Role of adipose differentiation-related protein in lung surfactant production: a reassessment

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Abstract **Based on data developed with the use of isolated lipid droplets from neonatal rat lung lipofibroblasts, we speculated previously that the droplet coat protein, adipose differentiation-related protein (ADFP), mediated the transfer of lipids into type 2 lung epithelial cells for the production of surfactant phospholipids. The present studies were designed to test the role of ADFP in this transfer with the use of ADFP-coated lipid droplets from CHO fibroblast cells and a cultured human lung epithelial cell line. We found no role for ADFP in the lipid transfer and conclude that a lipase associated with the lipid droplets hydrolyzes their core triacylglycerols, releasing fatty acids that are taken up by the epithelial cells.—**Magra, A. L., P. S. Mertz, J. S. Torday, and C. Londos. **Role of adipose differentiation-related protein in lung surfactant production: a reassessment.** ***J. Lipid Res.*** **2006.** 47: **2367–2373.**

Supplementary key words lipid droplets • surfactant phospholipid • PAT proteins

Intracellular neutral lipid droplets are coated with proteins of the PAT family (for Perilipin, ADFP, and TIP47) (1, 2). Perilipin was the first member identified and is expressed only in adipocytes and steroidogenic cells. Subsequently, a homologous protein, adipose differentiation-related protein (ADFP), was identified as a ubiquitously expressed lipid droplet-associated protein (3, 4). This protein has also been termed "adipophilin," although it is sparsely expressed in adipose cells despite high mRNA levels (3). A third protein, tail-interacting protein of 47 kDa (TIP47), is closely related in sequence to ADFP and associates with lipid droplets (5, 6). All three of these PAT genes contain a common sequence motif that represents a conserved pattern for lipid storage droplet proteins found in both vertebrates and invertebrates; thus, these proteins are likely derived from a common ancestral gene (7).

Previous studies demonstrated that *ADFP* mRNA expression in lung is second only to that in adipose tissue (3). This finding was surprising because lung is not known to store large quantities of neutral lipid, and ADFP expression is closely associated with the presence of neutral lipid storage droplets. Our group later found that high expression of ADFP occurs in lung lipofibroblasts (8). These cells are thought to store fatty acids in lipid droplets before their transfer to type 2 epithelial cells, which use the lipids to synthesize lung surfactant (9). Lipofibroblasts preloaded with radiolabeled fatty acids to form triacylglycerol (TAG) appeared to pass these fatty acids on to type 2 epithelial cells when the two cell types were cocultured (9). The type 2 cells then secreted radiolabeled fatty acids as part of the structure of surfactant phospholipids (9). Subsequently, we discovered that isolated ADFP-coated lipid droplets were an efficient substrate for the transfer of fatty acids into type 2 cells and incorporation into surfactant phospholipids (8). Surprisingly, antibodies against ADFP blocked this transfer, leading us to speculate that ADFP might mediate the transfer of lipids from the isolated droplets to the epithelial cells. This speculation was reinforced by the finding that the C terminus of TIP47 forms a four-helix bundle similar to the N terminus of apolipoprotein E (10). ADFP is also predicted to contain this structural element, which could facilitate the receptor-mediated uptake of ADFP-coated lipid droplets by a process similar to the uptake of very low density lipoproteins through apolipoprotein E binding to its receptor. In this study, we explored the possible role of ADFP in the transfer of lipids into cultured lung A549 epithelial cells. We found no direct role for ADFP in this process. Rather, a lipase(s) present in the lipid droplet preparation apparently hydrolyzes the droplet TAG, and the released fatty acids are taken up by the A549 cells, accounting for the intercellular transfer of lipids.

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EXPERIMENTAL PROCEDURES

Materials

Human lung carcinoma A549 cells and CHO-K1 cells were obtained from the American Type Culture Collection (Manassas, VA). FITC-conjugated AffiniPure goat anti-rabbit IgG and goat serum were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). RPMI 1640 and Ham's F12 medium were from Invitrogen (Carlsbad, CA). Penicillin/streptomycin (100×) and glutamine were from Biosource (Rockville, MD), and oleic acid was from Calbiochem (La Jolla, CA). Fatty acid-free BSA, FBS, paraoxon-ethyl (E600), triolein, egg yolk phosphatidylcholine (PC), soybean phosphatidylinositol (PI), and *Candida rugosa* lipase were from Sigma-Aldrich (St. Louis, MO). Bodipy 493/503 was obtained from Molecular Probes (Eugene, OR). Glass-bottom 35 mm (diameter) tissue culture dishes were purchased from MatTek Corp. (Ashland, MA). Triascin C was from Biomol (Plymouth Meeting, PA), and [9,10-^{3}H]oleic acid was from Perkin-Elmer (Boston, MA). CytoScint was from ICN (Irvine, CA), and pH 10.0 potassium carbonate standardization buffer was from Fisher Scientific (Hampton, NH). Complete protease inhibitor cocktail tablets (EDTA-free) were from Roche Diagnostics Corp. (Indianapolis, IN). Falcon 150 mm (diameter) tissue culture dishes were obtained from Becton Dickinson Labware (Franklin Lakes, NJ).

