Triglyceride and cholesteryl ester hydrolysis in a cell culture model of smooth muscle foam cells

Lisa K. Minor, George H. Rothblat, and Jane M. Glick[1]

Department of Physiology and Biochemistry, The Medical College of Pennsylvania, Philadelphia, PA 19129

Abstract Cultured rabbit aortic smooth muscle cells were converted to foam cells by exposure to sonicated lipid droplets of defined composition using an inverted culture technique. Uptake of the lipid droplets by the cells was shown to be dependent on the time of exposure to the droplets and on the mass of droplets presented to the cells. A comparison of the hydrolysis of triolein and cholesteryl oleate by cells that had been exposed to isotropic lipid droplets containing equimolar amounts of the two lipids revealed that the rate of hydrolysis of triglyceride was 3 to 4 times faster than that for cholesteryl ester. The hydrolysis of cholesteryl oleate from cells loaded with the isotropic droplets was approximately 1.5 times as fast as that from cells loaded with anisotropic droplets containing only cholesteryl oleate. A comparison of the hydrolysis of cholesteryl ester in the presence and absence of Sandoz compound 58-035, an inhibitor of acyl CoA:cholesterol acyl transferase, by cells loaded with isotropic droplets showed that about 30% of the free cholesterol liberated by hydrolysis was reesterified regardless of the mass of free cholesterol. We conclude that cultured smooth muscle cells have a greater capacity to hydrolyze triglyceride than cholesteryl ester, and that the rate of hydrolysis of cholesteryl ester appears to be related to the physical state of the droplet in which the cholesteryl ester is stored. In addition, it appears that the smooth muscle cells have a cholesteryl ester cycle that is inefficient in the reesterification of excess free cholesterol.—**Minor, L. K., G. H. Rothblat, and J. M. Glick.** Triglyceride and cholesteryl ester hydrolysis in a cell culture model of smooth muscle foam cells. *J. Lipid Res.* 1989. **30:** 189–197.

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The development of atherosclerotic plaque is a prolonged, multifactorial process wherein macrophages and smooth muscle cells accumulate large deposits of cellular lipid and thus acquire the phenotypic character of foam cells. The origin of the lipid in foam cells has been variously attributed to LDL (1), modified LDL such as malondialdehyde-LDL (2, 3), β-VLDL (4, 5), postprandial lipoproteins (6), extracellular lipid (7, 8), and macrophage lipid droplets (9), all of which are likely to play a contributing role. The intracellular lipid droplets in foam cells are thought to be in a dynamic state, wherein the lipid is constantly hydrolyzed and reesterified (10, 11). While the predominant lipid in plaque is cholesteryl ester (primarily cholesteryl oleate), a substantial amount of free cholesterol is also present. Although lipoproteins such as β-VLDL and postprandial lipoproteins might be expected to provide triglyceride as well as cholesteryl ester, relatively little triglyceride is found in plaque (12, 13).

Recent work in our laboratory has focused on an hypothesis regarding the mechanism underlying the formation of smooth muscle foam cells. We proposed that smooth muscle cells in the vessel wall might acquire lipid inclusions that were originally synthesized by macrophages (9). To test this hypothesis, we developed a cell culture model to demonstrate that cultured smooth muscle cells can become foam cells by the uptake of lipid droplets isolated from cultured macrophages. We also demonstrated that the lipid in these inclusions is metabolically available to the smooth muscle cells. An intriguing observation in these early experiments (9) was made using polarizing light microscopy which suggested selective clearance of triglyceride by the smooth muscle cells. In the present study, we have used sonicated lipid droplets as a model of the cellular inclusions to compare more quantitatively the relative metabolism of cholesteryl esters and triglyceride in smooth muscle cells.

MATERIALS AND METHODS

Materials

Oleic anhydride, cholesteryl oleate, triolein, egg phosphatidylcholine, phosphatidylserine, and BSA (essentially fatty acid-free from Fraction V) were purchased from Sigma. Coprostanol was purchased from Steraloids, Inc. Heat-inactivated fetal bovine serum and minimal essential medium (MEM) were purchased from Gibco. [7-

Abbreviations: LDL, low density lipoproteins; VLDL, very low density lipoproteins; MEM, minimum essential medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ACAT, acyl CoA:cholesterol acyltransferase; EC, esterified cholesterol.

[1]To whom correspondence should be addressed.

^{3}H]Cholesterol was purchased from ICN; trioleoyl [^{3}H]glycerol, cholesteryl [^{14}C]oleate, and tri[^{14}C]oleoylglycerol were purchased from Amersham. [7-^{3}H]Cholesteryl oleate was prepared from [7-^{3}H]cholesterol and oleic anhydride as described (14) and was purified prior to use by thin-layer chromatography using silica gel G plates developed in benzene–hexane 60:40 (v/v). Thin-layer chromatography plates were from Anasil. Peridochrome Triglyceride Assay Kit was purchased from Boehringer Mannheim Diagnostics. Sandoz compound 58-035, an inhibitor of acyl CoA:cholesterol acyl transferase (15), was a gift from Dr. John Heider.

