Catabolism of human very low density lipoproteins in vitro: a fluorescent phospholipid method for monitoring lipolysis

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Summary The catabolism of human plasma very low density lipoproteins (VLDL) by purified bovine milk lipoprotein lipase has been measured in vitro using a fluorescent phospholipid as a method to monitor lipolysis. Dansyl phosphatidylethanolamine (DPE) was incorporated into VLDL to form DPE-VLDL, and the rate of catabolism was followed by measuring the increase in fluorescence at 490 nm after the addition of the enzyme. The studies were performed with VLDL isolated from 20 normal individuals. In addition, the VLDL from 8 mildly obese subjects with primary hypertriglyceridemia (Type IV phenotype) was studied. With this in vitro system and with a constant amount of lipoprotein lipase, the rate of lipolysis did not differ in normal and in these hypertriglyceridemic subjects. Furthermore, there was no correlation between the rates of hydrolysis and the plasma levels of triglyceride or high density lipoprotein cholesterol.—**Taskinen, M-R., J. D. Johnson, M. L. Kashyap, K. Shirai, C. J. Glueck, and R. L. Jackson.** Catabolism of human very low density lipoproteins in vitro: a fluorescent phospholipid method for monitoring lipolysis. *J. Lipid Res.* 1981. **22:** 382–386.

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The triglyceride-rich lipoproteins, very low density lipoproteins (VLDL) and chylomicrons, are hydrolyzed at extrahepatic tissues by endothelial-bound lipoprotein lipase (LpL) (1–3). The characteristic feature of LpL is that it is stimulated by apolipoprotein C-II (apoC-II), a 78-amino acid residue polypeptide (Ref. 4, for review). Subjects with a hereditary apoC-II deficiency have been described (5–7). The characteristic feature of these subjects is that they have severe hypertriglyceridemia. The addition of normal plasma or apoC-II to apoC-II-deficient VLDL facilitates the hydrolysis of triglycerides in vivo and in vitro (5, 8). Subjects with familial hypertriglyceridemia (Types IV and V) have also been shown to have decreased concentrations of apoC-II per mg VLDL protein (9). Based on these findings, it has been suggested by a number of investigators that alterations of the amount of apoC-II in VLDL might modulate the rate and/or extent of catabolism (1–3, 9–13). A major factor limiting examination of the role of apoC-II in VLDL catabolism has been the lack of a practical technique to follow continuously the rate of triglyceride hydrolysis under controlled in vitro conditions.

In this report, we describe the application of a recent fluorescence technique (14, 15) to monitor in vitro the LpL-induced lipolysis of human VLDL from normal subjects and from patients with hypertriglyceridemia. With this technique we show that VLDL from normal subjects and from subjects with hypertriglyceridemia are hydrolyzed in vitro at the same rate by lipoprotein lipase.

MATERIALS AND METHODS

Subjects

The studies were carried out in 20 healthy volunteers (14 males, 6 females) and in 8 patients (all males) with hypertriglyceridemia (Type IV phenotype). The patients were selected from the outpatient Cincinnati Lipid Research Clinic. **Table 1** summarizes the fasting plasma triglyceride, cholesterol, and HDL-cholesterol values for the normal and hypertriglyceridemia subjects. All normal control subjects had plasma cholesterol less than 231 mg/dl and plasma fasting triglyceride below 150 mg/dl; HDL-cholesterol ranged from 43 to 89 mg/dl. None of the subjects had diabetes, hepatic disorder, or anamnestic heavy intake of alcohol. The mean age of the normal subjects was 29 ± 2 years and that of the patients 46 ± 3 years. None of the controls were obese, the mean value of the Quetelet index ($W/H^2 \times 1000$) being 2.11 ± 0.04 (mean ± SEM). The Quetelet index in the hyperlipoproteinemic patients was 2.62 ± 1.10. Informed consent was obtained, following a protocol approved by the University of Cincinnati Faculty Committee on Human Research.

Blood sampling and analytical methods

Venous blood (50–80 ml) was drawn after a 12-hr fast into EDTA tubes. Plasma triglyceride, cholesterol, and HDL cholesterol were determined utilizing methods and standardized procedures of the Lipid Research Clinic laboratory (16).

