

Uptake of very low density lipoprotein triglyceride by bovine aortic endothelial cells in culture

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Abstract Primary monolayers of calf aortic endothelial cells were presented with isolated human very low density lipoproteins that had been labeled with radioactive triglyceride. The cells were observed to take up triglyceride over a 24 hr period; incorporation increased with exogenous lipoprotein concentrations, and up to 60% of the triglyceride taken up was converted to other cell lipids within 24 hr. When [2-³H]glyceryl tri[1-¹⁴C]oleate-labeled very low density lipoprotein was used, the ³H/¹⁴C ratio in the cell triglyceride was always similar to that of the exogenous lipoprotein triglyceride. Moreover, no significant hydrolysis of the exogenous very low density lipoprotein triglyceride was observed during the time of exposure to the cells. Similar experiments using doubly-labeled triglyceride exposed to endothelial cells in triglyceride-phospholipid liposome preparations also resulted in incorporation of the exogenous triglyceride without evidence of extracellular hydrolysis. The results indicate that primary monolayers of endothelial cells in culture are able to incorporate and metabolize very low density lipoprotein triglyceride. However, triglyceride does not appear to be significantly hydrolyzed during uptake, suggesting an absence of lipoprotein lipase activity in these cells.

Supplementary key words endothelium · triglyceride metabolism · lipoprotein lipase · liposomes

The endothelium *in vivo* plays a significant role in the metabolism of triglycerides. The triglyceride-bearing lipoproteins, very low density lipoproteins (VLDL), and chylomicrons, have been observed to bind to endothelium (1, 2); triglyceride is taken up by these cells and transported to the underlying tissues primarily through the action of lipoprotein lipase (1, 3). Because of their proximity to other components of the vessel wall, it is difficult to study the specific aspects of endothelial cell triglyceride uptake and metabolism *in vivo*. In addition, *in vivo* studies have not elucidated whether lipoprotein lipase is synthesized by or simply sequestered in endothelium (4).

In recent years, investigators have developed methods for the establishment of cell cultures of vascular endothelium that appear to retain the charac-

teristics of endothelium *in vivo* (5–9). The availability of adequate amounts of endothelial cells *in vitro* allows the study of specific properties of the metabolism of these cells isolated from other components of the vessel wall. The present study is a characterization of triglyceride uptake from VLDL by endothelial cells *in vitro*. VLDL containing doubly-labeled triglycerides have been used in order to study the hydrolysis and metabolism of triglycerides by these cells.

MATERIALS AND METHODS

Endothelial cell cultures

Calf aortas were obtained through the courtesy of Cross Brothers Meat Packers, Inc. Philadelphia, PA. Thoracic aortas, approximately 20 cm long, were removed from the animals aseptically within one hour after death and immediately placed in sterile chilled Dulbecco's phosphate buffered saline (DPBS) supplemented with glucose (5 mg/ml), amphotericin B (2.5 µg/ml), penicillin (50 U/ml), and streptomycin (50 µg/ml). To isolate endothelial cells, the aortas were washed in DPBS and defatted by dissection. The paired intercostal vessels were tied and the lower ends of the vessels clamped. The vessels were then filled with 15 ml of a solution of collagenase (Type II, Worthington Biochemicals, Freehold, NJ) in medium 199 (M-199) (1 mg/ml) and the upper ends were clamped. The vessels were incubated at room temperature for 45 min and the contents were collected. This collagenase effluent usually contained

Abbreviations: VLDL, very low density lipoprotein; DPBS, Dulbecco's phosphate buffered saline; M-199, Medium 199; PE buffer, 0.05 M Na₃PO₄, 2 mM EDTA, pH 7.2; MEM, minimum essential medium (Eagle); TLC, thin-layer chromatography; FFA, free fatty acids.

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few cells and was discarded. The vessels were then washed four times with 10 ml of growth medium that consisted of M-199 as modified by Lewis et al. (5) supplemented with 20% fetal bovine serum, gentamicin (50 $\mu\text{g/ml}$), and amphotericin B (2.5 $\mu\text{g/ml}$). The pooled effluents containing the freed endothelial cells were distributed evenly among 25-cm² or 75-cm² glass or plastic Petri dishes, and the cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. After an initial lag period, cells grew with a generation time of approximately 65 hr, and confluency was reached in 5–7 days. All experiments were conducted on primary monolayers at the time that they reached confluency.

Cells established in this manner from calf aortas have been identified as endothelium by light and electron microscopic criteria, and the details of the growth and morphological characterization of these cultures have been described elsewhere (9). In addition, these cells have been shown to maintain biochemical criteria of endothelium in vivo, such as the synthesis of basement membrane collagen (10), the metabolism of serotonin (11), and the synthesis of factor VIII antigen (9).

Lipoproteins

VLDL was isolated from normal human plasma according to procedures described by Havel, Eder, and Bragdon (12) and modified by Marsh (13). Human plasma containing 2 mM EDTA was centrifuged for 30 min at 10,000 rpm in a Sorval (Norwalk, CT) Model RC2B refrigerated centrifuge. After removal of the chylomicrons, VLDL was isolated by centrifugation for 18 hr at 40,000 rpm in a Beckman (Spinco Div., Palo Alto, CA) Model L2-50 ultracentrifuge in 9-ml polycarbonate centrifuge tubes. The VLDL (top 1.0 ml) was resuspended in a solution of NaBr (ρ 1.006) in 0.05 M phosphate buffer, 2 mM EDTA, pH 7.2, (PE buffer) and recentrifuged. The rewash VLDL was dialyzed against 0.9% NaCl in 2 mM EDTA, pH 7.2, and, immediately before exposure to the cells, against minimum essential medium (MEM).

