Cholesteryl ester hydrolysis in J774 macrophages occurs in the cytoplasm and lysosomes

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Abstract The relationship of cholesteryl ester hydrolysis to the physical state of the cholesteryl ester in J774 murine macrophages was explored in cells induced to store cholesteryl esters either in anisotropic (ordered) inclusions or isotropic (liquid) inclusions. In contrast to other cell systems, the rate of cholesteryl ester hydrolysis was faster in cells containing anisotropic inclusions than in cells containing isotropic inclusions. Two contributing factors were identified. Kinetic analyses of the rates of hydrolysis are consistent with a substrate competition by co-deposited triglyceride in cells with isotropic inclusions. In addition, hydrolysis of cholesteryl esters in cells with anisotropic droplets is mediated by both cytoplasmic and lysosomal lipolytic enzymes, as shown by using the lysosomotropic agent, chloroquine, and an inhibitor of neutral cholesteryl ester hydrolase, umbelliferyl diethylphosphate. In cells containing anisotropic inclusions, hydrolysis was partially inhibited by incubation in media containing either chloroquine or umbelliferyl diethylphosphate. Together, chloroquine and umbelliferyl diethylphosphate completely inhibited hydrolysis. However, when cells containing isotropic inclusions were incubated with umbelliferyl diethylphosphate, cholesteryl ester hydrolysis was completely inhibited, but chloroquine had no effect. Transmission electron microscopy demonstrated a primarily lysosomal location for lipid droplets in cells with anisotropic droplets and both non-lysosomal and lysosomal populations of lipid droplets in cells with isotropic droplets. These results support the conclusion that there is a lysosomal component to the hydrolysis of stored cholesteryl esters in foam cells.—Avart, S. J., D. W. Bernard, W. G. Jerome, and J. M. Glick. **Cholesteryl ester hydrolysis in J774 macrophages occurs in the cytoplasm and lysosomes. *J. Lipid Res.* 1999.** 40: **405–414.**

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Foam cells laden with lipid, primarily cholesteryl ester, are a hallmark of atherosclerotic lesions. These foam cells are of monocyte-derived macrophage and smooth muscle cell origin (1–3). In the atherosclerotic lesion, excess cellular lipid accumulates in intracellular droplets that have been shown to be both cytoplasmic and lysosomal (4, 5). In addition, the physical state of the cholesteryl ester in these inclusions can be either anisotropic (liquid crystalline) or isotropic (liquid) (6, 7), a property that has been proposed to affect the ability of the foam cells to clear excess cholesteryl ester (6).

Brown and Goldstein (8) have extensively studied cellular cholesteryl ester metabolism in mouse peritoneal macrophages as a foam cell model. They found that these cells take up acetylated low density lipoproteins (acLDL) and hydrolyze the lipoprotein cholesteryl esters in lysosomes. The resulting free cholesterol is transported to the cytoplasm where it is then re-esterified by acyl CoA:cholesterol acyltransferase (ACAT) and stored in cytoplasmic droplets. These cholesteryl esters are in a dynamic state, undergoing continuous hydrolysis by neutral cholesteryl ester hydrolase, with re-esterification of the resulting free cholesterol by ACAT (9). This laboratory has previously demonstrated the presence of a similar cholesteryl ester cycle in Fu5AH rat hepatoma cells and J774 and P388 murine macrophage cells (10). The subcellular site of hydrolysis of ACAT-derived cholesteryl ester in both mouse peritoneal macrophages (9) and Fu5AH hepatoma cells (11) has been shown to be extra-lysosomal. Treatment of either type of cell with the lysosomotropic agent, chloroquine, had no effect on the hydrolysis of stored ester, indicating that these esters are not hydrolyzed by acid cholesteryl ester hydrolase. Another compound, umbelliferyl diethylphosphate, has been demonstrated to be a specific inhibitor of neutral cholesteryl ester hydrolase in cultured

Abbreviations: acLDL, acetylated low density lipoproteins; ACAT, acyl CoA:cholesterol acyltransferase; BSA, bovine serum albumin; CPT-cAMP, 8-(4-chlorophenylthio)-adenosine 3′:5′-cyclic monophosphate; FBS, fetal bovine serum; POPC, palmitoyl-oleoyl phosphatidylcholine; UDeP, umbelliferyl diethylphosphate.

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Fu5AH cells (11), in which hydrolysis of stored cholesteryl ester was completely inhibited by umbelliferyl diethylphosphate, confirming that endogenously synthesized, cytoplasmic cholesteryl ester is hydrolyzed by neutral cholesteryl ester hydrolase.

Earlier studies of cholesteryl ester hydrolysis in Fu5AH rat hepatoma cells containing ACAT-derived cholesteryl esters showed that the esters were hydrolyzed more rapidly in cells containing cholesteryl esters in a liquid, isotropic state than in cells whose cholesteryl ester stores were in a liquid–crystalline, anisotropic state (10, 12, 13). Similarly, when smooth muscle cells (14) and J774 cells (15) were loaded with sonicated lipid droplets of known composition and physical state, cholesteryl oleate in isotropic droplets was hydrolyzed more rapidly than cholesteryl oleate in anisotropic droplets. Therefore, we concluded that the rate of hydrolysis of cholesteryl esters is related to the physical state of the cholesteryl ester (12, 13, 16), perhaps due to the potentially greater accessibility of substrates contained in less-ordered, liquid droplets. Studies characterizing the lipid inclusions in J774 macrophage foam cells have shown that these inclusions have a higher cholesteryl palmitate:cholesteryl oleate mole ratio than inclusions isolated from Fu5AH cells (17). Additionally, differential scanning calorimetry suggested an increased potential for the formation of microcrystals of cholesteryl palmitate in the core of the inclusions isolated from J774 cells (18, 19). These data led us to hypothesize that the influence of a highly ordered physical state of lipid droplets would impede the hydrolysis of cholesteryl esters in J774, perhaps more profoundly than had been observed in the other cell systems. However, the kinetics of cholesteryl ester hydrolysis in J774 foam cells containing either anisotropic or isotropic inclusions reported herein are quite different from those obtained in the other systems. To attempt to account for this difference, we examined the effect of cell compartment-specific inhibitors of cholesteryl ester hydrolysis. The results of the studies presented here lead to the conclusion that there is a limited capacity to hydrolyze cholesteryl esters in J774 macrophage foam cells. Cholesteryl esters in cells loaded with anisotropic cholesteryl ester inclusions are hydrolyzed more rapidly than those in cells with isotropic inclusions for two reasons. In cells with anisotropic inclusions, a portion of the cholesteryl ester can be diverted from the cytoplasmic cholesteryl ester cycle to lysosomes where it is hydrolyzed by acid cholesteryl ester hydrolase, thus increasing the available lipolytic capacity. Moreover, in cells with isotropic inclusions, triglyceride presents a competing substrate that effectively decreases the rate of cholesteryl ester hydrolysis.