Plasma VLDL was isolated by ultracentrifugation in a Beckman L5-65 B ultracentrifuge using a Type

Abbreviations: VLDL, very low density lipoproteins; HDL, high density lipoproteins; LpL, lipoprotein lipase; DPE, dansyl phosphatidylethanolamine.

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50.2 Ti rotor at 48,000 rpm for 18 hr at 8°C at plasma density. VLDL was separated by aspiration and respun in a 50 Ti rotor at the same density and then dialyzed against 0.01 M Tris-HCl, 0.9% NaCl, 0.01% sodium azide, pH 7.4.

Determination of in vitro lipolysis of VLDL

Lipoprotein lipase (LpL) was purified from skimmed bovine milk by affinity chromatography on heparin-Sepharose as described previously (17); the purified enzyme had a specific activity of 60 mmol fatty acid released/hr per mg using triglyceride emulsified in Triton X-100. The standard assay mixture contained 0.378 μmol of triolein (Sigma) and 2 nCi of tri[1-^{14}C]oleoylglycerol (New England Nuclear, 50 mCi/mmol) emulsified with 0.02% Triton X-100, 3% fatty acid-free bovine serum albumin (Sigma), and 2.0 μg of apoC-II, in a final volume of 0.25 ml of 50 mM Tris-HCl, pH 8.6. Incubations were carried out for 30 min at 37°C. The enzyme was stored in small aliquots (1 ml) in 50% glycerol–0.01 M Tris-HCl, pH 7.5, at −70°C and thawed at −20°C before use.

In vitro lipolysis of VLDL by milk LpL was determined by following the changes in the intensity of DPE-VLDL fluorescence induced by LpL (14). DPE was obtained from Molecular Probes, Inc., Plano, TX, and stored in absolute ethanol (25 mg/ml). Labeling of VLDL was carried out by adding DPE to VLDL (2.0 mg triglyceride/ml) at a 1:40 molar ratio of DPE to triglyceride. VLDL was diluted if necessary with a standard buffer of 0.05 M Tris-HCl containing 0.9% NaCl, 0.01% sodium azide, pH 7.4. DPE-lipoprotein solutions were vortexed for 15 sec and then sonicated at 23°C for 6–8 min with a Cole-Parmer (Model 8846-50) bath sonicator. The reaction mixture contained DPE-VLDL (either 0.3 or 0.6 mg triglyceride/ml) in 1.0 ml total volume of standard buffer. Fatty acid-free serum albumin (Sigma) was added to a final concentration of 3% (w/v). The reaction was started by the addition of 5 μl of 1.0 mg/ml purified milk LpL. The reaction mixture was excited at 340 nm and the fluorescence intensity was monitored at 490 nm on a Perkin-Elmer 650-10S ratio recording spectrofluorometer for 30 min at 24°C. Changes of DPE-VLDL fluorescence were expressed as the ratio of the fluorescence intensity at a given time (F) to the initial fluorescence intensity (F_0) (i.e., F/F_0), or as the rate of the fluorescence increase in arbitrary fluorescence units per minute (u/min). The initial rates were calculated from the ascending linear part of the fluorescence intensity curve.

In some experiments, the extent of lipolysis was determined by measuring the release of ^{14}C-labeled fatty acids from VLDL labeled with tri[^{14}C]oleoylglycerol. Tri[1-^{14}C]oleoylglycerol (New England Nuclear, sp act 84.1 mCi/mmol) was incorporated into VLDL by the method of Fielding (18); 4–6 μCi of carboxyl [^{14}C]trioleoylglycerol was used per 10 mg of VLDL triglyceride. Free fatty acids released were extracted by the method of Nilsson-Ehle, Tornquist, and Belfrage (19).

TABLE 1. Plasma lipid concentrations and fluorescence changes of VLDL from normal and hypertriglyceridemic subjects

Measurement	Normals (n = 20)	Type IV (n = 8)
Plasma triglycerides (mg/dl)	85 ± 8	489 ± 62
Plasma cholesterol (mg/dl)	170 ± 7	233 ± 13
HDL-cholesterol (mg/dl)	57 ± 3	33 ± 3
VLDL triglyceride/protein	4.4 ± 0.3	4.7 ± 0.3
F/F_0 at 30 min	3.4 ± 0.1	3.3 ± 0.1
Initial rate (units/min)	9.6 ± 0.7	9.3 ± 0.6

The numbers represent mean values ± SEM.

