Remodeling of lipid droplets during lipolysis and growth in adipocytes*

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Background: Micro lipid droplets (mLDs) appear in adipocytes upon lipolytic stimulation. LDs may grow by spontaneous, homotypic fusion.

Results: Scavenging of fatty acids prevents mLD formation. LDs grow by a slow transfer of lipids between LDs.

Conclusion: mLDs form due to fatty acid overflow. LD growth is a controlled process.

Significance: Novel mechanistic insights into LD remodeling are provided.

SUMMARY

Synthesis, storage, and turnover of triacylglycerols (TAGs) in adipocytes are critical cellular processes to maintain lipid and energy homeostasis in mammals. TAGs are stored in metabolically highly dynamic lipid droplets (LDs), which are believed to undergo fragmentation and fusion under lipolytic and lipogenic conditions, respectively. Time-lapse fluorescence microscopy showed that stimulation of lipolysis in 3T3-L1 adipocytes causes progressive shrinkage and almost complete degradation of all cellular LDs but without mLDs. However, mLDs were rapidly formed after induction of lipolysis in the absence of bovine serum albumin (BSA) in the culture medium that acts as a fatty acid scavenger. Moreover, mLD formation was blocked by the acyl-CoA synthetase inhibitor Triacsin C implicating that mLDs are synthesized de novo in response to cellular fatty acid overload. Using label-free coherent anti-Stokes Raman scattering (CARS) microscopy, we demonstrate that LDs grow by transfer of lipids from one organelle to another. Notably, this lipid transfer between closely associated LDs is not a rapid and spontaneous process but rather occurs over several hours and does not appear to require physical interaction over large LD surface areas. These data indicate that LD growth is a highly regulated process leading to the heterogeneous LD size distribution within and between individual cells. Our findings suggest that lipolysis and lipogenesis occur in parallel in a cell to prevent cellular FA overflow. Furthermore, we propose that formation of large LDs requires a yet uncharacterized protein machinery mediating LD interaction and lipid transfer.

Most eukaryotic organisms deal with a typically fluctuating food supply by storing or mobilizing lipids as an energy source. Malfunction of the synthesis or degradation of fat stores is linked to prevalent diseases, such as obesity, type II diabetes, or various forms of lipodystrophy (1). In mammals, adipose tissue functions as the major energy depot of the body. Excess fatty acids (FA) and sterols are stored as neutral lipids (mostly as triacylglycerol, TAG, as well as sterol esters, SE) in cytosolic lipid droplets (LDs), which are mobilized by regulated lipolytic breakdown in response to specific nutritional needs of the cell or organism. The LD surface consists of a phospholipid monolayer harboring a set of enzymes and regulatory proteins that catalyze the highly metabolically controlled synthesis and mobilization of fat stores. Compartmentation of nonpolar neutral lipids into LDs ensures their physiological accessibility and, thus, plays a central and critical role in lipid homeostasis. Neutral lipid turnover is accompanied by a significant remodeling of LDs and both appearance of micro-lipid droplets (mLDs) during breakdown of neutral lipids as well as growth of the organelles by fusion events have been discussed as fundamental processes required for efficient mobilization and storage of fat in adipocytes (2-6).

A number of studies have reported that in differentiated murine (3T3-L1) adipocytes, large perinuclear LDs fragment and disperse into smaller mLDs in response to lipolytic stimulation (7-10). Such a fragmentation process is expected to drastically increase the surface-to-volume ratio of the LDs, leading to more efficient degradation of stored neutral lipids by LD-associated lipases. Dispersion of LDs was also found to affect the localization of perilipin, one of the main regulators of LD turnover, as well as of hormone-sensitive lipase (HSL). Both proteins are restricted to a subpopulation of dispersed LDs and it was suggested that mLDs that emerge under lipolytic conditions may represent an active pool of LDs from which neutral lipids are mobilized (10). Controversially, however, a recent time-lapse study using label-free coherent anti-Stokes Raman scattering (CARS) microscopy showed that in differentiated 3T3-L1 adipocytes mLDs appeared scattered throughout the cytosol upon lipolytic stimulation, but were not detected at specific regions neighboring large LDs as would be expected if they originated by LD fragmentation. It was suggested that these LDs may instead derive from other organelles, such as the endoplasmic reticulum (ER), rather than from existing LDs. On the other hand, ‘nano’
LDs (nLDs), which may not be detectable by CARS microscopy, may split off from larger LDs and subsequently fuse to give rise to mLDs that are found dispersed in the cytosol (11). Furthermore, controversy also exists concerning the mode of LD growth. Homotypic interaction between LDs of 3T3-L1 adipocytes may indeed cause fusion of the organelles: this process does not require TAG synthesis but depends on microtubules and the motor protein dynein (12,13). Furthermore, it was suggested that SNARE proteins mediate LD fusion (14). However, in another study fusion of LDs could not be observed in the same cell type under conditions of induced lipid synthesis. According to this study, nascent LDs may form at the cellular periphery and move towards larger perinuclear ‘storage’ LDs. Such LDs enlarge during their movement without observable fusion events, presumably by synthesis of new TAG directly on the LD (15). Both diacylglycerol (DAG) and diacylglycerol acyltransferase 2 (DGAT2) catalyzing the conversion of DAG to TAG were detected in vicinity of lipid droplets both in 3T3-L1 and COS7 cells suggesting that LDs may also grow by biosynthesis of TAG near LDs and direct incorporation into existing LDs (16). Moreover, it was shown that LDs of rat hepatoma cells grow by incorporation of esterified cholesterol into existing lipid droplets rather than by fusion events (17). A recent analysis of LD fusion in murine adipocytes using time-lapsed light microscopy showed that fast organelle fusion can be stimulated by various drugs, which, however, appears to be a rare event in untreated cells (18). Finally, in addition to de novo synthesis and fusion, LDs may also grow by a dynamic interaction and gradual (regulated) transfer of TAG between nascent and preformed LDs, as shown in primary mouse hepatocytes. In these cells, transient fusion and fission events may occur upon contact of two closely associated LDs (19).