MATERIALS AND METHODS

Materials

Cholesterol, cholesteryl oleate, oleic acid, cholesteryl methyl ether, chloroquine, 8-(4-chlorophenylthio)-adenosine 3′:5′-cyclic monophosphate (CPT-cAMP), gentamicin, and heat-inactivated fetal bovine serum (FBS) were purchased from Sigma Chemical Co. 1,2-[^{3}H]cholesterol, which was purchased from DuPont-New England Nuclear, was repurified before use as previously reported (20). Palmitoyl-oleoylphosphatidylcholine (POPC) was purchased from Avanti Polar Lipids. Compound 58035 was a gift from Dr. John Heider of Sandoz. Umbelliferyl diethylphosphate (UDeP) was a gift from Dr. Daniel Quinn of the University of Iowa. Bovine serum albumin (BSA) (Reagent Pure Powder) was purchased from Intergen. Tissue culture plasticware and media, β-glycerophosphate, lead nitrate, ScintiVerse BD, and Gelman instant thin-layer chromatography polysilicic acid gel-impregnated glass-fiber sheets (ITLC-SA) were purchased from Fisher Scientific. Agarose gels (1%) for electrophoresis were purchased from Bio-Rad. Sodium cacodylate, uranyl acetate, EM bed-812, osmium tetroxide, glutaraldehyde, and other materials used for electron microscopy studies were purchased from Electron Microscopic Sciences. All other materials were either reagent grade or the purest commercial grade available and were purchased from Fisher Scientific.

Cell culture

Cell cultures were maintained at 37°C, in a humidified 95% air, 5% CO_2 atmosphere. All media were buffered with 24 mm bicarbonate and contained gentamicin (50 μg/ml). Stock cultures of the J774.1 (21) murine macrophage cell line were grown in T-75 flasks in RPMI-1640 supplemented with FBS (final concentration 10% (v/v)), and the medium was changed three times a week. For experiments, floating cells were isolated from the medium by low-speed centrifugation (1000 rpm for 10 min). The cells were resuspended in medium containing 10% (v/v) FBS and dispensed into 6-well 35-mm diameter plates at a density of 1×10^6 cells/ml, using 2 ml of medium/well.

Lipoproteins and lipid dispersions

Human low density lipoproteins (LDL) were isolated by density ultracentrifugation (d 1.006 and 1.067 g/ml) (22) from plasma obtained with informed consent from normal donors. Acetylated LDL (acLDL) was prepared according to the method of Fraenkel-Conrat (23) and acetylation was verified by the demonstration of increased electrophoretic mobility using 1% agarose gels. Cholesterol-rich phosphatidylcholine dispersions with a cholesterol:phospholipid molar ratio >2 were prepared using POPC, as previously described (12). The acLDL and cholesterol dispersions were sterilized before use by filtration using a 0.45 μm Millipore filter.

Esterified cholesterol storage and hydrolysis

To address factors regulating the hydrolysis of endogenously synthesized cholesteryl esters, J774 cells containing liquid–crystalline (anisotropic) esterified-cholesterol inclusions were prepared using the procedure of McCloskey, Rothblat, and Glick (20). Cells were incubated for 48 h with RPMI-1640 containing 225 μg dispersion cholesterol/ml, 50 μg acLDL protein/ml, 10 mg BSA/ml, and 0.5 μCi [^{3}H]cholesterol/ml. Cells containing liquid (isotropic) inclusions were prepared by incubating the cells in the above medium supplemented with oleic acid (200 μg/ml) added as the potassium salt. Over the course of the loading period, unlabeled lipoprotein cholesteryl esters are internalized and degraded in lysosomes. The free cholesterol derived from this source equilibrates with the radiolabeled free cholesterol provided in the loading medium and becomes a substrate for ACAT, leading to deposition of cholesteryl ester in the form of cytoplasmic droplets. After the loading period, cells were incubated overnight with RPMI-1640 containing 10 mg/ml BSA to allow the equilibration of the specific activities of the intracellular cholesterol pools. This equilibration period ensured that residual lipoprotein-derived cholesteryl ester was cleared from the ly-

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sosomes and that all of the cholesteryl ester stores were endogenously synthesized by ACAT. After this incubation, a set of plates (n = 3 or 4) was harvested to determine the initial lipid content and the specific radioactivity of cholesterol in the free and esterified pools as described below. The remaining monolayers were washed three times and further incubated in RPMI-1640 containing 1 mg/ml BSA and 1 μg/ml Sandoz 58035 which inhibits ACAT activity (24). This treatment allowed the assessment of net cholesteryl ester hydrolysis because re-esterification by ACAT of free cholesterol generated by the hydrolysis of cholesteryl esters was prevented. Although cellular free-cholesterol increased, no visual evidence of toxicity was observed nor were there any differences in monolayer protein contents between control and 58035-treated groups. Chloroquine was added to medium dissolved in RPMI-1640. 58305, UDeP and CPT-cAMP were added to medium dissolved in dimethylsulfoxide (DMSO); the final concentration of DMSO in the medium was <0.5%.

Analytical procedures

Two different methods were used to analyze the cultures, an isopropanol extraction of the lipids from the washed cell monolayer, as modified from McCloskey et al. (20), and a sodium dodecyl sulfate solubilization of the cell monolayer into the incubation medium as previously described by Bernard et al. (25). Lipids were extracted from the detergent-solubilized material by the method of Bligh and Dyer (26). The distribution of radiolabel between free and esterified cholesterol was determined by thin-layer chromatography of the lipid extracts using ITLC-SA plates developed in a solvent system of petroleum ether–diethyl ether–glacial acetic acid 85:15:1 (v/v/v). The bands on the plates were cut out after visualization with iodine vapor and comparison with lipid standards. Radioactivity in the bands was quantitated using ScintiVerse BD cocktail in a Beckman LS5000TD liquid scintillation counter. Aliquots of the isopropanol extracts from the cell monolayers were used to determine the masses of free and total cholesterol using gas–liquid chromatography with cholesteryl methyl ether as an internal standard (27); the mass of esterified cholesterol was calculated by difference. The data reported for initial masses of free and esterified cholesterol were determined by gas–liquid chromatography. The isopropanol extracts were also subjected to thin-layer chromatography as described above to determine the radioactivity in the free and esterified cholesterol pools. These data were used to calculate the specific activity of the cholesterol in each of the pools. Fractional hydrolysis of esterified cholesterol (the percent of the initial esterified cholesterol hydrolyzed) was calculated using the changes in the distribution of radiolabeled cholesterol in the two pools, and the decreases in esterified cholesterol mass were calculated from the fractional hydrolysis data using the measured specific activity. The mass of triglyceride in the isopropanol extracts was measured using an assay kit from Boehringer-Mannheim. A commercial software package (Prism v2, GraphPad Software) was used for statistical analysis of the data. Phospholipid phosphorous content of the lipid dispersions was determined by the method of Sokoloff and Rothblat (28). After extraction of the lipids from the cell monolayer, protein content of the monolayer was determined by the method of Markwell et al. (29) using BSA as a standard.