Preparation of sonicated lipid droplets

Isotropic lipid droplets were prepared by combining 15 mg cholesteryl oleate, 20 mg triolein, 1 mg phosphatidylcholine (each stored in hexane), and 0.1 mg phosphatidylserine (stored in hexane–chloroform 1:1 (v/v) in a sterile 50-ml round-bottom Corex test tube. Either [7-^{3}H]cholesteryl oleate or [^{3}H]triolein was added to give specific activities of approximately 875 nCi/mg for triolein and 650 nCi/mg for cholesteryl oleate. The solvents were evaporated under N_2 and 15 ml MEM buffered with 12.5 mM HEPES, pH 7.4, was added to the tube, which brought the level of the solution to 3.5 cm from the bottom of the tube. This solution was placed in a boiling water bath for 10 min to melt the lipids and was immediately sonicated using a Branson Sonifier Cell Disrupter Model 350 with a microtip attachment. The tip of the microtip was placed 1 to 1.5 cm from the top of the solution and sonication was for 3 min at a setting of 2 to 3 with the output registering between 20 and 30 using the 50% pulse setting. During sonication, the temperature was not controlled. Anisotropic lipid droplets were prepared in a similar manner except that 30 mg of cholesteryl oleate was used, and the triolein was omitted. This procedure produced lipid droplets with diameters of approximately 1 μm. The diameters of two representative droplet preparations as measured in a Coulter N4 Laser Sizer were 0.948 ± 0.12 μm and 1.57 ± 0.41 μm. Free cholesterol contents of the droplet preparations were always less than 1% of the total cholesterol content.

Analytical and preparative procedures

Lipids were extracted from solutions using the method of Bligh and Dyer (16) and from cell monolayers with isopropanol as described by McCloskey, Rothblat, and Glick (17). Free and esterified cholesterol masses were determined by gas–liquid chromatography using coprostanol as an internal standard (18), and triglyceride mass was determined using Boehringer Mannheim Diagnostics Peridochrome Triglyceride Assay Kit with triolein as a reference standard. Phospholipid concentrations were determined by the method of Sokoloff and Rothblat (19). The distribution of radiolabel among glycerides or between free and esterified cholesterol was determined using thin-layer chromatography on ITLC-SA plates developed in petroleum ether–ethyl ether–acetic acid 85:14:1 (v/v/v). HDL was prepared from human plasma by the method of Hatch and Lees (20). ApoHDL/phosphatidylcholine acceptor particles were prepared as previously described (21). Protein concentrations were determined by the Markwell et al. modification (22) of the procedure of Lowry et al. (23) using BSA as a standard. Protein in cell monolayers was solubilized by incubation of the monolayer with 1 ml of the Lowry A solution (22). The physical state of the lipid droplets was determined using polarizing light microscopy (24).

Lipid loading of cultured cells

Rabbit aortic smooth muscle cells were established in culture and maintained as previously described (9). Cells to be loaded with lipid droplets were grown to confluence on glass coverslips (22 × 22 mm). To initiate uptake, cells were incubated with droplets as described previously (9). Briefly, each coverslip was inverted over a plastic ring in a fresh 35-mm plate and 1 ml MEM containing 10% fetal bovine serum and an appropriate amount of sonicated lipid droplets was added to each dish. Additional MEM with 10% fetal bovine serum was added to fill the space below the monolayer, so that droplets floated to the surface and came into direct contact with the cells. Because the actual volumes varied slightly from dish to dish, the amount of lipid added is expressed as μg of esterified cholesterol per dish rather than as a concentration. The dishes were then incubated for various periods at 37°C in a humidified incubator. In experiments where droplet uptake alone was being measured, the coverslips were rinsed three times with phosphate-buffered saline (PBS) (25) at the end of the incubation period; lipids were then extracted, and the protein contents of the monolayers were determined as described above. Aliquots of the isopropanol extracts were used to determine the total cell-associated radioactivities, the distribution of radiolabel among glycerides or between free and esterified cholesterol, and masses of triglyceride and free and esterified cholesterol. The extent of lipid uptake was determined both by mass and by radioactivity using the specific activity of the lipid in the sonicated droplets.

