

# Triglyceride depletion in THP-1 cells alters cholesteryl ester physical state and cholesterol efflux

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**Abstract** To study macrophage lipid droplet composition and the effects of TG on cholesteryl ester (CE) physical state, hydrolysis, and cholesterol efflux, a technique was developed to remove the majority of accumulated TG with minimal effect on CE content. THP-1 macrophages were incubated with acetylated LDL, and the accumulated TG was depleted by incubation with the acyl-CoA synthetase inhibitor triacsin D in the presence of albumin. Before TG removal, all cellular lipid droplets were isotropic as determined by polarizing light microscopy. When the TG concentration was reduced, anisotropic lipid droplets were visible, indicating a change in physical state, and suggesting that TG and CE originally accumulated in mixed lipid droplets. This change in physical state of lipid droplets was associated with slower rates of CE hydrolysis and cholesterol efflux. Although lipid droplets within the same cell had a similar physical state after TG depletion, there was considerable variability among cells in the physical state of their lipid droplets. **In conclusion, THP-1 macrophages store accumulated CE and TG in mixed droplets, and the proportion of CE to TG varies among cells. Reducing accumulated TG altered CE physical state, which in turn affected hydrolysis of CE and cholesterol efflux.**—Lada, A. T., M. C. Willingham, and R. W. St. Clair. Triglyceride depletion in THP-1 cells alters cholesteryl ester physical state and cholesterol efflux. *J. Lipid Res.* 2002. 43: 618–628.

**Supplementary key words** triglyceride • lipid droplet • physical state • macrophage • cholesteryl ester • macrophage foam cell

Atherosclerosis is characterized by the accumulation of cholesterol, particularly cholesteryl ester (CE), in the intima of arteries (1). Macrophage derived foam cells are the principal cellular components of fatty streaks, the earliest stage of atherosclerosis (1–3). Macrophages contribute to the formation of lesions by accumulating large amounts of CE through what is believed to be the uptake of modified lipoproteins by a variety of mechanisms, including the scavenger receptor pathway (4–6). In both the atherosclerotic lesion and in cells isolated from atherosclerotic arteries, CE is the major lipid component with TG typically comprising 10% or less of the total lipid, which includes CE, free cholesterol (FC), TG, and phospholipid (PL) (7–12).

CE can exist in several physical states including liquid (isotropic), cholesteric and smectic liquid-crystalline (anisotropic), and crystalline (13, 14). CE in a liquid state has been reported to be hydrolyzed faster than when in a liquid-crystalline state, and the increased rate of hydrolysis can lead to an increase in the rate of cellular cholesterol efflux if a cholesterol acceptor is present in the culture medium (15–17). TGs have been shown to greatly influence the physical state of CE. In the presence of high concentrations of cellular TGs, CE becomes more fluid and is hydrolyzed and effluxed more rapidly from cells than when very low levels of TGs are present (16, 17). Small amounts of TG can alter the physical state of CE, with as little as 2–3% (w/w) TG abolishing the cholesteric phase, and a concentration of 25% significantly reducing the smectic liquid-crystalline transition temperature (7, 18, 19). In general, 1% of lipid content as TG lowers the smectic transition temperature by about 1°C (18). Therefore, the levels of TG present in atherosclerotic lesions are below or at the lower range of concentrations at which TG should begin to affect the physical state of CE.

When studying such processes as the hydrolysis of CE and the subsequent efflux of cholesterol from macrophages in vitro, it is important to mimic the lipid content of the atherosclerotic lesions in vivo. Previous studies have shown in both cultured cell lines, such as THP-1 cells and primary human monocyte-macrophages, that incubation with either acetylated low density lipoprotein (AcLDL) or VLDL results in the accumulation of both CE and TG (20–26). Depending on cell type and lipoproteins used, the TG concentration can often be much higher than the CE concentration. Thus, hydrolysis of CE and cholesterol efflux studies done using such cells may not accurately reflect the situation in the atherosclerotic lesion.

For studies reported in this paper, TG concentrations in

Abbreviations: ACAT, acyl coenzyme A:cholesterol acyltransferase; AcLDL, acetylated low density lipoprotein; EC, esterified cholesterol; FC, free cholesterol; PPACK, D-phenylalanyl-L-prolylarginine chloromethyl ketone.

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THP-1 macrophage foam cells loaded with CE were reduced in order to more closely model macrophage foam cells of the atherosclerotic plaque. The purpose of these studies was to then examine the effect of the removal of accumulated TG on CE physical state, hydrolysis, and cholesterol efflux. In addition, by combining the biochemical analysis of these cells with phase contrast and polarizing light microscopy, we were able to make observations concerning the composition of cellular lipid droplets, and differences in the distribution of CE and TG among cells.

## MATERIALS AND METHODS

### Materials

Triacsin D was a generous gift from Dr. Satoshi Omura, The Kitasato Institute, Tokyo, Japan. CP113 was a gift from Pfizer, Inc., Groton, CT. RPMI 1640 cell culture medium, penicillin-streptomycin, L-glutamine, vitamins, and PBS were purchased from Cellgro by Mediatech, Herndon, VA. FBS was purchased from Atlanta Biologicals, Norcross, GA and was heat inactivated at 56°C for 30 min before use.  $\beta$ -Mercaptoethanol, Falcon culture dishes (35 mm), acetic anhydride, isopropanol, hexane, ether, iodine, microscope slides, 25 mm circular cover slips, and TLC plates were purchased from Fisher Scientific, Suwanee, GA. Glucose, PMA, EDTA, essentially fatty acid free BSA, Nile Red, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich, St. Louis, MO. *n*-phenylalanyl-L-prolylarginine chloromethyl ketone (PPACK), and aprotinin were purchased from Calbiochem Corporation, San Diego, CA. PMSF and Triglycerides-GB enzymatic assay kit were purchased from Roche Diagnostics, Indianapolis, IN. [1,2-<sup>3</sup>H(N)]cholesterol, and [<sup>14</sup>C]oleic acid were purchased from NEN Life Science Products, Boston, MA. CytoScint scintillation cocktail was purchased from ICN Biomedicals, Inc, Irvine, CA. Stigmasterol was purchased from Steraloids, Wilton, NH.

### Cell culture

The human THP-1 monocyte-macrophage cell line was used for these studies. Cells were grown in the monocytic form in suspension at 37°C in 5% CO<sub>2</sub> in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2- $\beta$ -mercaptoethanol (50  $\mu$ M), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), glucose (1.5 mg/ml), and vitamins. This medium without FBS will be referred to as base medium. To convert THP-1 monocytes to the macrophage phenotype, 1  $\times$  10<sup>6</sup> cells were plated into 35 mm culture dishes in base medium containing 10% FBS and 50 ng/ml PMA. For all experiments, PMA was added to the medium for the entire experiment to insure that the cells remained fully differentiated into the macrophage phenotype. The cells were incubated with PMA for 72 h before use in experiments.

### Lipoprotein isolation

LDL was isolated from the plasma of healthy human volunteers using a protocol approved by the University's Clinical Research Practices Committee. Blood was drawn into tubes containing EDTA (1 mg/ml), aprotinin (25 U/ml), and PPACK (1  $\mu$ M), and plasma was separated by centrifugation at 2,750 rpm for 30 min. PMSF was added to the plasma at a final concentration of 87  $\mu$ g/ml plasma. LDL was isolated from human plasma by sequential ultracentrifugation. The plasma was overlaid with saline-EDTA at 1.006 g/ml and centrifuged for 20 h in a Beckman L8-55R ultracentrifuge at 36,000 rpm in a SW-40

VLDL. The bottom layer was adjusted to  $d = 1.08$  with solid KBr and overlaid with saline-EDTA, which had been adjusted to  $d = 1.063$  g/ml with solid KBr and centrifuged for 24 h at 36,000 rpm in a SW-40 rotor to float the LDL. LDL was dialyzed extensively against a solution containing 0.9% NaCl and 0.01% EDTA. The above procedures were carried out at 4°C. The isolated lipoproteins were sterilized by filtration through a Millipore filter (0.45  $\mu$ m) and stored at 4°C under N<sub>2</sub>. LDL was acetylated by the acetic anhydride method of Basu et al. (27), and the level of acetylation was assessed by the trinitrobenzene sulfonic acid assay (28).