Microscopy

The physical state of lipid inclusions in whole cells was assessed using polarizing light microscopy as previously described (30). The ultrastructural localization of stored neutral lipid in J774 foam cells was determined by electron microscopy. To accomplish this, cholesteryl ester-loaded cells were incubated overnight in medium containing 10 mg/ml BSA. The cell monolayers were then washed three times in phosphate-buffered saline and the cells were fixed for 10 min in 4% glutaraldehyde in 0.1 m cacodylate buffer containing 0.1 m sucrose at room temperature, pH 7.2. After fixation and a 30-min wash in cacodylate/sucrose buffer, the cells were rinsed with 0.5 m Tris-maleate buffer, pH 5.0, followed by a 5-min wash in the same buffer. The cells were then stained for acid phosphatase using a modification (5) of the Gomori lead precipitation reaction (31). The reaction mixture contained 0.25% β-glycerophosphate as the substrate and 0.08% lead nitrate as the trapping agent. Cells incubated in the same medium without β-glycerophosphate served as the control. After a 1-h incubation in either the reaction or control medium, the monolayers were washed in Tris-maleate buffer. The cells were then postfixed in 1% osmium tetroxide in cacodylate/sucrose buffer for 30 min. After postfixation, the cells were washed in cacodylate/sucrose buffer, scraped from the dish, and collected by centrifugation. The cell pellets were stained in 25% ethanol saturated with uranyl acetate for 10 min, followed by dehydration in ethanol. Cell pellets were then embedded in epoxy resin (EM bed-812), and thin sections (80 nm) of the embedded material were viewed using a Philips EM400 operating at 80 keV. Quantitation was carried out using standard point count stereology (32) as previously used by us to quantify lysosomal lipid volumes (33). Fifteen fields from each of three separate experiments were analyzed for each condition, and the mean volume density of lysosome and cytoplasmic inclusion lipid per cell was computed.

RESULTS

We examined the effect of the physical state of cellular lipid inclusions on the rate of hydrolysis of endogenously synthesized cholesteryl esters by preparing J774 cells with either anisotropic (liquid–crystalline) or isotropic (liquid) lipid inclusions. The different physical states were produced by incubating cells in medium containing a mixture of acLDL and cholesterol-rich phospholipid dispersions containing radiolabeled free cholesterol, as described in Materials and Methods, without or with oleic acid. In the absence of oleic acid, cellular lipid inclusions were anisotropic, displaying a cross formée when viewed under polarizing light microscopy, while inclusions in cells loaded in the presence of oleic acid were isotropic. The mass of lipid stored in a typical experiment is shown in **Table 1**. In the presence of oleic acid the mass of total cholesterol stored is slightly higher, the percent of cellular cholesterol that is esterified is increased as was previously demonstrated (34), and there is an 8-fold increase in the mass of triglyceride stored. To examine the rates of hydrolysis under the two conditions, the loaded cells were incubated with lipid-free medium containing the ACAT inhibitor, Sandoz 58035, which prevents re-esterification of cholesterol liberated by hydrolysis of stored cholesteryl ester (24). Hydrolysis of esterified cholesterol was assessed by measuring the difference in cellular esterified cholesterol content between the beginning and end of the period during which the cells were incubated in the presence of 58035. Because our intent was to compare rates of hydrolysis of cholesteryl ester in cells with the two types of inclusions, it was important to establish that the rate of hydrolysis was linear over the time period examined. The data in

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TABLE 1. Lipid mass in J774 foam cells

	Total Cholesterol	Free Cholesterol	Esterified Cholesterol	Triglyceride
	μg/mg cell protein			
Anisotropic	132.0 ± 1.1	51.1 ± 3.3	80.9 ± 3.1	12.8 ± 1.1
Isotropic	147.2 ± 6.6	36.1 ± 0.9	110.8 ± 6.6	101.1 ± 7.0

J774 cells containing anisotropic or isotropic lipid inclusions were prepared as described in Materials and Methods. After an overnight incubation in RPMI-1640 containing 10 mg/ml BSA, lipids were extracted from the cell monolayers with isopropanol. Lipid and protein analyses were performed as described in Materials and Methods. Data are the mean ± standard deviation (n = 3).

Fig. 1 show that the decrease in the percent of cellular cholesteryl ester is linear over 24 h in cells with both types of cellular lipid inclusions, and 12 hours was chosen as the time point used in subsequent experiments to measure the rates of hydrolysis.

Preliminary experiments were conducted wherein the level of cholesteryl ester loading was varied by varying the concentration of the cholesterol-loading mixture and rates of hydrolysis were examined. In light of previous results in other cells systems (10, 12–15), the data (not shown) were anomalous in that rates of hydrolysis of cholesteryl esters in anisotropic inclusions were more rapid than those in isotropic inclusions at cellular cholesteryl ester contents <100 μg/mg cell protein and were similar at higher cellular contents. Because data points from single experiments were not sufficient to use for kinetic analyses, data from a number of experiments were pooled and are shown in **Fig. 2**. As was observed in the preliminary experiments, the rates of hydrolysis of anisotropic cholesteryl esters appear, with few exceptions, to be more rapid than that of isotropic cholesteryl esters for any initial cholesteryl ester load. Similarly, the plots of fractional hydrolysis for the data sets (Fig. 2, panel B) lead to a similar conclusion. While the extrapolation of the linear regression lines describing the fractional hydrolysis intersects at high cholesteryl ester loads, there are insufficient data at these very high contents of cellular cholesteryl ester to determine whether hydrolysis in cells with isotropic inclusions does indeed become more rapid, or whether the rates of hydrolysis in the two systems approach each other as a limit. Because the rates measured were initial rates of hydrolysis, the data were analyzed using a non-linear regres-

