Long-term live cell microscopy studies of lipid droplet fusion dynamics in adipocytes

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Abstract During the adipogenic differentiation process of mesenchymal stem cells, lipid droplets (LDs) grow slowly by transferring lipids between each other. Recent findings hint at the possibility that a fusion pore is involved. In this study, we analyze lipid transfer data obtained in long-term label-free microscopy studies in the framework of a Hagen-Poiseuille model. The data obtained show a LD fusion process in which the lipid transfer directionality depends on the size difference between LDs, whereas the respective rates depend on the size difference and additionally on the diameter of the smaller LDs. For the data analysis, the viscosity of the transferred material has to be known. We demonstrate that a viscosity-dependent molecular rotor dye can be used to measure LD viscosities in live cells. On this basis, we calculate the diameter of a putative lipid transfer channel which appears to have a direct dependence on the diameter of the smaller of the two participating LDs.—Jüngst, C., M. Klein, and A. Zumbusch. Long-term live cell microscopy studies of lipid droplet fusion dynamics in adipocytes. J. Lipid Res. 2013. 54: 3419–3429.

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Lipid droplets (LDs) are lipid-rich structures which are found in many prokaryotic and eukaryotic cells. Depending on the cell type, their sizes can vary greatly, with diameters ranging from some tens of nanometers in most cells to several tens of microns in adipocytes. Despite these size differences, the general structure of LDs is similar: A core of lipids, which can also contain a minor amount of proteins (1), is shielded against the cell plasma by a membrane consisting of a monolayer of phospholipids. The phospholipid membrane itself contains a variety of proteins, which can be bound by different mechanisms (2). Proteins associated with the LD membrane mediate many different processes ranging from triacylglyceride (TAG) synthesis (3) to lipolysis (4). The most important function of LDs, however, is storage of lipids required for membrane synthesis and energy generation. Work of the last decade has shown that LDs can be regarded as true cellular organelles (5–7). Their importance is reflected by the fact that a number of human diseases, such as obesity or type II diabetes (8, 9), are directly related to malfunction of LD synthesis, storage, and degradation. In addition, recent work has revealed that LDs are involved in infectious diseases, e.g., caused by hepatitis C virus (10) and by Chlamydia trichomatis (11).

A lot of research efforts have recently been dedicated to unraveling the mechanisms behind the morphological changes of LDs which occur during adipogenic differentiation, the fundamental biological process converting stem cells into adipocytes (12). During the differentiation, the number of LDs in a cell and their sizes change significantly. In the end, only one large unilocular LD remains in the cell. The question of how the volume of individual LDs increases has been the subject of a longstanding debate (13). Two different hypotheses have been discussed. The first assumes that the LDs grow by direct incorporation of lipids. Because electron microscopy always shows LDs in close proximity to the endoplasmic reticulum (ER) (14), one obvious possibility is that LDs grow by transfer of lipids from the ER. Alternatively, lipids could be synthesized directly at the LD membrane. This possibility is supported by the recent finding that during oleate loading, glycerol-3-phosphate-acyltransferase 4 (GPAT4), an important enzyme in TAG synthesis, relocates from the ER to LDs (15). The second possibility for LD growth is transfer of material between different LDs. This process has been described in a number of recent publications (16–18), and is considered to be a homotypic fusion if all the material is finally taken up by one LD. It is important to note that homotypic fusion of LDs is different from other fusion processes in cells (19). Most
cellular organelles have an aqueous content. During a fusion of these organelles, it is therefore possible to adjust their volume with their surface area by influx of cytoplasmic fluid. This is important because the surface area of two fusing vesicles exceeds the surface needed to accommodate their volume. By contrast, significant volume exchange between cytoplasm and fusing LDs is impossible, because of their hydrophobic content (Fig. 1). Thus, LD fusion with preservation of volume requires the removal of excess membrane material. Fusion between LDs is readily induced by a variety of drugs. Drug-induced fusion is a fast process which is completed on a second timescale (20). By contrast, spontaneous fusion between LDs has been described as a “rare event” (21), even if there have been some previous reports on its observation (22, 23). The reason for spontaneous fusion between LDs having been observed only rarely, most likely is that previous attempts to visualize it in live cells have been performed on a timescale of tens of minutes at most (24). Recent long-term imaging experiments, however, have shown that LDs can indeed grow by absorption of several smaller LDs in slow fusion processes which require hours (16–18). During this time, a defined contact point between the LDs is maintained. It has been found that Fsp27 (fat-specific protein of 27 kDa), a protein of the CIDE family, is recruited to the contact point between the two fusing LDs (16). Earlier work has already shown that Fsp27 plays a crucial role in LD growth (25–28). Thus, it was found that suppression of Fsp27 expression in adipocytes leads to cells which do not contain one unilocular LD, but instead possess many smaller LDs (26, 29). By contrast, expression of Fsp27 in various cell types leads to the appearance of a small number of large LDs (27, 28). Direct evidence that Fsp27 is important for the fusion of LDs is given by the fact that within a few minutes, it is recruited to the contact point between two LDs starting to fuse (16). This is possible because Fsp27 is usually found in the whole LD membrane, such that it can easily diffuse to the contact point, fluorescence recovery after photobleaching (FRAP) measurements then show that at the contact point diffusion of Fsp27 is suppressed, hinting at a tethering function of Fsp27 (16).