RESULTS

In our previous reports (14, 15), we have shown that the lipoprotein lipase-induced fluorescence changes in DPE-VLDL from normal subjects provide an accurate means of monitoring continuously the lipolysis of VLDL. In VLDL isolated from normal

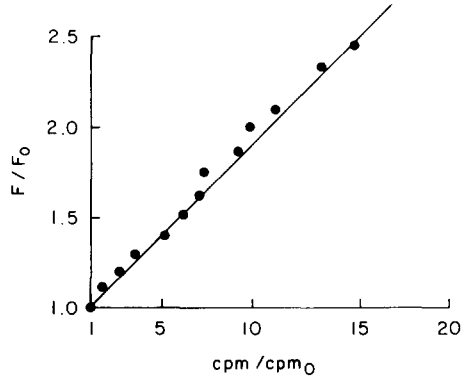

Fig. 1. Relationship of the fluorescence enhancements in DPE-labeled VLDL to the release of [^{14}C]oleic acid. The reaction mixture contained DPE, tri[^{14}C]oleoylglycerol-labeled VLDL (0.3 mg/ml in triglyceride) from a Type IV subject, and 3% fatty acid-free bovine serum albumin in a total volume of 10 ml of 50 mM Tris-HCl, pH 7.4, 0.9% NaCl, 0.01% NaN_3. The reaction was initiated by the addition of 30 μl of bovine milk lipoprotein lipase (0.5 mg/ml). At various times, the fluorescence intensity was read (F) and compared to the initial fluorescence before the LpL addition (F_0). At the time the fluorescence was read, 0.5 ml of the incubation mixture was extracted for [^{14}C]oleic acid as described in Materials and Methods. The fluorescence increase over basal (F/F_0) was then plotted versus the cpm over cpm basal (cpm/cpm_0) at various time points. The individual points were taken between zero and 37 min. At the final point, the reaction was >95% complete.

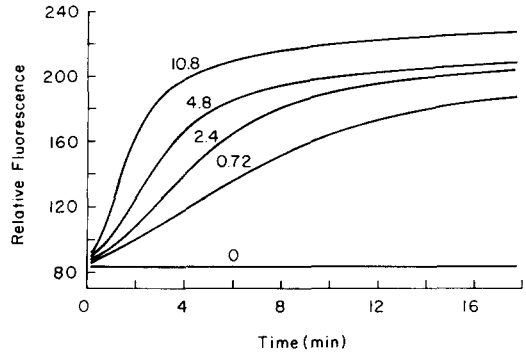

Fig. 2. The effect of LpL concentration on fluorescence increases in DPE-labeled VLDL from a Type IV subject. Each reaction mixture contained DPE-labeled VLDL (0.3 mg/ml), 3% fatty acid-free bovine serum albumin in 1.0 ml total volume of 50 mM Tris-HCl, pH 7.4, 0.9% NaCl, 0.01% NaN_3. At time zero, bovine milk LpL (0.5 mg/ml) was added to give LpL concentrations of 0, 0.72, 2.4, 4.8, or 10.8 μg/ml. The fluorescence increase at 490 nm, after the addition of LpL, is shown as a function of time.

subjects, the fluorescence increase in DPE-VLDL, at any point in time, was directly related to the extent of lipolysis (i.e., the amount of free fatty acid released).

Fig. 1 shows the direct linear relationship ($r = 0.99$) between the increase in DPE-VLDL fluorescence and the increase in the release of [^{14}C]oleic acid at different points in time during the lipolysis of VLDL isolated from a Type IV subject. Thus, the fluorescence increases provide a direct method of continuously monitoring lipolysis of VLDL from both normal and Type IV subjects.