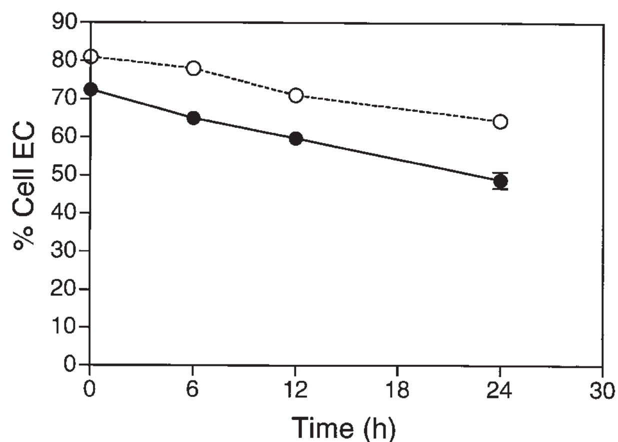

Fig. 1. Hydrolysis of esterified cholesterol in J774 foam cells. Cells containing lipid inclusions were prepared by incubation for 48 h with medium containing 225 μg dispersion cholesterol/ml, 50 μg acLDL protein/ml, 10 mg BSA/ml, and 0.5 μCi [^{3}H]cholesterol/ml, without (for anisotropic, ●) and with (for isotropic, ○) 200 μg/ml oleic acid. After an additional overnight incubation in medium containing 10 mg/ml BSA, the cells were incubated in RPMI-1640 containing 1 mg/ml BSA plus 1 μg/ml 58-035. After 6, 12, and 24 h, cultures were harvested, lipids were extracted, and the percent esterified cholesterol was determined as described in Materials and Methods. These are data from the experiment described in Table 1, and each point represents the mean ± standard deviation, n = 3. Where error bars are not visible, they are within the area of the symbol.

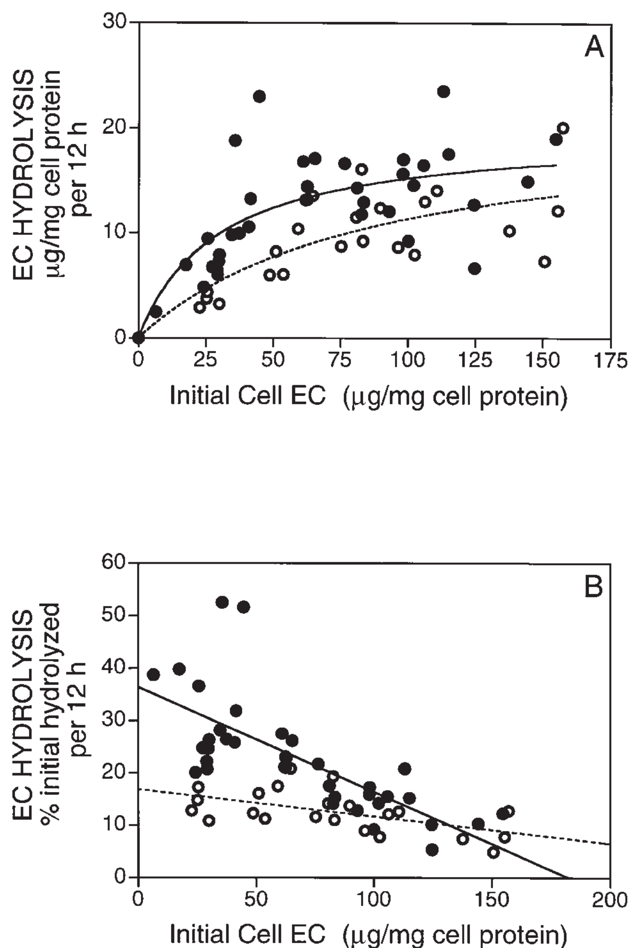

Fig. 2. The relationship between the hydrolysis of esterified cholesterol and the initial mass of esterified cholesterol in J774 cells. Panel A shows data for the rate of esterified cholesterol hydrolysis, and panel B shows the fractional hydrolysis. J774 cells containing either anisotropic esterified cholesterol (●) or isotropic esterified cholesterol (○) were prepared, and experiments were carried out as for the experiment described in Fig. 1, using a 12-h incubation with 58035. The data were pooled from numerous experiments, and each point is the mean of ⩾3 samples. The curves in panel A are computer-generated nonlinear regression lines fit to the Henri-Michaelis-Menten equation, and the lines in panel B are computer-generated linear regression lines.

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sion fitted to the Henri-Michaelis-Menten equation describing a first-order enzymatic reaction. The computer-fitted curves suggest that the hydrolytic process saturates regardless of the physical state of the lipid inclusions, and analyses of the two sets of data indicate very similar V_{max} values of 19.7 and 21.2 μg esterified cholesterol hydrolyzed per 12 h, but different apparent K_ms of 29.7 and 87.1 μg esterified cholesterol per mg cell protein for anisotropic and isotropic cholesteryl esters, respectively. Although there is considerable scatter in both sets of data, that for cells with anisotropic inclusions shows a poorer fit, both by the nonlinear regression analysis and by linear regression analysis of data transformed using a Lineweaver-Burk plot (not shown). The poorer fit is consistent with the hypothesis that more than one hydrolytic pathway may be operating on these lipid inclusions.

Hydrolytic pathways known to be capable of hydrolyzing cholesteryl esters can be divided operationally by subcellular compartment, i.e., lysosomal versus cytoplasmic. Lysosomal hydrolysis is usually associated with the hydrolysis by lysosomal acid lipase of exogenous cholesteryl esters delivered by receptor-mediated endocytosis of lipoproteins or phagocytosis of lipid droplets (8, 35). Cytoplasmic hydrolysis is associated with the hydrolysis, by one or possibly more neutral cholesteryl ester hydrolases, of endogenous cholesteryl esters, i.e., those synthesized within the cell by ACAT. In the case of mouse macrophages, the neutral cholesteryl ester hydrolase is thought to be hormone-sensitive lipase (36). To test the hypothesis that more than one hydrolytic system was operating in cells with anisotropic inclusions, the relative contributions of lysosomal and neutral hydrolases were examined using inhibitors that are selective for each of these processes. As we have previously demonstrated that umbelliferyl diethylphosphate (UDeP) selectively inhibits neutral cholesteryl ester hydrolase activity in intact Fu5AH cells (11), we examined the effect of this compound in J774 cells. Cells containing esterified cholesterol in either anisotropic or isotropic inclusions were incubated for 12 h in media containing 58035 in the absence or presence of increasing concentrations of UDeP. **Figure 3** shows the data pooled from three independent experiments. The hydrolysis of isotropic esterified cholesterol was completely inhibited by UDeP at concentrations of 10 μm or greater. However, even at a concentration of 100 μm, UDeP did not completely inhibit the hydrolysis of anisotropic esterified cholesterol, and incubation of the cells with UDeP at concentrations greater than 100 μm resulted in cellular toxicity as determined by microscopic examination. Data pooled from these and additional experiments comparing the extent of inhibition of cholesteryl ester hydrolysis in the presence of 100 μm UDeP in cells with either anisotropic or isotropic inclusions are shown in **Table 2**. Virtually complete inhibition (97%) of hydrolysis was observed in cells with isotropic inclusions; however, only partial inhibition (approximately 60%) was achieved in cells with anisotropic inclusions.