An interesting finding in the reports on slow LD fusion is that the transfer of material always takes place from the smaller to the larger LD (16, 17, 30). This makes it likely that the dynamics of lipid transfer between fusing LDs have an important hydrodynamic component. Because the pressure inside the smaller LD is larger than that of the larger LD, this pressure difference can be seen as the driving force of the lipid transfer during the fusion process. The fact that a single contact point is maintained over the whole duration of the fusion process nevertheless shows that fusion is a protein-regulated process. It is thus likely that the transfer of lipids takes place through a pore between the LDs. Indeed, it has been postulated that Fsp27 might have a tethering function in the formation of a pore (31, 32). At the contact point, Fsp27 interacts with the LD-associated protein Plin1 (32, 33). It has been reasoned that this interaction then could lead to the opening of a pore which allows transfer of neutral lipids between the LDs (32). Assuming that the fusion rate is not limited by the required reduction or increase of the surface of the donor or acceptor LD, the lipid flow rate should then be mainly dependent on the diameter of the putative fusion pore and the pressure difference between the fusing LDs.

In this contribution, we report quantitative experimental data on the dynamics of the fusion of LDs in differentiating adipose-derived stem cells. Our analysis is based on long-term imaging experiments of LD fusion using coherent anti-Stokes Raman scattering (CARS) microscopy, a label-free imaging technique with high sensitivity for lipids (34). Quantitative analysis of the CARS microscopy data directly yields the lipid transfer rates between the fusing LDs. Experimental evidence from this and earlier work suggests that during the fusion process, material is transported through a pore. We therefore present an extension of an earlier model describing the volume transfer during fusion as driven by Laplace’s surface tension (17) by a simple Hagen-Poiseuille model. The model is tested using the observed volume transfer rates, which are used to calculate fusion pore sizes. For this purpose, however, one needs to determine the viscosity of the LD content which is transferred. Because this should be a complex mixture of different lipids, this information must be gathered from cellular measurements. In order to determine the viscosity inside LDs of living cells, we employed a new technique based on molecular rotors (35). This approach is based on measuring the fluorescence lifetime of a BODIPY derivative. After demonstrating that this dye efficiently labels LDs and that it can be used to measure their viscosity and their temperature in live cells, we use the derived viscosities to calculate the sizes of putative pores between fusing LDs and discuss the applicability of the theoretical model employed. It is found that the observed lipid transfer rates cannot solely be determined by the pressure difference between the participating LDs. Additional possible rate-limiting factors are discussed.
EXPERIMENTAL PROCEDURES