We have previously shown that the rate of lipolysis of normal VLDL, as monitored by this fluorescence method, is strictly dependent on lipoprotein lipase concentration (14, 15). **Fig. 2** shows a similar effect of enzyme concentration on the rates of the fluorescence increases in DPE-labeled VLDL from a Type IV subject. Clearly the initial rates of these fluorescence changes increase dramatically with increasing LpL concentration and therefore with increasing rates of lipolysis. Under fixed substrate conditions and after each reaction is complete, the final F/F_0 ratios were all ~3.2–3.5. Thus the rate of the fluorescence increase in DPE-labeled normal and Type IV VLDL provide an accurate representation of the rate of lipolysis of these particles while the final F/F_0 is indicative of the final extent of lipolysis.

Comparison of the rate of lipolysis of VLDL isolated from normal and Type IV hypertriglyceridemic subjects. Before these comparisons could be made, it was first necessary to establish the interassay variability. In these experiments, LpL concentration was held constant and the daily variation in the fluorescence method was determined by monitoring the fluorescence changes in the same standard DPE-VLDL reference solution on consecutive days for the 2-week period. The mean value of F/F_0 at 30 min for the 14 experiments was 3.54 ± 0.05, representing an interassay variation of <2%. Thus, LpL activity and the fluorescence method itself had insignificant variation during the course of these experiments.

Table 1 compares the plasma lipids and lipoproteins isolated from 20 normal and 8 Type IV hypertriglyceridemic patients; the values shown are within the expected limits. In the next experiment, we labeled VLDL from normals and Type IV patients with DPE and monitored the changes in fluorescence intensity of these DPE-VLDL's with lipolysis. The assays were performed at a concentration of VLDL-triglyceride of 0.6 mg/ml, since this concentration represents the normal physiologic condition. This concentration of triglyceride also corresponds to a value lower than the K_m (14). Using this concentration of VLDL-triglyceride, a concentration of enzyme (5 μg) was chosen such that lipolysis was essentially complete in 30 min. Table 1 compares the fluorescent enhancements and rates of fluorescence increase in the 20 normal and 8 hypertriglyceridemic subjects. The mean value of F/F_0 in the 20 normals was 3.4 ± 0.1. The initial rates of the normals had a mean value of 9.6 ± 0.7. In the 8 Type IV subjects, these values corresponded to a mean F/F_0 of 3.3 ± 0.1 and initial rates of 9.3 ± 0.6. Thus, the mean values of both F/F_0 and the initial rates are not significantly different in Type IV and in control subjects. The similarity of the F/F_0 values at the completion of lipolysis (30 min) in normal and Type IV VLDL indicate that the reactions went to a similar extent of completion in each case. The similarity of the initial rate of the fluorescence changes in VLDL from normal and Type IV subjects indicate that the rate of lipolysis of Type IV VLDL does not differ significantly from the rate of lipolysis of normal VLDL. No correlation was found between the rates of in vitro lipolysis and plasma levels of triglyceride or HDL-cholesterol ($r = -0.15$ NS and $r = +0.18$ NS, respectively).

DISCUSSION

The level of plasma triglycerides is controlled by a variety of metabolic and endocrine factors which are involved in the synthesis and catabolism of chylomicrons and VLDL. In familial Type I hypertriglyceridemia (20, 21) or in apoC-II deficiency (5–7), the absence of lipoprotein lipase or its activator protein is clearly responsible for the hyper-

triglyceridemia. Much less is known concerning the metabolic factors which regulate lipolysis in normal individuals or subjects with other forms of hypertriglyceridemia. One of the major limitations in determining these factors has been the lack of a method to measure accurately the rate of lipolysis. The purpose of the present study was to determine whether there were differences in the rates of lipolysis of VLDL from normal subjects and from a few select subjects with hypertriglyceridemia. To monitor lipolysis, we have used a sensitive fluorescent phospholipid (DPE) assay procedure, which has recently been developed in this laboratory (14). The standard assay conditions were DPE-VLDL (0.6 mg triglyceride/ml) and a fixed amount of lipoprotein lipase, the amount being that which gave 80–90% hydrolysis in 30 min. With these conditions, the interassay variation was shown to be negligible. As shown in the results, VLDL triglycerides from 20 normal subjects were hydrolyzed at the same rate. Furthermore, the rate of lipolysis of VLDL from 8 mildly obese subjects with hypertriglyceridemia (Type IV phenotype) was not significantly different from normal VLDL.