Because treatment of cells containing anisotropic esterified cholesterol with UDeP did not completely inhibit the hydrolysis of the endogenously synthesized cholesteryl esters, experiments were performed using the lysosomotropic agent chloroquine to determine whether there is a lysosomal contribution to the hydrolysis of these esters. Cells containing esterified cholesterol in either the anisotropic or isotropic physical state were incubated for 12 h with medium containing 58035 in the absence or presence of chloroquine (50 μm), and pooled data from numerous individual experiments are shown in Table 2. As

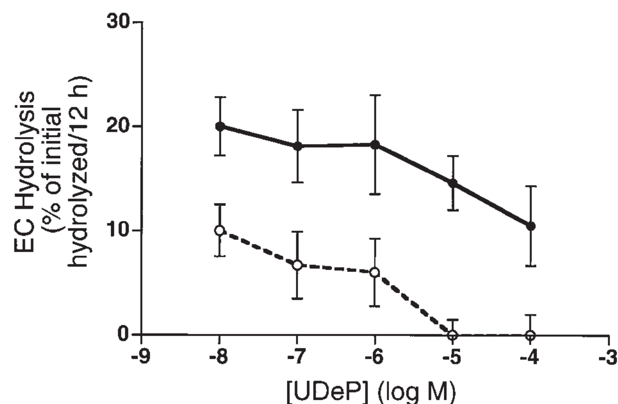

Fig. 3. Inhibition of esterified cholesterol hydrolysis in J774 cells by umbelliferyl diethylphosphate (UDeP). Cells containing esterified cholesterol in anisotropic inclusions (●) or isotropic inclusions (○) were prepared as described in the legend to Fig. 1. After an overnight incubation in medium containing 10 mg/ml BSA, the cells were incubated for 12 h in RPMI-1640 containing 1 mg/ml BSA plus 1 μg/ml 58-035 or the same medium containing the indicated concentrations of UDeP. Cells were harvested, and analyses and calculations were performed as described in Materials and Methods. Data are the mean ± standard deviation of nine samples pooled from three different experiments. Lipid loading in the experiments was as follows (all sets of values are μg free and esterified cholesterol per mg cell protein, respectively): Anisotropic: (1) 134 ± 5 and 46 ± 3; (2) 57 ± 6 and 46 ± 5; and (3) 147 ± 9 and 159 ± 14. Isotropic: (1) 74 ± 3 and 99 ± 4; (2) 89 ± 4 and 181 ± 7; and (3) 43 ± 3 and 78 ± 8.

TABLE 2. Effect of umbelliferyl diethylphosphate (UDeP) or chloroquine on esterified cholesterol hydrolysis in J774 cells containing either anisotropic or isotropic esterified cholesterol

	% of Hydrolysis Observed with 58-035 Alone	
Physical State	58-035 + UDeP	58-035 + Chloroquine
Isotropic	3.0 ± 9.2 (n = 16)[a]	99.6 ± 29.1 (n = 39)
Anisotropic	38.3 ± 26.5 (n = 30)[a]	69.6 ± 25.4 (n = 78)[a]

J774 cells containing anisotropic or isotropic esterified cholesterol were prepared as described in Materials and Methods. After an overnight incubation in RPMI-1640 containing 10 mg/ml BSA, the cells were incubated for 12 h in medium containing 1 mg/ml BSA and 1 μg/ml 58-035 or the same medium containing either 100 μm UDeP or 50 μm chloroquine. Analyses and calculations were performed as described in Materials and Methods. Data are the mean ± standard deviation pooled from 8 experiments (anisotropic) and 4 experiments (isotropic) for the UDeP treatment and 20 experiments (anisotropic) and 12 experiments (isotropic) for the chloroquine treatment; n, the total number of samples.

[a] Significantly different ($P < 0.001$) from 58-035 alone.

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expected from the results of UDeP experiments, chloroquine had no inhibitory effect on the hydrolysis of esterified cholesterol in cells containing isotropic inclusions. In contrast, in cells containing anisotropic esterified cholesterol, chloroquine inhibited hydrolysis by approximately 30%. Interestingly, the inhibitory effect of chloroquine in cells loaded with anisotropic inclusions varied widely among individual experiments. Statistically significant inhibition of the hydrolysis of anisotropic esterified cholesterol by chloroquine could be demonstrated in only 10 out of 20 individual experiments. In these experiments the mean inhibition was 43% and the range was 19–90%. It is important to note that the variability was inter-experimental and not intra-experimental. The etiology of this variability in response to chloroquine has not yet been identified. A number of possible explanations have been explored, including *1*) initial esterified cholesterol mass, *2*) differences among acLDL and dispersion preparations, *3*) the time of the post-incubation with lipid-free medium (up to 24 h), *4*) different sources or concentrations of chloroquine, *5*) a different lysosomal inhibitor (methylamine), *6*) variations in experimental protocol including time of equilibration or initial plating density of the cells, but none of these was found to eliminate the variability (data not shown). In summary, our data show that lysosomal hydrolysis of endogenously synthesized esterified cholesterol can occur in J774 macrophage foam cells containing anisotropic inclusions. Because both UDeP and chloroquine had each been shown to be capable of partially inhibiting cholesteryl ester hydrolysis, we examined the effect of co-incubation of cells containing either isotropic or anisotropic inclusions for 12 h with medium containing 58035 alone, or 58035 with the two agents, separately or in combination. As shown in **Fig. 4**, in cells containing anisotropic inclusions, partial inhibition of hydrolysis was observed with either UDeP or chloroquine; however, incubation of cells with both UDeP and chloroquine resulted in complete inhibition of the hydrolysis of esterified cholesterol. In cells containing isotropic inclusions, chloroquine had no effect and UDeP completely inhibited hydrolysis, consistent with data shown above.

