Evidence that newly synthesized esterified cholesterol is deposited in existing cytoplasmic lipid inclusions

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Abstract **Esterified cholesterol (EC) and triglyceride (TG) can be stored in cells as cytoplasmic inclusions. The physical state of the EC in these lipid droplets varies from liquid to liquid crystalline, depending on a number of factors, including the amount of TG co-deposited in the inclusion. The lipid in these droplets undergoes turnover via hydrolysis and resynthesis. We determined whether newly synthesized lipid is incorporated into existing cytoplasmic droplets, forms a discrete cytoplasmic droplet, or forms a small inclusion that fuses with an existing droplet. This was accomplished by monitoring the physical state of the lipid within the cytoplasmic inclusions following sequential deposition of TG and EC. Fu5AH cells were initially grown in media containing oleic acid to produce TG-rich, isotropic inclusions. The cells were then incubated with medium containing free cholesterol-phospholipid dispersions to promote synthesis and deposition of EC. To inhibit cytoplasmic TG hydrolysis, the lipase inhibitor, diethylumbelliferyl phosphate (UBP), was added at the time of cholesterol enrichment. The phase behavior of lipid droplets isolated from the lipid-rich cells was determined using polarizing light flow cytometry and microscopy. An anisotropic droplet population (EC-rich inclusions) was not detected, although there was an increase in cellular EC mass and no change in cellular TG mass. Therefore, under conditions where there is no turnover of cytoplasmic TG, newly synthesized EC is incorporated into existing TG inclusions.**—Kellner-Weibel, G., B. McHendry-Rinde, M. P. Haynes, and S. Adelman. **Evidence that newly synthesized esterified cholesterol is deposited in existing cytoplasmic lipid inclusions. *J. Lipid Res.* 2001.** 42: **768–777.**

Supplementary key words lipid droplet • triglyceride • lipase • hepatoma

The composition of lipid inclusions recovered from atherosclerotic lesions has been well characterized (1–3), and similar inclusions form in a number of cells in culture (4–8). In tissue culture cells, it is possible to modify the lipid component of cytoplasmic lipid droplets by supplementing the medium with fatty acids and/or cholesterol to produce co-deposition of triglycerides (TG) and esterified cholesterol (EC) (5). The physical state of the EC in the inclusion varies from liquid to liquid crystalline (1, 6–8), and is influenced by the nature of the acyl group in the EC, as well as the amount of TG in the droplet (5). It has also been demonstrated that the rate at which the EC can be hydrolyzed by a neutral cholesteryl ester hydrolase is influenced by the physical state of the EC in the inclusion, and therefore affects the rate of EC clearance from cells (5).

The current literature suggests similar routes of formation of TG and EC containing cytoplasmic inclusions, because both diaclyglycerol acyltransferase (DGAT), which catalyzes the final step in TG synthesis, and acyl CoA:cholesterol acyltransferase (ACAT), which is responsible for the esterification of unesterified (free) cholesterol (FC), are located at the endoplasmic reticulum (9, 10). One possible model of inclusion formation is that molecules of newly synthesized TG or EC initially accumulate within the bilayer of the endoplasmic reticulum (ER) (11). Continued accumulation at this site would lead to the formation of a lens of lipid that eventually grows to form a droplet containing a core of EC and/or TG and a surface of ER membrane phospholipid (12). This nascent droplet then pinches off and becomes the primary storage site for cytoplasmic EC and TG. This model suggests that as droplets form, TG and EC can be packaged into the same droplet and, in fact, EC and TG are both present in inclusions isolated from human (13) and animal tissues (1) and from cells in culture (5). Although there are considerable data on lipid droplet composition, there are many aspects of cytoplasmic lipid droplet formation that remain unclear. In many cell types, including macrophage foam

Abbreviations: EC, esterified cholesterol; TG, triglyceride; FC, unesterified (free) cholesterol; ACAT, acyl CoA:cholesterol acyltransferase; UBP, diethylumbelliferyl phosphate; PBS, phosphate buffered saline; BSA, bovine serum albumin; DLP, delipidized serum protein.

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cells and steriodogenic cells, droplets can vary within a single cell in both size and physical state (4, 8, 14, 15). We currently do not know whether droplets grow in size through the fusion of independent droplets or whether newly synthesized molecules of EC and TG are added to existing droplets. Additionally, it is not clear whether there are differences in the sorting and packaging of EC and TG into cytoplasmic droplets. The present studies were designed to investigate the sequential addition of TG and EC into Fu5AH rat hepatoma cytoplasmic lipid inclusions to better understand the mechanism of droplet formation.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS), bovine serum albumin (BSA) (essentially fatty acid free), gentamicin, oleic acid, triolein (TO), cholesteryl oleate (CO), and FC were purchased from Sigma Chemicals (St. Louis, MO). Organic solvents and glycerol were products of Fisher Scientific (Pittsburgh, PA). Tissue culture flasks and plates were obtained through Corning (Corning, NY) or Falcon (Lincoln, NJ). [1,2-^{3}H]Glycerol and [1,2-^{3}H]cholesterol were purchased from New England Nuclear (Boston, MA). Tissue culture media were obtained from Gibco (Grand Island, NY). 1-Pyerenedodecanoic acid was purchased from Molecular Probes (Eugene, OR). 1-Palmitoyl-2-oleoyl phosphatidylcholine (PC) was purchased from Avanti Polar Lipids (Alabasten, AL). FC and PC dispersions were made by the method of Arbogast et al. (16). Delipidized serum protein (DLP) was prepared as previously described (17). Diethylumbelliferyl phosphate (UBP) (18–20) was synthesized according to the procedure of Dr. Daniel Quinn (University of Iowa, Iowa [unpublished]). CP-113,818 was a generous gift from Pfizer Pharmaceuticals (Groton, CT).