In this study, we applied high-resolution long-term 4D live cell imaging of murine adipocytes and human adipose-derived stem cells to monitor the breakdown as well as the formation of LDs. Our results demonstrate that efficient degradation of LDs is not accompanied by fragmentation and dispersion of LDs in 3T3-L1 adipocytes, but rather leads to FA overflow that initiates formation of new LDs. This nLD formation can be prevented by excess BSA in cell culture medium to sequester lipolysis-derived FA, or by inhibiting FA activation by Triacsin C, even in the absence of extracellular FA acceptors. Long-term monitoring of LD growth during adipocyte cultivation revealed a slow transfer of neutral lipids between closely associated LDs via a ‘bridge’ between adjacent LDs and without apparent spatial interaction over large LD surface areas.

**EXPERIMENTAL PROCEDURES**

**Cell culture**– Cells were cultured in glass bottom dishes with 50 mm diameter (MatTek Corp., Ashland, MA). 3T3-L1 fibroblasts were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 4.5 g/l glucose and L-glutamine (Gibco, Invitrogen Corp., Carlsbad, USA) supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich) and antibiotics (DMEM +/-) under standard conditions (37°C, humidified atmosphere, 5% CO2). Two days after confluency, medium was changed to DMEM +/- containing 10 µg/ml insulin (Sigma-Aldrich, St. Louis, MO), 0.25 µM dexamethasone (Sigma-Aldrich), and 500 µM isobutyl-methylxanthine (IBMX) (Sigma-Aldrich). After 3 and 5 days, medium was changed to DMEM +/- containing 10 µg/ml and 0.05 µg/ml insulin, respectively. The day before the experiment, cells were incubated without insulin over night. Experiments were performed on day 8 or 9 after initiation of differentiation. For electron microscopy cells were cultured on collagen coated (1% collagen) Alcar film (Gröpl, Inc., Tulln, Austria) placed in the glass bottom dishes.

Human adipose-derived stem cells (Invitrogen) were grown in complete MesenPro RS Medium (Invitrogen) and after reaching confluency the medium was changed to Complete Adipogenic Differentiation Medium (Invitrogen). For long-term experiments cells were seeded in glass bottom dishes with 35 mm diameter (Ibidi, Germany) with an additional Culture-Insert (Ibidi) to enable 4D CARS imaging over more than a week without the need for changing the medium.

**Lipolytic stimulation of murine adipocytes** and inhibition of long chain fatty acyl CoA synthetase– For stimulation of lipolysis 10 µM forskolin (Sigma Aldrich) was added to the medium. To study the effect of bovine serum albumin (BSA) on LD formation during lipolysis, 3T3-L1 cells were incubated with 10 µM forskolin (in DMEM), either containing 2% fatty acid free BSA or no BSA. Incorporation of FAs into TAG was inhibited by addition of 5 µM Triacsin C (Sigma-Aldrich). First, cells were...
treated with Triacsin C for 2h in DMEM+/+, then, the medium was replaced by fresh DMEM containing 5 µM Triacsin C, 10 µM forskolin, and either 2% fatty acid free BSA (control) or no BSA. A total medium volume of 2 ml was used in all experiments.

**Analysis of acyl-CoA levels in Triacsin C treated differentiated 3T3-L1 cells by mass spectrometry**– Cells were differentiated for 8 days and subsequently pretreated for 2h with various concentrations of Triacsin C (1 µM, 5 µM, 10 µM or 20 µM) to test the efficacy of the drug to inhibit fatty acid activation. After inhibitor treatment, cells were further incubated for one hour in fresh DMEM again containing various concentrations of Triacsin C and 2% BSA. Acyl-CoAs were determined by on-line solid phase extraction liquid chromatography-mass spectrometry as previously described (20). In brief, buffer-suspended cells (250µl of 50% 0.1 M KH₂PO₄ and 50% 2-propanol, prechilled to 4°C) were spiked with internal standard mix (0.25nmol ¹³C₁₆-palmitoyl-CoA, 0.25nmol ¹³C₁₈-stearoyl-CoA, 0.1nmol ¹³C₁₈-oleoyl-CoA per sample). After addition of 15µl of saturated aqueous (NH₄)₂SO₄ solution and 0.25ml acetonitrile, the cell suspension was homogenized on ice for 10–20 s using an ultrasonic homogenizer. The homogenate was vigorously mixed and centrifuged at 2,500 × g for 10 min at 4 °C, and the supernatant was transferred to autosampler vials. Extracts were stored at −80°C, prior to analyses on an Ultimate 3000 System (Dionex, LC Packings, Sunnyvale, CA) consisting of an autosampler with cooled tray and a column oven with a switching unit coupled to an LTQ Orbitrap XL (Thermo Scientific, Waltham, MA). A Phenomenex Strata X 2.0 × 20-mm cartridge (Torrance, CA) and a Waters XBridge column (2.1 × 50 mm, 2.5 µm) (Milford, MA) were used for on-line solid phase extraction and as analytical column, respectively. Positive electrospray ionization-mass spectrometry was performed by high resolution mass spectrometry (scan range 500–1500 m/z, resolution 60,000).