### Modulation of cellular lipid composition

THP-1 macrophages were maintained in culture in the presence of PMA for 3 days before they were loaded with lipid by incubation with AcLDL. Cells were incubated with 50–100  $\mu$ g AcLDL protein per ml culture medium for the periods of time indicated in the figure legends. AcLDL was added in base medium containing 2% FBS as indicated in the figure legends.

To evaluate the incorporation of fatty acids into cellular lipids, [<sup>14</sup>C]oleate was first prepared in a fatty acid to albumin molar ratio of 6 to 1. The final concentration in each culture dish containing 2 ml culture medium was 1  $\mu$ Ci/ml [<sup>14</sup>C]oleate, with a final oleate concentration of 0.17 mM. THP-1 macrophages were incubated with [<sup>14</sup>C]oleate for 24 h in base medium containing 10% FBS over a range of triacsin D concentrations (0–25  $\mu$ M). Cellular lipids were extracted overnight with 2 ml isopropanol, and extracted lipids were separated by TLC. PL, TG, and CE bands were scraped and counted for <sup>14</sup>C dpm. Percent inhibition of [<sup>14</sup>C]oleate incorporation into cellular lipids was determined by comparing <sup>14</sup>C dpm incorporated with no triacsin D present with the <sup>14</sup>C dpm incorporated at each concentration.

Incubation with triacsin D and albumin was used to modulate the cellular TG concentration. First, THP-1 macrophages were loaded with lipids through incubation with AcLDL as indicated above. The AcLDL-containing medium was removed, the cells were washed with base medium, and incubated with the indicated concentrations of triacsin D added in base medium containing 400  $\mu$ M BSA.

### Measurement of cellular lipid and protein content

After removing the culture medium, the cells were washed three times with cold PBS, allowed to dry, and the lipid extracted overnight with 2 ml isopropanol containing 20  $\mu$ g stigmasterol as an internal standard. The isopropanol extract was analyzed for FC and total cholesterol (TC) content by gas-liquid chromatography by the procedure of Ishikawa et al. (29) as modified by Klasek et al. (30). Esterified cholesterol (EC) mass was calculated as the difference between total and FC. Cellular TG concentrations were determined by enzymatic assay on a portion of the isopropanol extract using a kit from Roche Diagnostics (Triglycerides-GB, Cat. No. 450032). Protein was measured in cells and lipoproteins by the method of Lowry et al. (31). Cell viability was determined by a colorimetric assay, which measures the reduction of MTT to form a blue formazan product (32), as modified by Denizot and Lang (33).

### Cholesterol efflux

AcLDL was labeled with [<sup>3</sup>H]FC (10  $\mu$ Ci/mg AcLDL protein) as described previously (34). THP-1 cells were lipid loaded through an incubation with [<sup>3</sup>H]FC-labeled-AcLDL for 6 days in base medium containing 2% FBS with one medium change after 3 days. Cells were then washed with base medium and equilibrated in medium containing 1% BSA for 24 h. After the equilibration period, 2 ml of efflux medium consisting of 10% FBS and 1.25  $\mu$ g/ml of the ACAT inhibitor CP113 were added. Dur-

ing a 24 h efflux period, at time points of 4, 8, 12, and 24 h, 200  $\mu$ l aliquots of the efflux medium were taken. The aliquots were centrifuged to pellet any cell debris, and a 100  $\mu$ l aliquot of the supernatant fluid was taken for scintillation counting. In addition, 50  $\mu$ l from the supernatant fluid was used to determine the percent FC in the efflux medium after extraction of lipids by the method of Bligh and Dyer (35) and separation by TLC. Cellular isopropanol lipid extracts were analyzed for cholesterol and TG mass, and for FC and CE  $^3\text{H}$  dpm after separation by TLC. Percent cholesterol efflux was calculated by dividing total [ $^3\text{H}$ ]FC in the efflux medium by the sum of [ $^3\text{H}$ ]FC and [ $^3\text{H}$ ]EC present in the cells at zero time. Percent CE hydrolysis after 24 h was calculated by dividing the [ $^3\text{H}$ ]FC formed from hydrolysis of CE by the [ $^3\text{H}$ ]CE present in cells at 0 time.

### Microscopy

THP-1 cells were plated on 25 mm round glass cover slips in 35 mm culture dishes. Prior to microscopic examination, the cover slips were removed from the dish and placed inverted on a glass slide. Cells were examined at room temperature using a neofluor (NA 1.3) oil immersion objective with both phase contrast and polarizing optics on a Zeiss Axioplan microscope. Polarizing filters were crossed at  $180^\circ$ , and cells were examined for the appearance of a formée cross, which is a positive sign of birefringence and is consistent with lipid droplets being in the liquid-crystalline or anisotropic physical state (7, 9, 13, 14, 36, 37). To evaluate the percentage of cells that contained formée crosses, slides were prepared as described above, and 10 randomly selected fields from each slide were counted for the number of cells in the field using a  $100\times$  objective. The two polarizing filters were then crossed, and the number of cells in the same fields that contained formée crosses was counted. The percent of cells containing formée crosses was calculated by dividing the number of cells containing formée crosses by the total number of cells present. In addition, within an individual cell, the percentage of lipid droplets that displayed formée crosses was determined. Using three slides from each treatment group, 10 cells from each were randomly selected. Cellular lipid droplets within a single cell were first counted using a  $100\times$  objective, and then formée crosses visible with polarizing microscopy in the same cell were counted. These values were then used to calculate the percentage of lipid droplets within individual cells that contained formée crosses.

To stain cells with Nile Red, a fluorescent vital stain for neutral lipid, cells were first rinsed three times with PBS. Two milliliters of PBS was added to each dish, and 20  $\mu$ l of a 10  $\mu\text{g}/\text{ml}$  Nile Red solution in acetone was added for a final concentration of 100 ng/ml. Cells were incubated for 15 min at  $37^\circ\text{C}$ . The cover slip was removed and placed inverted on a glass slide as indicated above. Nile Red fluorescence was examined at 490 nm excitation and 520 nm emission (38).

### Statistical analysis

All data are presented as the mean of triplicate samples  $\pm$  SD. ANOVA was used to determine if a difference was present among groups, and the Student-Newman-Keuls Test was used to determine differences between groups. Differences less than  $P = 0.05$  were considered to be significant.

## RESULTS

When macrophages, including THP-1 cells, are incubated with AcLDL, both TG and CE accumulate in the cells. In order to more accurately reflect the lipid compo-

sition of foam cells in vivo, triacsin D, an inhibitor of the enzyme acyl-CoA synthetase, was utilized to remove accumulated TG from these lipid-loaded cells. In order to confirm the inhibitory activity and specificity of triacsin D in THP-1 macrophages, cells were incubated with [ $^{14}\text{C}$ ]oleate and triacsin D (0–25  $\mu\text{M}$ ), for 24 h, and the [ $^{14}\text{C}$ ]oleate incorporation into PLs, TGs, and CEs was measured. As is shown in **Table 1**, triacsin D inhibited oleate incorporation into TGs by greater than 98% at concentrations greater than 12.5  $\mu\text{M}$ . At the same concentrations, oleate incorporation into CE was inhibited by greater than 90% and PL about 50%.

