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STIMULATION OF FATTY ACID UPTAKE AND TRIGLYCERIDE SYNTHESIS IN HUMAN CULTURED SKIN FIBROBLASTS AND ADIPOCYTES BY A SERUM PROTEIN

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SUMMARY: Lipoprotein deficient serum has been shown to enhance lipid synthesis in cultured normal human skin fibroblasts incubated in the presence of oleate-albumin. The factor responsible is nondialyzable and trypsin sensitive. The stimulation is proportional to the concentration of lipoprotein deficient serum in the media and is present at all oleate concentrations and incubation times assayed. The protein has been partially purified by column chromatography to yield a Peak II fraction which stimulates triglyceride synthesis in both fibroblasts and isolated human adipocytes. The stimulation is dependent on the concentration of protein fraction and increases to an apparent saturation level of 200% in fibroblasts. Triglyceride synthesis, however, increases to a much greater extent in adipocytes and did not demonstrate saturation at the maximum Peak II protein concentration assayed. These results suggest that human serum contains a protein which stimulates fatty acid uptake and esterification by adipose tissue.

Chylomicrons transport dietary fatty acids as triglyceride from the intestine, through plasma, either to tissues such as skeletal or cardiac muscles where the fatty acids are consumed to generate energy, or to adipose tissue, where they are stored as triglycerides. This process of energy storage consists of two principal steps: first, the triglycerides must be hydrolyzed in plasma, and second, the FFA thus liberated must enter the cell, be activated, and then coupled in a tightly linked sequence to glycerol-phosphate with the end product being triglyceride. The process is complex, and except for its initial steps, not well understood. Entry

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Abbreviations used: FFA, Free Fatty Acid; LDL apoB, Low Density Lipoprotein apoprotein B; LPD Serum, Lipoprotein Deficient Serum; PBS, Phosphate Buffered Saline.

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of fatty acids into cells is usually thought to occur by passive diffusion (1,2), although more recently evidence for a transport mediated process has also been reported (3,4,5). As well, one or more fatty acid binding proteins may also be involved in intracellular fatty acid transport (6,7). Accordingly we have examined fatty acid incorporation into cellular triglyceride with particular regard to the potential role plasma proteins may play in this process.

MATERIALS AND METHODS

Subjects: Plasma samples were obtained from 6 male control subjects, all of whom had plasma lipids and lipoprotein lipids within the normal range as defined by the Lipid Research Clinic survey (8); all also had a plasma LDL apoB<120 mg/dl. Their average age was 43 ± 6 years (29-65 years). Plasma cholesterol and triglyceride were measured enzymatically (Beckman Instruments Company, California) and LDL and HDL cholesterol by the methods of the Lipid Research Clinic. LDL apoB was measured by radial immunodiffusion (9). All subjects had forearm skin biopsies from which fibroblasts were cultured. Adipocytes were obtained from 5 patients undergoing elective abdominal surgery; all 5 patients were normolipidemic, and all had normal LDL apoB levels. Their average age was 43 ± 6 years (27-60 years).

Measurement of Lipid Synthesis in Cultured Skin Fibroblasts: Fibroblasts were maintained in Eagle's Minimum Essential Medium supplemented with 10% fetal calf serum and 100 IU/ml of penicillin streptomycin and were used between passages 5 and 15. Prior to experiments, cells were dissociated in 0.25% trypsin and plated into 60 mm dishes at a concentration of $1x10^5$ cells/dish in 2.0 ml media. On day 6, the medium was changed to a preincubation serum free medium consisting of 1:1 Dulbecco's MEM and Ham's F12 supplemented with insulin, biotin, calcium pantothenate, transferrin, triiodothyronine and hydrocortisone (10). To this was added either 1.25 mg/ml fatty acid free human serum albumin (Sigma Chemicals) or 2.5 mg/ml human LPD serum. LPD serum was defined as the serum fraction with a density greater than 1.21 gm/ml where lipoproteins had been removed by ultracentrifugation. All serum used had normal levels of lipids and LDL apoB as defined above. LPD serum was then respun, defibrinated,dialyzed and adjusted to 50 mg protein/ml. FFA concentration in LPD serum was measured enzymatically (Wako Chemical, Japan) and the amount corrected for in all experiments.