If the in vitro system adequately emulates the in vivo situation, then the hypertriglyceridemia seen in the subjects with Type IV hyperlipoproteinemia probably cannot be accounted for by a decreased rate of catabolism due to the VLDL itself. It is possible that milk lipoprotein lipase has properties that are different from tissue lipoprotein lipase and that the in vitro system may not truly reflect the in vivo situation. A more likely possibility to explain the hypertriglyceridemia in these subjects is that the rate of lipolysis in vivo is regulated by the amount of lipoprotein lipase itself. In this regard, measurements of adipose tissue lipoprotein lipase in subjects with Type IV hyperlipoproteinemia have been reported to be low or slightly decreased (22, 23). The results shown in Fig. 2 clearly demonstrate that even slight changes in lipoprotein lipase have dramatic consequences on the rate of VLDL lipolysis and, thus, could account for the hypertriglyceridemia. It should also be mentioned that we have carried out these studies with the total VLDL fraction. We have not attempted to study the rates of lipolysis of isolated subfractions. It is possible that a subgroup of hypertriglyceridemic individuals may have VLDL with an abnormal composition and an abnormal lipolytic rate compared to normals. For example, it is well known (24, 25) that VLDL composition varies with the size of the lipoprotein particle. With the fluorescence method described in this report, it will now be possible to determine whether the compositional differences found in VLDL from subjects with hypertriglyceridemia are reflected by differences in the rates of lipolysis. Experiments along these lines are currently in progress.

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REFERENCES

1. Smith, L. C., H. J. Pownall, and A. M. Gotto, Jr. 1978. The plasma lipoproteins: structure and metabolism. *Ann. Rev. Biochem.* **47:** 751–777.
2. Scow, R. O., E. J. Blanchette-Mackie, and L. C. Smith. 1976. Role of capillary endothelium in the clearance of chylomicrons: a model for lipid transport from blood by lateral diffusion in cell membranes. *Circ. Res.* **39:** 149–162.
3. Schaefer, E. J., S. Eisenberg, and R. I. Levy. 1978. Lipoprotein apoprotein metabolism. *J. Lipid Res.* **19:** 667–687.
4. Fielding, C. J., and R. J. Havel. 1977. Lipoprotein lipase. *Arch. Pathol. Lab. Med.* **101:** 225–229.
5. Breckenridge, W. C., J. A. Little, G. Steiner, A. Chow, and M. Poapst. 1978. Hypertriglyceridemia associated with deficiency of apolipoprotein C-II. *N. Engl. J. Med.* **298:** 1265–1273.
6. Cox, D. W., W. C. Breckenridge, and J. A. Little. 1978. Inheritance of apolipoprotein C-II deficiency with hypertriglyceridemia and pancreatitis. *N. Engl. J. Med.* **299:** 1421–1424.
7. Yamamura, T., H. Sudo, K. Ishikawa, and A. Yamamoto. 1979. Familial type I hypertriglyceridemia caused by apolipoprotein C-II deficiency. *Atherosclerosis.* **34:** 53–65.
8. Catapano, A. L., P. K. J. Kinnunen, W. C. Breckenridge, A. M. Gotto, Jr., R. L. Jackson, J. A. Little, L. C. Smith, and J. T. Sparrow. 1979. Lipolysis of apoC-II deficient very low density lipoproteins: enhancement of of lipoprotein lipase action by synthetic fragments of apoC-II. *Biochem. Biophys. Res. Commun.* **89:** 951–957.
9. Kashyap, M. L., L. S. Srivastava, C. Y. Chen, G. Perisutti, M. Campbell, R. F. Lutmer, and C. J. Glueck. 1977. Radioimmunoassay of human apolipoprotein C-II: a study in normal and hypertriglyceridemic subjects. *J. Clin. Invest.* **60:** 171–180.
10. Schonfeld, G., S. W. Weidman, J. L. Witztum, and R. M. Bowen. 1976. Alterations in levels and interrelations of plasma apolipoproteins induced by diet. *Metabolism.* **25:** 261–275.
11. Schonfeld, G., P. K. George, J. Miller, P. Reilly, and J. Witztum. 1979. Apolipoprotein C-II and C-III levels in hyperlipoproteinemia. *Metabolism.* **28:** 1001–1010.