VLDL was labeled with radioactive triglycerides by incubation of the isolated lipoprotein with triglyceride-coated Celite according to the method described by Avigan (14) and Brenneman and Spector (15). The radioactive triglyceride was dissolved in petroleum ether and added to Celite (Johns-Manville, Denver, CO). One to two μCi of ¹⁴C-labeled triolein and 10–20 μCi of ³H-labeled triolein (see Radioactivity below) were added per mg of Celite, and the petroleum ether was evaporated under nitrogen. VLDL preparations containing 100–1000 $\mu\text{g/ml}$ tri-

glyceride were incubated with the triglyceride-coated Celite for 2 hr at room temperature (1 mg Celite/ml VLDL). After incubation the suspension was centrifuged at 10,000 g for 10 min at 4°C and the supernatant solution was passed through a 5 μm Millipore filter. Under these conditions approximately 2–5% of the available radioactivity was incorporated into VLDL.

In some experiments glyceryl tri[9,10-³H]oleate was added to the VLDL by a solvent exchange technique. The isotope (10 μCi) was dissolved in a small amount of benzene and the mixture was added to 20 ml of VLDL (100 $\mu\text{g/ml}$) in MEM. This was stirred at 37°C for 1 hr, and then filtered through a 5 μm Millipore filter. Using this method approximately 15–25% of the label was incorporated into the VLDL.

Triglyceride–phospholipid dispersions were prepared according to a modification of the method of Brecher et al. (16). A 70 mg quantity of egg yolk lecithin (Sigma Chemical, St. Louis, MO) and 7 mg of glyceryl trioleate (Supelco, Bellefonte, PA) containing 50–100 μCi of ³H or 5–10 μCi of ¹⁴C were dissolved in chloroform, evaporated under nitrogen, and resuspended in 7 ml of DPBS. The mixture was agitated with a Vortex mixer for 2 min and then sonicated in an ice bath for 2–10 min intervals using a Branson sonifier (Branson Instruments, Stamford, CT Model S-125) equipped with a 16 cm \times 2 mm probe at an output setting of 6. This procedure produced an opalescent preparation that was stable under nitrogen at 4°C for at least 1 week.

VLDL and liposome preparations were fractionated by gel filtration chromatography according to the method of Sata et al. (17). Columns, 90 \times 1.5 cm, containing agarose A-50m (BioRad, Richmond, CA) were eluted with 1 M NaCl in PE buffer. Samples were applied in a volume of approximately 5 ml and eluted in 3 ml fractions. Total volume of the column was determined using tritiated water, and the column was standardized using human lipoprotein preparations. Lipoprotein electrophoresis was performed by the agarose gel method of Noble (18).

Cell incubations

Confluent monolayers in 25 cm²- or 75 cm²-Petri dishes (6×10^4 cells/cm²; 13 μg cell protein/cm²) were rinsed with DPBS and the medium was replaced with 3 or 6 ml of MEM containing the indicated amount of VLDL or liposome preparations. Cultures were incubated during the indicated period of time at 37°C in a humidified atmosphere of 5% CO₂. At the termination of incubation, monolayers

were rinsed five times with DPBS and the cell material was mopped with a rubber policeman into a small amount of distilled water. An aliquot was taken for determination of cell protein (19) and lipids were extracted and assayed as described below. For experiments using spent medium, the medium was removed from confluent monolayers and filtered through 0.22 μm Millipore filter. It was then mixed with the VLDL preparation and incubated at 37°C in a humidified atmosphere of CO₂ in air. All experiments were conducted on duplicate dishes and repeated three to five times. Labeled VLDL and liposomes were prepared fresh for each experiment and different amounts of ³H and ¹⁴C were used. Therefore initial ³H/¹⁴C ratios varied, preventing averaging of results of similar experiments. Ranges of experimental values are included in legends for each table.

Assay of cell lipids

Serum or cell lipids were extracted with a 2:1 solution of acidified chloroform–methanol containing 0.1 mg of alpha-tocopherol, and the extract was washed according to the method of Folch, Lee, and Sloane Stanley (20). The extract was evaporated to dryness in a stream of nitrogen and redissolved in petroleum ether. Triglycerides and other lipid subfractions were isolated by thin-layer chromatography on plates of silica gel G, 250 μm , in a solvent system of petroleum ether–ethyl ether–acetic acid 75:25:1. Lipid subclasses were quantitated by the sulfuric acid charring procedure of Marsh and Weinstein (21) as modified for thin-layer chromatography by Kritchevsky et al. (22). When the lipids in the cell culture medium were assayed, the medium was lyophilized and resuspended in a small volume of distilled water. Lipid was then extracted and assayed in a similar manner to that of the cells.

Radioactivity

Glyceryl tri[1-¹⁴C]oleate (30 mCi/mmol), glyceryl tri[9,10-³H]oleate (100 mCi/mmol) and [2-³H]glyceryl trioleate (50 mCi/mmol) were obtained from Amersham-Searle, Arlington Heights, IL, and their purity confirmed by thin-layer chromatography. For uptake and time course experiments, glyceryl tri[9,10-³H]oleate was used; for experiments designated doubly-labeled triglyceride, a mixture of glyceryl tri[1-¹⁴C]oleate and [2-³H]glyceryl trioleate was used.

Radioactivity was assayed in a Packard (Downer's Grove, IL) liquid scintillation spectrometer equipped with an external standard for quench correction. Lipid extracts were counted in a scintillation solution containing toluene, 2,5-diphenyloxazole (6 g/

liter) and 1,4-bis-[2-(4 methyl-5-phenyloxazolyl)]-benzene (200 mg/liter). Media, cell suspensions, and areas of the thin-layer chromatography plate were counted in a 2:1 mixture of the above scintillation solution and Triton X-100.

RESULTS

Characterization of triglyceride-labeled VLDL

Assay of the unlabeled VLDL isolated after re-floitation indicated an average lipid/protein ratio of 7.6; greater than 60% of the lipid was triglyceride, approximately 15% was phospholipid, and 22% was sterol ester. These values were consistent with those previously described for human VLDL preparations (23).