On day 7, [^{14}C] oleate (52.6 mCi/mmol New England Nuclear) was evaporated to dryness, resuspended in a solution of 10mM sodium oleate complexed to albumin (11) (molar ratio 6:1), and added to cells at the required concentration. In some instances, D- [6-^{3}H(N)]-glucose (33.2 Ci/mmol, New England Nuclear) was also used as a tracer. Oleate incorporation into lipids was measured following incubation in a 5% CO_2 humid incubator usually for four hours. The cells were then washed, harvested by scraping, collected in PBS buffer, counted, and extracted in chloroform: methanol: 1N HCL (20:10:0.25). Lipid extracts were separated by thin layer chromatography on Silica Gel G plates in hexane: ether: acetic acid (75:25:1) along with standards and visualized in iodine vapour, recovery was 95% complete. The spots were scraped and counted in a liquid scintillation counter. Results were expressed per mg cell protein as measured by a modified Lowry assay (12).

In experiments using partially purified serum fractions, cells were prepared as described but the preincubation serum free medium consisted of 1:1 Dulbecco's MEM and Ham's F12 plus hormone supplement but without either LPD serum or albumin. The next day, 10 uM [^{14}C] oleic acid and specified amounts of the protein fraction were added. After 4 hours, the cells were harvested and analyzed as described above.

Adipocytes: Fresh adipose tissue was obtained from subcutaneous tissue of patients undergoing abdominal surgery. The tissue (2g to 6g) was cleaned, washed, minced, and digested in Kreb's bicarbonate buffer supplemented with glucose, albumin and

collagenase type II (Sigma Chemicals, Missouri) (2 ug/ml, 10 mg/g of tissue) for 1 hour (13). Following this, cells were filtered,washed by centrifugation and counted in a Neubauer counting chamber. Aliquots (usually 100 ul) of 1 x 10^6 cells were incubated for 2 hours in 1 ml Krebs bicarbonate buffer supplemented with 10 uM labelled sodium oleate, 2mM glucose and 2.5 ug/ml insulin in a 5% CO_2:95% O_2 atmosphere at 37°C. The protein fraction was added at the specified concentration. Following incubation, lipids were extracted with 3 ml isopropanol:heptane (1:1), washed with 2 ml Isopropanol:heptane: 0.025% KOH (4:1:3), separated by thin layer chromatography, and counted. Results are expressed per mg cell protein.

Serum Fractionation: Serum was fractionated first by affinity chromatography on an Affigel Blue (150-300 μ) column (Bio-Rad laboratories, California) at 1g serum protein/100 ml gel equilibrated in 0.02 M phosphate buffer pH 7.1. The bound peak was eluted with 3M NaCl in 0.02M phosphate pH 7.1, concentrated in 50% PEG and dialyzed against 0.02M phosphate buffer. This fraction was then applied to a Sephadex G75 (40-120 μ)column (Pharmacia, Sweden) equilibrated in the same buffer from which two peaks emerged with activity confined to the second peak. This was subsequently concentrated and again dialyzed against 0.02M phosphate buffer.

RESULTS

We first examined the effect of medium composition on oleate incorporation into total lipids in fibroblasts. The data are shown in Figure 1. Albumin concentration in the medium was maintained constant at 1.25 mg/ml while the non albumin protein in LPD serum was increased successively from 0 to 1.25 mg/ml. Note that oleate incorporation into total lipid was a linear function of non-albumin LPD serum protein, increasing from 7.5 to 21.6 nmoles oleate/mg cell protein over this range. On the other hand, LPD serum pretreated with trypsin did not stimulate oleate incorporation into lipids whereas dialyzed LPD serum did.

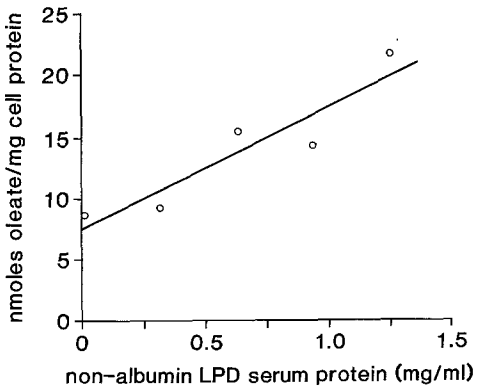

FIGURE 1. Stimulation by LPD serum of oleate incorporation into fibroblast lipids. Cells (in triplicate) were incubated for 4 hours in the presence of increasing amounts of LPD serum supplemented with human albumin (to a total concentration of 1.25 mg/ml albumin) and 100 μM [^{14}C] oleate complexed to albumin (specific activity 948 dpm/nmole). Results are expressed as the average nmoles oleate incorporated into total lipid per mg of cell protein.