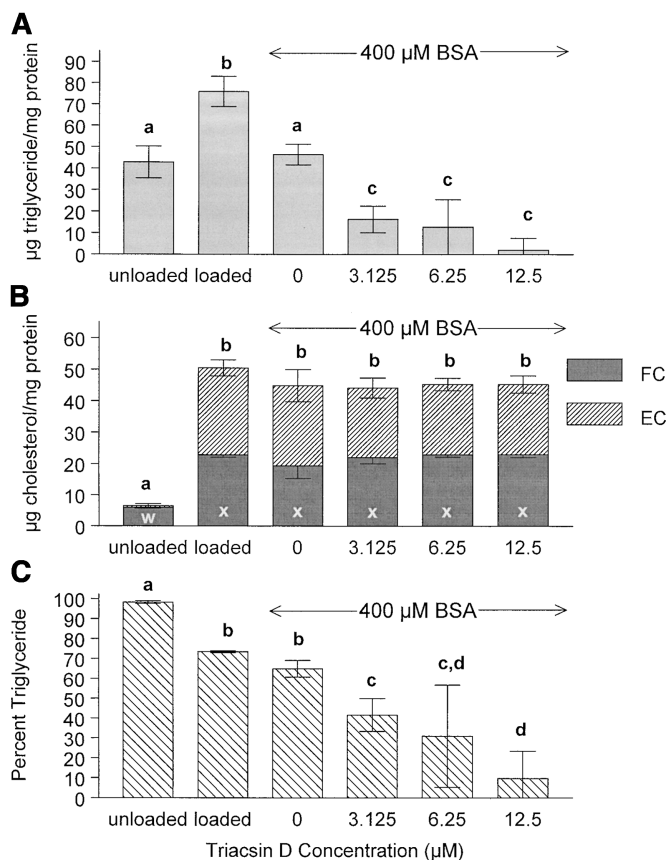
THP-1 macrophages were lipid loaded by incubation for 6 days with AcLDL, after which fresh medium containing triacsin D and 400  $\mu\text{M}$  BSA was added for 24 h. Before incubation with AcLDL, the cellular TG concentration was 43  $\mu\text{g}$  per mg cell protein and increased nearly 2-fold to 76  $\mu\text{g}/\text{mg}$  cell protein during the loading period with AcLDL (**Fig. 1A**). With no triacsin D present, reflecting the effect of BSA alone, there was removal of an amount of TG equivalent to that gained during the loading period. The addition of triacsin D resulted in additional depletion of TG concentrations with over 90% being removed at the highest concentration (12.5  $\mu\text{M}$ ) of triacsin D used. The depletion of TG by BSA alone and BSA combined with triacsin D had a minimal effect on the FC and CE content of the cells with no significant reduction at any of the concentrations used (**Fig. 1B**). **Figure 1C** demonstrates the effect of adding triacsin D together with BSA on the percentage of neutral lipid (sum of TG and EC mass) that was TG. The decrease in the percent neutral lipid as TG over increasing concentrations of triacsin D was due to the significant loss of TG combined with the minimal effect on CE. At the highest concentration of triacsin D used, 12.5  $\mu\text{M}$ , the percent neutral lipid as TG was reduced to less than 10%, which mimics macrophage foam cells from atherosclerotic lesions. Cell viability during this process was measured using the MTT assay and the measurement of cell protein per dish. There were no differences in either of these measurements before or after TG removal (data not shown).

TABLE 1. Percent inhibition of [ $^{14}\text{C}$ ] oleate incorporation into PL, TG, and CE by triacsin D

Triacsin D Concentration ( $\mu\text{M}$ )	Percent Inhibition of Oleate Incorporation		
	PL	TG	CE
6.25	38.6% $\pm$ 2.3	95.6% $\pm$ 0.3	78.9% $\pm$ 0.6
12.5	42.0% $\pm$ 0.5	98.2% $\pm$ 0.1	88.1% $\pm$ 2.0
25	55.9% $\pm$ 1.7	99.3% $\pm$ 0.1	91.5% $\pm$ 0.6

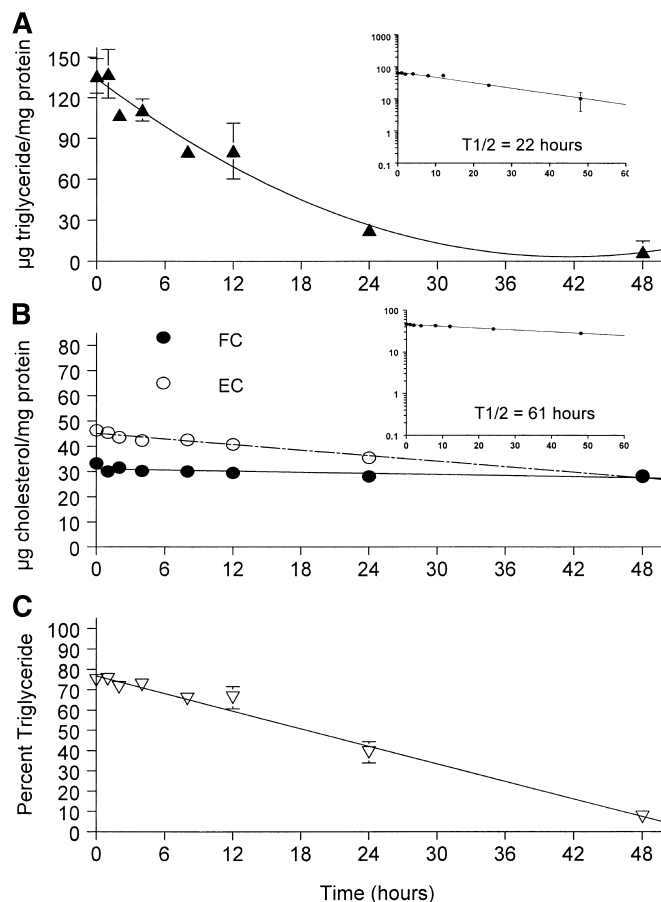
THP-1 macrophages were incubated for 24 h in base medium containing 10% FBS and 1  $\mu\text{Ci}/\text{ml}$  [ $^{14}\text{C}$ ]oleate, with a final oleate concentration of 0.17 mM. Either 0, 6.25, 12.5, or 25  $\mu\text{M}$  triacsin was added for the 24 h incubation. After incubation, cellular lipids were extracted in isopropanol and the lipids were separated by TLC. The PL, TG, and CE bands were scraped from the plates and the  $^{14}\text{C}$  dpms per lipid fraction was measured. Percent inhibition of [ $^{14}\text{C}$ ]oleate incorporation into each lipid fraction was determined by comparing the dpm/mg cell protein incorporated into each lipid fraction at each concentration of triacsin D with the amount incorporated when no triacsin was present. The data are expressed as mean  $\pm$  SD,  $n = 3$ .





**Fig. 1.** Effect of triacsin D and albumin on esterified cholesterol (EC) and TG mass when incubated with lipid loaded THP-1 cells. THP-1 macrophages were incubated with 50 µg/ml acetylated low density lipoprotein (AcLDL) in medium containing 2% FBS for 6 days to load the cells with cholesteryl esters (CEs) and TGs. Following lipid loading, the AcLDL medium was removed, and medium containing the indicated concentration of triacsin D and 400 µM BSA was added. After 24 h, the cells were analyzed for (A) TG mass, and (B) FC, TC, and EC mass as described in the methods. "Unloaded" represents THP-1 cells harvested after initial 3 day plating before incubation with AcLDL. "Loaded" represents cells harvested after 6 day incubation with AcLDL. The percent of total neutral lipid as TG was calculated by dividing the TG mass by the mass of the total neutral lipid (TG+EC), and multiplying by 100 (C). Significant differences ( $P < 0.05$ ) are represented by different letters. The data are expressed as mean  $\pm$  SD,  $n = 3$ .