Cell culture

Fu5AH rat hepatoma cells were routinely grown in Eagle's medium (EMEM) containing 5% calf serum and 50 μg/ml gentamicin. Before each experiment, Fu5AH cells were grown for 18 h on DLP (5 mg/ml) in EMEM to lipid-deplete the cells. To enrich the cells with TG, oleic acid was dissolved in 0.5 ml 0.5 M KOH and BSA in EMEM (10% of the final volume), and this was added to EMEM to yield a final fatty acid:albumin mole ratio of 5:1. The mixture was heated at 45°C for 1 h and then diluted to the final volume (pH = 7.4) with EMEM containing calf serum (1% final concentration). This medium was incubated with cells for 48 h. To cholesterol-enrich the cells, FC/PC dispersions (250 μg cholesterol/ml, FC:PC mole ratio >2) were added to EMEM containing 1% calf serum, and the mixture was incubated with the cells for 10, 24, or 48 h. Where indicated, incubations contained 120 μg/ml UBP to block cellular EC and TG hydrolysis.

Inclusion isolation

To isolate cellular lipid inclusions, monolayers of Fu5AH cells containing inclusions were washed twice with phosphate-buffered saline (PBS) and scraped into PBS (3 ml per 100-mm dish). The cells were disrupted using a ball-bearing homogenizer (three passes over a ball bearing = 0.2487 in.) (21). Homogenization by this procedure resulted in reproducible isolation of intact lipid inclusions. The structure and composition of lipid inclusions isolated from cells disrupted with the ball bearing homogenizer were compared with those of inclusions isolated from cells disrupted using ultrasonication (5), and there were no significant differences (W. Gray Jerome, personal communication). Homogenates were centrifuged at 26,000 rpm in an SW40Ti rotor for 1 h. The floating lipid inclusions were removed with a syringe, dispersed in PBS, and kept at 4°C until analyzed (see below).

Preparation of synthetic lipid droplets

Lipid droplets were prepared as described by Minor, Rothblat, and Glick (22). Briefly, anisotropic droplets were made by adding 15 ml PBS to a 50-ml round-bottom Corex glass tube containing 30 mg CO plus 1 mg PC (mole ratio PC:CO, 1:35). The tube was placed in boiling water for 10 min to melt the lipids. The mixture was then sonicated using a tapered microtip attachment on a Branson Sonifier Cell Disrupter Model 350 for 3 min at room temperature, followed by 6 min in an ice water bath (settings at 50% duty cycle and 4.5 output). The physical state of the droplets was confirmed by polarizing light microscopy. Isotropic droplets were made similarly with the addition of 20.7 mg TO together with CO and PC (mole ratio PC:CO:TO, 1:35:18).

Microscopy

Microscopic examination of lipid inclusions in whole cells and isolated droplets was performed using polarizing light microscopy under oil at 1,000× magnification with a Zeiss light microscope with a Vivitar polarizing lens. Inclusions were identified as anisotropic by the appearance of the cross forme (4).

Flow cytometry

Isolated inclusion samples were analyzed on a FACSVantage (Becton-Dickinson, San Jose, CA) equipped with an argon ion laser that emitted 200 mW of 488 nM light. Scattered light was collected in the forward and right angle directions (FSC and SSC, respectively). Depolarized side scatter (dSSC) was measured using the strategy described by Grooth et al. (23) using a depolarizing filter (Melles Griot, Irvine, CA) and a 485/10 bandpass filter proximal to the FL1 photomultiplier tube. The polarizing filter was oriented orthogonally to the incoming polarized light. Data acquisition and analysis were performed using CellQuest software (Becton-Dickinson, San Jose, CA). The percentage of anisotropic and isotropic droplets was evaluated using gating criteria based on a forward scatter versus depolarized side scatter dot plot containing 10,000 events. Before each experiment, synthetic anisotropic and isotropic lipid droplets were analyzed by flow cytometry to identify each population and account for any day-to-day variation. Isotropic and anisotropic droplet populations were defined on the basis of FSC and dSSC profiles of 10,000 droplets per sample. Since the isotropic and anisotropic profiles were not mutually exclusive, a region encompassing greater than 95% of isotropic droplets was chosen as the criterion for analysis of all subsequent samples within an experiment. The area of overlap of the anisotropic and isotropic populations, on average, represented 7% of the total. All samples were run at 37°C and room temperature to control for possible changes in physical state due to temperature differences. Room temperature data are shown for all figures and are not significantly different from the data collected at 37°C. Incorporation of 1-pyrenedodecanoic acid was measured using multiline ultraviolet (UV) excitation (50 mW) and emission at 380 nm (380/15 bandpass).

Quantitation of lipids

Cellular lipids were quantitated using the thin-layer chromatographic method of Brasaemle et al. (24). Briefly, lipids were extracted from monolayers with isopropanol and applied to silica gel H plates that were impregnated with ammonium sulfate (Analtech). Plates were developed in hexane:diethyl ether:formic acid (90:10:1, v/v/v) and allowed to air dry for 30

min. The plates were then charred at 160°C for 2 h. Spots corresponding to various lipid classes were detected using a Molecular Dynamics Densitometer, and relative densities were determined using ImageQuant software (Molecular Dynamics). Sample densities were compared with standards developed on the same plate.

Lipid exchange and droplet fusion studies

Fluorescently labeled CE inclusions were created by incubating Fu5AH cells for 18 h with EMEM media containing FC-PC dispersions (250 μg cholesterol/ml), 5% calf serum, and 8 μM 1-pyrenedodecanoic acid. To determine whether the fluorescent fatty acid was incorporated into EC, fluorescently labeled droplets were isolated as described above and the lipids were extracted using the method of Bligh and Dyer (25). The lipid extract was dried under nitrogen and dissolved in 0.1 ml chloroform/methanol (1:1, v/v). The resolubilized lipids were applied to silica gel H plates that were impregnated with ammonium sulfate (Analtech), and the plate was developed using the solvent system described above. Fluorescent lipid classes were identified using a UV light source and compared with lipid standards run on the same plate. Standard lipid classes were detected by exposing the plate to iodine. To determine whether there was lipid exchange between populations of droplets mixed together in vitro, the inclusions were isolated from whole cells as described above. Inclusions containing fluorescently labeled lipids were incubated with isolated TG inclusions that did not contain the fluorescent label in PBS for up to 24 h at 37°C. In a parallel set of experiments, high-speed supernatant was added to the mixture of fluorescently labeled and nonlabeled droplets at a 1:1 (v/v) ratio. The percent of fluorescently labeled inclusions was monitored by flow cytometry as described above.