Cell culture

A549 cells were maintained in RPMI medium supplemented with 10% (v/v) FBS. CHO cells were cultured in Ham's F12 medium supplemented with 10% (v/v) FBS, 1.176 g/l sodium bicarbonate, and 2 mM glutamine. The media for all cells were supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were maintained at 37°C in a humidified atmosphere of 5% (v/v) CO_2.

CHO cell lipid loading and fat cake isolation

CHO cells were plated at a density of 6×10^5 cells per 150 mm tissue culture dish. After the cells were confluent, the medium was replaced with F12 medium supplemented with 400 μM oleic acid complexed to 66.7 μM BSA (6:1, mol/mol) and incubated overnight to promote triglyceride accumulation. The cells were rinsed three times with PBS, scraped into 5 ml of ice-cold PBS per dish, and pelleted by centrifugation (300 *g* for 5 min at 4°C). The pellet was resuspended in 6 ml of ice-cold hypotonic lysis solution containing 10 mM Tris, 1 mM EDTA, pH 7.4, 10% (v/v) glycerol, 0.02 mg/ml pancreas extract, 0.02 mg/ml chymotrypsin, 0.5 μg/ml thermolysin, 0.02 mg/ml trypsin, and 0.33 mg/ml papain. The cells were lysed by freeze/thaw cycle (−80°C for 45 min), followed by 10 passages through a 23 gauge needle. The resulting lysates were layered onto 4 ml of a sterile 80% (w/v) sucrose solution and centrifuged at 165,000 *g* in a Beckman Coulter Optima XL100K ultracentrifuge for 1 h at 4°C to isolate the floating lipid layer (hereafter referred to as fat cake).

Antibodies

For ADFP immunofluorescence and fat cake incubation, an anti-ADFP IgG fraction raised in rabbits against the N-terminal 26 amino acids of murine ADFP and isolated on a protein A column (Bio-Rad Laboratories) was used. The secondary antibody used for immunofluorescence was FITC-conjugated AffiniPure goat anti-rabbit IgG.

Incubation of A549 cells with lipid droplets and an anti-ADFP antibody

A549 cells were plated at a density of 2.5×10^5 cells/dish in glass-bottom 35 mm dishes and allowed to adhere. Cells were subsequently serum-starved by incubating overnight in RPMI medium containing 0.2% (w/v) BSA. Fat cake was isolated from CHO cells and incubated in the presence or absence of 2 or 20 μg/ml rabbit anti-ADFP IgG fraction for 1 h at 4°C with constant rotation. The fat cake with ADFP antibody was then added to the A549 cells for overnight incubation at 37°C in a 5% (v/v) CO_2 humidified atmosphere. Lipid droplet formation in the A549 cells as a result of fat cake feeding was observed with a Zeiss LSM 510 Meta confocal microscope.

Immunofluorescence and confocal imaging

CHO or A549 cells were plated in glass-bottom 35 mm dishes at a density of 2.5×10^5 cells/dish and allowed to adhere overnight. The cells were lipid-loaded as described previously or incubated overnight in cell medium supplemented with 0.2% (w/v) BSA. After lipid loading or serum starvation, the cells were fixed in 3% (v/v) paraformaldehyde in PBS for 1 h at room temperature. After fixation, cells were rinsed two times with PBS (pH 7.4). Nonspecific binding was blocked for 2 h with 3 mg/ml goat serum in PBS with 0.1% (w/v) saponin and 0.75 mg/ml glycine. Dishes were then rinsed two times in PBS with 0.1% (w/v) saponin.

For detection of ADFP, the cells were incubated overnight at 4°C with a polyclonal rabbit anti-ADFP IgG fraction at a 1:100 dilution. Cells were then rinsed three times with PBS containing 0.1% (w/v) saponin and incubated with a FITC-conjugated goat anti-rabbit IgG antibody at a 1:50 dilution in the presence of 3 mg/ml goat serum for 1 h in the dark at room temperature. The cells were subsequently rinsed three times with PBS containing 0.1% (w/v) saponin before viewing. All staining was observed with a Zeiss LSM 510 Meta confocal microscope.

Lipid droplet staining

To detect intracellular lipid droplets, A549 cells were incubated with Bodipy 493/503 at a final concentration of 1 μg/ml in PBS for 5 min at room temperature. Cells were then washed two times in PBS and viewed in 0.2% (w/v) RPMI medium.