Hydrolysis of cellular lipids

To quantitate the cellular hydrolysis of lipid derived from droplets, cells were loaded with lipid as described above. At the end of the loading period, monolayers were washed three times with MEM containing 0.1% BSA and were incubated for an additional 2 hr with MEM containing 1.0% BSA to allow complete uptake of any adsorbed lipid droplets. The monolayers were washed once with

MEM containing 1.0% BSA, and 2 ml of the same medium was added. For measurements of cholesteryl ester hydrolysis, 1 μg/ml Sandoz 58-035 was included unless otherwise indicated. Some of the dishes were harvested to determine the concentrations of cellular esterified cholesterol and triglyceride present at the initiation of the experiment by the isopropanol extraction method outlined above and by the detergent solubilization method outlined below. The remaining dishes were incubated for various periods and were harvested for lipid analysis. A detergent solubilization method was used to avoid loss of cellular material which might occur by washing the monolayer. For this procedure, the monolayer and medium in each dish were harvested as a unit by the addition of 50 μl of 10% sodium dodecyl sulfate to the medium. The dishes were then agitated for 1 hr at room temperature to produce a slurry resulting from detergent solubilization of the cells into the medium. Lipids were extracted from an aliquot of the slurry using the method of Bligh and Dyer (16). An internal standard of free [^{14}C]cholesterol and esterified [^{14}C]cholesterol of known ratio (for cholesterol determinations) or [^{14}C]triglyceride (for triglyceride measurements) was included in these extractions. The distribution of radiolabel was determined by thin-layer chromatography as detailed above. The hydrolysis of esterified cholesterol was estimated from the reduction in the percentage of radiolabeled esterified cholesterol during the incubation period. As there was essentially no unlabeled cholesteryl ester in the smooth muscle cells, we were able to determine absolute rates of cholesteryl ester hydrolysis by monitoring the change in the percentage of radiolabeled esterified cholesterol in the presence of 1 μg/ml Sandoz 58-035, a concentration that has been shown to be effective in preventing the esterification of free cholesterol by ACAT (acyl CoA:cholesterol acyl transferase) (15). In preliminary experiments (data not presented), determination of the masses of free and esterified cholesterol agreed with the estimations using radioisotope within 5%. Therefore, mass analyses were not routinely performed at the end of each experiment.

The loss of triglyceride was estimated by comparing the amount of the triglyceride present at the initiation and at the end of the hydrolysis phase. To determine the absolute rate of triglyceride hydrolysis, 1% BSA was included in the incubation media. Preliminary experiments (data not presented) demonstrated no significant difference between these isotopically derived values and actual mass determinations when the albumin concentration in the culture medium was 1% or greater. At lower albumin concentrations there was a divergence between the isotopic values and those obtained by direct mass determinations, indicating that the fatty acids liberated by triglyceride hydrolysis were reesterified if they were not efficiently removed.

RESULTS

We were interested in further characterizing and comparing the metabolism of both triglyceride and esterified cholesterol delivered to smooth muscle cells by the inverted culture technique. However, the use of lipid inclusions isolated from cultured macrophages presented a number of technical problems. Among these were our inability to control rigorously the lipid composition of the cellular inclusions and the inability to selectively and specifically radiolabel individual lipid classes. To obviate these problems, we prepared sonicated lipid droplets of defined chemical composition to serve as models for the isolated cellular inclusions. The droplets were similar in size to those isolated from the J774 (26) or Fu5AH rat hepatoma (27) cells and, like cellular inclusions, were amenable to manipulation of physical state; when cholesteryl oleate was used as the sole neutral lipid, the physical state of the droplets was anisotropic, whereas isotropic droplets were obtained when equimolar cholesteryl oleate and triolein were present in the droplet. As the micrographs in **Fig. 1** demonstrate, incubation of smooth muscle cells with the sonicated droplets for 24 hr using the inverted culture technique resulted in extensive incorporation of lipid into the smooth muscle cells. The data in **Fig. 2** show that the incorporation of lipid, as monitored by the uptake of radiolabeled esterified cholesterol by the cells, was time-dependent during the first 6 hr with little or no additional uptake observed in the subsequent 18 hr. The uptake of lipid was also dependent on the mass of droplet lipid in the culture dish (**Fig. 3**). Uptake of droplets by the smooth muscle cells was relatively efficient, with approximately 15 to 30% of the available extracellular lipid being incorporated. Intracellular esterified cholesterol concentrations achieved were comparable to those previously obtained using lipid inclusions isolated from the J774 macrophages (9).

The mechanism of uptake of cellular lipid inclusions by smooth muscle cells using the inverted culture technique has not been established. The experiments described above using sonicated lipid droplets clearly speak against the involvement of any inclusion-associated cellular proteins in the uptake process. However, these studies were conducted using culture medium containing fetal bovine serum, and it was possible that serum proteins might become associated with the lipid droplets and contribute to their uptake. To test this possibility, washed smooth muscle cell monolayers were incubated with sonicated isotropic or anisotropic droplets in the presence or absence of serum. As the data in **Table 1** indicate, the presence of serum in the incubation media had no influence on the extent of cellular lipid accumulation.