Cell culture of mesenchymal stem cells

For the microscopy experiments, human adipose-derived stem cells (Invitrogen, USA) were seeded in Petri dishes with glass bottoms (Ibidi, Germany). For long-term observations, small inserts (Ibidi) restricting the cell growth to four small areas of 2 × 1.5 mm were placed into the dish prior to seeding. The cells were seeded exclusively inside these areas with a concentration of 500,000 cells/ml. This allows reaching confluence, which is necessary for adipogenic differentiation, with a relatively low number of cells. In addition, the low number of cells relative to the large volume of medium permits long-term observation without the need for frequent medium change. After reaching confluence, which was most often the case after overnight incubation, the culture medium (MesenPro RS medium, Invitrogen) was exchanged against the differentiation medium (StemPro adipocyte differentiation medium, Invitrogen) and the cells were cultivated for another 3–5 days at 37°C at 5% CO₂. After this first differentiation medium, Invitrogen) and the cells were cultured against the differentiation medium (StemPro adipocyte differentiation medium, Invitrogen) and the cells were cultivated for another 3–5 days at 37°C at 5% CO₂. After this first differentiation phase, the cells were placed on the microscope and the long-term measurements were started. Fluorescence lifetime imaging (FLIM) measurements for determining the LD viscosity were done with cells which were incubated in the differentiation medium for approximately 7–10 days.

CARS microscopy and data analysis

Adipogenic differentiation of human mesenchymal stem cells was imaged using a home-built CARS microscope based on a confocal microscope (Leica TCS SP5, Leica Microsystems, Germany). As excitation sources, an Er: fiber-based laser system with a pulse repetition rate of 40 MHz was employed (Femtobre pro, Topica, Germany). The system consists of two branches delivering nearly jitter-free synchronized pulses with pulse durations of approximately 3 ps (36). As a pump beam, we used the frequency fixed laser output at 777 nm, whereas the frequency tunable laser output was set to 998 nm and was used as a Stokes pulse. This corresponds to a vibrational resonance frequency of 2,850 cm⁻¹, typical for symmetric CH₂-stretch vibrations. Incident laser powers were 62 mW (pump) and 6.5 mW (Stokes), respectively, before the microscope. The excitation light was focused with a 40× air objective (0.85 NA, Leica). The CARS signal was collected in transmission geometry with an air condenser (0.55 NA, Leica) and filtered by suitable filters (641/75 and 680/SP, Semrock, USA) before detection. Long-term three-dimensional (3D) imaging was performed by recording a z-stack of the sample with a distance of 0.38 μm between each image every 30 min for a total duration of up to 120 h. Based on these data, rendering of the LD volume was done using the Imaris software package (Bitplane, Switzerland). For the derivation of dV/dt values, the volumes of the LDs were determined in each recorded image frame. Due to the nonlinear dependence of the optical transfer function on the number of scattering molecules, no image deconvolution was applied. For LDs with radii between 1 μm and 3 μm, this leads to an absolute overestimation of the radii of less than 10% for the smaller and less than 5% for the larger LDs, respectively.

Synthesis of the molecular rotor BODIPY-C₁₂

BODIPY-C₁₂ was synthesized according to the literature (37, 38). Briefly, a mixture of 17.3 ml of pyrrole (250 mmol) and 0.726 g 4-dodecyloxybenzaldehyde (2.5 mmol) were degassed for 20 min under a nitrogen atmosphere. InCl₃ (55.5 mg, 0.25 mmol) was added and the mixture was stirred at room temperature. The reaction was quenched after 1.5 h by addition of 300 mg NaOH (7.5 mmol) powder and stirred for another hour. Subsequently, the reaction mixture was filtered with kieselguhr. The excess of pyrrole was removed using a rotary evaporator. The resulting oil (1.43 g) was used without further purification.

5-(4-Dodecyloxyphenyl)dipyrromethane (500 mg, 1.23 mmol) thus obtained was dissolved in toluene and 280 mg 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ, 1.23 mmol) was added. The mixture was stirred for 5 min at room temperature and turned dark red. Triethylamine (1.2 ml, 8.61 mmol) and BF₃-etherate (1.1 ml, 8.61 mmol) were added and the mixture was stirred for another hour at room temperature. The dark green precipitate was then separated from the solution and washed several times with toluene. The strongly fluorescing toluene solutions were then in turn washed several times with water and dried with Na₂SO₄. Finally, the solvent was removed under reduced pressure and the product was purified using column chromatography. Two hundred milligrams (0.44 mmol, 36% yield) of the product were obtained.