We have previously shown that cAMP analogs stimulate esterified cholesterol hydrolysis in intact J774 cells, an observation consistent with the evidence that hormone-sensitive lipase plays a role in this process (36). Hajjar (37) using in vitro assays concluded that lysosomal cholesteryl ester hydrolase is stimulated by cAMP in smooth muscle cells. We performed experiments to determine whether cAMP can affect both hydrolytic systems in J774 cells. J774 cells containing esterified cholesterol in either anisotropic or isotropic inclusions were incubated for 12 h in media containing 58035 alone or 58035 plus CPT-cAMP, UDeP, or chloroquine and various combinations of these compounds as described in **Table 3**. As shown, CPT-cAMP stimulated esterified cholesterol hydrolysis and UDeP and chloroquine each partially inhibited hydrolysis in J774 cells containing anisotropic inclusions, in agreement with previous data (see above and ref. 38). When chloroquine and CPT-cAMP were used together, chloroquine inhibition was masked by the stimulation of residual (presumably cytoplasmic) hydrolysis by CPT-cAMP. In contrast, when cytoplasmic hydrolysis was blocked with UDeP, no stimulatory effect of CPT-cAMP on the residual lysosomal component was observed. These data demonstrate that CPT-cAMP stimulates esterified cholesterol hydrolysis in

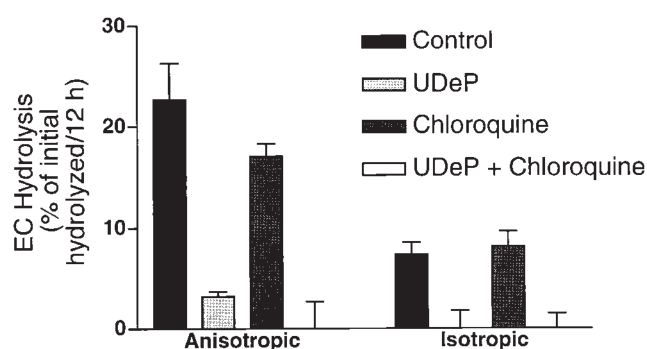

Fig. 4. The effect of umbelliferyl diethylphosphate (UDeP) and chloroquine on esterified cholesterol hydrolysis in J774 cells. Cells containing esterified cholesterol in either anisotropic or isotropic inclusions were prepared as described in Materials and Methods. After an overnight incubation in medium containing 10 mg/ml BSA, the cells were incubated for 12 h in RPMI-1640 containing 1 mg/ml BSA plus 1 μg/ml 58-035 or the same medium supplemented with 100 μm UDeP, 50 μm chloroquine, or both. Cells were harvested, and analyses and calculations were performed as described in Materials and Methods. Data are the mean ± standard deviation of quadruplicate samples. Lipid loading for the cells with anisotropic inclusions was 90 ± 5 and 61 ± 4 μg free and esterified cholesterol per mg cell protein, respectively. For cells with isotropic inclusions, the values were 50 ± 3 and 82 ± 2 μg free and esterified cholesterol per mg cell protein, respectively.

TABLE 3. Esterified cholesterol hydrolysis in J774 cells containing either anisotropic or isotropic esterified cholesterol in the presence of agents affecting cholesteryl ester hydrolysis

Treatment	Physical State: Anisotropic	Physical State: Isotropic
	% initial esterified cholesterol hydrolyzed	
Control (58-035 alone)	27.9 ± 0.3	19.4 ± 1.2
Chloroquine	22.8 ± 0.6[a]	23.3 ± 3.2
UDeP	6.2 ± 1.5[a]	7.2 ± 2.2[a]
CPT-cAMP	39.0 ± 1.7[a, c]	49.8 ± 2.6[a]
Chloroquine + UDeP	2.2 ± 1.0[a]	7.0 ± 3.4[a]
CPT-cAMP + chloroquine	36.9 ± 5.6[b, c]	N.D.
CPT-cAMP + UDeP	8.5 ± 2.4[a]	6.7 ± 2.0[a]

J774 cells containing anisotropic or isotropic esterified cholesterol were prepared as described in Materials and Methods. After an incubation with lipid-free medium, the cells were incubated for 12 h in medium containing 1 mg/ml BSA and 1 μg/ml 58-035 or the same medium also containing CPT-cAMP (100 μm), UDeP (100 μm), chloroquine (50 μm), or various combinations as shown. The initial masses of free and esterified cholesterol in cells with anisotropic inclusions were 83 ± 6 and 77 ± 9, respectively, and those for cells with isotropic inclusions were 46 ± 2 and 82 ± 3, respectively. Analyses and calculations were performed as described under Materials and Methods. Data are mean ± standard deviation, n = 4; N.D., not determined.

[a] Significantly different from control at $P < 0.001$.
[b] Significantly different from control at $P < 0.05$.
[c] Not significantly different from each other.

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J774 cells containing anisotropic inclusions solely by increasing the hydrolysis catalyzed by neutral cholesteryl ester hydrolase and has no effect on lysosomal hydrolysis. The data obtained for J774 cells containing isotropic inclusions were as expected from our previous experiments. CPT-cAMP stimulated esterified cholesterol hydrolysis, UDeP inhibited hydrolysis to the same degree whether or not CPT-cAMP was present, and chloroquine had no effect.

In light of the evidence indicating that a portion of endogenously synthesized cholesteryl ester can be hydrolyzed in lysosomes, we examined the subcellular location of lipid in cholesteryl ester-laden J774 cells using electron microscopy. After an overnight incubation with lipid-free medium (equivalent to the beginning of the hydrolysis period for the other experiments), cells with either anisotropic or isotropic esterified cholesterol were fixed with glutaraldehyde, stained for acid phosphatase activity to identify lysosomes using cytochemical methods, and postfixed with osmium tetroxide and analyzed by electron microscopy as described in Materials and Methods. As shown in **Fig. 5**, there was extensive lipid accumulation in cells containing either anisotropic or isotropic esterified cholesterol. In cells with anisotropic inclusions (panel A), this lipid appeared to be primarily in large, pleiomorphic lysosomes as identified by a positive acid phosphatase reaction. Quantitative electron microscopy confirmed that 84 ± 3% (range 78–92% for three experiments) of the lipid accumulation was lysosomal. In cells containing isotropic esterified cholesterol (panel B), lipid droplets are present primarily as cytoplasmic lipid inclusions, but surprisingly, in light of the data using the selective inhibitors of neutral or acid hydrolysis, an appreciable amount of cellular lipid appeared to be present in lysosomes. The portion of cellular lipid in lysosomes ranged from 12 to 40% (mean = 21 ± 8%), and individual lysosomes were smaller and less complicated in appearance than those in cells with anisotropic inclusions.