The ability to make sonicated droplets of known lipid composition that were efficiently taken up by smooth

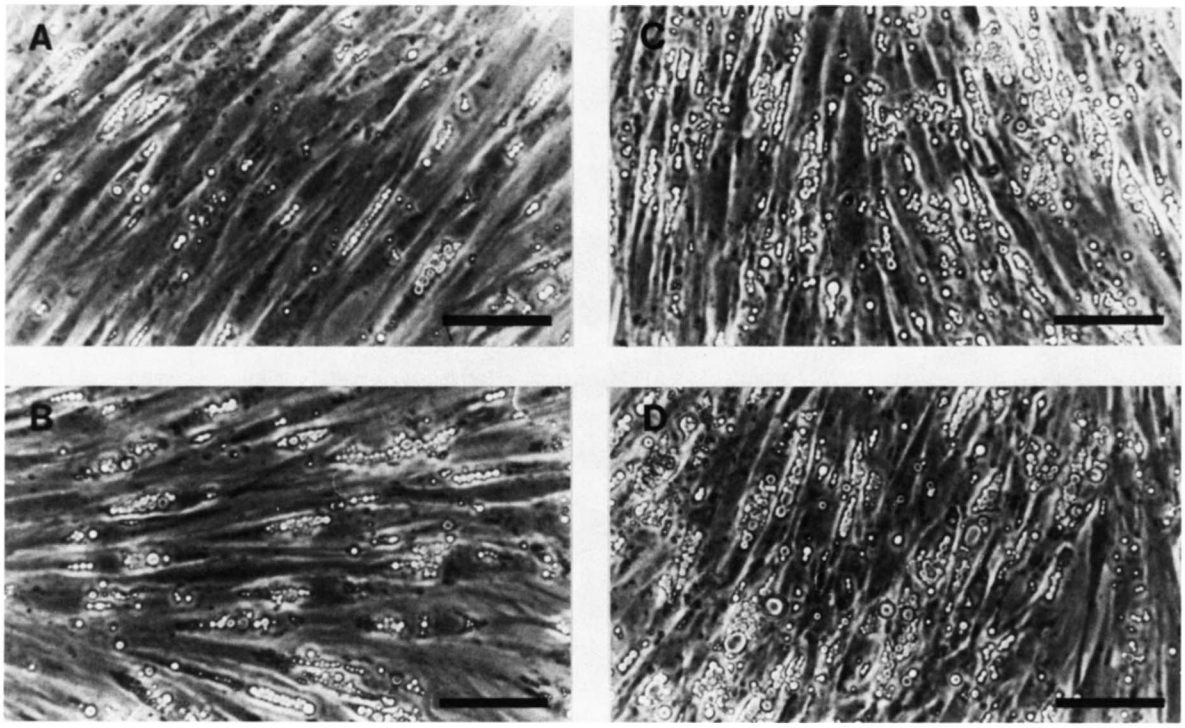

Fig. 1. Uptake of sonicated lipid droplets by rabbit aortic smooth muscle cells. Rabbit aortic smooth muscle cells grown to confluence on coverslips were incubated as described in Materials and Methods for 24 hr with the indicated masses of esterified cholesterol per dish as sonicated isotropic lipid droplets; A: 50 μg; B: 100 μg; C: 150 μg; and D: 250 μg. Monolayers were washed three times with MEM containing 0.1% BSA and were placed in MEM with 1.0% BSA for photomicroscopy. Bars indicate 50 μm.

muscle cells provided the opportunity to address two questions: *1*) Are the neutral lipids within droplets incorporated in stoichiometric amounts? and *2*) Are these lipids differentially metabolized? To determine the relative incorporation of esterified cholesterol and triglyceride, smooth muscle cells were incubated with isotropic droplets doubly labeled in both the triglyceride and cholesteryl ester moieties. At various intervals over a period of 18 hr, cells were harvested and lipids were extracted. After 1 hr, the ratio of [^{3}H]triolein to [^{14}C]cholesteryl oleate was not significantly different from that in the droplet preparation (3.77 vs. 3.86, respectively). Thus it appears that the droplets were taken up intact. However, as shown in **Fig. 4,** the ratio of [^{3}H]triolein to [^{14}C]cholesteryl oleate decreased over the 18-hr period, again suggesting that the triglyceride was being selectively hydrolyzed from the droplets.