TABLE I. LIPID SYNTHESIS IN FIBROBLASTS: LPD SERUM VS NON-LPD SERUM

	TRIGLYCERIDE	PHOSPHOLIPID	TOTAL LIPID
LPD serum	170 ± 16	56 ± 9	230 ± 24
No LPD serum	100 ± 10	61 ± 9	170 ± 16
	p .025	pNS	p .05

Fibroblasts from 5 different cell lines were incubated (in duplicate) for 24 hours in the presence of 100 μM ^{14}C oleate complexed to albumin (specific activity 923 dpm/nmole) plus 1.25 mg/ml albumin, with or without 1.25 mg/ml of non-albumin LPD serum. Results are expressed as average ± SEM nmoles oleate incorporated per lipid per mg cell protein.

We next examined the effect of oleate concentration on fibroblast lipid synthesis over a 4 hour time period. Oleate (bound to albumin) was increased systematically from 25 μM to 300 μM; but at each point, albumin concentration was the same in both the LPD serum and non-LPD serum (albumin only) samples. LPD serum protein was maintained at 1.25 mg/ml. FFA uptake and incorporation into lipids increased as the concentration of oleate in the medium increased. However at each point, oleate incorporation into cell lipid was significantly higher in the LPD serum medium (data not shown). Similarly, at all incubation times examined up to 30 hours, the difference between LPD serum and non-LPD serum medium was maintained (data not shown).

The effect of LPD serum on synthesis of different lipid classes is shown in Table I. Cultured skin fibroblasts from 5 normals were incubated in duplicate for 24 hours at a concentration of 100 μM oleate and 1.25 mg/ml albumin with and without 1.25 mg/ml non-albumin LPD serum. Note that triglyceride synthesis was on average 85% higher in the presence of LPD serum ($p < .05$) but there was no significant difference in phospholipid synthesis where triglyceride and phospholipid synthesis consituted 94% of oleate incorporation. Additional experiments using [^{3}H] glucose as a concurrent tracer confirmed that most of the triglyceride was being synthesized from glycerol-3-phosphate based on the close agreement between the observed ratio of ^{14}C oleate acid: ^{3}H glucose in triglyceride of 4.9, compared to the theoretical ratio of 6.0.

The possibility that triglyceride hydrolysis was diminished in the LPD serum enriched medium was also examined. In these experiments, cells were preincubated with 100 uM [^{14}C] oleate with 1.25 mg/ml albumin for 24 hours and then incubated in either 1.25 mg/ml albumin or 1.25 mg/ml albumin + 1.25 ml/ml non-albumin LPD serum media and the release of labelled fatty acid observed for 2 hours. During this time, there was no significant difference in FFA release (19.1% ± 2.2 vs 19.6% ± 1.8 pNS). Further experiments using labelled glucose also showed that there was no difference in total cellular uptake of glucose between cells incubated in albumin - only media or cells incubated in albumin-LPD serum media eliminating the possibility that differences in glycerolipid synthesis might be due to such a phenomenon (910 ± 70 with LPD serum vs 800 ± 115 without LPD serum, nmoles glucose/mg cell protein, pNS). Similarly this effect cannot be attributed to a different rate of cell growth since the level of [^{3}H] thymidine incorporation into cells was the same.

As outlined in the methods, serum was fractionated by being passed first through an Affi-Gel Blue column and then a Sephadex G 75 column. Lipid syntnesis stimulating activity was confined to the second peak eluted from the Sephadex G 75 column and this fraction contains multiple bands when analyzed by SDS-poly-acrylamide gel electrophoresis by the method of Laemmli (14). However reaction of this fraction with antialbumin antibody (Behring Diagnostic, W. Germany) was negative. Stimulatory activity in this peak II fraction was demonstrated with both fibroblasts and adipocytes (figures 2 and 3). In figure 2, fibroblasts preincubated for 24 hours in serum free medium were then incubated for 4 hours with 10 μM oleate with peak II protein present in concentrations varying from 0 to 6 μg/ml. Note that triglyceride synthesis rises rapidly to an apparent maximum of 200%. In figure 3, isolated human adipocytes were incubated for 2 hours in the presence of 10 μM oleate and varying concentrations of the peak II protein fraction. Throughout the range examined, from 0 to 30 μg/ml of peak II protein fraction, triglyceride synthesis rose linearly. At the maximum concentration assayed the total increase was 300%. No significant incorporation of the tracer into phospholipid or cholesterol ester was observed however.