Fluorescent labeling of the cells

The staining of the LDs with the molecular rotor BODIPY-C₁₂ was done according to the literature (39). In brief, a stock solution with a concentration of 1 mg/ml in methanol was prepared and 10 μl of this solution was added to the living cells in petri dishes containing 2 ml of medium. After 30 min incubation at 37°C and 5% CO₂, the cells were washed twice with PBS buffer (Invitrogen). Directly before FLIM measurements, fresh differentiation medium was added.

FLIM measurements

For the viscosity measurements, the fluorescence lifetime of the BODIPY-C₁₂ dye was determined. For this purpose, we employed a homebuilt FLIM setup based on an inverted microscope (Axiovert 200, Zeiss, Germany) equipped with a water immersion objective (1.25 NA, 1.3, LCI Plan-Neofluar, Zeiss). The fluorescence signal was collected in a backscattering geometry, focused on a confocal pinhole, filtered with a longpass filter (488 LP, Semrock), split in two rays with a beamsplitter, and detected with two avalanche photodiodes (SPCM-AQR-14, Perkin-Elmer). Fluorescence lifetimes were determined using TCSPC electronics (Hydraharp 400, PicoQuant, Germany). Lifetime determinations were done using the Symphotime software (PicoQuant). For fluorescence lifetime measurements of BODIPY-C₁₂ solutions, one droplet of the solution was pipetted onto a glass coverslip and the signal was detected at a distance of 10 μm above the glass-liquid interface. For temperature-dependent lifetime measurements, an indium tin oxide (ITO) coverslip was used instead. The temperature of the ITO coverslip was then resistively heated to temperatures of up to 160°C (40). FLIM measurements in live cells were done on the microscope with the Petri dishes either being held at room temperature or at 37°C using a commercial heating stage (Tokai Hit, Japan).

RESULTS

Quantification of LD fusion by CARS microscopy

In order to directly observe the fusion of LDs, we performed long-term 3D CARS microscopy experiments on unlabeled living cells 3–5 days after the addition of the differentiation medium. The cells were continuously imaged for a minimum of 24 h and a maximum of 120 h. In all experiments, we detected fusion events between LDs. Figure 2A shows selected images of the exemplary observation of several subsequent fusion processes between different
LDs which were observed during 96 h (cf. supplementary Movie I). The right part of Fig. 2A shows the rendered color coded LDs and their movement over time. One should note that this movement is most likely due to the motion of the whole cell and not caused by active transport. Whereas LDs in nonadipogenic cells are actively transported via the tubulin network (41), LDs in adipocytes are surrounded by intermediate filaments (42). After nearly 96 h, the number of LDs in the cells has significantly decreased, while the remaining LDs have a much larger volume. Because the cells were imaged in three dimensions, the data can be used to derive the volume changes of the observed LDs as a function of time (Fig. 2B). One important result is that the fusion events at later stages are the fastest. This is expected because in these cases the pressure differences between the LDs are also the largest and this factor is supposed to be the main driving force of the lipid transfer (16). The temporal dynamics of single fusion events, however, frequently show a clear decrease of the rate of transferred LD volume during the course of a fusion (cf. Fig. 2B and Fig. 3). This observation is surprising because it contradicts the aforementioned assumption that the fusion rate solely depends on the pressure difference between the fusing LDs, which should increase during a single fusion event. One should note here, that this effect can only be seen at early differentiation stages, when the pressure difference between the fusing LDs is not yet very large, because the LD sizes are comparable. Inspection of individual fusion events furthermore shows that the lipid transfer is always directed from a smaller LD to a larger LD. In addition, the data show that “acceptor” LDs, which in earlier fusion processes have received material from other LDs, can in later fusion events donate their material to another acceptor by becoming a “donor” (see Fig. 3 and supplementary Movie II) (17).