A series of experiments was conducted to obtain more quantitative information on the relative hydrolysis of triglyceride and esterified cholesterol incorporated into smooth muscle cells exposed to lipid droplets containing equimolar cholesteryl oleate and triolein. A comparison of time courses of hydrolysis for triolein and cholesteryl oleate incorporated into smooth muscle cells in isotropic droplets is shown in **Fig. 5.** It is obvious that, in these representative experiments, triglyceride was hydrolyzed much faster than the esterified cholesterol. The extent of hydrolysis of triglyceride was sufficient to demonstrate that kinetics of hydrolysis of triglyceride were first order; however, this was not obvious for cholesteryl ester where the magnitude of hydrolysis was much lower. Previous ex-

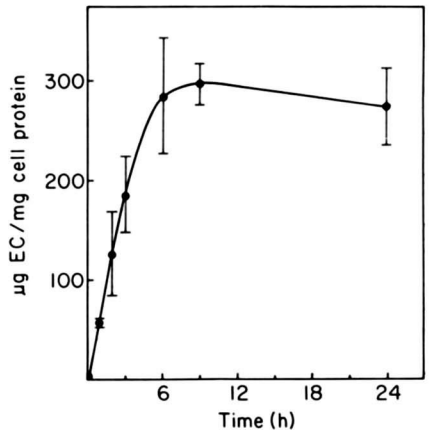

Fig. 2. Uptake of sonicated lipid droplets as a function of time. Rabbit aortic smooth muscle cells grown to confluence were incubated with sonicated isotropic lipid droplets containing 150 μg per dish esterified cholesterol (EC) radiolabeled in the cholesterol moiety. After various intervals, the monolayers were washed and the lipids were extracted and quantitated by radioisotope as described in Materials and Methods. Data are the mean ± standard deviation, n = 4.

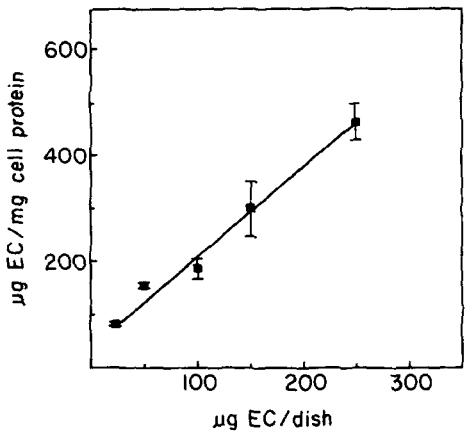

Fig. 3. Relationship between the mass of lipid incorporated by rabbit aortic smooth muscle cells to the mass of lipid provided to the cells. Rabbit aortic smooth muscle cells grown to confluence on glass coverslips were incubated for 16 hr with increasing amounts of sonicated isotropic lipid droplets. Cells were harvested, lipids were extracted, and protein contents were determined as described in the text. The uptake of esterified cholesterol (EC) was calculated using the uptake of radiolabeled cholesteryl ester and the specific activity of cholesteryl ester in the original droplet preparation. Data points are the mean ± standard deviation, n = 4.

perience in examining cholesteryl ester hydrolysis in the Fu5AH hepatoma cell line (28) had indicated that relative hydrolysis cannot be rigorously assessed from individual experiments since the rate of hydrolysis is a function of the initial lipid content in the cells. For this reason, it was important to choose conditions whereby hydrolysis could be assessed over a range of initial lipid contents. As large numbers of samples were required, it was necessary to evaluate hydrolysis using a single time point. The data in Fig. 5 suggested that 24-hr data were satisfactory for evaluating the hydrolysis of both lipids, and, thus, this time point was used in experiments where hydrolysis was evaluated from a single time point.

The data in **Fig. 6,** which are compiled from a number of experiments wherein cells were loaded with varying amounts of lipid, show that the masses of both triglyceride and esterified cholesterol hydrolyzed in 24 hr were directly related to the initial intracellular concentration of the respective lipid. At equivalent cellular concentrations, the mass of triglyceride hydrolyzed was 3 to 4 times that of esterified cholesterol. Also shown is the hydrolysis of cholesteryl oleate from anisotropic droplets (i.e., containing only cholesteryl oleate). It is obvious that the amount of esterified cholesterol hydrolyzed from isotropic droplets was greater than that from anisotropic droplets at all concentrations, indicating that the physical state of the droplet influences the rate of hydrolysis of esterified cholesterol in smooth muscle cells. In addition, these data demonstrate that the physical state of the lipid droplets was not critical to uptake since cholesteryl ester in both types of droplets was incorporated to similar extents.

In the experiments described above, ACAT was inhibited with Sandoz 58-035 to allow measurement of the absolute rate of cholesteryl ester hydrolysis. To determine the potential for esterified cholesterol hydrolysis from smooth muscle cells under more physiological conditions, i.e., when ACAT was active, experiments similar to those described above were performed in the absence of Sandoz 58-035. In all cases, there was net hydrolysis of cholesteryl ester, despite the presence of active ACAT. As shown in the inset to Fig. 6, the mass of cholesteryl ester hydrolyzed in the absence of Sandoz 58-035 was less, and it appears that approximately 30% of the free cholesterol liberated by the hydrolysis of cholesteryl ester was reesterified.