Inhibition of lipid droplet formation with triascin C

A549 cells were plated at a density of 2.5×10^5 cells/dish in 35 mm glass-bottom dishes. The cells were serum-starved for 7 h by culturing in RPMI medium containing 0.2% (w/v) BSA before the addition of 2.5 μM triascin C (an acyl-CoA synthetase inhibitor) and 100 μl of CHO fat cake to 1 ml of medium. Control cells received either 100 μl of fat cake or no additions. After an overnight incubation at 37°C in a 5% (v/v) CO_2 humidified atmosphere, A549 cells were viewed with a Zeiss LSM 510 Meta confocal microscope.

Preparing micelles

Phospholipid-coated TAG micelles were prepared according to Holm et al. (11). Twelve milligrams (13.4 μmol) of triolein was mixed with 1.2 mg of phospholipids in a small Erlenmeyer flask. The phospholipids were a mixture of PC and PI at a 3:1 ratio (w/w) of PC/PI. Solvents were evaporated, and 7.2 ml of 0.1 M potassium phosphate, pH 7.0, was added to the dried lipids. Samples were sonicated for two cycles with a Misonix Sonicator 3000 at a setting of 4 (12 W output). Each cycle consisted of a 1 min pulse at room temperature followed by a 1 min rest period. Samples were then cooled on ice followed by sonication on ice, four cycles of 30 s each with 30 s between each cycle. Samples were kept

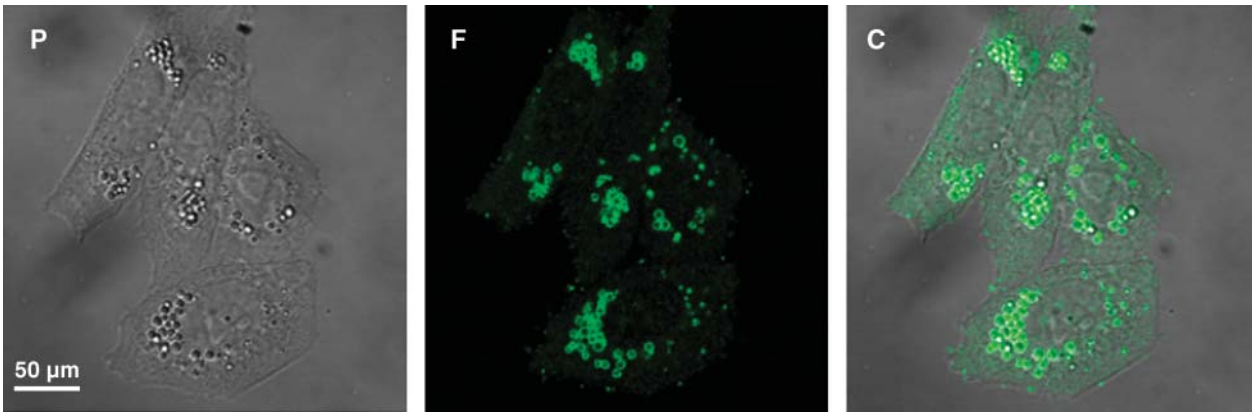

Figure 1. Detection of adipose differentiation-related protein (ADFP) associated with lipid droplets in CHO cells. CHO cells were lipid-loaded overnight, fixed, and stained with an IgG fraction of a rabbit anti-ADFP antiserum (1:100 dilution) as described in Experimental Procedures. The secondary antibody was a FITC-conjugated AffiniPure goat anti-rabbit IgG (1:50 dilution). ADFP, detected by the green staining, appears as rings around the lipid droplets. P, phase contrast; F, fluorescence; C, combined phase-contrast and fluorescence images.

on ice between pulses. After sonication was complete, 0.8 ml of 20% (w/v) defatted BSA in 0.1 M potassium phosphate, pH 7.0, was added and the solution was mixed. Micelle size and integrity were checked by preparing slides followed by analysis with a Zeiss LSM 510 Meta confocal microscope. A portion of the micelle preparation was treated with 440 U/μl *C. rugosa* lipase to induce hydrolysis. One hundred microliters of the micelle preparations in 1 ml of RPMI medium with 0.2% (w/v) BSA was added to A549 cells plated in 35 mm glass-bottom dishes for overnight incubation at 37°C in a humidified 5% (v/v) CO_2 atmosphere.