**Figure 2A** shows the time course of TG removal during incubation with triacsin D and BSA. THP-1 cells were first lipid loaded for 6 days with AcLDL, the lipoproteins were removed, and medium containing 12.5 µM triacsin D and 400 µM BSA was added for up to 48 h. Slight, but non-significant decreases in TG mass occurred by 4 h. By 8 h there was a significant 40% decrease in TG mass, an 83% decrease by 24 h, and a 95% decrease after 48 h. As can be seen from the insert to Fig. 2A, under these conditions, the depletion of cellular TG mass was log linear. The loss of CE from the cells was also log linear, but considerably less EC mass was removed than TG mass (Fig. 2B). Since triacsin D is an effective inhibitor of both TG and CE synthesis (Table 1), it was possible to calculate the  $T_{1/2}$  for hydrolysis of both lipids. In this experiment, the  $T_{1/2}$  for hydrolysis of TG was 22 h while the  $T_{1/2}$  for hydrolysis of CE was 61 h. In **Table 2**, the values for the  $T_{1/2}$  for the hydrolysis of both TGs and CE from this experiment as well as two similar experiments are shown. In addition, the initial concentration of each lipid is given, as well as the percentage of neutral lipid that was TG. There were differences in the  $T_{1/2}$  values for both TG and CE hydrolysis among the experiments, and they did not correlate with either the initial concentration of TG or CE, or the percent neutral lipid as TG. The results clearly demonstrate, however, that in each experiment cellular TGs turned over more rapidly than CEs, which agrees with the results from Minor et al. (39), which demonstrated that cellular TGs turn over three to four times faster than CEs. As seen in Fig. 2C, the percent neutral lipid as TG dropped over the time course



**Fig. 2.** Time course for TG depletion of lipid loaded THP-1 cells by incubation with triacsin D and albumin. THP-1 macrophages were incubated with 75 µg/ml AcLDL in medium containing 2% FBS for 6 days to load the cells with CEs and TGs. Following lipid loading, the AcLDL medium was removed, and medium containing 12.5 µM triacsin D and 400 µM BSA was added. Cells were incubated for the indicated times, harvested and analyzed for (A) TG mass, and (B) FC, TC, and EC mass as described in Materials and Methods. The µg/mg cell protein for the cellular TC, FC, EC, and TG values before incubation with AcLDL were, TC = 10, FC = 9, EC = 1, and TG = 47.5. The percent neutral lipid as TG was calculated as described in the legend to Fig. 1 (C). The inserted graphs in A and B represent the same data with a log scale on the y-axis. The data are expressed as mean  $\pm$  SD,  $n = 3$ . Where SD error bars are not seen, they are contained within the symbol.

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TABLE 2. Half time for hydrolysis of TG and CE under conditions where re-synthesis of TG and CE was inhibited

Expt.	Initial TG Concentration (μg/mg)	Initial EC Concentration (μg/mg)	Initial TG Percent (TG/TG + EC)	TG T <sub>1/2</sub> (h)	EC T <sub>1/2</sub> (h)
I	190.8 ± 10.5	61.9 ± 4.6	75.5	14.4	43.8
II	147.8 ± 18.6	48.7 ± 7.7	75.2	25.3	51.8
III	136.1 ± 12.7	46.3 ± 0.4	74.6	21.8	61.4
			average	20.5	52.3

THP-1 macrophages were incubated with 75–100 μg/ml AcLDL in medium containing 2% FBS for 6 days. Following the AcLDL incubation, the AcLDL medium was removed, and medium containing 12.5 μM triacsin D and 400 μM BSA was added for up to 48 h. During the 48-h incubation, cells were harvested at various time points and analyzed for TG, FC, TC, and EC mass as described in the methods. The T<sub>1/2</sub> for the hydrolysis of TGs and CEs is given for three experiments with different initial cellular lipid concentrations at the start of the triacsin D and BSA incubation. The percent neutral lipid as TG using the initial concentrations of TG and EC was calculated as described in the legend to Fig. 1C. The data for TG and EC mass are given as μg lipid/mg cell protein and are expressed as mean ± SD, n = 3. The data in the last row are from the experiment shown in Fig. 2.

to below 10% after 48 h, again reflecting the greater loss of TG compared with CE. Thus, while triacsin D is not a specific inhibitor for TG synthesis at the concentration used, it results in the selective removal of TGs due to the more rapid rate of hydrolysis of cellular TGs compared with CEs.

In order to explore the effects of TG removal on the physical state of cellular lipids, cells were examined by polarizing light microscopy both before and after TG removal. THP-1 cells were first incubated with AcLDL for 7 days followed by incubation with medium containing 12.5 μM triacsin D and 400 μM albumin for 24 h. Cellular lipid concentrations were measured after each stage and values are given in the legend to Fig. 3. Under phase contrast, lipid filled cells after 7 days of incubation with AcLDL can be seen containing refractile lipid droplets (Fig. 3A). Using polarizing light microscopy, the field is dark, indicating only the presence of isotropic droplets of CE, which would be expected in cells containing 60% of neutral lipid as TG (Fig. 3B). This image is typical of the entire slide; that is, no formée crosses were visible in any of the cells. After 24 h of TG removal, lipid filled cells remain (Fig. 3C), and under polarizing light microscopy formée crosses are now seen, consistent with the presence of anisotropic droplets of CE (Fig. 3D), despite TG still representing 27% of the neutral lipid (Fig. 3D).

To quantify the percentage of cells that displayed formée crosses after TG removal, fields of cells were selected at random, and the total number of cells visible in a field and the number that contained formée crosses were counted. As stated above, before TGs were depleted, no cells displayed formée crosses. After TG removal, however, 67% of cells contained formée crosses. In addition, for cells that displayed formée crosses, virtually all (>90%) of the lipid droplets within those cells displayed a formée cross. The general trend observed was that within individual cells there either were no visible formée crosses, or the majority, if not all the lipid droplets displayed a formée