Triglyceride hydrolysis

Fu5AH cells were incubated for 18 h on DLP to lipid-deplete the cells and subsequently incubated for 24 h with fatty acid rich media as described under "Cell Culture," with the addition of 5-μCi/ml [1,2-^{3}H]glycerol to the loading medium. To measure hydrolysis, the radiolabeled TG-enriched cells were then incubated with EMEM containing 0.2% BSA and 0.1% unlabeled glycerol for 0 and 48 h. Some incubations contained 120 μg/ml UBP to block cellular TG hydrolysis. At each time point, lipids were extracted with isopropanol and separated using silica gel 60 F_{254} TLC plates with a mobile phase of petroleum ether:ethyl ether:acetic acid (90:10:1, v/v/v). Lipids were detected by exposure of the plate to iodine. Spots corresponding to TG were scraped and analyzed by liquid scintillation counting. Cellular [^{3}H]TG was determined at 48 h and compared with the amount of cellular [^{3}H]TG at time zero.

Esterified cholesterol hydrolysis

Fu5AH cells were incubated for 18 h on DLP to lipid-deplete the cells, and subsequently incubated for 24 h with cholesterol-rich media as described under "Cell Culture," with the addition of 0.5 μCi/ml [^{3}H]cholesterol to the loading medium. The radiolabeled EC-enriched cells were then incubated with EMEM containing 0.2% BSA plus the ACAT inhibitor CP-113,818 (2 μg/ml) for 0 and 48 h. Some incubations also contained 120 μg/ml UBP to inhibit cellular EC hydrolysis. At each time point, lipids were extracted with isopropanol, separated, and detected as described in "Triglyceride Hydrolysis." Spots corresponding to EC were scraped and analyzed by liquid scintillation counting. Cellular [^{3}H]EC was determined at 48 h and compared with the amount of cellular [^{3}H]EC at time zero.

Data analysis

Values are expressed as means ± SD unless otherwise indicated. Calculations were performed using Quatro Pro version 6, Novell. Data were graphed and statistical analysis performed using an unpaired Student's *t*-test (GraphPad Prism version 2.01, GraphPad software). The criterion for significance was set at $P \leq 0.05$.

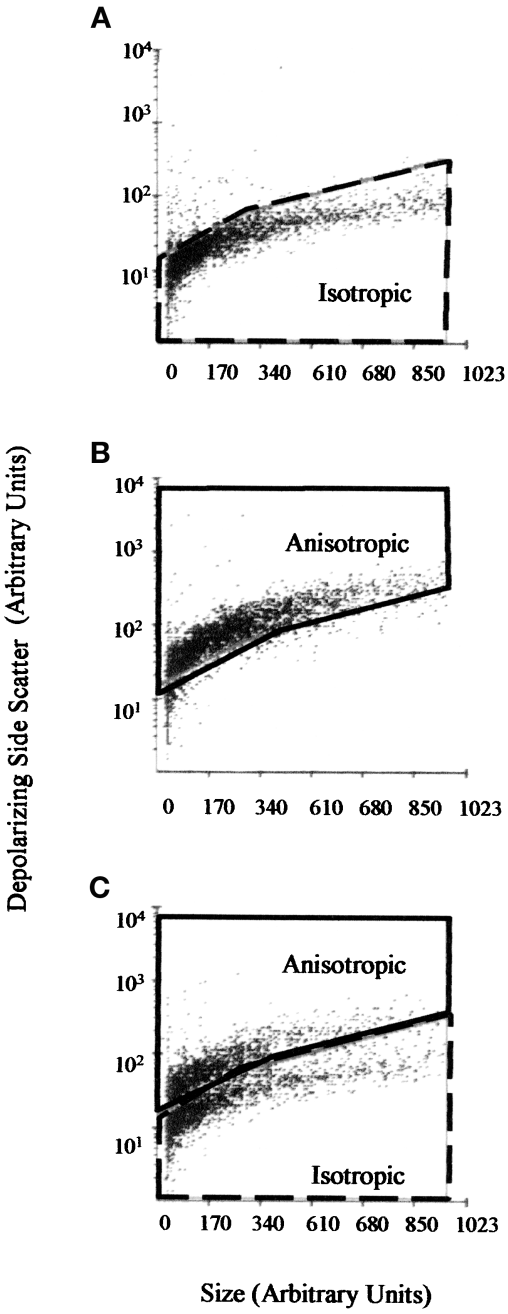

Fig. 1. Flow cytometry of artificial lipid droplets; depolarizing light side scatter unprocessed data. Synthetic lipid droplets consisting of phosphatidylcholine (PC), cholesteryl oleate (CO), and triolein (TO) (isotropic, A) or PC and CO (anisotropic, B) were analyzed by flow cytometry to determine whether this technique could discriminate between isotropic and anisotropic inclusion populations. Droplet populations were defined as described in Materials and Methods. Figure 1C is the unprocessed data from a droplet sample comprising of a 1:1 ratio of the droplets from (A) and (B). The flow cytometry technique differentiated between the two populations with a 7% error.