When the labeled VLDL was assayed, the Celite method of labeling resulted in 2–5% of the radioactivity being incorporated into the VLDL. When VLDL was labeled by the solvent exchange method, 15–25% of the label was incorporated into the VLDL, after the preparation was filtered to remove un-exchanged triglyceride emulsions. Upon TLC, greater than 95% of the radioactivity was triglyceride in both preparations. The VLDL labeled by the Celite method and the solvent exchange method appeared to be incorporated in the cells in a similar fashion (Fig. 2), although most of the studies employed VLDL labeled by the Celite method. This method was preferable because it did not expose the VLDL to solvent.

Fig. 1A shows a gel filtration chromatography elution pattern of the triglyceride-labeled VLDL on a column of Agarose A-50m. The VLDL preparation was prepared by the Celite method using glyceryl tri[9,10-³H]oleate. Absorbance measurements indicated that the VLDL eluted in a biphasic peak corresponding to the pattern described for VLDL by Sata et al. (17). Determination of radioactivity in the column fractions indicated that the distribution of radioactivity corresponded to the absorbance pattern; thus the VLDL preparations appeared to be uniformly labeled with triglyceride. When doubly-labeled triglyceride was used as a tracer, the ³H- and ¹⁴C-labeled materials cochromatographed.

In order to assess the ability of this triglyceride-labeled VLDL to serve as a substrate for cellular triglyceride metabolism, the VLDL was exposed to suspensions of rat epididymal adipocytes (supplied by Dr. F. DeMartinis) and triglyceride hydrolysis was assayed by determination of free fatty acid. The data (Table 1) indicated that during the incu-

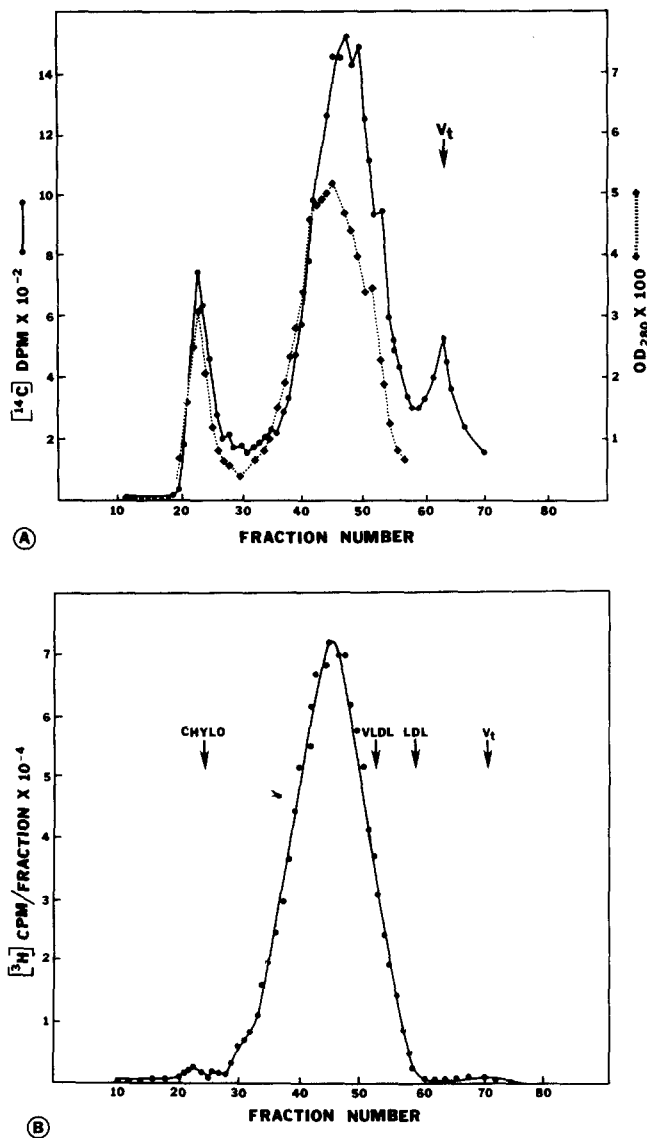


Fig. 1. Gel filtration chromatography elution pattern of VLDL and liposome preparations. Material was applied in 5 ml of DPBS containing 10% sucrose and eluted in 3 ml fractions of 1 M NaCl in PE buffer. *A.* Human VLDL preparation isolated and washed by flotation and labeled with glyceryl tri[9,10-³H]oleate by the Celite method. *B.* Phosphatidylcholine-glycerol tri[9,10-³H]oleate liposome preparation 10:1 (w/w).

bation period there was a rapid hydrolysis of the VLDL triglyceride. At 3 hr this corresponded to 10% of the original triglyceride in the absence of serum and 18% of the original triglyceride in the presence of serum, which either stabilized the adipocyte preparation or served as an acceptor for free fatty acid. These results indicated that when the VLDL was mixed with 10^6 adipocytes, a cell number considerably less than that used for the endothelial cell incubations, the VLDL was an acceptable substrate for the measurement of lipase activity.

Uptake of VLDL triglyceride by endothelial cell cultures

Fig. 2 shows results of experiments when triglyceride-labeled VLDL was exposed to primary monolayers of calf aortic endothelial cultures for up to 24 hr. A continuous uptake of triglyceride occurred during this time period. A lower concentration of VLDL triglyceride led to a similar pattern of accumulation but at a decreased level. The data also show that triglyceride from VLDL labeled by both Celite and solvent exchange techniques appeared to be taken up by the cells in a similar manner.