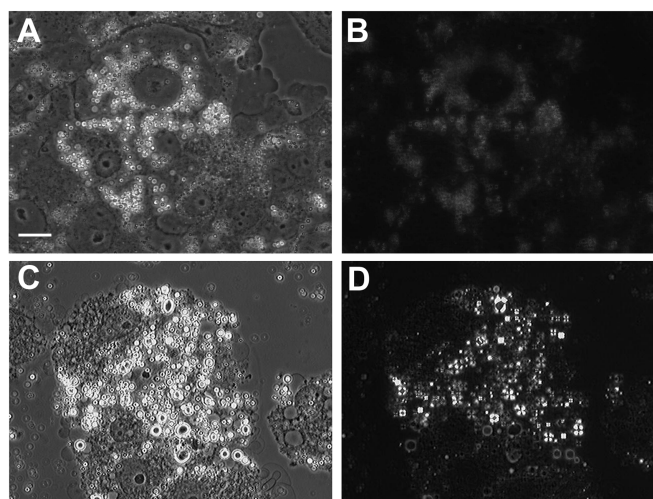
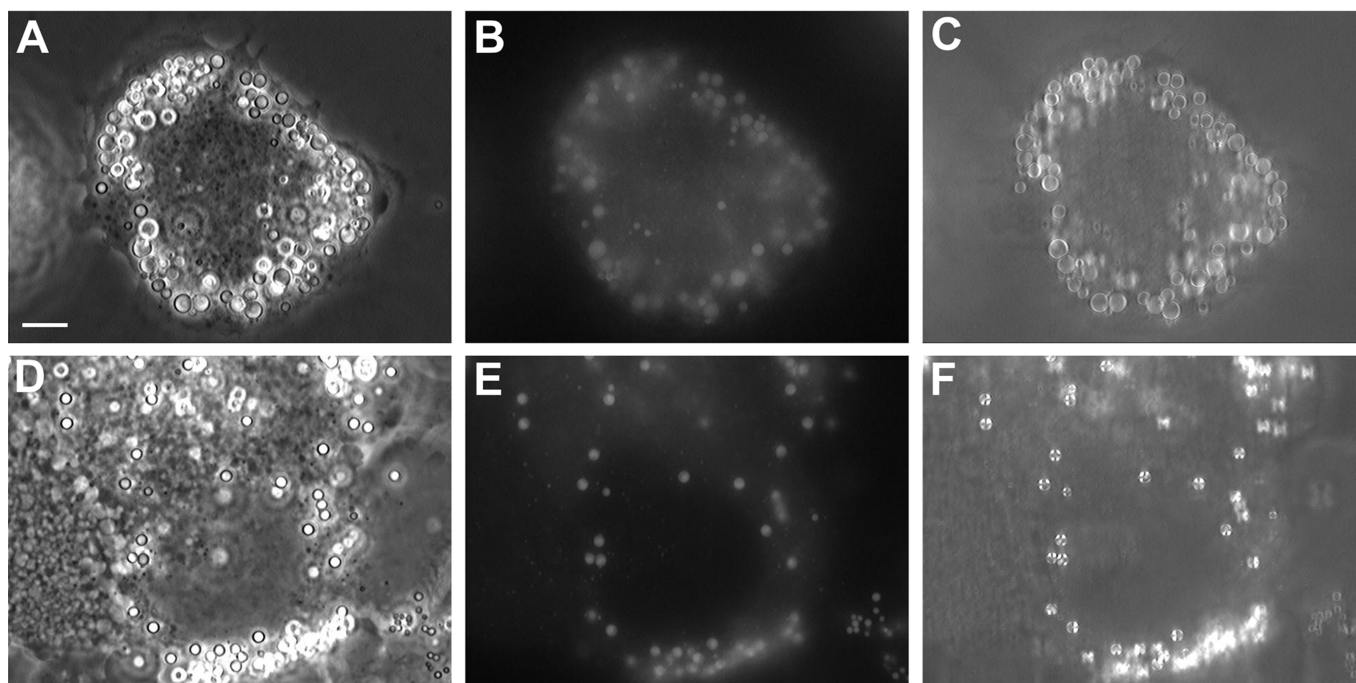


Fig. 3. THP-1 cells viewed under phase contrast and polarizing light microscopy before and after TG removal. THP-1 macrophages were grown in 35 mm dishes on a 25 mm glass cover slip and incubated for 7 days with 100 μg/ml AcLDL in medium containing 2% FBS. Following lipid loading, the AcLDL medium was removed, and medium containing 12.5 μM triacsin D and 400 μM BSA was added for 24 h. Cells were analyzed for TG, FC, TC, and EC mass as described in the Materials and Methods. Lipid content of cells after AcLDL incubation was 163 μg/mg TG, 57 μg/mg FC, 117 μg/mg EC, with 60% of neutral lipid as TG. Lipid content after 24 h TG removal was 30 μg/mg TG, 61 μg/mg FC, 82 μg/mg EC, with 27% of neutral lipid as TG. All lipid values represent the mean of three replicate samples. After each incubation, the glass cover slip was removed and placed inverted on a glass slide as described in Materials and Methods. A and B are cells viewed after the AcLDL loading period, before TG removal. The same fields were examined under phase contrast (A) and polarizing optics (B). C and D represent a lipid-loaded cell viewed after 24 h of TG removal. Identical fields were examined under phase contrast (C) and polarizing optics (D). All observations were done with a 100× objective for a total magnification of the image shown equal to 667×. The bar in the lower left corner of A is 6 μm.

cross. This trend was consistently seen during the examination of multiple fields from replicate samples over the course of three experiments.

The fluorescent neutral lipid stain, Nile Red, was used to confirm that the inclusions displaying formée crosses were indeed lipid droplets. This fluorescent dye allowed for the examination of the same field with phase contrast, polarizing, and fluorescent microscopy. Cells from the experiment shown in Fig. 3 were stained with Nile Red after the AcLDL incubation and the 24 h TG removal period. In Fig. 4, the top panels are the phase (Fig. 4A), fluorescent (Fig. 4B), and polarizing (Fig. 4C) images from the same field of cells after the AcLDL incubation period, and the lower panels are the phase (Fig. 4D), fluorescent (Fig. 4E), and polarizing (Fig. 4F) images after the 24 h TG removal period. Cellular lipid droplets visible under phase contrast in Fig. 4A and D stain positive with Nile Red, as seen in Fig. 4B and E. In Fig. 4C, cells that did not undergo TG removal did not display formée crosses, similar to the results shown in Fig. 3. In Fig. 4F, formée crosses are visible in TG depleted cells corresponding to the Nile Red positive lipid droplets seen in Fig. 4E. Therefore, structures that dis-



**Fig. 4.** THP-1 cells viewed under phase, fluorescent, and polarizing light microscopy after Nile Red staining. THP-1 cells were grown in 35 mm dishes on a 25 mm glass cover slip as described in Fig. 3. After each incubation, cells were stained with Nile Red as described in the Materials and Methods, and the glass cover slip was removed and placed inverted on a glass slide. A–C are the same field viewed following the AcLDL incubation under phase, fluorescent, and polarizing optics respectively. D–F are the same field viewed following the 24 h incubation with 12.5  $\mu\text{M}$  triacsin D and 400  $\mu\text{M}$  BSA under phase, fluorescent, and polarizing optics respectively. All observations were done with a 100 $\times$  objective for a total magnification of the image shown equal to 550 $\times$ . The bar in the lower left corner of A is 6  $\mu\text{m}$ .

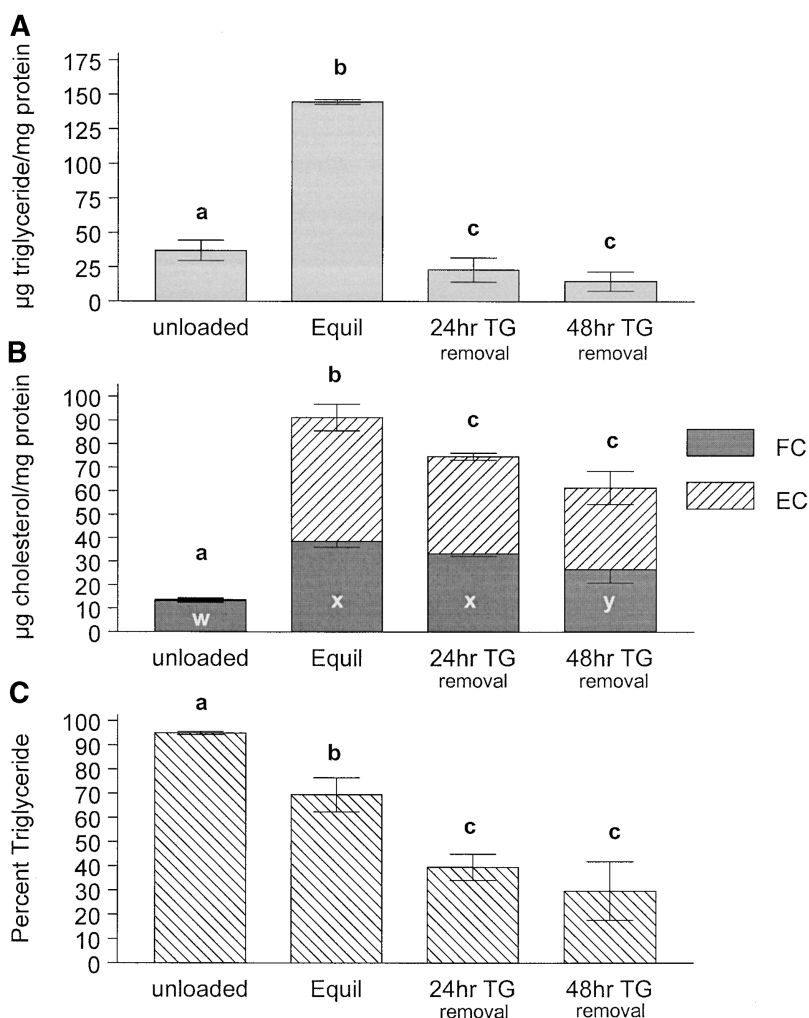
played formée crosses were lipid droplets, indicated by positive Nile Red staining, and were not an artifact produced during sample preparation. In addition, the absence of formée crosses in Fig. 4C indicates that Nile Red is not affecting the physical state of lipid droplets causing the artificial production of formée crosses.