RESULTS

Validation of the use of flow cytometry for analysis of lipid droplet phase behavior

The physical state of lipids within a cytoplasmic droplet reflects the chemical composition of the lipids. dSSC was used in these studies to differentiate between isotropic (liquid) and anisotropic (liquid crystal) droplet populations. To validate this method of detection, synthetic lipid droplets were made consisting of PC and CO (1:35 mole ratio PC:CO, anisotropic) or PC, CO, and TO (1:35:18 mole ratio PC:CO:TO, isotropic) as described in Materials and Methods. The physical state of these artificial droplets was confirmed using polarizing light microscopy. Aniso-

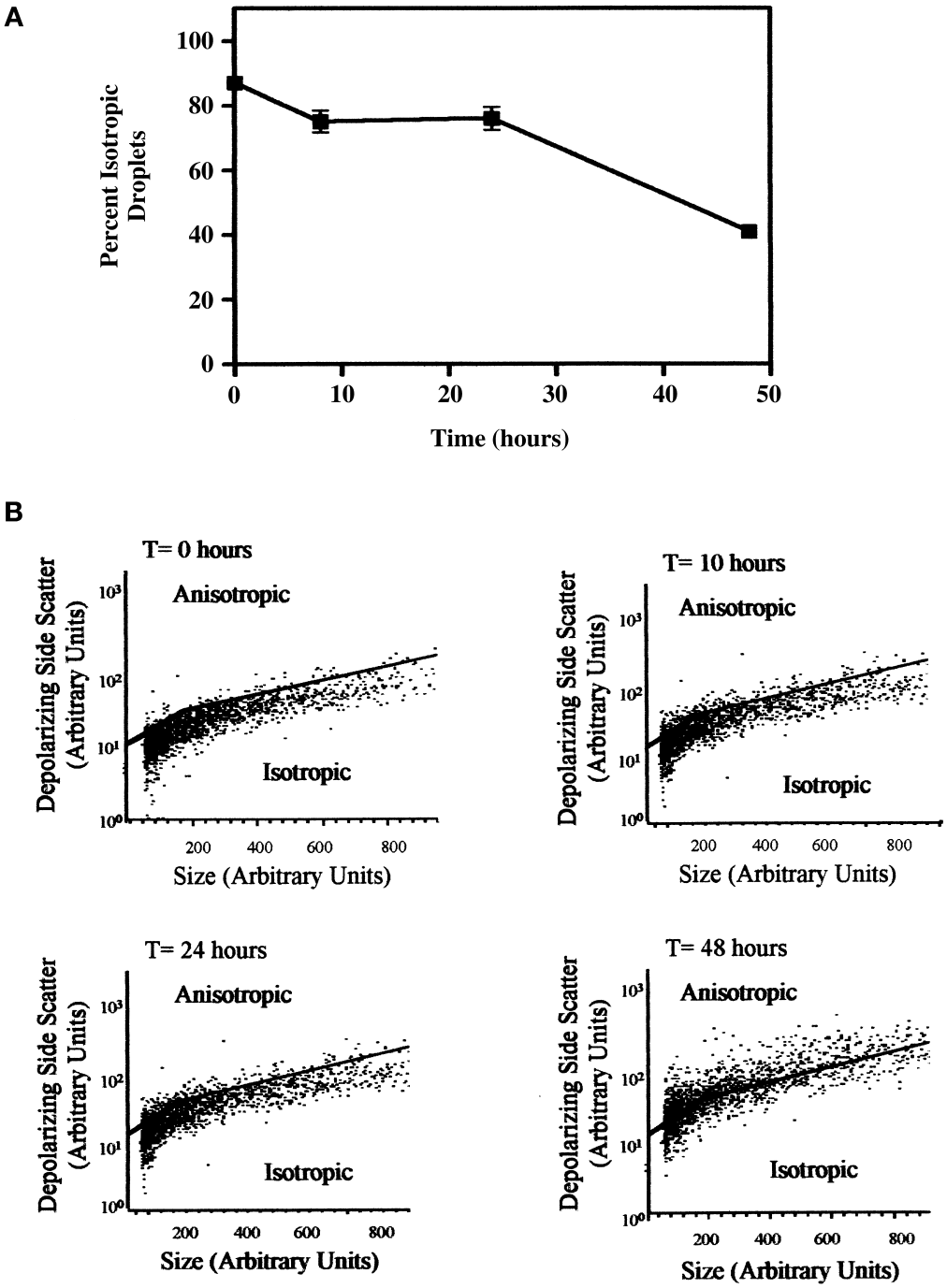

Fig. 2. The effect of cholesterol enrichment on the physical state of cellular TG inclusions. Fu5AH cells were enriched with TG by incubating the cells for 48 h in media enriched with oleic acid. After an equilibration period, the cells were incubated with cholesterol-enriched media for up to 48 h to promote the synthesis and deposition of EC. At each time point, the cells were disrupted with a ball-bearing homogenizer, cytoplasmic lipid inclusions were isolated by ultracentrifugation, and the physical state of the droplets was determined by flow cytometry. A: Percent isotropic droplets calculated from three separate experiments. B: Unprocessed data for each time point from a representative experiment. Cellular TG and EC mass were determined for each time point (Table 1).

tropic and isotropic droplets were analyzed separately using a FACSVantage (Becton-Dickinson) flow cytometer. **Figure 1** shows the data collected from the isotropic (A) and anisotropic (B) inclusions. The droplets were then mixed in a 1:1 ratio (Fig. 1C) and analyzed. From the mixed sample we determined that we could accurately discriminate between the two populations with an average error of 7%. Similar results were obtained when using anisotropic and isotropic droplet populations isolated from cellular material (data not shown). In subsequent experiments, standards were run to define the isotropic and anisotropic populations before each experiment.

Sequential deposition of TG and cholesteryl ester in Fu5AH cells

Fu5AH cells were enriched with TG by exposing them to medium enriched with oleic acid for 48 h. This treatment produced isotropic cytoplasmic TG inclusions evidenced by microscopic examination with polarizing light (data not shown). The cells were then exposed to medium containing FC-PC dispersions plus 1% calf serum for 10, 24, and 48 h to promote the synthesis and deposition of EC. Analysis by flow cytometry of isolated inclusions revealed that the droplet population shifted from 90% isotropic to a mixture of isotropic (41%) and anisotropic (59%) inclusions at 48 h (**Fig. 2**). The apparent 10% anisotropic droplet population at time 0, where there is no detectable level of EC mass, is a reflection of the error intrinsic to this technique when detecting anisotropic and isotropic droplet populations (see Materials and Methods). **Table 1** indicates that the mass of TG decreased over time (50% loss at 48 h), while the mass of EC increased from an undetectable level at time 0 to 13.1 μg/well at 48 h. The loss of cellular TG mass complicates the interpretation of these data because we cannot discern whether the emergence of the anisotropic population is due to a separate population of EC-rich droplets being formed or the existing droplets are becoming EC-enriched because of TG loss. However, it is clear, based on the TG:EC mole ratio values (Table 1), that a population of droplets greatly enriched with EC is produced. At 48 h the TG:EC mole ratio is 0.7:1.0 (Table 1). If all of the droplets had this mole ratio, they would all be isotropic, because only 2–8% TG will abolish the anisotropic quality of an inclusion (26–28).