**Biochemical analysis of fatty acid release**– For determination of free FAs (FFAs), aliquots of the corresponding media were collected and FFA content was determined using a commercial analysis kit (WAKO Chemicals GmbH, Neuss, Germany). Cells were lysed in 0.3 M NaOH/0.1% SDS and protein concentration was determined using BCA reagent (Pierce Biotechnology, Rockford, IL).

**Fluorescence microscopy**– Imaging of fluorescein labeled structures was performed on a Leica SP5 confocal microscope using a 40x NA 1.25 oil immersion objective. LDs of 3T3-L1 cells were labeled by adding LD540 (21) to the culture medium (final concentration 1 µg/ml). The neutral lipid-specific dye LD540 was a kind gift from Christoph Thiele, Max Planck Institute of Molecular Cell Biology and Genetics, Germany. Labeling of neutral lipids was typically achieved after 10-15 min of incubation. LD540 fluorescence was excited at 561 nm and emission was detected between 570-620 nm. For 4D live cell imaging cells were incubated in glass bottom dishes directly on the microscope stage using a PECON S-2 stage incubator (PECON, Inc., Germany) at 37°C and 5% CO₂. 3D image data were acquired in 30 min time intervals and with a voxel size of 90x90x300 nm (x/y/z), 100 nm TetraSpeck™ microspheres (Invitrogen, Inc.) were used as sub-resolution particles for determination of detection capabilities of the fluorescence imaging system (λexc/λem: 561 nm/570-620 nm).

**CARS microscopy**– Coherent anti-Stokes Raman Scattering (CARS) microscopy of 3T3-L1 cells was performed using a commercial setup consisting of a picosecond laser source and an optical parametric oscillator (OPO; picoEmerald; APE, Germany; HighQ Laser, Austria) integrated into a Leica SP5 confocal microscope (Leica Microsystems, Inc.). Detection of the CARS signal was achieved using 650/210 and 770/SP emission filters. The microscope was equipped with a non-descanned detector (NDDs) for acquisition of signals in forward (F-) CARS mode (22). To detect neutral lipids/LDs the laser was tuned to 2845 cm⁻¹, thus enabling imaging of CH₂ symmetric stretching vibrations. For imaging a Leica 1.25 NA, 40x oil objective was used. For long-term experiments of 3T3-L1 cells, z-stacks of images were taken at different time intervals, as indicated, with a voxel size of 90x90x300 nm (x/y/z).

4D imaging of human adipose-derived stem cells was performed on a ‘homebuilt’ CARS setup, based on a multiphoton microscope (Leica TCS SP5) and an Er: Fiber laser source (Toptica FemtoFiber pro) at a repetition rate of 40 MHz. The experiments were performed at 2845 cm⁻¹ resonance frequency using the Stokes beam tuned to 998 nm with a power of 6.5 mW and the pump laser tuned to 777 nm with a power of 62 mW. A Leica 0.85 NA, 40x air objective was used for focusing the excitation beams. Data were recorded in transmission type geometry (F-CARS) and were collected by a Leica 0.55 NA
Transmission electron microscopy (TEM)—Adipocytes were fixed in 0.1 M cacodylate buffer (pH 7.2) containing 2.5% glutardialdehyde and 2% paraformaldehyde for 30 min at room temperature. Cells were rinsed twice in 0.1 M cacodylate buffer, specimens were dehydrated in a series of 50, 70, 90, and 100% cold acetone for 30 min each. Preparations were infiltrated by 2:1, 1:1, and 1:2 mixtures of 100% acetone and agar 100 epoxy resin (Gröpl, Inc., Austria) and pure agar 100 epoxy resin for 4 h. Finally, cells were placed in agar 100 epoxy resin at room temperature for 8 h, transferred into embedding molds, and polymerized at 60°C for 48 h. Ultrathin sections (70 nm) were cut into embedding molds, and polymerized at 60°C for 8 h, transferred in 0.1 M cacodylate buffer for 1 h and postfixed in 2% osmium tetroxide in the same buffer for 1 h. After rinsing four times for 10 min in 0.1 M cacodylate buffer, specimens were dehydrated in a series of 50, 70, 90, and 100% cold acetone for 30 min each. Preparations were infiltrated by 2:1, 1:1, and 1:2 mixtures of 100% acetone and agar 100 epoxy resin (Gröpl, Inc., Austria) and pure agar 100 epoxy resin for 4 h. Finally, cells were placed in agar 100 epoxy resin at room temperature for 8 h, transferred into embedding molds, and polymerized at 60°C for 48 h. Ultrathin sections (70 nm) were cut with a Leica Ultracut UC6 and stained with lead citrate for 5 min and with uranyl acetate for 15 min. Images were taken on a Tecnai 20 transmission electron microscope (Fei, Inc., USA).