Lipase assays of CHO fat cake

A 50 mg/ml stock solution of E600, a general lipase inhibitor, was prepared by diluting E600 in ethanol. Fat cake was isolated from ten 175 cm^2 flasks of CHO cells incubated overnight with

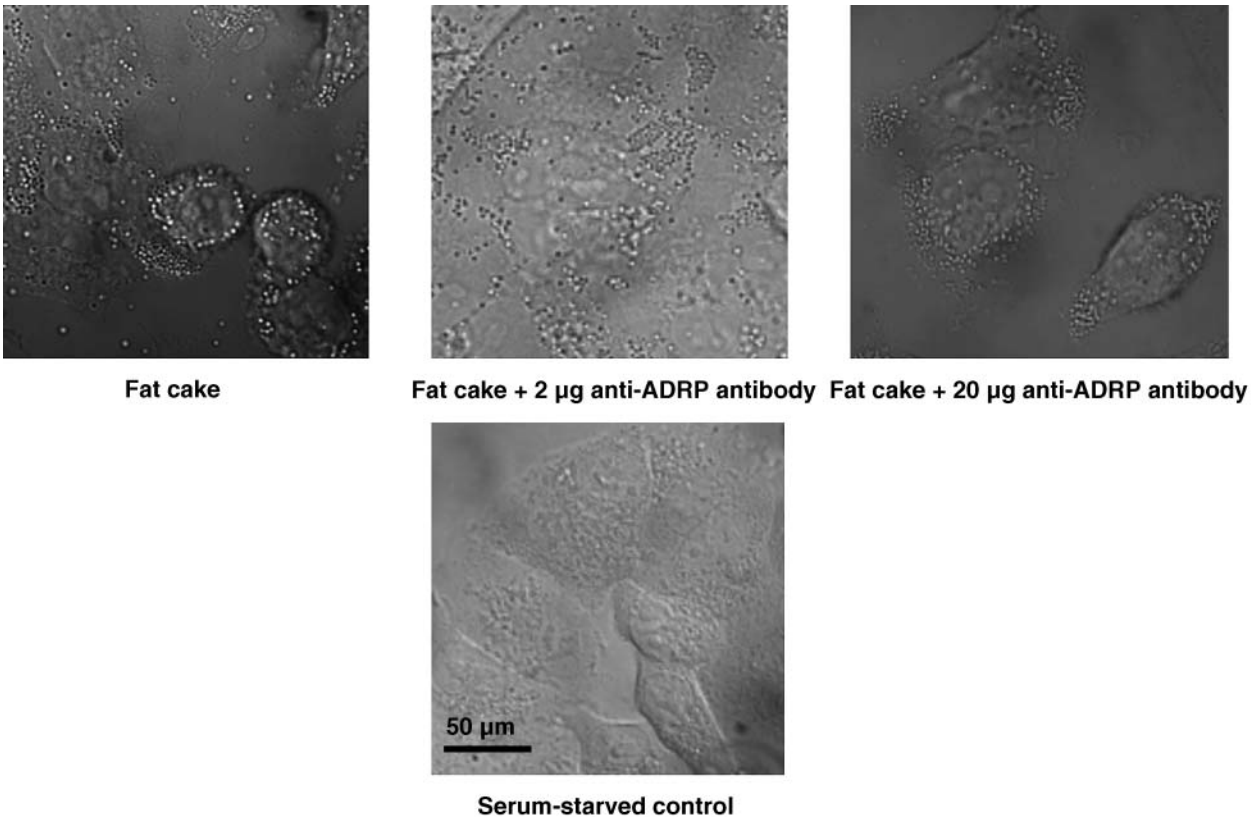

Figure 2. Preincubation of fat cake with an antibody specific for ADFP is unable to prevent lipid transfer to A549 cells. Fat cake harvested from CHO cells was preincubated with the indicated concentrations of rabbit anti-ADFP IgG fraction for 1 h. The treated fat cake was given to serum-starved A549 cells at 37°C, and the cells were viewed after ~17 h.

F12 medium containing oleic acid/BSA (6:1, mol/mol) supplemented with 2.5 μCi/ml [^{3}H]oleic acid. The volume of the fat cake was split in two portions, and 1.5 mM E600 (final concentration) was added to one half of the sample. Both samples were incubated at 37°C, and aliquots of the sample were taken at different time points. Fatty acids were extracted at each time point according to Belfrage and Vaughan (12) by adding 3.25 ml of a methanol-chloroform-heptane mixture (1.41:1.25:1, v/v/v) to 200 μl of sample (100 μl of fat cake/100 μl of distilled water). Samples were mixed well, and 1.0 ml of potassium carbonate buffer, pH 10, was added. Samples were centrifuged at 370 *g* in a Jouan C4.12 centrifuge for 15 min. Radioactivity in 0.5 ml aliquots of the upper phase containing [^{3}H]oleic acid released by endogenous lipase activity was measured in a Packard A1600 liquid scintillation counter.