TABLE 1. Mass of cholesteryl ester and triglyceride in Fu5AH cells

Time	EC	TG	TG:EC
h	*μg/well*	*μg/well*	*mole ratio*
0 h	ND	26.0 ± 4.0	—
10 h	4.4 ± 0.7	33.1 ± 5.9	5.5:1.0
24 h	10.2 ± 0.6	27.7 ± 1.6	2.0:1.0
48 h	13.1 ± 1.6	12.4 ± 0.5*	0.7:1.0

Fu5AH cells were TG-enriched as described in Fig. 2, and cellular TG and EC mass were measured chromatographically after extraction from the monolayer by isopropanol as described in Materials and Methods (time 0 h). After a 48-h period of EC enrichment, cellular TG and EC mass were determined (time 48 h). Mole ratios were calculated from experimentally determined masses. ND = not detected.

* Significantly different from T = 0 h value.

Diethylumbelliferyl phosphate inhibits TG and cholesteryl ester hydrolysis

The continual turnover of TG in this system, evidenced by loss of TG mass, confounded the interpretation of the phase behavior data. We could not determine whether there was a new EC-rich inclusion population formed, or the loss of TG from the cytoplasmic compartment had an impact on the physical state of the droplets. To address this problem, we included the lipase inhibitor UBP in some incubations. UBP was previously reported to block hydrolysis of EC and TG in various cells (18–20, 29). To determine whether UBP inhibited lipid hydrolysis under our experimental conditions, Fu5AH cells were incubated with oleic acid-enriched medium containing [^{3}H]glycerol as described in Materials and Methods. The amount of

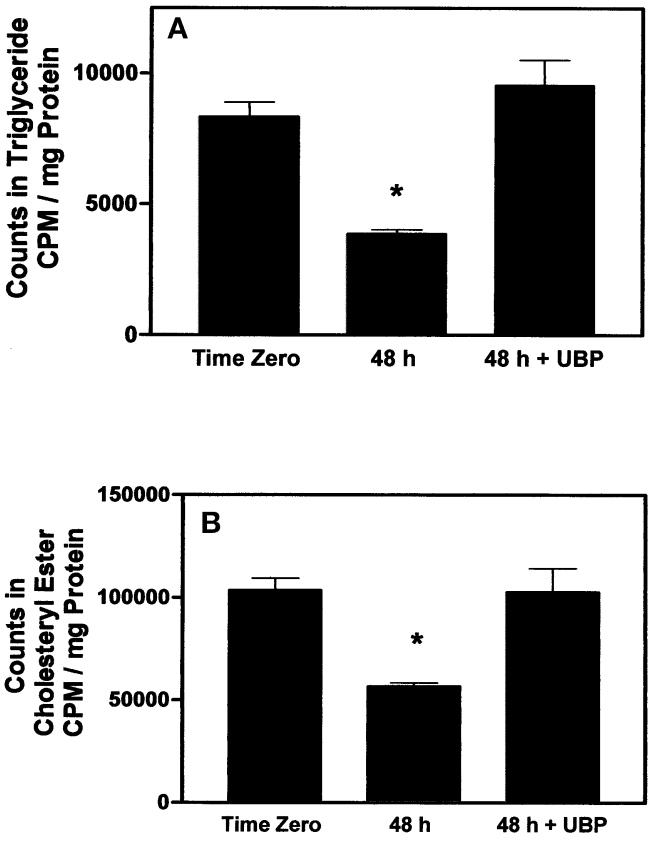

Fig. 3. The effect of the cellular lipase inhibitor UBP on TG and EC hydrolysis. A: Fu5AH cells were incubated with media containing oleic acid and [^{3}H]glycerol to promote the synthesis and deposition of [^{3}H]TG. TG-rich cells were then exposed to chase medium containing cold glycerol ± UBP (120 μg/ml) for 0 and 48 h. Total [^{3}H] TG was determined at both time points. B: Fu5AH cells were incubated with media containing FC/PL and [^{3}H]cholesterol to promote the synthesis and deposition of [^{3}H]EC. EC-rich cells were then exposed to chase medium containing the ACAT inhibitor CP-113,818 (2 μg/ml) ± UBP (120 μg/ml) for 0 and 48 h. Total [^{3}H]EC was determined at both time points. Data represent N = 3 for a representative experiment. * Significantly different from time zero values ($P \leq 0.05$).

[^{3}H]glycerol incorporated into cellular TG was determined (time 0). The monolayers were then incubated in EMEM medium containing 0.1% unlabeled glycerol in the presence or absence of 120 μg/ml UBP for 48 h. **Figure 3A** demonstrates that in the absence of UBP, there is loss of [^{3}H]TG, indicating hydrolysis. However, in the presence of UBP there is no loss of labeled TG at 48 h. Similarly, Fig. 3B indicates that UBP inhibits the hydrolysis of cellular EC when cells are labeled with [^{3}H]cholesterol. In preliminary studies, we determined that UBP does not interfere with EC and TG synthesis and does not cause cellular toxicity (measured by adenine release) up to 48 h at 120 μg/ml (data not shown). Control incubations of Fu5AH cells, which did not contain a triglyceride load, were cholesterol-enriched in the presence of UBP. Cholesterol enrichment promoted the synthesis and deposition of EC, and the resulting inclusions were anisotropic (data not shown), indicating that UBP does not interfere with anisotropic droplet formation. Thus, UBP effectively inhibits the hydrolysis of cytoplasmic TG and EC in our experimental system.