The quantitative analysis of the obtained data also indicates that the fusion of LDs is indeed the only process significantly contributing to an increase of LD size. This can be seen by analyzing the overall volume of LDs after several fusion processes (Fig. 2C). The right side of Fig. 2C shows the volume increase of the remaining large LD from Fig. 2A after nine fusion events (same color coding as in

**Fig. 2.** A: Exemplary long-term CARS microscopy experiment of the adipogenic differentiation of mesenchymal stem cells in which several LD fusion events were captured (cf. supplementary Movie I). One main acceptor (red) finally incorporates the whole volume of several donor LDs. The images on the left are taken from a time series recorded over 96 h. The right part shows the rendered volumes of all fusing LDs. In addition, the spatiotemporal trajectory of the main acceptor is depicted. In order to visualize the temporal progress, the trajectory is color coded from blue to red. Scale bar: 15 μm. B: Volume change of the LDs depicted in (A) during 96 h calculated by volume rendering using the Imaris Software package (Bitplane). Overall, only the main acceptor LD (red) increases its volume, starting from around 100 μm³ up to nearly 900 μm³. All the other LDs serve as donors by transferring their complete volume to the acceptor LD. The lower part of (B) is a zoom into the region marked in light blue. C: Comparison between the surface increase of the main acceptor LD and the net surface provided by the fusing LDs (left) and comparison between the volume increase of the main acceptor LD and the net volume of all fusing LDs (right). The graph shows that the volume increase of the main acceptor LD is due to fusion events. The black LD from Fig. 2B is not included, because its content is transferred via the purple LD.
Fig. 3. Zoom into Fig. 2B demonstrating that the lipid transfer rates are not increasing over time, but instead are decelerating. Furthermore this example illustrates the ability of LDs to change from acceptor to donor and vice versa. In this case the LD depicted in purple initially accepts material from the LD in black, before donating its content to the LD shown in red.

Fig. 2A). The overall volume increase of the large LD thus very closely corresponds to the added volume of all the other LDs which fused with the remaining LD. The net growth of the latter thus appears to be mainly due to LD fusion, even if it cannot be excluded that direct synthesis and incorporation of triacylglycerides and equilibrium with the fusion and budding of smaller LDs (43) play a certain role. At the same time, the sum of the surface area of all the fusing LDs is nearly a factor of three larger than the surface increase of the final large LD (left side of Fig. 2C). A similar behavior with conservation of LD volume and decrease in LD surface area has earlier been observed for drug-induced fusion events (20).

Measurements of LD viscosities and temperatures in living cells

For the quantitative description of LD fusion processes, it is mandatory to know the viscosity of the material transferred. Because the composition of the interior of fusing LDs is not known, we performed experiments allowing us to measure LD viscosities directly in living cells. The measurements are based on the recently published dye BODIPY-C_{12} (35, 38). BODIPY-C_{12} is a BODIPY derivative which carries an alkoxy-substituted phenyl ring as substituent at the carbon bridging the two ring systems (inset of Fig. 4A). It has been reported that the fluorescence lifetime of BODIPY-C_{12} is sensitive to the viscosity of the surrounding medium. This is explained by the viscosity-dependent rotation of the substituent which serves as a radiationless decay channel for the electronically excited state. An increase in solvent viscosity leads to the suppression of the rotation and, consequently, to an increase in the dye’s fluorescence lifetime. As a calibration measurement, we determined the fluorescence lifetimes of BODIPY-C_{12} in pure triolein at different temperatures for which the viscosities are known (45). We measured the lifetimes from room temperature up to 160°C (see Fig. 4A). As expected, the lifetime changes in an exponential manner from approximately 2 ns at room temperature to approximately 0.5 ns at 160°C. It should be noted that in the original publication, the calibration measurements were done with methanol/glycerol mixtures of varying concentrations (38) resulting in a dependence which we could also reproduce (cf. supplementary Fig. I). The two calibration methods, however, clearly result in different calibration curves, most probably because of the influence of the polarity of the surrounding medium. We assume that for the measurement of the viscosity inside LDs, the calibration with pure triolein is more accurate. Early work on the dependence of the quantum yield $\Phi_i$ of triphenylmethane dyes as examples for molecular rotors on the solvent’s dynamic viscosity $\eta$ yield