To study the effect of TG depletion on cholesterol efflux, THP-1 cells were first incubated with [ $^3\text{H}$ ]FC labeled AcLDL for 6 days, and then equilibrated in 1% BSA medium for 24 h. To one set of dishes, efflux medium containing 10% FBS and the ACAT inhibitor CP113 was added. To a second set of dishes, TG removal medium was added consisting of 12.5  $\mu\text{M}$  triacsin D and 400  $\mu\text{M}$  BSA for either 24 or 48 h. Following the TG removal period, efflux medium was added for 24 h. In this way, the rates of cholesterol efflux in cells that contained different concentrations of TG could be compared. In addition, to determine the effect of triacsin D on cholesterol efflux, after the 48 h TG removal period, one set of dishes was given efflux medium containing 10% FBS and CP113, while another set was given efflux medium containing 10% FBS and 12.5  $\mu\text{M}$  triacsin D in place of CP113. **Figure 5A** shows the cellular TG concentrations during each stage of the experiment. Cellular TG was reduced from 145  $\mu\text{g}/\text{mg}$  protein to 23 and 15  $\mu\text{g}/\text{mg}$  protein after the 24 and 48 h TG removal periods respectively. During this same time, a small but significant amount of EC was also lost (Fig. 5, compare bars “equil” and “24 h TG removal” and “48 h TG removal”). In addition, the distribution of  $^3\text{H}$  dpm in

FC and CE at each stage was similar to that of the mass values shown in Fig. 5B (data not shown). In this experiment, the TG removal process resulted in the percent neutral lipid as TG being reduced only to 39% after 24 h and 30% after 48 h (Fig. 5C).

The percent cholesterol efflux from these cells is shown in **Fig. 6A**. In addition to comparing efflux in cells that had undergone TG removal for 0, 24, or 48 h, efflux was also compared with either the ACAT inhibitor CP113 or triacsin D in the efflux medium. At all time points, the percent cholesterol efflux was significantly higher in cells which had not undergone TG removal, and there were no differences among the other three groups, demonstrating that reduction in the percent neutral lipid as TG from 69% to either 39% or 30%, was sufficient to significantly reduce the rate of cholesterol efflux. The presence of triacsin D in the efflux medium in place of CP113 did not affect cholesterol efflux. For all conditions, at all time points, over 90% of the cholesterol appearing in the efflux medium was FC (data not shown). The FC and EC specific activities at each stage of the experiment are shown in Fig. 6B. After lipid loading, the specific activity of FC was approximately twice that of EC. During the 24 h equilibration phase, the specific activities of FC and EC approached one another and remained stable during efflux in the presence of either CP113 or triacsin D. Thus, differences in cholesterol efflux before and after TG removal cannot be explained by differences in the proportion of  $^3\text{H}$  labeled FC and EC in the cells.



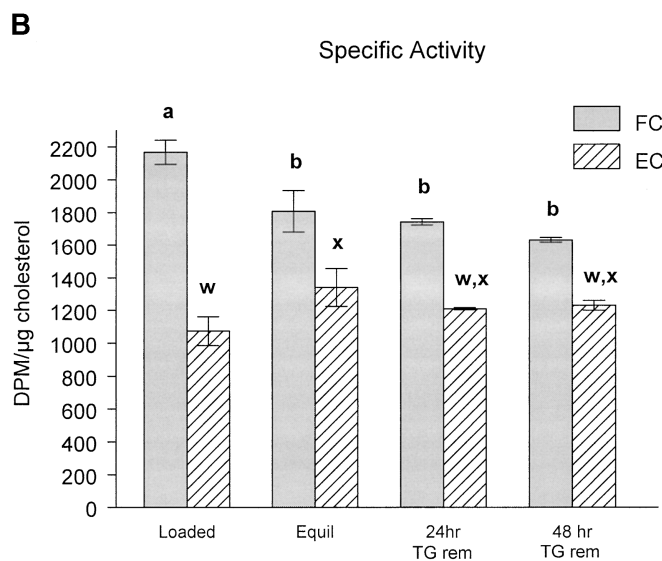
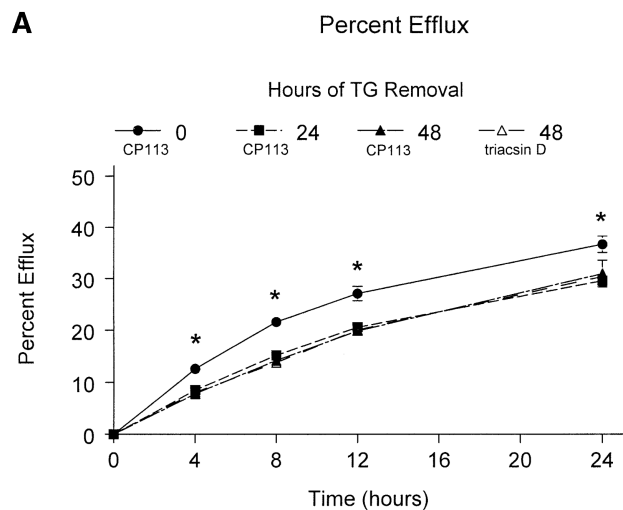


**Fig. 5.** Lipid composition of THP-1 cells before cholesterol efflux. THP-1 macrophages were first incubated with 100  $\mu\text{g}/\text{ml}$  [ $^3\text{H}$ ]FC-AcLDL in medium containing 2% FBS for 6 days to load cells with CEs and TGs. Cells were then equilibrated in medium containing 1% BSA for 24 h. Following equilibration, to one set of cells, efflux medium containing 10% FBS and 1.25  $\mu\text{g}/\text{ml}$  CP113 was added for 24 h. To a second set, TG removal medium containing 12.5  $\mu\text{M}$  triacsin D and 400  $\mu\text{M}$  BSA was added for either 24 or 48 h, after which efflux medium was added for 24 h. Efflux medium added after the 24 h TG removal period was the same as described above. After the 48 h TG removal period, one set of dishes received efflux medium consisting of 10% FBS and 1.25  $\mu\text{g}/\text{ml}$  CP113, and a second set of dishes received medium consisting of 10% FBS and 12.5  $\mu\text{M}$  triacsin D. After each stage, cells were harvested and analyzed for TG mass (A), FC, TC, and EC mass (B) as described in Materials and Methods. The percent neutral lipid as TG was calculated as described in the legend to Fig. 1 (C). “Unloaded” represents THP-1 cells harvested before incubation with AcLDL. “Equil” represents cells harvested after the 1% BSA equilibration period. “24 h TG removal” and “48 h TG removal” represent cells to which TG removal medium was added following the 1% BSA equilibration period for 24 or 48 h respectively. Significant differences ( $P < 0.05$ ) are represented by different letters. The data are expressed as mean  $\pm$  SD,  $n = 3$ .

