin contamination.
* $2p<0.05$ for percent of total counts compared to no fatty acid infused.
† $2p<0.05$ for DPM/g/hr compared to no fatty acid infused.

particular interest that FFA was observed to stimulate VLDL cholesterol synthesis (2). Our laboratory demonstrated also that the rate limiting enzyme in cholesterol biosynthesis, HMG-CoA reductase, was stimulated by exogenous oleic acid (10,11). The stimulation of hepatic cholesterol biosynthesis by fatty acids is probably related to the function of cholesterol in transporting TG in the VLDL. Esterification of fatty acids to TG and PL is proportional to fatty acid availability. Interestingly, however, the stimulation of secretion of TG in VLDL exceeds the direct proportional secretion of the other lipid components. This non-proportionality results in the elaboration of larger VLDL particles in an effort by the liver to conserve VLDL surface components, C and PL, or because synthesis and availability of these are limited. Several studies have suggested that exogenous FFA may stimulate VLDL apoprotein synthesis concomitant with TG synthesis as measured with the perfused liver or cultured hepatocytes (4,5,6,8,9,25,26). The increased incorporation of

[^{3}H]leucine into the VLDL apoprotein coincident with increased secretion of VLDL protein mass when oleic acid was added to the medium, suggests that fatty acid does indeed stimulate the synthesis of VLDL apoprotein necessary for transport of VLDL lipids. Our data also suggest that this stimulation of synthesis might be specific for apo E. However, in separate experiments, we observed that secretion of apo E into the total perfusate was not altered by infusion of oleic acid (166 μmol/hr), but rather that the percent of apo E in VLDL apoproteins was increased. These data suggest an alternative explanation in addition to the possible stimulation of VLDL apoprotein synthesis by fatty acid. It is possible that additional apoprotein (apo E) is acquired during formation or after secretion of the larger VLDL particles by the liver, perhaps because apo B synthesis is limited and/or specific properties of apo E (i.e. affinity to the larger VLDL particles).

In most of the previous studies, de novo synthesis of apoprotein was not measured, but rather increase in secretion of total protein mass. It has been suggested that the increase in VLDL apoprotein secretion accompanying the stimulated secretion of TG resulted from a transfer of protein constituents from the higher density lipoprotein to the VLDL (27). Davis and Boogaerts (28) as well as Patsch et al (27), were not able to demonstrate any stimulation of the incorporation of labeled amino acids into VLDL protein by fatty acids in cultured hepatocytes, as we have with the more functionally intact perfused organ. The coupling of lipid and protein synthesis and VLDL formation and secretion, may have been lost in the cultured cell.

The experiments reported here suggest that fatty acid may act as a positive signal for the de novo biosynthesis or secretion of specific VLDL lipid transport proteins. The stimulation by oleate of incorporation of [^{3}H]leucine into VLDL apo E suggests an important role for this apoprotein in the synthesis and/or secretion of the VLDL by the liver. Whether the increased incorporation of labeled apoproteins into VLDL results from increased biosynthesis of specific apoproteins or from redistribution and increased binding to the VLDL particle remains to be determined.

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