Imaging-based quantification of LD size—A z-stack of images of a selected cell was projected into a single 2D image using maximum-intensity projection. The diameter of ~50 LDs was measured using the line measure feature implemented in ImageJ (23), and surface and volume data were calculated.

Statistical analysis of FA release—Data are represented as mean values and standard deviation from three independent experiments. Group differences were calculated using unpaired Student’s t-test (two-tailed). Levels of statistical significance were considered $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***)

RESULTS

Lipolytic stimulation of 3T3-L1 adipocytes causes rapid shrinkage of LDs without detectable LD fragmentation—To investigate the effect of lipolysis on LD size and subcellular distribution, differentiated 3T3-L1 adipocytes were labeled with LD540, stimulated with forskolin and analyzed over time by 4D live cell imaging. Already within 30 min after initiation of lipolysis, a clear shrinkage of LDs was detectable. Notably, all monitored LDs showed a progressive response to lipolytic stimulation, independent of their size or subcellular position (Figure 1). Moreover, degradation of LD contents appeared to be correlated with the size of the organelles. Computed values for the half-life of the volume of selected LDs during lipolysis indicate that smaller LDs are faster degraded than larger LDs (Figure 3A). Five hours after stimulation of lipolysis, LDs in most cells were almost completely depleted. Quite notably, no fragmentation of existing LDs was detectable under our experimental conditions, neither at the beginning of the experiment nor at later stages, when the TAG stores were almost entirely depleted (Figures 1 and 2A). Similarly, no mLDs were observed in differentiated 3T3-L1 adipocytes that were stimulated with isoproterenol and IBMX (Figure 2B), demonstrating that changes in LD morphology during lipolysis were independent of the type of stimulus. As shown in a control experiment with 100 nm fluorescent sub-resolution beads, LDs of similar size should be clearly detectable with the microscope system used (Figure 3B). This indicates, that mLDs either do not occur or that their size is below the detection limit of the used microscope system. Notably, no increase in fluorescence background during lipolysis was observed, as would be expected upon appearance of large numbers of small nano particles. Interestingly, although cell volumes significantly shrank over time during stimulated lipolysis, most LDs appeared to remain at their relative position within the cell, suggesting that they are associated with other subcellular structures (Figure 2A).

mLDs are synthesized de novo—To test whether LD dynamics and morphology during lipolysis are influenced by the ability of cells to sequester excess FAs that derive from lipolysis, we omitted BSA from the culture medium which acts as FA scavenger. In the absence of BSA, existing LDs indeed decreased in size upon forskolin-stimulated lipolysis indicating activation of lipolysis (Figures 4A-B). However, FA release from adipocytes was reduced by ~90%, compared to cells cultivated in the presence of BSA (Figure 5A). Notably, the absence of BSA led to the rapid formation of a large number of small LD (<1µm) within 30 min after initiation of lipolysis. These LD grew significantly in size (~1.3µm) within the following 60-90 min (Figures 4A-C). Thus, mLD formation is strongly influenced by the presence of the FA acceptor BSA in the culture.
medium during stimulated lipolysis. These newly emerging mLDs were not specifically detectable on the surface of existing LDs but were rather dispersed throughout the cell. In addition, they did not significantly change their relative subcellular position during their growth (Movie S1). Furthermore, transmission electron micrographs acquired upon lipolytic stimulation in the absence of BSA show a large number of mLDs (>1µm) but also of nano LDs (nLDs, <1µm). Again, both nLDs and mLDs are not specifically detected on the surface of larger LDs but occur dispersed in the cell (Figure 6).

Taken together, these data suggest that formation of mLDs results from cellular FA overload in the absence of BSA in the media, which may trigger TAG (and LD) synthesis to prevent FA toxicity. Since FAs released from TAG by lipolytic degradation require activation with coenzyme A prior to (re)incorporation into lipids, we next tested whether mLD formation during stimulated lipolysis is dependent on the activity of acyl-CoA synthetases. For this purpose, we analyzed mLD formation in the presence of Triacsin C, an inhibitor of acyl-CoA synthetase (24-28). To evaluate the efficacy of Triacsin C treatment of 3T3-L1 cells, we first determined the effect of the drug on acyl-CoA synthetase activity. For this purpose, we analyzed mLD formation in the presence of Triacsin C, an inhibitor of acyl-CoA synthetase (24-28). To evaluate the efficacy of Triacsin C treatment of 3T3-L1 cells, we first determined the effect of the drug on acyl-CoA synthetase activity. For this purpose, we analyzed mLD formation in the presence of Triacsin C, an inhibitor of acyl-CoA synthetase (24-28). To evaluate the efficacy of Triacsin C treatment of 3T3-L1 cells, we first determined the effect of the drug on acyl-CoA synthetase activity.