During cholesterol efflux, CE synthesis was inhibited by the addition of either the ACAT inhibitor CP113 (40) or triacsin D (Table 1) to the efflux medium, which allowed for the calculation of CE hydrolysis. The relationship of CE hydrolysis to cholesterol efflux in the same cells is shown in **Table 3**. There was a significantly higher rate of CE hydrolysis in cells that had not undergone TG removal than in those that had for either 24 or 48 h. This higher rate of CE hydrolysis is reflected by a higher rate of cholesterol efflux. In all cases, the percent cholesterol efflux measured in the same cells was greater than the percent CE hydrolyzed, con-

sistent with the efflux of some preexisting [ $^3\text{H}$ ]FC which had a higher specific activity (Fig. 6B) as well as the efflux of [ $^3\text{H}$ ]FC derived from hydrolysis of [ $^3\text{H}$ ]CE.

**Figure 7** shows that the proportion of CE and TG is not equal among cells in the same culture dish. The cells shown in Fig. 7 were first incubated with AcLDL for 6 days, and then incubated with TG removal medium for 24 h. The lipid content of the cells after TG removal is given in the figure legend. Figure 7A shows a field under phase contrast in which several lipid loaded cells can be seen containing refractile lipid droplets. In this experiment, after



**Fig. 6.** Effect of TG concentration on cellular cholesterol efflux. Cholesterol efflux was compared in lipid-loaded cells, which did not undergo TG removal, and those that had as described in Fig. 5. In A, percent efflux was calculated by dividing the amount of [ $^3\text{H}$ ]FC dpm in the medium at each time point by the total  $^3\text{H}$  dpm present in the cells at zero time. The mean ( $n = 3$ )  $^3\text{H}$  dpm present in cells at zero time after 0, 24, and 48 h of TG removal was 148,960, 112,054, and 101,870 dpm/mg cell protein respectively. The value for 48 h of TG removal when triacsin D was included in the efflux medium was 100,196. A: An asterisk represents significant differences ( $P < 0.05$ ). Where error bars are not visible, they are contained within the symbol. The data are expressed as mean  $\pm$  SD,  $n = 3$ . B: Shows cellular FC and EC specific activity at each stage of the experiment. Significant differences ( $P < 0.05$ ) among FC values are indicated by the different letters, a–b, and among EC values are indicated by the different letters, w–x.

TG removal, TG still comprised 48.3% of the neutral lipid in the culture dish. Nevertheless, anisotropic lipid droplets were visible in some cells but not others (note formée crosses in cells in only the top half of the field in Fig. 7B and C). Figure 7B and C are the same field viewed with polarizing light microscopy in which the plane of focus was altered. Due to the 3-D structure of cellular lipid droplets, the pattern of formée crosses in the upper portion of

**TABLE 3.** Percent hydrolysis of CE and efflux of FC after 24 h following different times of TG removal

Hours of TG Removal	Percent EC Hydrolysis After 24 h	Percent FC Efflux After 24 h
0 (CP113)	26.5 $\pm$ 0.5 <sup>a</sup>	36.7 $\pm$ 1.6 <sup>a</sup>
24 (CP113)	20.4 $\pm$ 1.3 <sup>b</sup>	29.6 $\pm$ 0.3 <sup>b</sup>
48 (CP113)	18.4 $\pm$ 1.4 <sup>b,c</sup>	31.0 $\pm$ 2.6 <sup>b</sup>
48 (triacsin D)	15.9 $\pm$ 1.8 <sup>c</sup>	30.4 $\pm$ 1.0 <sup>b</sup>

THP-1 cells were treated during the efflux phase of the experiment as described in the legend to Fig. 5. The percent CE hydrolysis was calculated from the 24 h cholesterol efflux time point by dividing the FC produced through CE hydrolysis by the EC present at 0 time. The mean ( $n = 3$ ) dpm/mg protein of EC present at 0 time for the 0, 24, and 48 h (with CP113) and 48 h (with triacsin D) time points was 74,458, 51,830, 44,202, and 43,476 respectively. The 24 h percent efflux time point is the same as that in Fig. 6. Significant differences ( $P < 0.05$ ) among the groups are represented by different letters (a–c). The data are expressed as mean  $\pm$  SD,  $n = 3$ .

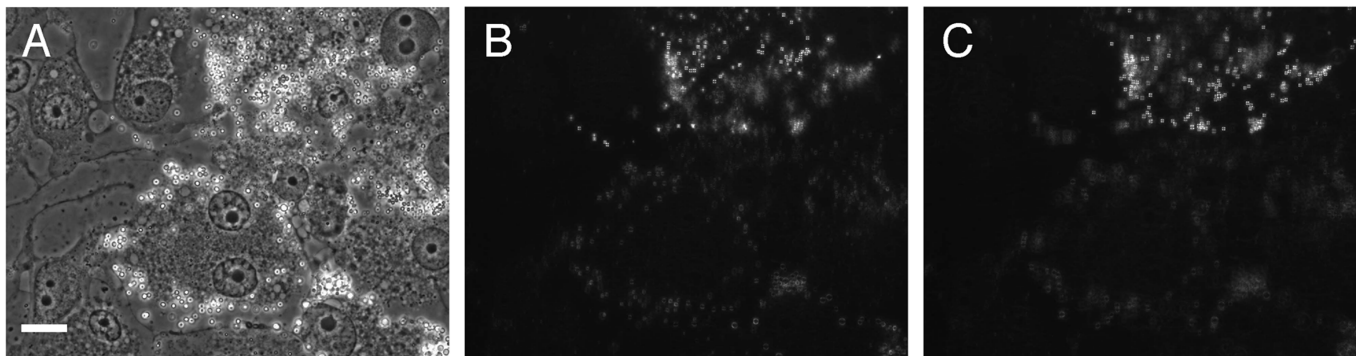
the field changes as the plane of focus was altered, but anisotropic droplets appear in each field. In contrast, formée crosses were never visible in the cells in the lower portion of the field regardless of the plane of focus (Fig. 7B and C), confirming that during TG depletion, the lipid droplets of some cells become anisotropic, while under the same conditions the lipid droplets of adjacent cells remain isotropic.

## DISCUSSION

In atherosclerotic lesions and in foam cells isolated from lesions, the majority of the accumulated lipid has been reported to be CE and FC, with TG usually making up less than 10% of the total lipid (7, 8, 10–12). Studies by others have shown that when THP-1 macrophages and other cell types are incubated with lipoproteins in culture, both CE and TG accumulation occurs (20, 21, 22–26), and our data are consistent with these reports (Figs. 1, 2, and 5). Triacsin D in combination with albumin was used to remove accumulated TG. Triacsin D, (triacsin A–D), are compounds that have been shown to inhibit acyl-CoA synthetase (41, 42). This enzyme catalyzes the conversion of free fatty acids to their active thioesters, acyl-CoAs, which is a requirement for esterification of fatty acids in the synthesis of CE and TGs.