E600 experiment with A549 cells

Fat cake was harvested from twenty 150 mm dishes of lipid-loaded CHO cells. Ten of the dishes were harvested with 1 mM E600 in the PBS wash buffer and 0.5 mM E600 in the hypotonic lysis buffer. Cell lysates were layered on 80% (w/v) sucrose and centrifuged in a Beckman Coulter Optima XL-100K ultracentrifuge for 1 h at 165,000 *g* at 4°C. Isolated fat cake was brought up to 8.6 ml with lysis buffer containing 1 mM E600, incubated at room temperature for 2 h, and then layered on 80% (w/v)

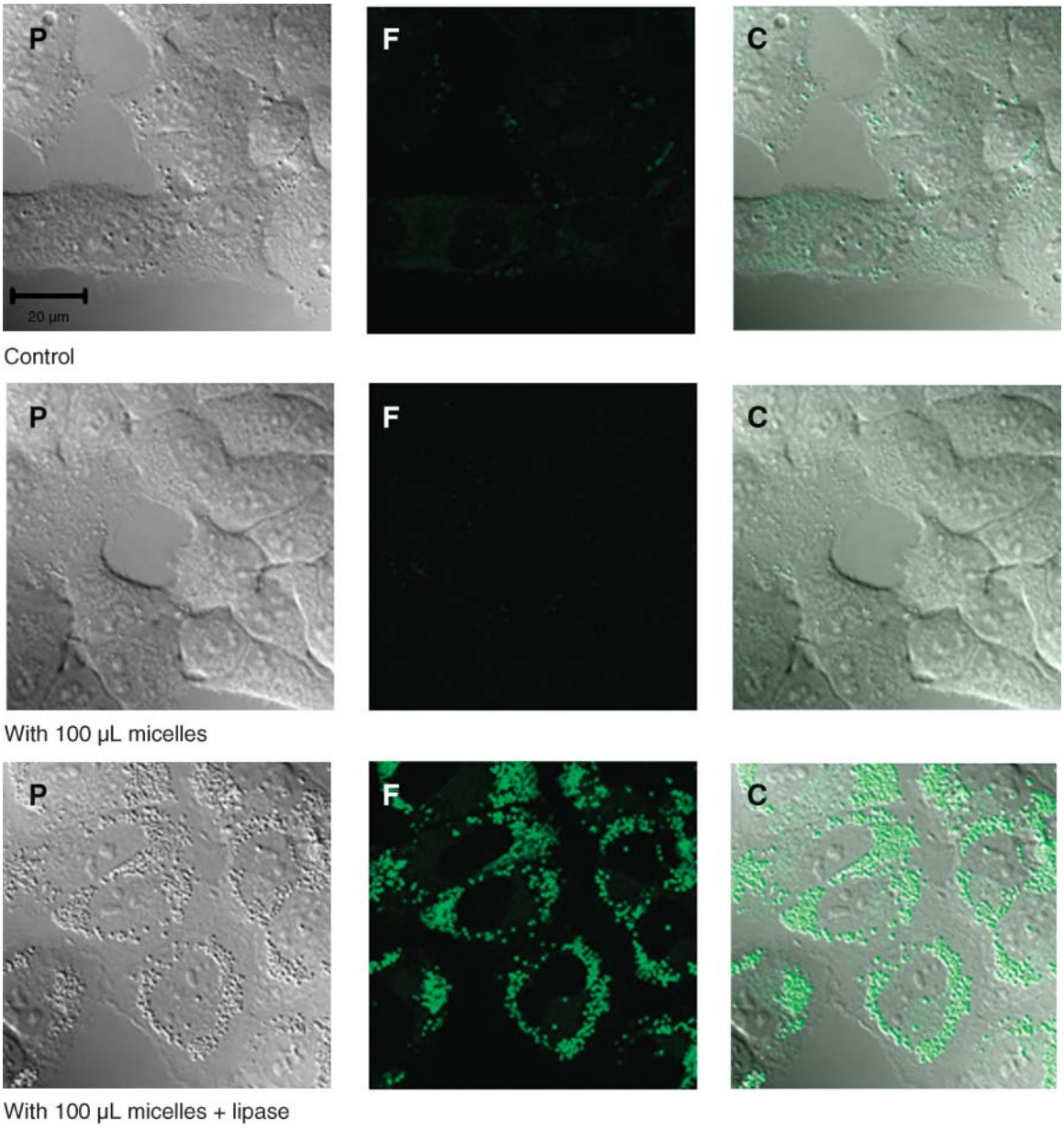

Figure 3. A549 cells are unable to accumulate intracellular lipid in the presence of synthetic micelles unless micelles are first treated with exogenous lipase. A549 cells were serum-starved overnight. Micelles consisting of triolein and phosphatidylcholine-phosphatidylinositol (3:1, w/w) were prepared by sonication as described in Experimental Procedures. A portion of this micelle preparation was treated with *C. rugosa* lipase for 1 h at 37°C to release free fatty acids. A549 cells were then incubated with 100 μl of the micelles overnight (treated with or without lipase). Control cells received only lipase in buffer. Neutral lipid droplets in the cells were detected by green staining with Bodipy 493/503. P, phase contrast; F, fluorescence; C, combined phase-contrast and fluorescence images.

sucrose for centrifugation for 1 h at 165,000 *g* at 4°C. The 10 remaining dishes of CHO cells were harvested without E600 in the buffers but treated the same otherwise except that the sample was kept on ice for the 2 h incubation between the centrifugations. Serum-starved A549 cells in 35 mm glass-bottom confocal dishes were fed with 100 μl of fat cake isolated with E600 or 100 μl of fat cake without E600. Control cells were treated only with lysis buffer (with or without E600). All cells were incubated for 19 h at 37°C in a 5% (v/v) CO_2 humidified atmosphere before viewing with the Zeiss LSM 510 Meta confocal microscope.