DISCUSSION

Studies on model macrophage foam cells in culture have demonstrated that cholesteryl esters are stored in the cytoplasm and are in a dynamic state, undergoing continuous hydrolysis and resynthesis in what is known as the cholesteryl ester cycle (9). A substrate cycle of this type is critical to facilitating rapid mobilization of stored cholesteryl esters (16). In the case of foam cells, hydrolysis may be viewed as the first step in reversing the foam cell phenotype as unesterified cholesterol is the only form of cholesterol that can leave the cell when an acceptor of cholesterol is available (39). Unlike the model foam cells studied to date, cellular lipid in atherosclerotic lesions appears to be both cytoplasmic and lysosomal, with lysosomal deposition being more prominent in older, more mature areas of the lesion (40). Although relatively little is known about the cholesteryl ester cycle in foam cells in atherosclerotic lesions, a factor that could potentially lead to the perpetuation of foam cells is a limitation or failure of the hydrolytic arm of the cholesteryl ester cycle. Pitas, Innerarity, and Mahley (41) have demonstrated that foam cells isolated from atherosclerotic lesions fail to clear cho-

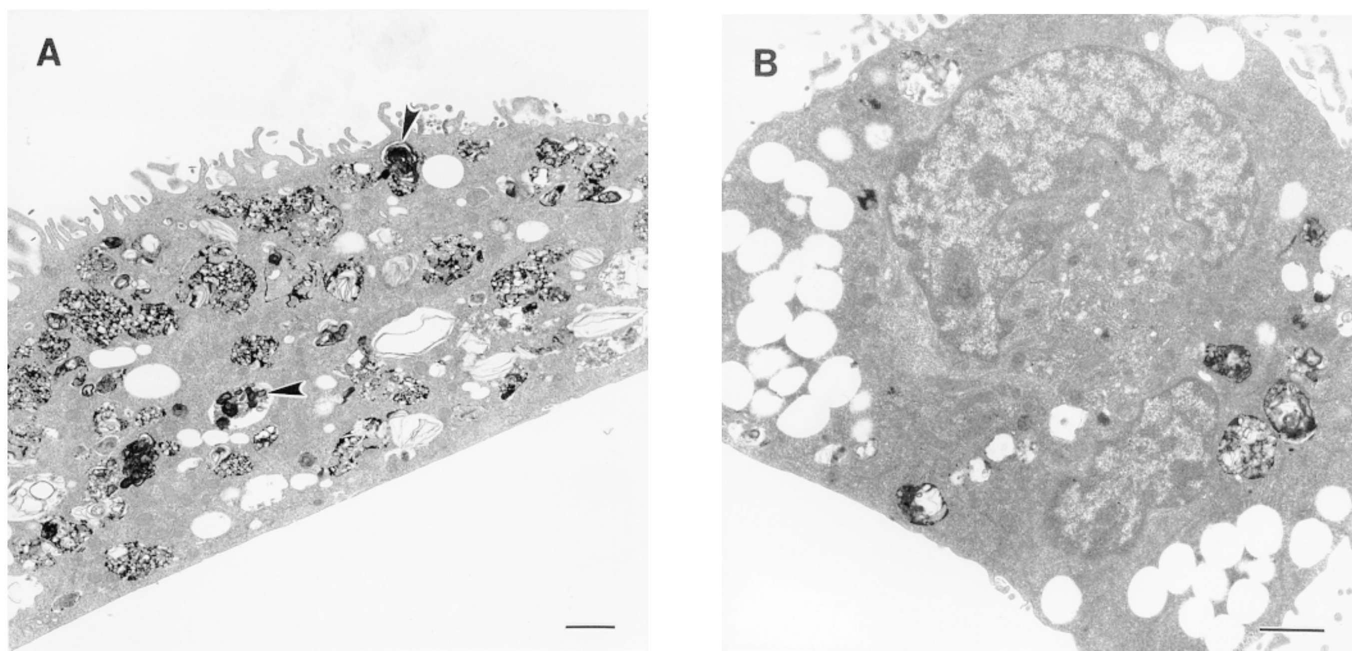

Fig. 5. Electron micrographs of J774 cells containing either anisotropic esterified cholesterol (panel A) or isotropic esterified cholesterol (panel B). Cells were prepared exactly as for the other experiments, except that radiolabeled cholesterol was not included. Thus, cells were loaded for 48 h with esterified cholesterol, post-incubated in medium with BSA, washed, fixed, and stained for acid phosphatase a ctivity as described in Materials and Methods. There was no post-incubation with 58-035. Lipid-filled lysosomes (arrowheads) are identified by positive acid phosphatase staining. A: 6,700× magnification, bar = 1 μm; B: 8,600× magnification, bar = 1 μm.

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lesteryl esters in the presence of HDL, although the reason for this was not explored. The data presented here demonstrate that J774 macrophage foam cells have a limited capacity for hydrolysis of intracellular cholesteryl ester and divert a portion of their lipid stores to lysosomes. Small and Shipley (6) have suggested that regression of atherosclerotic lesions may be influenced by the physical state of lipids in foam cells in that removal of cholesteryl ester stored in isotropic (liquid) inclusions should be more rapid than that stored in more ordered, anisotropic (liquid crystalline) inclusions. Using the ACAT inhibitor, Sandoz 58035, to prevent re-esterification of free cholesterol, we have examined rates of hydrolysis of cholesteryl esters and have demonstrated the validity of this hypothesis in several cell systems, including Fu5AH rat hepatoma cells containing endogenously synthesized cholesteryl esters, as well as smooth muscle cells and macrophages containing lipid droplets derived from phagocytic uptake of exogenously supplied lipid droplets (12–14). In each of these systems, cholesteryl esters were mobilized more rapidly in the cells with isotropic lipid droplets. Also, in each of these systems, the fractional hydrolysis was constant, and there was no evidence of saturation of hydrolysis regardless of cellular cholesteryl ester content. Thus, the enzymatic capacity to hydrolyze cholesteryl esters always exceeded the amount of substrate available to it. In contrast, the data reported herein, for J774 macrophages containing endogenously synthesized (i.e., ACAT-derived) cholesteryl ester droplets, show quite a different outcome. Regardless of cholesteryl ester load, the rate of hydrolysis of cholesteryl esters in cells with anisotropic (ordered) lipid droplets is faster than that in cells with isotropic droplets, although the calculated V_{max} values for each are quite similar. Fractional hydrolysis decreases with increasing cholesteryl ester load, and this is more obvious for cells with anisotropic cholesteryl ester droplets. Thus, for cells with either type of lipid droplet, there is a clear indication of saturation of the hydrolytic system suggesting that there is limiting cholesteryl esterase activity. A factor contributing to this may be a down-regulation of hormone-sensitive lipase activity in response to cholesterol as recently reported by two groups (42, 43). However, even in cell preparations with very high cholesteryl ester loads, we have never observed a complete loss of hydrolytic activity.