Similar to CEs, TGs also turn over in a cycle of hydrolysis and re-esterification of their fatty acids (43, 44). In smooth muscle cells, for example, TGs turn over three to four times faster than CEs (39). Assuming a similar relationship was present in THP-1 cells, the strategy used was to first load cells with lipid through incubation with AcLDL, remove the lipoproteins, and then add medium containing triacsin D and albumin. Triacsin D prevented the re-esterification of the fatty acids released from hydrolysis of TGs as they turned over (Table 1), and the albumin in the medium provided an acceptor for the free fatty acids released from the cells. Using this approach, it was possible to reduce TG concentrations in a time dependent manner, with minimal effect on EC concentrations





**Fig. 7.** Differences in lipid droplet physical state among cells. THP-1 cells were plated in 35 mm dishes on a 25 mm glass cover slip and incubated with 100  $\mu\text{g}/\text{ml}$  AcLDL in medium containing 2% FBS for 6 days, and then incubated with 12.5  $\mu\text{M}$  triacsin D and 400  $\mu\text{M}$  albumin for 24 h. Following the TG removal period, three dishes of cells were harvested and analyzed for TG, FC, TC, and EC mass as described in Materials and Methods, and the percent neutral lipid as TG was calculated as described in the legend to Figure 1. The average  $\mu\text{g}/\text{mg}$  cell protein for the cellular EC, FC, and TG values were EC = 57.0, FC = 32.9, and TG = 53.6. The percent neutral lipid as TG was 48.3%. In addition, from a fourth dish, the glass cover slip was removed and placed on a glass slide and viewed with phase contrast and polarizing light microscopy. The same field was viewed under phase contrast (A) and with polarizing light microscopy (B, C). B and C are the same field in which a different plane of focus was selected. The field was observed with a 100 $\times$  objective for a total magnification of the image shown equal to 433 $\times$ . The bar in the lower left corner of A is 6  $\mu\text{m}$ .

(Fig. 2). The more rapid reduction in TG content was due largely to its faster rate of hydrolysis compared with CE. In three separate experiments, the  $T_{1/2}$  for hydrolysis of TG was 2–3 times faster than for the hydrolysis of CE (Table 2), consistent with reports that TGs turn over faster than CEs in other cell types (39), and that THP-1 macrophages hydrolyze CEs slowly (24).

Our data also demonstrate that the reduction of cellular TG can alter the physical state of CE in lipid droplets. After incubation with AcLDL, cells accumulated both TG and CE, and no formée crosses were visible, indicating the presence of only isotropic lipid droplets. Anisotropic lipid droplets were visible after TG was removed, indicating a change in physical state, and demonstrating that initially cellular CEs and TGs were present in mixed lipid droplets, with enough TG present to cause CE to exist in an isotropic state. Lipid droplets are present in every mammalian cell, and typically are composed of a core of TG and CE surrounded by proteins (45, 46). However, this composition can vary widely. For example, in adipocytes, droplets are composed mainly of TG (46), while in foam cells of atherosclerotic lesions, droplets are composed almost entirely of CE (8, 47, 48). In addition, atherosclerotic lesions have been shown to contain anisotropic lipid droplets, indicative of very little TG (7, 9, 49–51). Thus, after incubation with AcLDL, lipid-loaded THP-1 macrophages differ from foam cells *in vivo* in that cellular lipid droplets contain much higher concentrations of TG, and no droplets are anisotropic. It is only after removal of some of the TG that the physical state of the CE in these THP-1 macrophages begins to resemble true foam cells from atherosclerotic lesions.


Polarizing light microscopy was used to determine the physical state of cellular lipid droplets in intact cells. When viewed using polarizing light microscopy, lipid droplets in an isotropic physical state appear dark, while those in an anisotropic state display a formée cross (7, 9,

13, 14, 36, 37). It should be noted that the polarizing light microscopy observations were made at room temperature, thus, they may not exactly reflect the physical state of mixed droplets at 37°C. Clearly, however, there are changes in the physical state of CE after TG removal that can be viewed at room temperature (Figs. 3 and 4). In addition, the physical state changes visualized at room temperature after TG removal were supported by differences in cholesterol efflux and CE hydrolysis measured at 37°C (Fig. 6, and Table 3).

The presence of cellular TG could affect cholesterol efflux by two possible mechanisms. First, by creating a more fluid CE physical state, the interaction between CE and the enzyme(s) responsible for hydrolysis could be facilitated, resulting in an increased rate of hydrolysis. The hydrolysis of CE is generally considered to be the rate-limiting step in removal of CE from cells (52, 53). Second, if a common enzyme is responsible for the hydrolysis of both CE and TG, accumulated TG could reduce CE hydrolysis through substrate competition. Since CE hydrolysis was enhanced in the presence of TG, our data would argue against this second possibility. The enhanced cholesterol efflux observed with higher cellular TG concentrations is consistent with that of others indicating that TG can alter CE physical state, and that the physical state of CE can affect cholesterol efflux (16, 17, 39, 54, 55). These results point to the importance of controlling cellular TG concentrations when carrying out studies on the clearance of CE from cells. Because of cell-to-cell variability in the TG and CE content of lipid droplets, it is difficult to determine a value to which TG should be reduced to eliminate the effect on the physical state of CE and efflux of cholesterol from cells. Results from Adelman et al. (17), however, indicate that the rate of cholesterol efflux will be proportional to the TG concentration. Thus, cellular TG probably should be reduced to the lowest level possible to eliminate its effects on cholesterol efflux and to more

closely mimic macrophage foam cells of atherosclerotic lesions.

Data from studies using isolated lipids indicate that only small amounts of TG (2–12% of total cellular lipid) may be necessary to alter CE physical state (18, 37, 56). However, after TG depletion, when TG represented 27% (Fig. 3) and even 48% (Fig. 7) of the neutral lipid in the entire dish of THP-1 cells, liquid-crystalline lipid droplets were visible. If TG and CE were evenly distributed among the droplets, especially with 48% of the neutral lipid as TG, it would be expected that all of the CE would be in the liquid state and no formée crosses would be seen. Two lines of evidence are presented in these studies that suggest that following TG depletion, CE and TG are not distributed equally among cells resulting in different proportions of these lipids among cells in the same dish. First, after TG was depleted, formée crosses were visible in 67% of cells, and within these cells, virtually all lipid droplets (>90%) displayed a formée cross. Within the same dish, some cells did not contain any formée crosses, while in others, formée crosses were visible in most, if not all, lipid droplets (Fig. 7). Together, these data indicate there is homogeneity in TG and CE content among lipid droplets within an individual cell, but considerable variability in the proportions of TG and CE among cells. It is not certain whether these cell-to-cell variations are due to differences in AcLDL uptake, or in the turnover of cellular lipids during the TG depletion period. Clearly, however, cells within the same dish had different lipid compositions despite having differentiated from a uniform monocytic cell line and incubated under identical conditions. In studies using Fu5AH hepatoma cells, others have suggested that there could be variability in the physical state of lipid droplets within the same cell (57). The reason for the discrepancy between the results in Fu5AH cells and our results in THP-1 macrophages is unclear, but most likely is a reflection of the different origins of Fu5AH cells and THP-1 cells. This conclusion agrees, however, with the observations made in atherosclerotic arteries (49, 50) and foam cells from Tangier Disease patients (36), that within cells, lipid droplets are fairly homogenous in their lipid content and physical state while there is considerable heterogeneity among cells.

In conclusion, these studies demonstrate, using a novel method to selectively deplete cells of TG, that lipid loading of THP-1 macrophages with AcLDL generates mixed intracellular lipid droplets containing both TG and CE. Lipid droplets within a cell have uniform physical state suggesting a similar composition. In contrast, there was cell-to-cell variability in lipid droplet physical state after TG depletion indicating differences among cells in the same dish in their lipid composition. The alterations in the physical state of cellular lipids after TG depletion reduced the rates of CE hydrolysis and the efflux of cholesterol from the cells. 

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