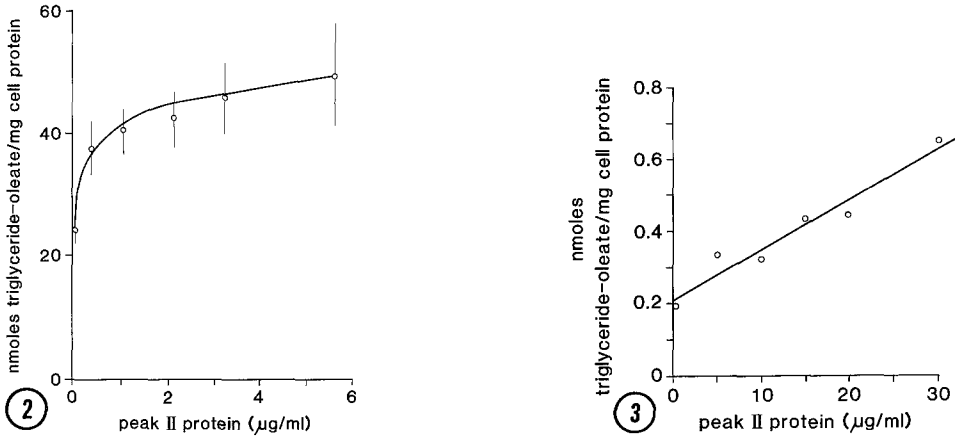

FIGURE 2. Effect of the Peak II serum protein fraction on triglyceride synthesis in fibroblasts.
Fibroblasts from 5 different cell lines (in duplicate) were incubated for 4 hours in albumin-free medium containing 10 μM [^{14}C] oleate (specific activity 9.9 dpm/pmole) and increasing amounts of the Peak II serum protein fraction. Results are expressed as average ± SEM nmoles oleate incorporated into triglyceride per mg of cell protein .

FIGURE 3. Effect of the Peak II serum protein fraction on triglyceride synthesis in adipocytes. Isolated adipocytes were incubated (in duplicate) for 2 hours in albumin-free buffer containing 10 μM [^{14}C] oleate (specific activity 183 dpm/pmole) and increasing amounts of the Peak II serum protein fraction. Results are expressed as average nmoles oleate incorporated into triglyceride per mg of cell protein.

DISCUSSION

The present experiments demonstrate the presence of a protein fraction in plasma which can stimulate the uptake and incorporation of extracellular free fatty acids into cellular triglycerides. The fraction is albumin free and the activity nondialyzable but sensitive to trypsin. The protein has been partially purified and shown to be active with both cultured skin fibroblasts and adipocytes. The degree of stimulation is concentration dependent with an upper limit evident in fibroblasts but not demonstrated in adipocytes. Its effect is entirely due to changes in synthesis since hydrolysis was unaffected.

Synthesis of adipocyte triglyceride is a complex process in which substrate must first enter the cell, be activated, and then coupled in a series of tightly linked reactions to form glycerolipids. Studies of this process have focused most intensively on the enzymes themselves with opinion still divided over which step or steps are rate limiting. However given the present results, substrate delivery to the enzyme may be an important regulatory step. This may involve mechanisms for

fatty acid transport across the plasma membrane (1-5) or within the cell itself. (6,7). Cytosol has previously been shown to contain one or more proteins that can stimulate acylation reactions though they have not all been characterized, nor has their mechanism of action been elucidated in detail (15,16). It is certainly possible, however, that the plasma protein fraction we have identified contains one of these. The importance of the present report, we believe, lies in the fact that, to our knowledge, it is the first demonstration of a human plasma protein component which can markedly stimulate intracellular triglyceride synthesis. This effect was evident in fibroblasts but far more pronounced in adipocytes, a difference which raises the possibility that it may be of physiological significance, and perhaps, even of relevance to the clinical problem of obesity. Clearly however before these hypotheses can be tested or the protein's mechanism of action elucidated, complete purification must be achieved.

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