VLDL presented to the cell in increasing concentrations resulted in an increasing cell triglyceride accumulation (**Fig. 3**). The data also indicated that, as exogenous VLDL triglyceride increased, the percent uptake decreased. Since percent uptake was a maximum of 3% of the total in the medium, the possibility arose that the uptake did not represent triglyceride accumulation but could be accounted for by the small amount of labeled free fatty acids that were present in all preparations. This was shown not to be the case, however, for experiments were conducted when excess unlabeled free fatty acid was added to the VLDL-containing medium (Table 6). Total cell accumulation did not change in these experiments.

Table 2 shows the influence of trypsinization on triglyceride uptake from labeled VLDL. Five rinses of the monolayer were used routinely, because assay of subsequent rinses indicated only 1 or 2% of the triglyceride taken up was released in subsequent rinses. When cells were trypsinized after the five

TABLE 1. Hydrolysis of VLDL triglyceride by adipocytes

Time	Serum	Free Fatty Acid	$\mu\text{gFFA}/10^6$ Cells
hr		dpm	%
0		507	2.5
1.5	No	755	5.1
3	No	1689	10.5
1.5	Yes	2225	13.5
3	Yes	3237	18.5

Adipocytes were isolated from epididymal fat pads of 6-month old male rats by collagenase digestion (36). The washed adipocyte preparation, in Krebs bicarbonate-Ringer solution containing 13 mg/ml albumin, was mixed with an equal volume of VLDL labeled with glyceryl tri[9,10-³H]oleate by the Celite method (final concentration 50 μg triglyceride/ml). The mixture was gently rotated at 37°C and, at the indicated times, 5 ml aliquots (approx. 5×10^5 cells) were removed and chilled. Adipocytes were isolated by centrifugation at 60 *g* for 1 min. The medium was then lyophilized, lipid was extracted, and radioactivity was determined in the free fatty acid fraction as described in Methods. Data are from one of four experiments. Original triglyceride radioactivity ranged from 10,500 to 60,300 dpm/ml and 3 hr values for FFA as percent of original triglyceride ranged from 10.5 to 22% in all experiments.

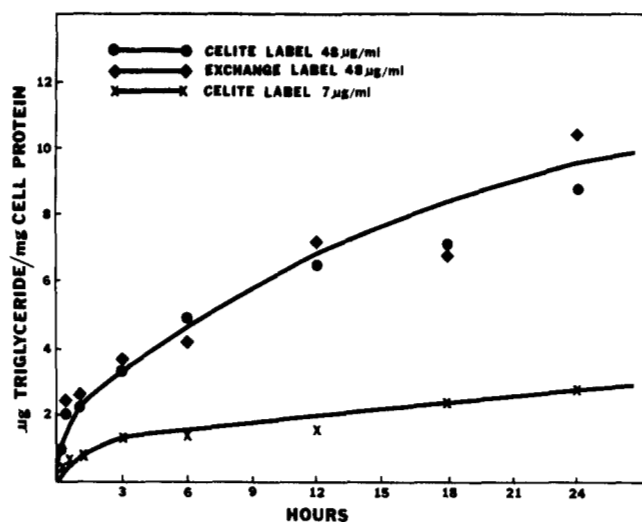


Fig. 2. Uptake of VLDL triglyceride by endothelial cell cultures. Confluent primary monolayers of calf aortic endothelial cells (in 60 mm Petri dishes; approx. 1.5×10^6 cells, 325 μg of protein) were transferred to 3 ml of MEM containing VLDL at the indicated concentration of triglyceride. VLDL was isolated by flotation and labeled with glyceryl tri[9,10- ^3H]oleate by the Celite or solvent exchange procedure as described in Methods. At the indicated times monolayers were rinsed five times with DPBS, the cells were harvested, and radioactivity was quantitated as described in Methods. Values were corrected for zero time radioactivity (approx. 10% of the 1 hr uptake) which represents the dpm in cells harvested immediately after they were exposed to the labeled VLDL.

rinses, the values for trypsinizable radioactivity indicated that only 2–10% of the triglyceride taken up was removed by trypsinization.

Hydrolysis and metabolism of VLDL triglyceride by endothelial cell cultures

In order to investigate cell triglyceride metabolism and whether the triglycerides were hydrolyzed in the medium or during the uptake process, the VLDL was labeled with a mixture of triglyceride containing ^3H in the glycerol moiety and ^{14}C in the fatty acid portion of the molecule ([2- ^3H]glyceryl [1- ^{14}C]trioleate). Assay of the $^3\text{H}/^{14}\text{C}$ was thus an indication of the extent of hydrolysis of the triglyceride. **Table 3** shows the distribution of the labeled VLDL triglyceride in the medium at the beginning of the experiments and after 24 hr exposure to endothelial cell cultures. The data indicated that there was no significant change in the distribution of label during the course of the experiment, suggesting that little or no triglyceride hydrolysis had occurred in the medium during exposure to the cells. Extent of hydrolysis in the medium was further assayed in experiments where either albumin or albumin plus unlabeled free fatty acids were added to the VLDL in order to provide an acceptor for any free fatty acids released. The data (**Table 4**) indicated that even