As shown in Figure 5A, Triacsin C treatment of 3T3-L1 cells led to a dose-dependent decrease in cellular acyl-CoA levels, indicative for efficient inhibition of ACS activity in vivo. Maximal inhibition was achieved at 5 µM Triacsin C (~ - 83%). Thus, the increase in FA release in response to Triacsin C treatment as shown in Figure 5A correlates with a reduction in ACS activity.

As shown in Figure 7, mLD formation was almost completely inhibited during stimulated lipolysis in cells treated with 5µM Triacsin C, even in the absence of the FA acceptor BSA in the medium. These data show that mLDs form de novo as a result of cellular FA overflow during stimulated lipolysis, and that TAG degradation and new synthesis may operate in parallel to control the level of (activated) FA in the cell.

LDs grow by controlled transfer of lipids between the organelle- The molecular mechanisms that govern LD growth are currently unclear and may occur by spontaneous homotypic fusion of individual LDs (4), by selective lipid transfer between LDs (19), or by TAG synthesis ‘on site’ that is catalyzed by LD-resident acyltranferases (16). Subpopulations of LDs in differentiated adipocytes vary significantly in size; large LDs are frequently observed in the vicinity of the nucleus, whereas the smaller LDs form a size gradient towards the cellular periphery, both in murine 3T3-L1 cells (Figure 8A) and in human adipose-derived stem cells (Figure 8B). Since neutral lipid-specific fluorescent dyes tend to promote LD fusion and influence intracellular LD movement upon microscopic inspection (Wolinski & Jüngst, personal observations) we applied label-free CARS microscopy for long-term analysis of LD growth and assembly in living cells. To accelerate LD growth, 3T3-L1 cells (day 7 after initiation of differentiation) were supplemented with oleic acid and analyzed by CARS microscopy for up to 16 hours. As shown in Figure 9 and Movie S2, several LDs grew by ‘absorbing’ the lipid content of other, in all cases, smaller LDs. ‘Importantly, complete lipid transfer between larger ‘acceptor’ LDs and closely associated smaller ‘donor’ LDs took up to several hours, indicative of a finely tuned process rather than fast spontaneous fusion.

To test whether this mechanism of LD growth is induced by excess FA supply or is also occurring in untreated cells, we analyzed LD dynamics in human adipose-derived stem cells in the absence of exogenous oleic acid. Similarly to the data obtained with 3T3-L1 adipocytes in the presence of oleic acid, LDs grew by absorption of the neutral lipid content of smaller LDs also in these cells and independent of exogenous FA supply. Notably, individual LD apparently took up the content of several other LDs, and ‘acceptor’ LDs in turn served as lipid ‘donors’ for others. Again, the complete absorption process of individual LDs was not a spontaneous and rapid event but rather took place over a time period of several hours. (Figure 10, Movie S3, Movie S4). In summary, these observations demonstrate that lipid transfer between LDs occurs in a slow and regulated process without interaction of larger areas of the LD surfaces.

DISCUSSION

Understanding LD dynamics and metabolism is of great biomedical interest in view of prevalent lipid-associated disorders. Notably, LD size distribution is typically rather heterogeneous in adult adipocytes, both in cultured cell lines and in primary fat cells such as in murine white adipose tissue (29-31). Currently it is unclear how this heterogeneity is established and how the size distribution affects
the metabolic fate of LDs. Using 4D live cell imaging, we have investigated in greater detail the dynamics and morphological alterations of LDs during stimulated lipolysis as well as in metabolically active but not proliferating cells. Virtually all LDs showed a progressive loss of lipids independent of their size or subcellular position and were almost totally depleted of lipids after 5 h persistent stimulation. We conclude that under our experimental conditions lipolytic activity is not restricted to a subpopulation of LDs. Indeed, smaller LDs appeared to shrink faster than larger LDs suggesting higher lipolytic activity on their surface, consistent with the increased surface-to-volume ratio and potentially increased density of lipase molecules on their surface. Thus, the intracellular redistribution of neutral lipids and the tendency to generate large LD may function as an additional mechanism to regulate the rate of lipolysis. This strategy may be optimized in primary adipocytes usually containing one very large and a number of small LD. However, the rate of degradation of LD contents is not only a function of size (surface to volume ratio), since individual LDs can occasionally be degraded significantly faster than LDs of similar size, which may be related to the differential localization of regulatory proteins. 3T3-L1 adipocytes grown in a 3D matrix develop large ‘core’ LDs and smaller peripheral LDs, which are specifically coated with perilipin (Plin1) (10). Since Plin1 is essential for the recruitment of hormone-sensitive lipase (HSL) to LDs (32), it has been proposed that peripheral LDs are more sensitive to lipolytic stimulation. However, Plin1 also affects the activity of adipose triglyceride lipase (ATGL), the rate-limiting enzyme of TAG hydrolysis (33,34). In its non-phosphorylated state, Plin1 binds the ATGL co-activator CGI-58. Upon protein kinase A (PKA) activation and phosphorylation of Plin1, CGI-58 is released and activates ATGL (35). Notably, mutations in Plin1 which fail to sequester CGI-58 have recently been shown to increase lipolysis and are associated with a novel subtype of partial lipodystrophy (36). Thus, at least under basal conditions, Plin1 can inhibit ATGL activity and the absence of this protein on ‘core’ LDs may actually promote lipolysis. Moreover, the localization of Plin1 and HSL in 3T3-L1 cells is controversial since both proteins have been shown to localize to all detectable LDs under standard culture conditions (10,37,38).