An explanation for the apparently slower rate of hydrolysis in cells with isotropic lipid inclusions may be the presence of a competing substrate, triglyceride, in the lipid droplets. Our previous work (13, 14) on other cell systems had not given any indication of such a competition, however the enzymes mediating hydrolysis in those studies were different. For cells loaded with lipid droplets by phagocytosis, hydrolysis was mediated by lysosomal acid lipase (35). The exact nature of the enzyme mediating hydrolysis of cytoplasmic lipids in liver-derived cells such as the Fu5AH hepatoma is not known but is probably different from that in macrophages (16, 44). Moreover, in each of these cases, hydrolytic activity never appeared to be saturated (12, 14). Hormone-sensitive lipase, the enzyme thought to hydrolyze cytoplasmic cholesteryl esters in mouse macrophages (36, 44), is able to hydrolyze both triglycerides and cholesteryl esters with a fairly similar activity (45). If cholesteryl ester hydrolysis were mediated solely by one enzymatic activity and the decreased rate of hydrolysis of cholesteryl esters were due strictly to competition by triglyceride, analysis of data from cells with isotropic and anisotropic lipid inclusions should show similar apparent V_{max} but the apparent K_m for cholesteryl ester should be higher in the former case. Such a difference in apparent K_ms is observed (87.1 μg versus 29.7 μg esterified cholesterol per mg cell protein for cells with isotropic and anisotropic lipid inclusions, respectively), while the V_{max} values are very similar.

Another hypothesis, suggested by the higher degree of variability in the data set describing rates of hydrolysis of anisotropic cholesteryl esters, is that more than one enzyme system contributes to hydrolysis in these cell preparations. Previous work in this laboratory had demonstrated that a novel compound, UDeP, was capable of inhibiting neutral cholesteryl esterase activity in intact Fu5AH hepatoma cells and in cell homogenates (11). Exposing intact J774 macrophage foam cells containing either isotropic or anisotropic cholesteryl esters to increasing amounts of UDeP revealed that cholesteryl ester hydrolysis could be completely inhibited in cells with isotropic droplets but not in cells with anisotropic droplets. These data affirmed our hypothesis that more than one hydrolytic system was operating in these cells but did not speak to the nature or cellular location of the additional activities(11). As chloroquine effectively inhibits lysosomal hydrolysis in intact cells, we examined the sensitivity to chloroquine of cholesteryl ester hydrolysis in cells with each type of lipid droplet. Significant chloroquine sensitivity was not observed in cells with isotropic droplets, suggesting that lysosomal hydrolysis does not play a quantitatively important role in these cells. However, the results of studies using cells with anisotropic lipid inclusions were different; in 10 of 20 individual experiments addressing this issue, inhibition of the hydrolysis of cholesteryl esters by chloroquine was statistically significant. In experiments where both chloroquine and UDeP were used and chloroquine sensitivity could be demonstrated, a combination of the two inhibitors completely inhibited hydrolysis. When a cAMP analog was added, only the cytoplasmic activity was stimulated, consistent with the concept that cytoplasmic cholesteryl ester hydrolysis in macrophages is mediated, at least in part, by hormone-sensitive lipase and demonstrating a lack of sensitivity of macrophage lysosomal lipase to cAMP.

The presence of lipid in the lysosomes of cholesteryl ester-laden J774 cells was also demonstrated using electron microscopy, and the fraction of cellular lipid observed in lysosomes of cells loaded with either anisotropic or isotropic lipid droplets was considerably higher than might have been predicted from the data using lipase inhibitors. Our failure to detect chloroquine-sensitive cholesteryl ester hydrolysis in cells with isotropic inclusions may be due to a lack of sensitivity in our measurements. In cells with isotropic inclusions, lysosomal lipid, comprised

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of both cholesteryl ester and triglyceride, accounted for approximately 20% of cell lipid, and complete inhibition of a lysosomal component of that size would be within the standard deviation of the measured overall fractional hydrolysis. The variability of the sensitivity to chloroquine in cell preparations with anisotropic inclusions was obviously unsatisfying. As described above, examination of a number of potential factors failed to account for the variability. However, variations in the proportion of lysosomal lipid and the limitations of the measurements may have been contributing factors.

These data raise the question of why cytoplasmically synthesized lipid droplets should find their way to lysosomes at all. One possible explanation may relate to a combination of compositional and physical state phenomena in cells with anisotropic lipid droplets. To prepare cells with anisotropic lipid inclusions, cells were exposed to a mixture of acLDL and free cholesterol-rich dispersions containing POPC, and the resulting population of cholesteryl esters is composed of approximately 30 to 40% cholesteryl palmitate and similar proportions of cholesteryl oleate (18). As the method of producing cells with isotropic droplets rests on adding oleic acid to the same loading medium, it is not surprising that approximately 80% of the cholesteryl esters are present as cholesteryl oleate (unpublished observations). One possible explanation for lysosomal targeting is that hormone-sensitive lipase is unable to hydrolyze cholesteryl palmitate, requiring that residual cholesteryl esters be hydrolyzed lysosomally. Preliminary experiments comparing cholesteryl ester distribution as a function of time of hydrolysis do not show a selective retention of cholesteryl palmitate, but confirmation of this will require more rigorous analysis than has presently been done. A different, but related possibility is the potential for formation of microcrystalline domains of cholesteryl palmitate within the liquid crystalline mixture of the two cholesteryl esters, as has been demonstrated for model mixtures of cholesteryl palmitate and cholesteryl oleate (17). While calorimetric examination of cellular lipid inclusions or emulsified particles containing the two cholesteryl esters revealed less crystallization than was observed in bulk mixtures, the formation of such crystals in cells cannot be excluded. If microcrystals form in cellular droplets, then the high degree of order of the microcrystalline domains might preclude hydrolysis and target the droplets to lysosomes where the cholesteryl esters become a substrate for lysosomal lipase. Even in lysosomes, the highly ordered physical state of the lipid may render the cholesteryl ester a relatively poor substrate.

The inability of macrophage hormone-sensitive lipase to efficiently hydrolyze stored cholesteryl esters may influence the progression of atherosclerotic lesions in a number of ways. One factor may simply relate to low abundance of the enzyme. Although hormone-sensitive lipase activity is easily detectable in mouse macrophages (42, 46–48), the activity is much lower than that seen in adipose or steroidogenic cells (49). Similarly, the abundance of hormone-sensitive lipase mRNA in mouse macrophages is low (50). Although there is one report that concludes that hormone-sensitive lipase is not present in human macrophages (51), a more recent study convincingly confirms that the mRNA is indeed present, although at low abundance (52). There is no evidence that hormone-sensitive lipase can be up-regulated in macrophages in response to lipid loading, as is seen during adipocyte differentiation (53), and there have been two recent reports describing a decrease in hormone-sensitive lipase in response to cholesterol in macrophages (43, 54). The data presented here clearly demonstrate a limited capacity for hydrolysis of cholesteryl esters in macrophages. Another factor that this study identifies is a competition of cholesteryl ester hydrolysis by co-deposited cellular triglyceride, which may be a contributing factor in foam cell formation and maintenance in individuals eating a high-fat diet. In addition, the observation that a portion of cellular cholesteryl esters can be shunted to lysosomes and lead to lysosomal lipid storage could also contribute to the progression of an atherosclerotic lesion, as it has been shown that excess deposition of lipid in lysosomes can lead to the formation of cholesterol crystals and cell death (55).

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