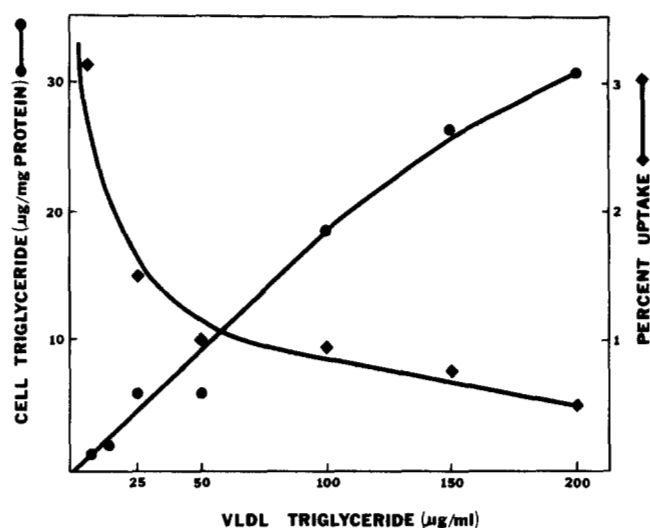


Fig. 3. Confluent primary monolayers of calf aortic endothelial cells in 100 mm Petri dishes (approx. 4.5×10^6 cells, 1 mg of protein) were transferred to 6 ml of MEM containing labeled VLDL at the indicated concentrations. VLDL was isolated by flotation and labeled with glyceryl [9,10- ^3H]trioleate by the Celite method. The VLDL preparation (500 $\mu\text{g}/\text{ml}$) was labeled and indicated dilutions were prepared in MEM. An aliquot of the original VLDL was lyophilized and the lipid was extracted. Both quantity and radioactivity in the major lipid subclasses were determined as described in Methods. After 6 hr monolayers were rinsed five times with DPBS, the cells were harvested, and radioactivity was quantitated as described in Methods. Percent uptake was expressed as total dpm in the cell monolayer/total dpm triglyceride in the medium VLDL.

under these conditions there was no indication of hydrolysis during the 6 or 24 hr exposure of the VLDL triglyceride to endothelial cell cultures, eliminating the possibility that free fatty acid was hydrolyzed but was immediately taken up by the cells.

In addition, triglyceride hydrolysis was assessed using medium that had been previously exposed to confluent monolayers of endothelial cultures for at least 3 days. Controls included analogous medium obtained from fibroblast cultures and fresh medium that had not been previously exposed to cells. The

TABLE 2. Influence of trypsinization on triglyceride incorporation by endothelial cultures

Incorporation	No. 1	No. 2	No. 3	No. 4
	<i>dpm/mg</i>			
After 5 washes	2424	944	8828	7720
After trypsinization	2372	904	7948	7220
% Released	2%	4%	10%	7%

Duplicate confluent monolayers of primary calf aortic endothelial cells were exposed to triglyceride-labeled VLDL at 100 $\mu\text{g}/\text{ml}$ as described in Fig. 2. After washing, the cells in one dish were harvested and radioactivity was determined in cell triglyceride. Cells in the other dish were exposed to 3 ml of trypsin solution (0.25% trypsin, 0.05% EDTA in DPBS) for 5 min and centrifuged at 500 g for 10 min. Triglyceride radioactivity was then quantitated in the cell pellet.

TABLE 3. Distribution of radioactivity in medium exposed to endothelial cell cultures

Vehicle	Time of Exposure	Mono-glycerides	Di-glycerides	Free Fatty Acid	Tri-glycerides	³ H/ ¹⁴ C	% total ¹⁴ C	
VLDL	0	0.6	1.1	0.6	98	1.70		
	24	0.9	3.4	1.3	94	1.82		
Liposomes	0	1.4	2.7	3.3	93	1.76		
	24	0.9	2.1	1.5	95	1.60		

VLDL (100 μg triglyceride/ml MEM) was labeled with [2-³H]-glyceryl tri[1-¹⁴C]oleate by the Celite method. Dispersions of phosphatidylcholine and [2-³H]glyceryl tri[1-¹⁴C]oleate 10:1 (w/w) were prepared by sonication and diluted in MEM to 100 μg/ml triglyceride. Both preparations were exposed to primary monolayers of calf aortic endothelial cells for 24 hr. Aliquots of both preparations at the beginning (Time 0) and end (Time 24) of the experiment were lyophilized, the lipid was extracted, and radioactivity was determined in lipid subfractions isolated by TLC. Total ¹⁴C in the medium in the case of the VLDL was 22,140 dpm/ml at T₀ and 20,770 at T₂₄. Liposome triglyceride was 453,000 dpm/ml at T₀ and 447,000 at T₂₄. Data are from one of seven experiments, in which percent triglycerides in the medium ranged 92–99%.

media that had been exposed to cells were filtered in order to remove any cellular material. The media were mixed with triglyceride-labeled VLDL and incubated at 37°C for up to 72 hr. The data (not shown) indicated that over the 3 day period there was no evidence of hydrolysis of triglyceride of VLDL mixed with fresh medium or with the medium exposed to either type of cell culture. These data along with the assays of media during the time of the experiments strongly suggest that no lipolytic activity was released into the medium by endothelial cell cultures.

To further explore the uptake and metabolism of triglyceride by endothelial cell cultures the ³H/¹⁴C ratios were measured in the accumulated cell tri-

TABLE 4. Free fatty acid in medium containing triglyceride-labeled VLDL

Addition	Time	Free Fatty Acid	
		dpm	%
None	0	804	2.4
	6 hr.	733	2.7
None	24 hr.	730	2.4
	6 hr.	1071	3.7
Alb.	6 hr.	726	2.7
	24 hr.	1267	3.7
Alb. & FFA	6 hr.	804	2.2
	24 hr.		

VLDL (100 μg triglyceride/ml) was labeled with glyceryl tri[9, 10-³H]oleate by the Celite method. The preparation was diluted 1:1 in MEM, the MEM containing either 10 mg/ml albumin, or 10 mg/ml albumin (fatty acid poor; Schwartz–Mann) and 100 μg/ml oleic acid. The latter was prepared by heating the MEM–albumin to 60°C and adding fatty acid as their sodium salts in a small volume of 0.5 N NaOH. The VLDL preparations were exposed to monolayers of endothelial cells as described in Fig. 3. At 6 and 24 hr, medium was removed from replicate cultures and lyophilized. Percent free fatty acid is dpm FFA/total lipid dpm in medium. Data are representative of three experiments in which dpm FFA ranged from 503 to 2307 and values for percent FFA varied from 1.0 to 3.7%.