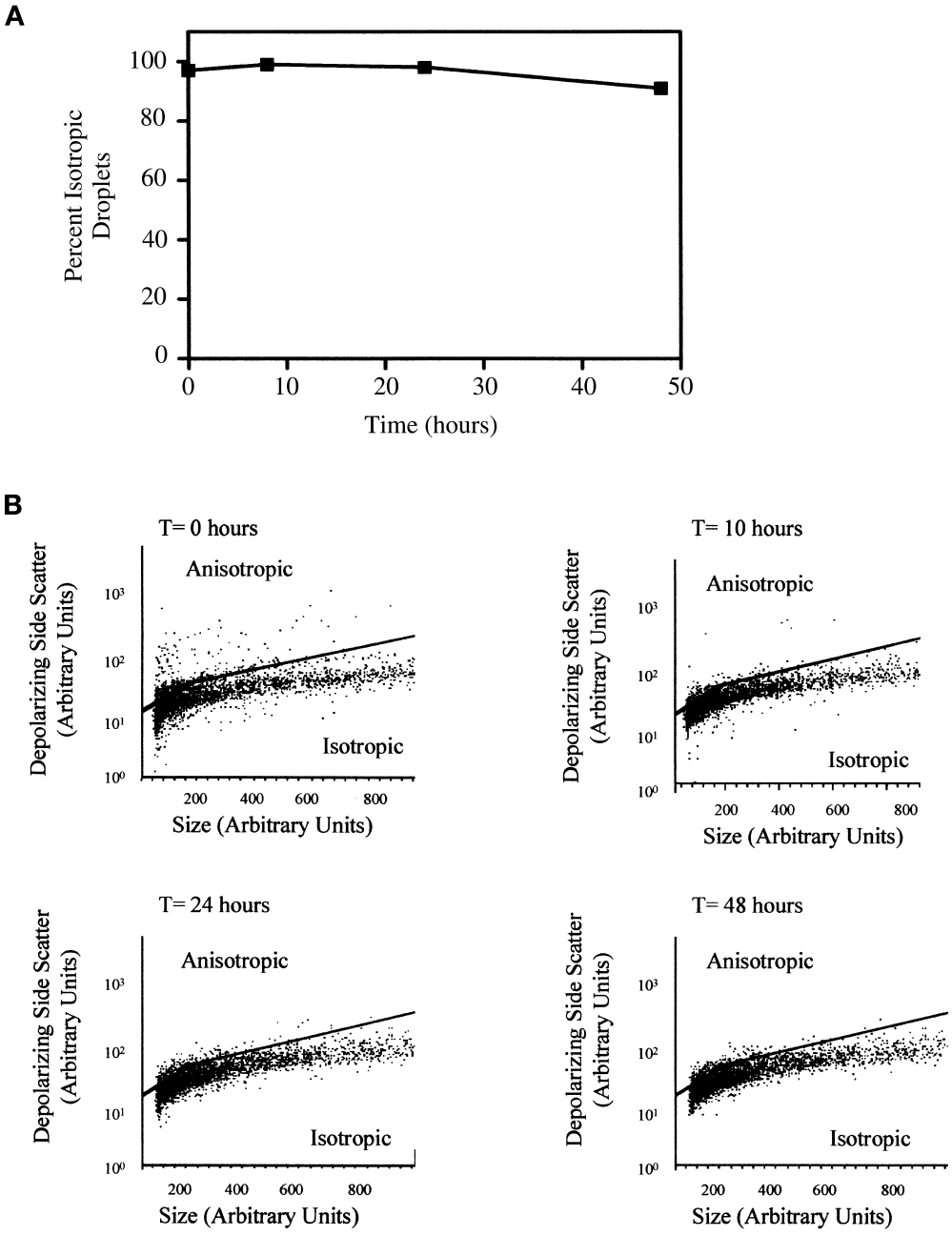

Fig. 4. The effect of cholesterol enrichment on the physical state of cellular TG inclusions in the presence of UBP. Fu5AH cells were treated as described in Fig. 2 with the addition of UBP (120 μg/ml) to the equilibration and cholesterol-enriched media. The physical state of the isolated lipid droplets was analyzed by flow cytometry. A: Percent isotropic droplets calculated from three separate experiments. B: Unprocessed data for each time point from a representative experiment. Cellular TG and EC mass were determined for each time point (Table 2).

Sequential addition of TG and esterified cholesterol to Fu5AH cells in the presence of UBP

To avoid the problem of TG mass loss due to hydrolysis during the EC-enrichment phase of the experiment, the lipase inhibitor UBP was included in our protocol. Fu5AH cells were TG-enriched as described above, and UBP (120 μg/ml) was added to the equilibration and to the FC-enriched medium. Under these conditions the mass of TG did not decrease and an anisotropic droplet population was not detected, even though the mass of cellular EC increased (**Fig. 4** and **Table 2**). If a separate population of inclusions containing primarily the newly synthesized EC was produced, these inclusions would be anisotropic. No anisotropic inclusions were detected by polarizing light flow cytometry or microscopy. This result is consistent with a system in which newly synthesized EC is incorporated into existing cytoplasmic TG, therefore retaining the isotropic quality of the droplet. To test our hypothesis further, we performed the converse of the above system and initially incubated Fu5AH with cholesterol to produce anisotropic EC droplets within the cells (91.4 ± 0.3% anisotropic at 0 h). Fatty acid-rich media was then added for up to 48 h in the presence of UBP to promote the synthesis and deposition of TG. The droplet population shifted to 22.0 ± 1.0% anisotropic at 48 h, indicating that the newly synthesized TG is incorporated into existing EC droplets, resulting in the conversion of the anisotropic droplets into the isotropic liquid state.

It is possible that the shift in physical state is due to droplet fusion or lipid transfer between inclusions after isolation and before analysis. To address this question, Fu5AH cells were cholesterol-enriched in the presence of the fluorescent fatty acid, 1-pyrenedodecanoic acid, which has been shown to be taken up by cells (30, 31) and incorporated into neutral lipids (32, 33). This treatment produced anisotropic inclusions containing fluorescently labeled EC and phospholipid (data not shown). The fluorescently labeled EC inclusions were isolated and mixed with isolated, nonlabeled TG inclusions (10 parts labeled EC inclusion to 1 part nonlabeled TG inclusion) at 37°C for up to 24 h. There was no change in the percent of droplets containing the fluorescent label or in the physical state of the droplets over the time course (**Fig. 5**).