12. Kane, J. P., T. Sata, R. L. Hamilton, and R. J. Havel. 1975. Apoprotein composition of very low density lipoproteins of human serum. *J. Clin. Invest.* **56:** 1622–1634.
13. Kashyap, M. L., L. S. Srivastava, B. A. Hynd, G. Perisutti, D. W. Brady, P. Gartside, and C. J. Glueck. 1978. The role of high density lipoprotein apolipoprotein C-II in triglyceride metabolism. *Lipids.* **13:** 933–942.
14. Johnson, J. D., M-R. Taskinen, N. Matsuoka, and R. L. Jackson. 1980. 5-Dimethylaminonaphthalene-1-sulfonyl phosphatidylethanolamine-labeled very low density lipoproteins: a fluorescent probe for monitoring lipolysis. *J. Biol. Chem.* **255:** 3461–3465.
15. Johnson, J. D., M-R. Taskinen, N. Matsuoka, and R. L. Jackson. 1980. On the mechanism of the lipoprotein lipase-induced fluorescence changes in 5-dimethylaminonaphthalene-l-sulfonyl phosphatidylethanolamine-labeled very low density lipoproteins. *J. Biol. Chem.* **255:** 3466–3471.
16. Manual of Laboratory Methods. 1972. Lipid Research Clinics Program, University of North Carolina, Chapel Hill, North Carolina.
17. Jackson, R. L., F. Pattus, and G. de Haas. 1980. Mechanism of action of milk lipoprotein lipase at substrate interfaces: effects of apolipoproteins. *Biochemistry.* **19:** 373–378.
18. Fielding, C. J. 1979. Validation of a procedure for exogenous isotopic labeling of lipoprotein triglyceride with radioactive triolein. *Biochim. Biophys. Acta.* **573:** 255–265.
19. Nilsson-Ehle, P., H. Tornquist, and P. Belfrage. 1972. Rapid determination of lipoprotein lipase activity in human adipose tissue. *Clin. Chim. Acta.* **42:** 383–390.
20. Havel, R. J., and R. S. Gordon, Jr. 1960. Idiopathic hyperlipidemia: metabolic studies in an affected family. *J. Clin. Invest.* **39:** 1777–1790.
21. Kashyap, M. L., L. S. Srivastava, R. C. Tsang, M-R. Taskinen, B. A. Hynd, G. Perisutti, D. W. Brady, C. J. Glueck, C. A. Ahumada, J. A. McCarthy, R. A. Sosa, and T. O. Reeds. 1980. Apolipoprotein C-II in type I hyperlipoproteinemia: a study in three cases. *J. Lab. Clin. Med.* **95:** 180–187.
22. Goldberg, A. P., D. M. Applebaum-Bowden, and W. R. Hazzard. 1979. Adipose tissue lipoprotein lipase activity in Type III hyperlipoproteinemia. *Metabolism.* **28:** 1122–1126.
23. Nikkila, E. A. 1978. Metabolic and endocrine control of plasma high density lipoprotein concentration: relation to catabolism of triglyceride-rich lipoproteins. *In* High Density Lipoproteins and Atherosclerosis. A. M. Gotto, Jr., N. E. Miller, and M. F. Oliver, editors. Elsevier/North-Holland Biomedical Press, Amsterdam, The Netherlands. 177–192.
24. Quarfordt, S. H., A. Nathans, M. Dowdee, and H. L. Hilderman. 1972. Heterogeneity of human very low density lipoproteins by gel filtration chromatography. *J. Lipid Res.* **13:** 435–444.
25. Sata, T., R. J. Havel, and A. L. Jones. 1972. Characterization of subfractions of triglyceride-rich lipoproteins separated by gel chromatography from blood plasma of normolipemic and hyperlipemic humans. *J. Lipid Res.* **13:** 757–768.