glyceride and in other major cell lipid classes after the cells had been incubated 6 and 24 hr with the doubly-labeled VLDL. Values for the sterol ester fraction were always close to background and were omitted. After 6 hr of exposure, 61% of the intracellular radioactivity was present as triglycerides (Table 5). However, there had also been some conversion of radioactivity, mainly to free fatty acids and phospholipid. (The value for ³H/¹⁴C in the fatty acid fraction is greater than zero because of ³H-labeled-diglyceride contamination.) By 24 hr only 46% of the accumulated radioactivity remained as

TABLE 5. Uptake and metabolism of doubly labeled VLDL triglyceride by endothelial cell cultures

	Phospholipid		Diglyceride		Free Fatty Acid		Triglyceride	
	%	³ H/ ¹⁴ C	%	³ H/ ¹⁴ C	%	³ H/ ¹⁴ C	%	³ H/ ¹⁴ C
Celite label								
Medium								1.70
Cells, 6 hr	17	1.0	7.9	1.8	11	0.26	64	1.68
Cells, 24 hr	34	1.2	8.3	2.2	13	0.25	45	1.85
Exchange label								
Medium								2.30
Cells, 6 hr	17	1.7	9.8	3.2	13	0.32	60	2.41
Cells, 24 hr	30	1.8	13	2.9	12	0.26	45	2.20

VLDL (100 μg/ml triglyceride) was labeled with [³H]glyceryl [1-¹⁴C]trioleate by either the Celite or solvent exchange method. Aliquots of the VLDL preparations were extracted and ³H and ¹⁴C were assayed in triglyceride and other major lipid subclasses as described in Methods. VLDL was exposed to endothelial monolayers and cells were harvested as described in Fig. 3. Percent distributions are expressed on the basis of the ¹⁴C values. Data are from one of four experiments in which initial ³H/¹⁴C varied from 0.45 to 2.30. Percent triglyceride at 6 hr ranged from 55 to 72% and at 24 hr from 28 to 46%.

triglyceride and 33% had been converted to phospholipids. Assay of the $^3\text{H}/^{14}\text{C}$ ratio indicated that at both time periods the $^3\text{H}/^{14}\text{C}$ in the cellular triglyceride was similar to that in the medium, indicating that the majority of the triglyceride entered the cell without hydrolysis.

In order to determine if the triglyceride had been hydrolyzed during uptake and then reesterified intracellularly, excess nonradioactive glycerol or glycerol-2-octadecyl ether, a nonhydrolyzable monoglyceride analog (24), was added to medium. If ^3H was released as glycerol or monoglyceride during hydrolysis it would be diluted before a rapid intracellular reesterification could occur. The data (Table 6) indicated that [^3H]glyceryl trioleate uptake was not decreased by the presence of either glycerol or monoether in the medium; similarly the $^3\text{H}/^{14}\text{C}$ in the cell triglyceride was unchanged, confirming that [^3H]glycerol or monoglyceride had not been released and then reesterified intracellularly.

The results of the studies of cellular uptake and metabolism of doubly-labeled VLDL triglyceride therefore indicated that confluent primary monolayers of calf aortic endothelial cells were able to metabolize triglyceride intracellularly to a significant extent. It indicated that the triglyceride taken up was indeed available for metabolism by the cells. However there was no indication of hydrolysis and reesterification as a mechanism of cell entry. In all cases a maintenance of the [$^3\text{H}/^{14}\text{C}$] suggested that the triglyceride was taken up into the cells intact.

Uptake of triglyceride from phospholipid-triglyceride liposome preparations by endothelial cell monolayers

The experiments using the triglyceride-labeled VLDL had indicated that although endothelial cells were able to take up and metabolize triglyceride, there was no evidence of hydrolysis of triglyceride either in the medium or during the uptake process. In order to confirm this observation, experiments were conducted where triglyceride was presented to the cells in an alternative form. A liposome preparation was chosen, since it had been demonstrated that cultured cells are able to incorporate liposomes and their contents (25).

Sonication of a phosphatidylcholine-triolein mixture as described above (Methods) easily resulted in a stable opalescent dispersion. When this preparation was chromatographed on a gel filtration column under conditions similar to those of lipoprotein preparations, the liposomes eluted in a single peak that was broader than the typical VLDL peak (Fig. 1B). The peak eluted with a retention volume smaller

TABLE 6. Effect of additional nonradioactive sources of glycerol or fatty acid on the incorporation of doubly labeled triglyceride by endothelial cell cultures

	^3H	^{14}C	$^3\text{H}/^{14}\text{C}$
	dpm/mg cell protein		
Exp 1. Control	3410		1.91
Glycerol (200×)	4530		1.88
Monoether (6×)	3450		1.92
Exp 2. Control		1240	1.28
Oleic acid (4×)		1072	1.47

VLDL (25 $\mu\text{g}/\text{ml}$ MEM) was labeled with [$2\text{-}^3\text{H}$]glyceryl tri[$1\text{-}^{14}\text{C}_1$]oleate by the Celite method. Glycerol was added at 500 $\mu\text{g}/\text{ml}$, representing a 200-fold molar excess of free glycerol compared to triglyceride glycerol. Octadecyl monoether was added at 50 $\mu\text{g}/\text{ml}$, representing an approximately 6-fold molar excess compared to triglyceride. Monoether was added in a small volume of warm ethanol. Oleic acid was added on albumin carrier as described in Table 4. Final concentration was 100 $\mu\text{g}/\text{ml}$, which is approximately a 4-fold molar excess compared to triglyceride free fatty acid. Cells were exposed to VLDL preparations, and ^3H and ^{14}C in cell triglyceride were measured.

than that of VLDL, indicating that the liposomes were somewhat larger than the VLDL. Our phosphatidylcholine-triolein liposomes appeared to be somewhat larger than those prepared from single phospholipids (26). This is most likely because of the presence of triglyceride.