TABLE 2. Mass of cholesteryl ester and triglyceride in Fu5AH cells in the presence of UBP

Time	EC	TG	TG:EC
h	*μg/well*	*μg/well*	*mole ratio*
0 h	ND	26.0 ± 4.0	—
10 h	4.3 ± 0.5	27.6 ± 5.1	4.7:1.0
24 h	7.7 ± 0.9	22.0 ± 1.9	2.1:1.0
48 h	16.8 ± 3.0	19.3 ± 3.3	0.9:1.0

Fu5AH cells were TG-enriched as described in Fig. 4, and cellular TG and EC mass were measured chromatographically after extraction from the monolayer by isopropanol as described in Materials and Methods (time 0 h). After a 48-h period of EC enrichment in the presence of UBP (120 μg/ml), cellular TG and EC mass were determined (time 48 h). Mole ratios were calculated from experimentally determined masses. ND = not detected.

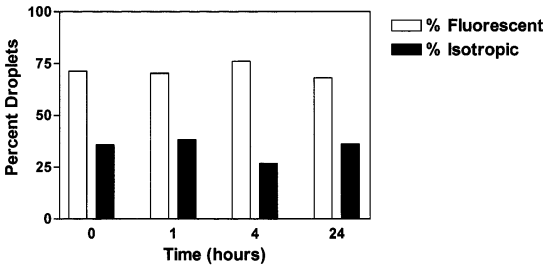

Fig. 5. Lack of lipid exchange in isolated inclusions. TG inclusions isolated from Fu5AH cells were mixed and incubated with EC inclusions containing a fluorescent label in the fatty acyl portion of the ester. The mixture was incubated at 37°C for 24 h. At each time point the physical state and the percent of the droplets containing the fluorescent label were determined by flow cytometry. Data represent the mean value for 10,000 events from a single representative experiment.

These data indicate that there was no lipid exchange between the droplets and no inclusion fusion. Furthermore, when high-speed supernatant was added to the mixture of fluorescently labeled and nonlabeled inclusions, there was no exchange of label over time (data not shown), indicating that there was not an active factor in the supernatant that would cause the fusion of lipid droplets in vitro.

DISCUSSION

Excess EC and TG are stored in many cell types as cytoplasmic lipid inclusions. These inclusions are metabolically active in that the lipid within them is constantly being turned over through hydrolysis and resynthesis (34). Recent advances in the study of inclusions in adipocytes and steroidogenic cells have provided a number of insights regarding the role of lipid inclusions in cell lipid homeostasis (24, 35, 36). However, many aspects of the origin and growth of lipid droplets are poorly understood. One theory on the origin of lipid inclusions is that droplets are formed as EC and/or TG accumulate within the bilayer of the endoplasmic reticulum (11, 12). This is an attractive hypothesis because both ACAT and DGAT, the enzymes responsible for the esterification of FC and the final step in TG synthesis, respectively, are located at the endoplasmic reticulum (9, 10). As the lipid is deposited in the bilayer, a lens develops that eventually pinches off to form a cytoplasmic droplet composed of a core of EC and/or TG surrounded by a phospholipid monolayer (**Fig. 6A**). In fact, inclusions isolated from human and animal tissues and cells in culture contain both EC and TG and vary in size (4, 37, 38). There are a number of mechanisms by which lipid droplets could grow in size: 1) the fusion of independent droplets (Fig. 6B); 2) the addition of molecules of newly synthesized lipid directly into an existing droplet (Fig. 6C); 3) the exchange of molecules of lipid between mature lipid droplets (Fig. 6D); 4) or budding off of droplets of varying size owing to differences in surface tension within the particular domain of the ER.

The present studies were designed to gain a better understanding of lipid droplet formation and growth. The

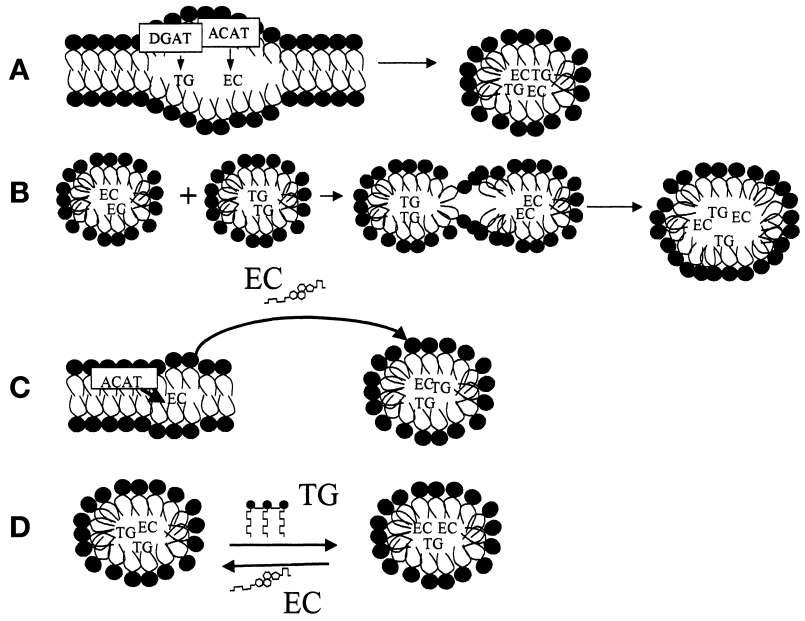

Fig. 6. Mechanisms of lipid inclusion growth. A: TG and EC synthesis may occur coincidentally, and therefore both lipids are packaged into the same inclusion. B: Individual inclusion may fuse. C: Newly synthesized lipid may be deposited into an existing inclusion by a transfer protein. D: Lipid may exchange between inclusions via a carrier protein.