RESULTS

The primary goal of this study was to determine whether ADFP has a role in the transfer of neutral lipids between cells. This project was based on an earlier study suggesting that lipid droplets from primary intracellular lipofibroblasts were transferred to type 2 lung epithelial cells via an ADFP-mediated process. To this end, we recovered lipid droplets from CHO fibroblast cells ("donor" cells) that were fed to A549 (13) lung carcinoma cells ("recipient" cells). CHO cells were selected for these studies because they produce moderately sized ADFP-coated lipid droplets after lipid loading with oleic acid complexes (**Fig. 1**) (14). Immunostaining with a polyclonal antibody raised against the N-terminal 26 amino acids of murine ADFP demonstrated that droplets in these cells are coated with ADFP. All such droplets stained positive with Bodipy 493/503 (data not shown). Serum-starved A549 cells contained few visible lipid droplets (**Fig. 2**), but after incubating these cells with the lipid droplets (fat cake) derived from lipid-loaded CHO cells, the A459 cells acquired abundant cytosolic lipid droplets (Fig. 2).

Previous work from our group demonstrated that the transfer of lipids from rat lung lipofibroblasts to rat type 2 epithelial cells could be abolished by the presence of an anti-ADFP antibody. However, the same antibody failed to diminish the apparent uptake of lipids into the A549 cells at two concentrations equal to or greater than those from the previous experiment (Fig. 2). To further examine the potential role of ADFP in the proposed lipid transfer process, we also tested for lipid uptake using synthetic lipid droplets prepared by sonicating TAG with a mixture of phospholipids (**Fig. 3**). Unlike the isolated lipid droplets recovered from CHO cells, incubation of A549 cells with the synthetic micelles resulted in no detectable acquisition of lipid droplets. However, preincubation of the micelles with a lipase from *C. rugosa* led to lipid droplet formation in the A549 cells. This result indicated that although ADFP may not play a key role in the transfer of lipid into A549 cells, there was a component of the isolated CHO fat cake necessary for the acquisition and accumulation of lipid into the cells.

Triascin C, an inhibitor of acyl-CoA synthases, blocked the appearance of lipid droplets in A549 cells when incubated with fat cake (**Fig. 4**). Such data suggested that the lipid droplets were being hydrolyzed before being reesterified and assembled into neutral lipid storage droplets by the A549 cells. Furthermore, we hypothesized that the lipid droplets were being hydrolyzed by a lipase contaminant in the fat cake before entering the A549 cells. In this case, the appearance of lipid droplets in the fed A549 cells would be the result of the released free fatty acids rather than an ADFP-mediated transfer process. To determine whether the CHO cell droplets contained a lipase, we incubated the isolated droplets in the presence or absence of E600, a general lipase inhibitor. Incubation of the fat cake with E600 greatly inhibited lipase activity as monitored by free fatty acid release (**Fig. 5**). Subsequently, preincubation of the CHO fat cake with E600 significantly inhibited the formation of lipid droplets in the fed A549 cells (**Fig. 6**), demonstrating that the isolated droplet preparation contained a contaminating lipase that liberated free fatty acids. In further experiments, we determined that preincubation of A549 cells with the lipase inhibitor did not prevent the lipid transfer, confirming that the lipase was associated with the isolated lipid droplets and not the A549 cells (data not shown). Accordingly, we concluded that the A549 cells imported fatty acids produced by lipase-