Fig. 4 shows the results of experiments in which liposome preparations containing labeled triglyceride were exposed to endothelial cell monolayers at concentrations of triglyceride/ml similar to the VLDL preparations. The time course of uptake of liposome

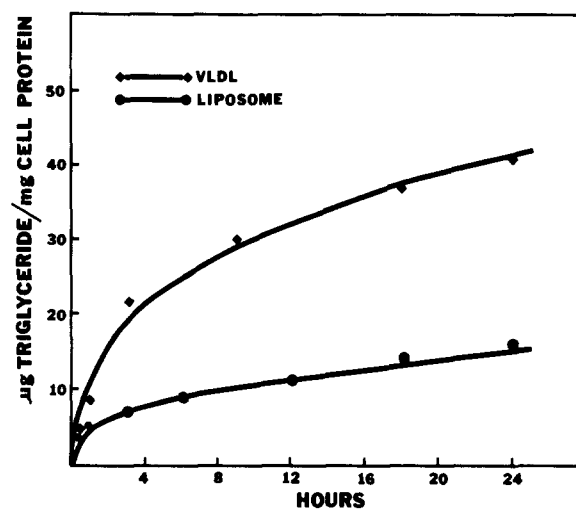


Fig. 4. Uptake of triglyceride from VLDL or liposome preparations by endothelial cells in culture. VLDL was isolated by flotation and labeled with glyceryl tri[$9,10\text{-}^3\text{H}$]oleate by the Celite method. Phosphatidylcholine-glyceryl tri[$9,10\text{-}^3\text{H}$]oleate dispersions 10:1 (w/w) were prepared by sonication as described. Each was exposed to the cells in MEM at 100 $\mu\text{g}/\text{ml}$ triglyceride. Radioactivity was assayed in confluent primary monolayers of calf aortic endothelial cells as described in Fig. 2.

triglyceride by the cell cultures was very similar to that of the VLDL triglycerides. However, when the two were exposed at equivalent triglyceride concentrations, the uptake of triglyceride from the liposome preparation appeared to be less than that from the VLDL.

The medium containing the doubly-labeled liposome preparation was monitored during the course of exposure to the cells. The data (Table 3) show that the liposome preparations contained a distribution of label similar to that of the labeled VLDL and no liposome triglyceride was hydrolyzed in the medium during the course of the experiment. Thus the liposome preparation did not stimulate the release of a lipolytic activity from the cultures into the medium.

Table 7 shows the values for cell lipids in experiments when the liposomes contained doubly-labeled triglyceride. When the $^3\text{H}/^{14}\text{C}$ ratio in the cell triglyceride was compared to that of the exogenous liposome preparation it was found that, after 6 or 24 hr of exposure, the ratio of label inside the cell remained similar to that of the original preparation. This again indicated that the triglyceride appeared to be entering the cell without hydrolysis. Measurement of radioactivity in other cell lipid classes indicated the cells were able to metabolize a portion of the triglyceride taken up from the liposome preparation; the extent of metabolism in these experiments appeared to be somewhat less than that observed from VLDL preparations, especially at 24 hr.

In control experiments additional nonradioactive sources of glycerol or monoglyceride were added to

TABLE 7. Uptake of [^3H]glyceryl tri[^{14}C]oleate from triglyceride-phospholipid liposomes by endothelial cell cultures

	6 hr.		24 hr.	
	%	$^3\text{H}/^{14}\text{C}^a$	%	$^3\text{H}/^{14}\text{C}^a$
Phospholipid	7.1	1.9	10.7	1.2
Diglyceride	11.2	2.7	11.3	2.2
Free fatty acid	13.2	0.50	12.9	0.42
Triglyceride	68.4	1.78	64.4	1.46

^a Liposome triglyceride $^3\text{H}/^{14}\text{C} = 1.76$.

Dispersions of phosphatidylcholine and [$2\text{-}^3\text{H}$]glyceryl [$1\text{-}^{14}\text{C}$]trioleate 10:1 (w/w) were prepared by sonication as described in Methods. They were diluted in MEM to 100 $\mu\text{g}/\text{ml}$ triglyceride, an aliquot was extracted, and ^3H and ^{14}C radioactivity were determined in triglyceride isolated by TLC. Dispersions were exposed to confluent monolayers of primary calf aortic endothelium. Percent distributions are expressed on the basis of the ^{14}C values. Total cell ^{14}C was 8,400 dpm at 6 hr and 12,400 at 24 hr. Experiment is one of three; percent triglyceride in all experiments ranged from 58 to 72%.

TABLE 8. Effect of serum or diluents on uptake of [^3H]glyceryl tri[^{14}C]oleate from liposomes

Additions	[^3H]Glyceryl Triooleate Uptake	
	dpm/mg	$^3\text{H}/^{14}\text{C}^a$
None	17,033	1.62
Glycerol (200 \times)	14,063	1.31
Monoether (6 \times)	14,749	1.63

^a Original medium $^3\text{H}/^{14}\text{C} = 1.73$.

Liposome preparations were prepared and exposed to the endothelial cells as described in Table 7. Diluents were added according to procedures described in Table 6. Monolayers were harvested and ^3H and ^{14}C radioactivity were determined in cell triglyceride. Data are representative of three experiments in which initial $^3\text{H}/^{14}\text{C}$ ranged from 1.23 to 2.97. Values for [^3H]glyceryl triooleate uptake in the presence of glycerol or monoether in all experiments were from 80 to 90% of the control value.

the medium in order to dilute out [^3H]glycerol or [^3H]monoglyceride taken up as a product of hydrolysis and then reesterified. The data (Table 8) indicated that neither glycerol nor the monoglyceride analog had an effect on the [^3H]glyceryl triooleate uptake from triglyceride-phospholipid liposomes in these cultures. In addition the $^3\text{H}/^{14}\text{C}$ in the cell triglyceride remained constant under all conditions and equivalent to that in the original medium.