As expected, the decrease of LD size in 3T3-L1 adipocytes in response to lipolytic stimulation resulted in a massive release of FA. Under standard lipolytic conditions, i.e. in the presence of the FA scavenger BSA in the culture medium, neither LD fragmentation nor the appearance of mLDs was observed, even though cellular lipid stores were almost fully depleted in the course of the experiment. On the other hand, 3T3-L1 cells rapidly produced a large number of mLDs upon stimulation of lipolysis if BSA was omitted from the medium, suggesting that mLD formation is influenced by the availability of extracellular FA acceptors promoting the cellular release of FAs. Moreover, mLD formation was virtually abolished in the presence of Triacsin C, an inhibitor of acyl-CoA synthetase, which is required for activation of (lipolysis-derived) FA and their esterification into TAG. The inhibitory effect of Triacsin C on FA activation is evident from the significant decrease of acyl-CoA levels and the increase of free fatty acid release under basal conditions (Figure 5A-B), and is also consistent with published data (39). We conclude that mLDs are newly synthesized organelles in response to cellular FA overload. Since FAs are toxic for living cells (40), re-esterification of mobilized FAs and deposition in TAG stores provides a mechanism to protect cells from FA-mediated lipotoxicity. Partial re-esterification of FAs was already shown under basal conditions (41) as well as during lipolysis in adipocytes (42). Re-import of released FAs was also suggested to cause LD dispersion under lipolytic conditions in 3T3-L1 cells (43).

Similarly, LDs in hepatocytes undergo rapid lipolysis but only a small portion of FAs is released or integrated into very-low-density lipoprotein. In these cells, most FAs are indeed recycled back to TAG and stored in LDs (44,45). Furthermore, metabolic modeling studies in yeast also suggest that lipolysis and lipogenesis may indeed operate in parallel (46). Together, these observations imply that mLDs are formed de novo during lipolysis when FA concentrations exceed the binding capacity of extracellular BSA. Thus, the amount of BSA in the culture needs to be critically considered. In addition, the amount of intracellular FA acceptors, such as FA binding proteins (FABPs), (47), might also play a role in buffering FAs and possibly influence the formation of mLDs during lipolysis.

Our study supports previous observations demonstrating that mLDs grow during lipolytic stimulation and are formed in all areas of the cell, presumably at the endoplasmic reticulum rather than at specific regions neighboring large LDs. It was hypothesized that mLDs may grow
by fragmentation of existing LDs into nLDs, which may not be detectable by light microscopy, and subsequent fusion giving rise to mLDs (48). Indeed, although such small LDs cannot be resolved as single objects by light microscopy, they are still detectable based on their fluorescence staining (49), and are expected to result in a strongly increased fluorescence ‘background’ signal. However, such an increase of the fluorescence signal was not detected in stimulated cells, neither in the presence nor in the absence of BSA. In addition, transmission electron microscopy showed a large number of mLDs and nLDs in cells stimulated for 1h in the absence of BSA; however, these small LDs localized at a distance from the large LDs rather than close to their surfaces, which would be expected if they were derived by fragmentation. It should be noted that formation of small LD was also observed under conditions of inhibited lipolysis, suggesting that LD fragmentation occurs independently of TAG degradation (7). This apparent discrepancy to our and other findings (43) remains to be resolved. Notably, mLDs did not appear to fuse but rather grew in size by de novo lipid deposition. Thus, we propose that fragmentation of larger LDs into nLDs and their subsequent fusion is not required to form mLDs, but rather resembles a mechanism compatible with de novo LD formation at specific LD/ER contact sites (16).

Spatially highly resolved light microscopy of LD dynamics in living cells is widely used due to the availability of intensely emitting, lipophilic fluorophores, such as BODIPY 493/503 or LD540 (21). We have, however, observed that fluorescent labeling may cause changes in the fusion behavior of LDs upon (extended) light exposure (Wolinski & Jüngst, pers. observation). To prevent such light-induced alterations we have employed coherent anti-Stokes Raman scattering (CARS) microscopy in this study to assess long-term LD dynamics. CARS is a nonlinear optical process that allows label free monitoring of molecular vibrations, and is therefore suited for the generation of molecule-specific contrast in unlabeled samples. CARS microscopy is highly effective for detection of lipids, due to the high density of CH₂ groups in lipid molecules (22,50,51). Optimized excitation conditions allow CARS microscopic observations of individual unstained cells over days without detectable cell damage (52). It should be noted that LDs of sizes below ~200nm are not visible by CARS under the chosen excitation conditions. Thus, we cannot determine whether sub-resolution LDs grow by a process that is different from the observed long-term lipid transfer between the fat storage organelles.