We wished to determine whether the free cholesterol generated from esterified cholesterol hydrolysis was available for efflux from cells exposed to an acceptor of free cholesterol. Smooth muscle cells with isotropic droplets were incubated for 24 hr in medium with or without Sandoz 58-035 in the presence or absence of apoHDL/phosphatidylcholine acceptor particles. At the concentration of acceptor particles used (500 µg phospholipid/ml), efflux of free cholesterol has been shown to be independent of acceptor concentration (21). The results presented in **Table 2** indicate that there was net hydrolysis of esterified cholesterol under all conditions. The radiolabeled free cholesterol generated was identical when either 58-035 or apoHDL/PC acceptor particles were present and was significantly greater than that generated in the absence of 58-035 and acceptor particles. When acceptors were present, approximately one-third of the labeled free cholesterol generated by hydrolysis of cholesteryl ester was found in the medium.

DISCUSSION

The ability of smooth muscle cells in culture to incorporate sonicated lipid droplets of controlled chemical composition allowed us to compare the kinetics of hydrol-

TABLE 1. Uptake of droplet esterified cholesterol by smooth muscle cells in the presence and absence of serum

Droplet Physical State	+ Serum	− Serum
	(nmol cholesterol/mg cell protein)	
Anisotropic	412 ± 114	466 ± 85
Isotropic	144 ± 27	137 ± 31

Rabbit aortic smooth muscle cells grown to confluence on glass cover slips were exposed for 5 hr to anisotropic lipid droplets (150 µg esterified cholesterol per dish, n = 5) or isotropic droplets (100 µg esterified cholesterol per dish, n = 6) radiolabeled with [^{3}H]cholesteryl oleate in either serum-free MEM or MEM with 10% fetal bovine serum. Uptake was determined as described in Materials and Methods. Values are the mean ± standard deviation. Uptake in the presence and absence of serum is not statistically different as determined by Student's *t*-test.

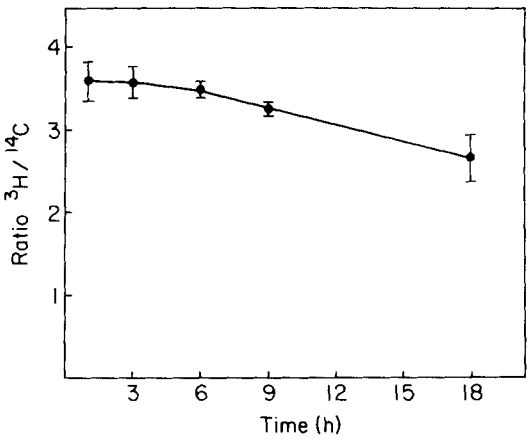

Fig. 4. Change in the isotope ratio of radiolabeled triolein to cholesteryl ester in droplets taken up by rabbit aortic smooth muscle cells. Rabbit aortic smooth muscle cells grown to confluence were exposed to sonicated isotropic lpid droplets containing trioleoyl [^{3}H]glycerol and [^{14}C]cholesteryl oleate at an isotope ratio of 3.86:1. At various intervals during the exposure to droplets, cells were harvested, and lipids were extracted as described in the text. An aliquot of the lipid extract was subjected to liquid scintillation counting and the ratio of ^{3}H to ^{14}C was determined. Data points are the mean ± standard deviation, n = 6.

ysis of esterified cholesterol and triglyceride taken into the cells in the same droplets. Our initial examinations of the time courses of hydrolysis of the two lipid esters revealed very different rates of hydrolysis; however, it was important to verify that this difference was not a fortuitous consequence of the initial masses of lipid in the cells. Compiling data from several experiments wherein the initial load of lipid was varied revealed that the hydrolysis of triglyceride was indeed much more rapid than that of cholesteryl ester at all concentrations studied. In fact, it was not possible to study the hydrolysis of triglyceride at levels comparable to the higher cholesteryl ester loads, probably because of hydrolysis of the triglyceride during the loading period as suggested by the experiment illustrated in Fig. 5. The data for the hydrolysis of cholesteryl esters are somewhat different in form from data previously reported for the hydrolysis of cholesteryl esters from Fu5AH hepatoma cells (28). In that experimental system, the hydrolysis of cholesteryl esters was linear with time and the rate of hydrolysis (mass cleared/unit time) increased linearly with the initial cellular content of cholesteryl ester. At any given cholesteryl ester content, the fraction of the cholesteryl ester cleared was always constant. In the present study the rate of hydrolysis of esterified cholesterol by the smooth muscle cells was also highly dependent on the initial esterified cholesterol content within the cells, yet the fractional hydrolysis of the esterified cholesterol was not constant and ranged from 7 to 30%. The rate of hydrolysis of TG was also dependent on the initial cellular triglyceride content, but the dependence of the fractional hydrolysis of triglyceride on initial triglyceride content is less clear. Values obtained for the hydrolysis of triglyceride compare favorably to those reported for macrophages (29) and rabbit aortic smooth muscle cells (30) clearing endogenously synthesized triglyceride. The hydrolysis of triglyceride by the Fu5AH hepatoma cell line has not been examined.