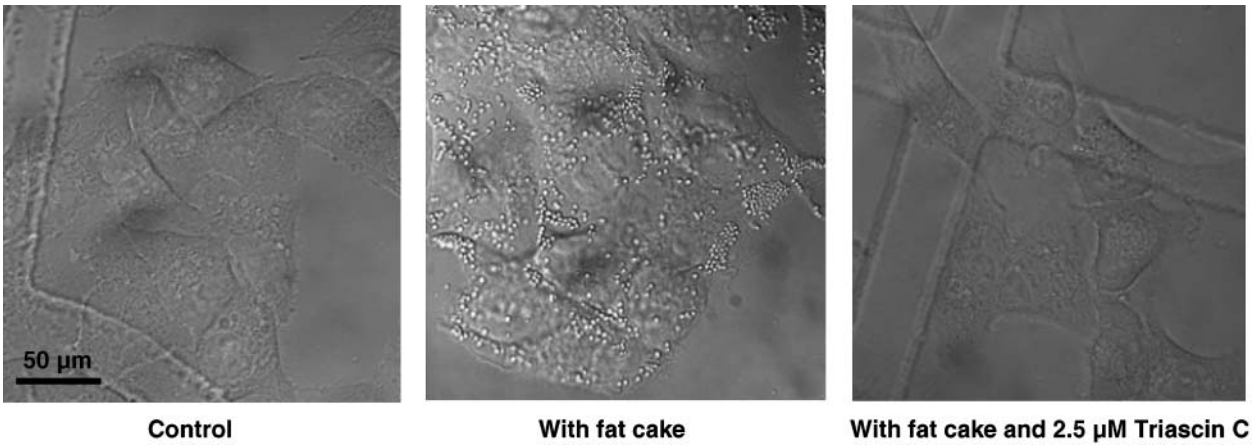

Figure 4. Triascin C inhibits lipid droplet formation in A549 cells incubated with fat cake. Fat cake was harvested from CHO cells as described previously. A549 cells were serum-starved for 7 h before incubating overnight with fat cake in the presence or absence of 2.5 μM triascin C.

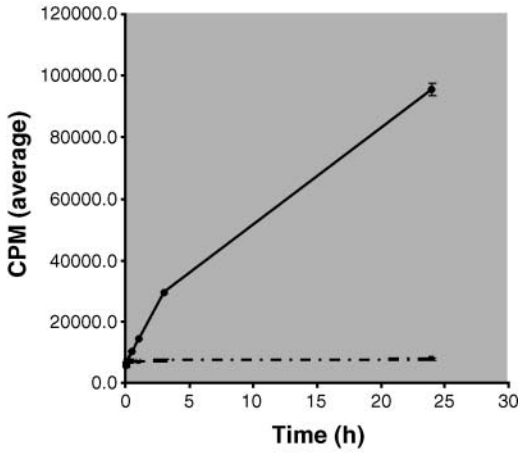

Figure 5. E600 inhibits free fatty acid release from CHO cell fat cake. CHO cells were lipid-loaded overnight in the presence of [^{3}H]oleic acid. The fat cake from these cells was incubated in the presence or absence of 1.5 mM E600 at 37°C. At the indicated time points, fatty acids were extracted from samples of the fat cake. The average of four trials was plotted with SEM. The solid line shows fat cake incubated without E600; the dashed line shows fat cake incubated with E600. Free fatty acids were monitored by ^{3}H release as detected by liquid scintillation counting.

mediated hydrolysis of TAG in the isolated lipid droplets from CHO cells. A protein in CHO fat cake that migrated at 50 kDa under SDS-PAGE was revealed to be adipose triglyceride lipase (ATGL) (15) by immunoblotting with a specific antibody; thus, ATGL may be the contaminating lipase (**Fig. 7**). The liberated fatty acids are subsequently packaged as TAG within ADFP-coated droplets by the A549 cells.

DISCUSSION

ADFP was identified initially as an early gene of adipocyte differentiation (16) and was subsequently found to be expressed in a wide variety of tissues and cultured cells (3, 4). Its relatively high mRNA expression in lung (3) was traced to lung lipofibroblasts (8), cells that are thought to package lipids for transfer to type 2 epithelial cells to be used for surfactant phospholipid synthesis (9). Lung lipofibroblasts are laden with intracellular neutral lipid droplets and express ADFP mRNA at levels that rival the expression in adipocytes (8). A primary mechanism for ADFP regulation is posttranslational stabilization by association with lipid droplets; thus, ADFP content reflects the neutral lipid content (14). In a previous study, we found that fatty acids derived from lipid droplets isolated from primary fetal rat lung lipofibroblasts were readily transferred into type 2 epithelial cells and incorporated into surfactant phospholipids (8). Moreover, this cell-to-cell transfer was blocked by incubation with an anti-ADFP antibody, leading to speculation that the lipid transfer was mediated by ADFP. However, in this study, the same antibody failed to interfere with the transfer of lipid from isolated CHO cell fat cake into A549 cells, even though the anti-ADFP antibody is able to clearly detect ADFP on CHO cell lipid droplets (Fig. 1). Given this finding, we established that the isolated CHO cell lipid droplets contained an associated lipase that liberated fatty acids from the droplets that were taken up by the A549 cells. This process would completely bypass ADFP, which indicates that ADFP played no role in the lipid transfer.