amount of TG within a cytoplasmic droplet influences the physical state of the droplet (5). Droplets that are enriched with EC have a positive sign of birefringence (anisotropic), evidenced by the appearance of the cross formée under polarizing light microscopy (4). Inclusions that are enriched with TG do not polarize light (isotropic), and addition of 2–8% TG to an EC droplet will abolish the anisotropic quality of the inclusion (26–28). We have taken advantage of the differing phase behavior of TG- and EC-enriched droplets by quantitating the physical state of the droplets using polarizing light flow cytometry and microscopy. We validated our flow cytometry methods by analyzing both synthetic anisotropic and isotropic inclusions and droplet populations isolated from Fu5AH rat hepatoma cells, created by manipulating cellular EC and TG levels (Fig. 1). Fu5AH cells have been used in these studies because previous work has shown that the phase behavior of the lipid inclusions within these cells is predictable, based on the composition of the inclusions and that the composition can be readily manipulated by varying the lipid components of the tissue culture medium in which the cells are incubated (5, 39). We used the ability of TG to modulate the phase behavior of EC as a tool in these experiments to investigate if newly synthesized EC will 1) insert into existing TG cytoplasmic droplets, or 2) form a discrete inclusion. In the first scenario, the inclusions in the cells would remain isotropic unless the cellular mass of EC reached greater than 92% of the combined TG and EC masses. In the second model, a separate, anisotropic population of droplets would be detected, because the newly formed inclusions would be primarily EC. Our initial experiments demonstrated that when Fu5AH cells containing cytoplasmic isotropic TG droplets were exposed to media that promotes the synthesis and deposition of EC, the phase behavior of the droplets changed from 87% isotropic before exposure to cholesterol-rich media to 41% isotropic 48 h after exposure. These data suggest that as EC is synthesized, it forms inclusions separate from those of the existing TG droplets, or forms droplets consisting of greater than 90% EC. However, there was a concurrent loss of cellular TG mass (50% at 48 h). Loss of TG mass is most likely due to the hydrolysis of cytoplasmic TG followed by tÎat if TG and EC are supplied to cells at the same time, they are packaged into the same droplet (Fig. 6A) (5, 39). To avoid cytoplasmic lipid turnover, we included the lipase inhibitor UBP. The current studies demonstrate that when cellular TG is not metabolically available, newly synthesized EC is mixed with existing cytoplasmic TG. This is evidenced by a lack of change in the phase behavior of the cytoplasmic inclusions, even though there is deposition of cellular EC mass equal to that of TG (Table 2). In addition, when UBP is present, there is not a loss of cellular TG mass (Table 2). Therefore, our data indicate that newly synthesized EC can be packaged into existing TG droplets under conditions where there is no cytoplasmic lipid turnover.

The mechanism by which newly synthesized EC is incorporated into preformed TG droplets is not known. One possibility is that nascent EC droplets are formed, bud off, and then fuse with existing TG droplets. However, this is unlikely because in the experiments that included UBP, we did not detect any anisotropic droplet population. In addition, the results obtained in these studies were not a function of droplet fusion after isolation, as there was no evidence of fusion or lipid exchange (Fig. 5) and our data indicate that the droplets were stable after isolation. The question of how the newly synthesized EC is translocated from the ER to the cytoplasmic lipid droplet remains. Previous studies suggested that some cytoplasmic lipid droplets are contiguous with the ER (40). In this model, the newly synthesized EC could migrate from the site of esterification directly to the lipid droplet through a membrane network. In addition, Reaven, Tsai, and Azhar (41) suggested that lipoprotein-derived EC might be transported

to various cellular locations through vesicles or intracellular membrane sheets. Another possibility for intracellular EC transport is that there is a sterol transfer protein that would shuttle the newly synthesized EC from the ER to the cytoplasmic lipid droplet, dock with the inclusion, and transfer the lipid into its core. A likely candidate for this model is sterol carrier protein$_2$ (SCP_2). SCP_2 has been implicated in intracellular transport of cholesterol and its metabolites to various locations within the cell (42–45). However, there is no direct evidence for SCP_2 translocating EC from the ER to a cytoplasmic compartment.

In addition to the question of how lipid reaches the cytoplasmic inclusion, it is not clear whether the number of inclusions within a particular cell increases with greater lipid mass, or whether there is a finite number of droplets that a cell synthesizes and that these droplets increase in size in response to lipid accumulation. In the present study, it was difficult to obtain an accurate measure of the number of droplets per cell and to determine whether that number changes with increasing lipid mass. However, using light microscopy, it is apparent that in Fu5AH cells, the number of lipid droplets per cell varies from cell to cell and inclusion size varies within a single cell (data not shown). The physical state of the lipid inclusions in cells in culture also varies within a single cell (4). This is in contrast to foam cell material isolated from atherosclerotic plaques, in which the inclusions within a cell are of the same physical state but the state varies from cell to cell (46). Although it is easy to manipulate cells in culture to produce cytoplasmic droplets of varying phase behavior, the physical state of the lipid inclusions in foam cells within a lesion is most likely a function of the microenvironment around the cells and the amount and type of lipid the cells are exposed to. Given our current understanding of the metabolically active lipid inclusion, it is reasonable to suggest that the droplets are in a dynamic state, constantly being degraded and resynthesized, regardless of whether they are in cells in culture or within an atherosclerotic plaque. Studies are currently being conducted to address these questions.

The idea that the cytoplasmic lipid droplet is simply a storage depot for excess lipid has been replaced with the knowledge that these inclusions are important to cellular cholesterol and TG homeostasis. The current study has provided insight into the growth of the lipid droplet in a hepatoma cell line. Under conditions where there is cytoplasmic lipid turnover, the interpretation of the data is more difficult; however, previous studies suggest that if TG and EC are synthesized simultaneously, they are packaged into the same cytoplasmic lipid droplet (Fig. 6A) (5, 39). Additionally, under the conditions of the current study, where cellular lipid turnover is occurring and cholesterol is supplied to the cell without additional fatty acid, it is reasonable to predict that the cellular inclusions produced are primarily EC. The data presented in this paper support a model of lipid droplet formation under conditions where cytoplasmic lipolysis is blocked, in which newly synthesized EC is preferentially inserted into existing TG inclusions rather than forming a new lipid droplet.

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