The reason for the very different rate of hydrolysis of neutral lipids, supplied to the cells in the same droplets, is not immediately obvious. One possibility is that the two lipids are hydrolyzed by the same enzyme that has different capacities for hydrolyzing cholesteryl esters and triglycerides. Several intracellular lipases have been characterized that have the ability to hydrolyze both triglycerides and cholesteryl esters, as well as other lipid esters (31–33). Two of the best characterized of these are purified hormone-sensitive lipase found in adipose tissue (31) and purified lysosomal acid lipase (32, 33). However, in both cases, the enzymatic activity toward triglycerides is similar to that toward cholesteryl esters. A second possibility is that the two lipids are hydrolyzed by different enzymes that have unique specificities for the individual lipid esters. Khoo et al. (34) have reported that J774 macrophages have a very active triglyceride lipase that appears to be distinct from the neutral cholesteryl ester hydrolase (35) in the same cells, but neither of these activities has been rigorously purified. These are precedents for the existence of lipases with a high degree of substrate specificity; both lipoprotein lipase and hepatic lipase are potent

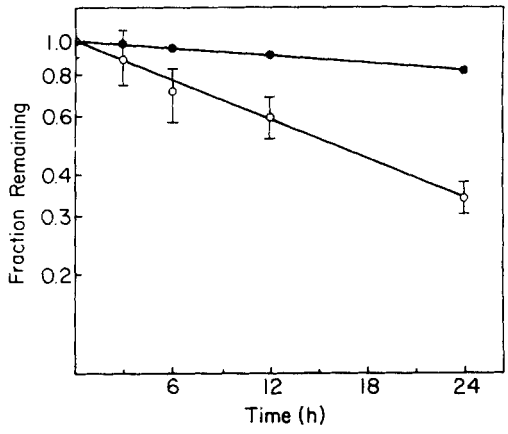

Fig. 5. Time course of hydrolysis of triolein and cholesteryl oleate in rabbit aortic smooth muscle cells loaded with sonicated isotropic lipid droplets. Confluent monolayers of rabbit aortic smooth muscle cells were incubated for 15 hr with sonicated isotropic lipid droplets (150 μg esterified cholesterol per dish) that contained either trioleoyl [^{3}H]glycerol or [7-^{3}H]cholesteryl oleate. The monolayers were then washed and incubated as described in the text. At the indicated intervals, cells were harvested, and lipids and protein were extracted and quantitated as described in Materials and Methods. Initial triglyceride content was 374 μg/mg cell protein and initial cholesteryl ester content was 628 μg/mg cell protein. The data for triglyceride hydrolysis (○) are the mean ± standard deviation, n = 4. Data points for cholesteryl ester (●) are the mean, n = 4, and the standard deviations for these values are included within the plotted point.

triglyceridases that also display phospholipase activity, yet have no cholesteryl ester hydrolase activity (36).

We were interested in exploring other factors that might affect the rate of cholesteryl ester hydrolysis in cells. Small and Shipley (37) predicted that the rate of hydrolysis of cholesteryl esters from foam cells might be restricted if the cholesteryl ester were present in an ordered (anisotropic) droplet. Previous work in this laboratory (27, 28) examining the hydrolysis of cholesteryl esters from Fu5AH cells containing either isotropic (liquid) or anisotropic (liquid crystalline) droplets showed that cholesteryl esters were cleared more rapidly from cells containing liquid droplets. In the present study, this also appears to be the case; over the entire range of initial cellular cholesteryl ester contents, the rate of hydrolysis was always higher for the cells with liquid droplets. Another factor that affects the net hydrolysis of cholesteryl esters in cells is the activity of ACAT. In most of the studies presented in this manuscript, ACAT was inhibited; however it was of interest to us to examine the influence of ACAT on the net hydrolysis