The anti-ADFP antibody inhibited the transfer of lipids in the earlier study, which led to the hypothesis that ADFP participated in the transfer of lipid. We can only speculate on why this same antibody failed to block the transfer in the present study. If, in the previous study, ADFP had no direct role in the transfer, and if, in that same study, the transfer was attributable to a lipase-induced release of fatty acids, we can assume that the ADFP-bound antibody blocked access of the putative lipase to the TAG within the droplet core. Correspondingly, the antibody must not have interfered with the lipase action in the present study. As with the A549 cells used here, we did examine the effect of feeding fatty acids to the primary rat epithelial

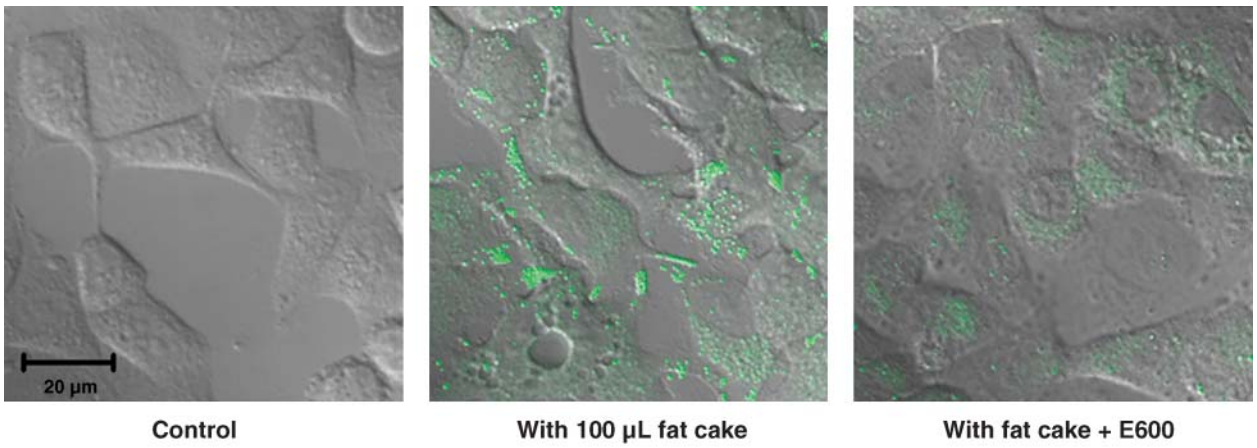

Figure 6. Effect of E600 on lipid droplet formation in A549 cells. Fat cake from CHO cells was harvested in the presence or absence of E600 at the indicated concentrations as described in Experimental Procedures. Serum-starved A549 cells were then incubated with 100 μl of the fat cake (treated with or without E600) overnight at 37°C, then stained with Bodipy 493/503.

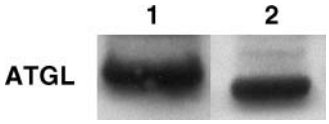

Figure 7. Identification of adipose triglyceride lipase (ATGL) in CHO cell fat cake. Fat cake from CHO cells was run on a 4–12% Bis-Tris SDS-PAGE gel, transferred to nitrocellulose, and probed with an antibody to rabbit anti-mouse ATGL (a gift from Rudolf Zechner) at a 1:5,000 dilution. The secondary antibody was a goat polyclonal rabbit IgG (Abcam, Cambridge, MA) conjugated to horseradish peroxidase at a 1:20,000 dilution. Lane 1, CHO cell fat cake sample; lane 2, positive control sample from the total protein from the fat pad of a wild-type C57Bl6J mouse.

cells, and the fatty acids were taken up and incorporated into ADFP-coated droplets within the primary type 2 epithelial cells (C. Schultz and C. Londos, unpublished data). Thus, we deem it likely that a contaminating lipase in the droplet preparation accounted for the droplet-to-epithelial cell transfer found previously. In this study, ATGL was identified in the CHO cell fat cake (Fig. 7). Liu et al. (17) showed previously that CHO cells contain a 47.3 kDa protein with a 168 amino acid long PATATIN domain, also likely to be ATGL. This may be the lipase responsible for hydrolyzing TAG in this study.

We also note that in this study, we used droplets isolated from CHO cell fibroblasts as donors and A549 cultured cells as recipients. We consider the A549 cell line to be a comparable model to the primary type 2 epithelial cells. A549 cells are a human cell line isolated from an alveolar cell carcinoma and have been shown to have many of the same characteristics as type 2 epithelial cells, including the ability to synthesize surfactant (18). This cell line has been widely used as a model to study type 2 epithelial cells.

One caveat to our earlier speculation that lipid droplets may be transferred from lipofibroblasts to type 2 epithelial cells was our failure to find any ADFP secreted into the medium (8), indicating that, unlike mammary epithelial cells (19), the lung lipofibroblasts do not secrete their lipid droplets. A remaining unanswered question is the reason for the high expression of *ADFP* mRNA in the lung lipofibroblasts. We deem it likely that because ADFP serves to package and store fatty acids as TAG (14), ADFP stores fatty acids in TAG for future use in surfactant phospholipid synthesis. This speculation is buttressed by the finding that ADFP mRNA levels increase dramatically in rats immediately before parturition (8).

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