Long-term monitoring during adipocyte cultivation using CARS revealed a slow transfer of neutral lipids between closely associated LDs. This process was not only observed between very large LDs but also between smaller LDs (≥1µm). Strikingly, this gradual process did not require physical interaction over large LD surface areas, thus implicating that associated LDs form a channel for lipid transfer. Most notably, lipid transfer between LDs took up to several hours, depending on the size of the LDs. In primary hepatocytes, a dynamic interaction between nascent and existing LDs was described that results in the transfer of neutral lipids into preformed LDs. It was hypothesized that a transient fusion and fission occurs during the contact of LDs leading to the transfer of lipids between smaller nascent and existing larger LDs (19). The thermodynamic driving force to suck up lipids into the larger LDs is clearly defined by the tendency to minimize surface tension, i.e. to reduce the surface to volume ratio. However, such a transfer of lipids would require an as yet uncharacterized protein machinery that mediates LD interactions, and may indeed include Rab (53) as well as SNARE (14) and motor proteins such as dynein (12,13). Rab18 GTPase is found both at ER membranes and on LDs in 3T3-L1 cells and other non-adipocyte cell lines (54-56), and it was suggested to represent a critical factor for establishing LD/endoplasmic reticulum associations (55). In addition, a role for Rab18 in lipogenesis and lipolysis was recently proposed. Overexpression of Rab18 in 3T3-L1 cells after insulin administration leads to an increase of TAG content and LD size, indicating that Rab18 may facilitate insulin-mediated lipid assembly into LDs. On the other hand, forskolin treatment of cells overexpressing Rab18 results in increased TAG hydrolysis in 3T3-L1 cells. Thus, Rab18 contributes in multiple ways to the regulation of lipid metabolism, perhaps as a mediator in the ER, by bringing LDs and ER structures together and thus facilitating lipid loading from the ER and/or LD fusion (57). Since LDs also harbor DGAT activity it could be speculated that lipid transfer between individual droplets also occurs by a de-acylation/FA activation/re-acylation cycle. Such a mechanism would explain the rather slow rate of lipid transfer between LDs and is compatible with the dual function of Rab18 GTPase both promoting
LD fusion and lipolysis. However, since LD associated DGAT2 harbors a transmembrane domain it is controversially whether this enzyme directly resides on the LD or LD-associated subdomains of the ER (58).

In conclusion, our findings suggest that formation of large LDs represents a regulated and slow physiological process in differentiating adipocytes whereas mLDS form rapidly in response to cellular FA overload and are synthesized to prevent FA toxicity. We propose that LD growth requires a distinct protein machinery that mediates LD interactions, forms a channel between LDs, and thus promotes the transfer of lipids. Indeed, elegant recent evidence demonstrates that Fsp27, a member of the cell death-inducing DFF45-like effector (CIDE) family of proteins, localizes to the LD-LD interface and is involved in mediating lipid transfer between adjacent lipid droplets, in 3T3-L1 cells (59,60).
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FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. 4D live cell imaging of LD consumption in 3T3-L1 adipocytes. All imaged LDs decrease their size significantly within 2h after stimulation of lipolysis by forskolin. In addition, the cell contract with progressing LD breakdown as indicated by the LD540 ‘background’ signal and decreased cell area. No fragmentation or dispersion of LDs is observed during lipolysis. *Left panel:* maximum-intensity projections of 3D data acquired at the indicated time points; *middle panel:* enlarged area enclosed by the white rectangular frame shown in the left panel; *right panel:* direct volume rendering representations. Bar=10µm.

FIGURE 2. Depletion of neutral lipid stores in 3T3-L1 adipocytes. A. Cell with almost totally depleted neutral lipid stores 5h after stimulation of lipolysis using forskolin. An individual LD appears to be faster degraded than other LDs of comparable size (solid arrows). LDs do not significantly alter their relative subcellular position to each other during lipolysis (open arrows). B. 3T3-L1 cells stimulated with isoproterenol and IBMX instead of forskolin. Similar to forskolin stimulation, LDs shrink but without detectable fragmentation or dispersion. Maximum-intensity projections of 3D data acquired at the indicated time points. Bar=10µm.

FIGURE 3. A. Half-life of the volume of LDs upon lipolytic stimulation in 3T3-L1 adipocytes. The volume of 46 selected LDs of an individual cell was calculated based on their diameter measured in maximum-intensity projections of an acquired 4D data set. The half-life of the volume of LDs in stimulated cells appears to be dependent on the size of LDs. Larger LDs reach T_{1/2} slower than smaller LDs. B. Evaluation of the microscope setup for detection of subresolution particles. 100nm fluorescent microspheres imaged with the same microscope setup as used for 4D imaging of 3T3-L1 cells. Groups of 100nm beads but also individual particles are clearly detected. Image represents an enlarged area of a larger image. Bar=1µm.