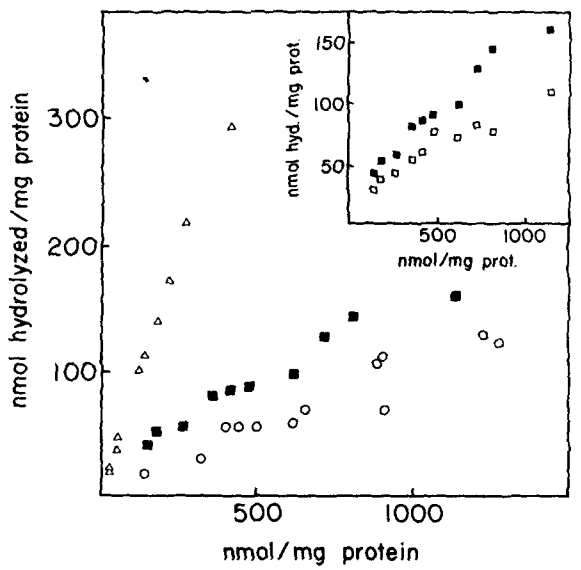

Fig. 6. Hydrolysis of triglyceride and cholesteryl ester by rabbit aortic smooth muscle cells as a function of initial cellular content of the respective lipids at the start of a 24-hr hydrolysis period. Rabbit aortic smooth muscle cells grown to confluence on glass coverslips were exposed for 16 hr to a range of concentrations (25 to 250 μg esterified cholesterol per dish) of appropriately radiolabeled sonicated lipid droplets to produce cell preparations containing the masses of lipid indicated on the x-axis. The cells were incubated an additional 24 hr and the extent of lipid hydrolysis was assessed and is shown on the y-axis as mass hydrolyzed per 24 hr. Data shown are compiled from two experiments examining triglyceride hydrolysis (△), three experiments examining cholesteryl ester hydrolysis from anisotropic (○) droplets, and two experiments examining cholesteryl ester hydrolysis from isotropic (■) droplets. The inset shows a replot of the data for hydrolysis of cholesteryl ester from isotropic droplets in the presence of 58-035 (■) and a plot of cholesteryl ester hydrolysis from the same sets of cells in the absence of 58-035 (□). Data points are the mean, n = 4 or 5. The average standard deviation for the lipid content data was 10.8% and for the hydrolysis rate data was 7.2%.

TABLE 2. Availability for efflux of free cholesterol derived from droplet cholesteryl ester

Conditions		Total	Monolayer	Medium
58-035	Acceptors	(μg labeled free cholesterol/mg cell protein)		
–	–	35.4 ± 10.3	25.0 ± 12.3	10.5 ± 2.1
+	+	60.6 ± 4.2	23.9 ± 1.0	36.7 ± 5.2
+	–	61.4 ± 1.5	52.0 ± 3.3	9.4 ± 4.9
–	+	57.1 ± 2.8	30.3 ± 0.6	26.7 ± 3.4

Rabbit aortic smooth muscle cells were loaded with sonicated anisotropic lipid droplets as described in Materials and Methods and were further incubated in MEM with 1.0% BSA for 24 hr in the presence and absence of 1 μg/ml 58-035 and in the presence or absence of apoHDL/phosphatidylcholine particles (500 μg phospholipid/ml) as indicated. The radiolabeled free cholesterol derived from hydrolysis of radiolabeled cholesteryl oleate during the 24-hr period was determined in the media and in monolayers. Values are mean ± standard deviation, n = 6.

of cholesteryl esters. The difference in the apparent cholesteryl ester hydrolysis in the presence and absence of ACAT (Fig. 6) speaks to the presence of a cholesteryl ester cycle in smooth muscle cells, although it appears to be relatively inefficient. There was net hydrolysis of cholesteryl ester even when ACAT was active, suggesting that ACAT does not esterify all of the free cholesterol generated by hydrolysis of cholesteryl esters. This is in contrast to data reported for other cell systems wherein ACAT appeared to maintain essentially constant cellular cholesteryl ester levels (10, 11). It is interesting to note that the difference between absolute cholesteryl ester hydrolysis (i.e., in the absence of ACAT activity) and net cholesteryl ester hydrolysis (i.e., when ACAT is active) was about 30% at all initial cellular cholesteryl ester contents. Thus, the inefficient recycling was not the result of saturation of ACAT, but appeared to be the result of a controlled delivery of free cholesterol to the ACAT substrate pool. The availability of the labeled free cholesterol for removal from the cells by efficient acceptors of free cholesterol (Table 2) suggests that this free cholesterol mixes effectively with the plasma membrane free cholesterol pool. In the absence of effective extracellular acceptors, free cholesterol accumulated in the cells.

During early development of atherosclerotic plaque, there is a significant increase in esterified cholesterol in the vessel wall with more modest increases in triglyceride and free cholesterol (12, 13). With progression, the proportion of triglyceride decreases, and there is an increase in free cholesterol, often seen as cholesterol monohydrate crystals (38). The data obtained in the present study are consistent with this pattern. The high capacity of smooth muscle cells to mobilize and clear triglyceride is consistent with the relatively low contribution of triglyceride in plaque. The continuing presence of cholesteryl ester is consistent with the apparently low capacity of smooth muscle cells to clear this ester, and the presence of free cholesterol in the lesion may be a result of an inefficient

cholesteryl ester cycle coupled with a spatial inaccessibility to efficient cholesterol acceptors.

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