DISCUSSION

These studies demonstrated that calf aortic endothelial cells were able to take up triglyceride when it is presented in the form of VLDL. This uptake increased with time and with increasing exogenous VLDL triglyceride concentrations. The source of the uptake was not the small amount of VLDL free fatty acid, since inclusion of unlabeled free fatty acid in the medium did not influence radioactive accumulation. It is unlikely that the triglyceride uptake was simply a nonspecific surface absorption, since the monolayers were rinsed five times and since trypsinization did not result in significant release of the radioactive triglyceride.

The amount of uptake at the higher VLDL concentrations was in the range of 100 μg of triglyceride per culture. This amount would not lead to significant accumulation as measured by chemical assay. Therefore, the data do not distinguish how much uptake was accumulation vs. an exchange process. Previous studies on cell triglyceride uptake from whole serum have indicated that some excretion of triglyceride can occur (28). However, measurable increases in cell triglyceride have been observed in

experiments using higher VLDL concentrations², indicating that net accumulation is occurring. Also, a portion of the triglyceride was shown to be metabolized inside the cell. This is also indicative of true cell uptake.

Triglyceride accumulation from serum has been demonstrated in other cell culture systems in this laboratory, including L cells (27) and human skin fibroblasts (28). In the latter case an elevation of cell triglyceride levels occurred in response to hypertriglyceridemic serum. Triglyceride uptake from VLDL has also been reported in ascites cells by Brenneman and Spector (15) and in human skin fibroblasts (29). The experimental evidence from cell culture systems thus suggest that cells *in vivo* may be able to incorporate intact triglycerides. This capability is probably overshadowed in most cases by the incorporation of fatty acid after lipoprotein lipase action; however, intact triglyceride uptake may be significant in tissues or situations where this enzyme is not present.

The results of the experiments using doubly-labeled triglyceride indicated that most of the triglyceride entered the cell intact. The ³H/¹⁴C ratio of the intracellular triglyceride was similar to that of the exogenous VLDL at all times. The ratio was not altered when glycerol monoether (nonhydrolyzable monoglyceride analog) was included to dilute out any [³H]glycerol or [³H]monoglyceride released by hydrolysis, ruling out a rapid hydrolysis and reesterification. In addition, no significant lipolytic activity appeared to be released into the medium from the endothelial cultures, as indicated by a lack of hydrolysis of the VLDL triglyceride during exposure to the cells or when incubated with spent medium. In control experiments, when VLDL containing doubly-labeled triglyceride was exposed to adipocytes, a rapid and easily measurable hydrolysis of triglyceride occurred, indicating this substrate can be used to measure lipolytic activity.

Experiments were also conducted presenting doubly-labeled triglyceride to cells in a liposome preparation. Liposomes, or phospholipid dispersions, have been shown to serve as a vehicle for uptake of a number of components in cultured cells (16, 25). Although the liposomes in these studies were not extensively characterized in terms of integrity and lamellar structure, they were shown to induce measurable cell triglyceride accumulation. Again, using this system of presentation, very little triglyceride hydrolysis occurred in the medium or during uptake.

² delaLlera, M. M., and B. V. Howard. Unpublished observations.

The results of all experiments using doubly-labeled triglyceride thus suggest that little or no lipase-like activity was present in the medium of bovine aortic endothelial cells in culture. Triglyceride was hydrolyzed inside the cell to some extent. This could be due to the presence of intracellular lipases or triglyceride hydrolases similar to those described in other cell cultures (30, 31). Alternatively, it is possible that in these isolated endothelial cells the hydrolysis is caused by lipoprotein lipase that is only active inside the cell under these conditions.

The mechanism of the observed uptake of intact triglyceride by the endothelial cells is not clear from these experiments, since no measurements of VLDL binding or uptake were made. It is possible that the triglyceride uptake occurs because endothelial cells can bind VLDL and then incorporate the intact VLDL into vacuoles where the triglycerides become available for cell metabolism. It is also possible that triglyceride associates with the cell simply by collision of the VLDL with the surface membrane. VLDL uptake has been quantitated by Bierman and Albers in several smooth muscle cultures (32) and observed by Brown and Goldstein in fibroblast systems (33). Uptake in the former system was of the order of 1% of the exogenous VLDL. Triglyceride uptake in the present studies was of a similar order of magnitude, supporting the possibility that triglyceride enters after VLDL binds to the cell surface (33). The observations that percent uptake decreased at increasing VLDL concentrations (Fig. 3), and that liposomes at equivalent medium triglyceride levels led to somewhat less accumulation also implicate a specific binding involved in triglyceride uptake. The mechanism of triglyceride uptake in these endothelial cultures must be further studied and the relationship between VLDL binding and triglyceride uptake explored. In addition, it will be important to investigate the problem using human endothelial cultures, since there is undoubtedly species specificity in lipoprotein-cell surface interactions.

The lack of evidence for lipoprotein lipase activity in the medium of cultures of aortic endothelial cells is of interest in relation to endothelial function *in vivo*. It is possible that the enzyme is present inside the cell and, under these conditions, only active on intracellular triglyceride. Another possibility is that the cells have lost this function through de-differentiation in the culture environment. However, all experiments were conducted with primary monolayers, which are only 3–4 population doublings *in vitro*. In addition, these cultures have been shown to retain other differentiated characteristics of endo-

thelium in vivo, such as Weibel-Palade bodies (9), synthesis of basement membrane collagen (10), and synthesis of factor VIII antigen (9). It is also possible that production or excretion of the enzyme must be induced, perhaps by more extensive cell division, or deprivation of exogenous free fatty acid. Lipoprotein lipase activity has been demonstrated in preparations of whole aorta (34). However, Vost (35) has shown in whole vessel perfusion studies that chylomicron triglycerides can enter the intima intact. Schotz, et al. (4), in studies of adipose tissue, have suggested that lipoprotein lipase is synthesized by adipocytes and then sequestered in endothelium. The results of the present studies suggest that an analogous system may exist in large vessels such as aorta. ■■

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