FIGURE 4. Rapid formation of mLDs in 3T3-L1 adipocytes after stimulation of lipolysis in the absence of the extracellular fatty acid acceptor BSA. A. Large numbers of small LDs (<1µm in diameter) are detected 30-60min after hormonal stimulation. B. Lipolysis is not inhibited in the absence of BSA (arrows; enlarged areas of the white rectangular fields shown in A). Bar=10µm. C. mLDs are formed in most cells of a cell population. Maximum-intensity projections of 3D data acquired at the indicated time points. Bar=20µm.

FIGURE 5. A. Dependence of FA release on extracellular fatty acid acceptors in 3T3-L1 adipocytes. >40-fold increase of FA release upon lipolytic stimulation compared to basal conditions in cells containing DMSO and BSA. ~6-fold increase of FA release upon lipolytic stimulation in the presence of Triascin C and BSA (*left panel*). 80-fold (cells in DMSO) and 50-fold (cells in DMSO+Triascin C) decrease of FA release of cells cultured without fatty acid acceptors (*right panel*) compared to cells cultured in the presence of BSA. Mean +/-SD, (3 independent experiments). B. Dose dependent effect of Triascin C on acyl-CoA levels in differentiated (day 8) adipocytes (see experimental procedures). Maximal inhibition was achieved at 5 µM Triascin C (~ -83%). Increasing concentrations of Triascin C (10 µM, 20 µM) did no further reduce acyl-CoA levels. Mean +/-SD, (2 independent experiments).

FIGURE 6. Ultrastructural analysis of nLDs and mLDs formed upon lipolytic stimulation in 3T3-L1 adipocytes incubated in the absence of BSA. 3T3-L1 adipocytes were treated with forskolin to stimulate lipolysis for 1h in the absence of BSA. Single TEM section showing a larger number of dispersed nLDs and mLDs (*left panel*). Such small LD are not detected on the surface of significantly larger LDs (>10µm), (*right panel*). Bar=1µm.

FIGURE 7. Inhibition of mLD formation in 3T3-L1 adipocytes upon lipolytic stimulation in BSA free medium using Triascin C. Cells degrade their lipid droplets to some extent (solid arrows) or contract without significant LD consumption (open arrows). mLDs are not detected within 2h of observation time. Bar=10µm. Maximum-intensity projections of 3D data acquired at the indicated time points.
FIGURE 8. Heterogeneity of LD size in differentiated adipocytes. 3T3-L1 cells (A) and human adipose-derived stem cells differentiated into adipocytes (B). Large LDs (arrows) are frequently located near the nucleus. LDs of various sizes are scattered throughout the cytoplasm. CARS images acquired at 2845 cm\(^{-1}\) (CH\(_2\) symmetric stretching vibration). Maximum-intensity-projection of acquired 3D data. Bar=10 µm.

FIGURE 9. Long-term lipid transfer between LD in 3T3-L1 adipocytes. Large LD grows by complete ‘absorption’ of a closely associated LD over hours. The lipid ‘donor’ gets smaller over time (solid arrows), whereas the lipid ‘acceptor’ grows over time (open arrows). Only small areas of the LD surfaces are closely associated during this process. The participating LDs do not significantly alter their rounded shape during lipid transfer. The process takes several hours. Imaging was started 12h after oleic acid treatment of the cells. CARS images were acquired at 2845 cm\(^{-1}\) (CH\(_2\) symmetric stretching vibration). Maximum-intensity projections of 3D data acquired at the indicated time points. Bar=10 µm.

FIGURE 10. LD growth in human adipose-derived stem cells induced to differentiate towards adipocytes is comparable to LD growth seen in 3T3-L1 cells. Lipid ‘acceptors’ can turn into lipid ‘donors’; lipid ‘donor’ (solid arrow, first panel) is almost completely absorbed over time by a closely associated lipid ‘acceptor’ below. Subsequently, this lipid ‘acceptor’ in turn serves as a lipid ‘donor’ (open arrow, second panel) for a LD, which simultaneously absorbs a second LD (solid arrow, second panel). Finally, this LD starts to absorb a third LD (solid arrow, last panel). Absorption of individual LDs takes more than 2h. Again, only small fractions of the LD surfaces are closely associated during this process. CARS images were acquired at 2845 cm\(^{-1}\) (CH\(_2\) symmetric stretching vibration). Maximum-intensity projections of 3D data acquired at the indicated time points. Bar=10 µm.
Figure 1
Figure 3

A

B

half life (min)

log LD volume (fl)
Figure 5

A

\[
\begin{align*}
\text{+BSA} & \\
\text{DMSO} & \quad \text{Triacsin C + DMSO} \\
\end{align*}
\]

FFA (nmol/h \* mg)

basal & ** \\
foroskolin stimulated & *** \\

-BSA

\[
\begin{align*}
\text{DMSO} & \quad \text{Triacsin C + DMSO} \\
\end{align*}
\]

FFA (nmol/h \* mg)

basal & ** \\
foroskolin stimulated & ** \\

B

long-chain acyl-CoA (nmol/mg)

\[
\begin{align*}
0 & \\
1 & \\
5 & \\
10 & \\
20 & \\
\end{align*}
\]

Triacsin C (\mu mol/l)
Figure 6.
Figure 9
Figure 10
Remodeling Of lipid droplets during lipolysis and growth in adipocytes
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