$$\Phi_i = A \eta^\alpha$$

where $A$ is a constant and the exponent $\alpha$ has a value between 1/3 and 2/3 (46). Using

$$\Phi_i = k_r \tau_i$$

with the fluorescence lifetime $\tau_i$ and the fluorescence emission rate $k_r$, this leads to

$$\tau_i = A k_r \eta^\alpha$$

which is found in the log-log representation in Fig. 4A. This curve can be used as a calibration curve for the determination of the local viscosity of the BODIPY-C_{12} labels in other measurements.

It has previously been postulated that BODIPY-C_{12} labels membranes of intracellular organelles (47). Because BODIPY dyes in general are very good labels for LDs (48), we however assumed that BODIPY-C_{12} with its additional long alkyl chain would accumulate in LDs. For this reason we labeled differentiated adipocytes with BODIPY-C_{12}. The comparison of the bright field image and the fluorescence image of the same cells clearly shows that BODIPY-C_{12} indeed efficiently stains LDs (Fig. 4B). This is also confirmed by costaining the LDs with a commercial LD marker (cf. supplementary Fig. II).

We next performed live cell FLIM experiments using BODIPY-C_{12} as a label. In these measurements, we found contrast for two different kinds of cellular structures, namely LDs with a lifetime of 1.6 ns, which corresponds to a viscosity of about 57 cP, and other organelle-like structures with a lifetime of more than 2 ns (Fig. 4C). A negative control without BODIPY-C_{12} shows that the latter structures are not LDs and that the signal originates from autofluorescence (cf. supplementary Fig. III). This is in agreement with another study where it was demonstrated that cells in an early stage of adipogenic differentiation, still without larger LDs, show a characteristic autofluorescence signal with a characteristic lifetime of more than 2 ns (49). To make sure that LDs could be distinguished from these other structures also in cells with smaller LDs, we stimulated LD production in HeLa cells by addition of oleic acid to the medium. Also, for this system, we found the same fluorescence lifetime of around 1.6 ns inside the LDs (cf. supplementary Fig. IV). The measured lifetime of 1.6 ns in LDs at 37°C differs slightly from the lifetime of pure triolein, which shows a lifetime of around 1.4 ns at the same temperature. It should be noted that bright spots
We have employed CARS microscopy in order to investigate the dynamics of LD fusion in living adipocytes. In this application, CARS microscopy offers two advantages over other microscopy approaches. First, it is a label-free technique that allows the examination of living cells under nearly physiological conditions. In this respect, it is of special importance that no labeling artifacts can occur. Second, it is possible to perform long-term observations using CARS microscopy, because the samples exhibit no photobleaching. It is thus possible to directly follow the differentiation of the same cells on the microscope for several days.

Similar to 3D fluorescence imaging data, CARS microscopy data can be analyzed in order to determine the volume of LDs and, from repeated imaging, their volume change as a function of time. This analysis gives some direct insights into the growth mechanisms of LDs. Most obviously, it shows that during adipogenic differentiation, LDs grow by a slow exchange of material between adjacent LDs. This process has recently been reported in a number of publications (16–18). Our data show that its result is the growth of one LD, while the other one is completely

**DISCUSSION**

**LD fusion**

We have employed CARS microscopy in order to investigate the dynamics of LD fusion in living adipocytes. In this application, CARS microscopy offers two advantages over other microscopy approaches. First, it is a label-free technique that allows the examination of living cells under nearly physiological conditions. In this respect, it is of special importance that no labeling artifacts can occur. Second, it is possible to perform long-term observations using CARS microscopy, because the samples exhibit no photobleaching. It is thus possible to directly follow the differentiation of the same cells on the microscope for several days. Similar to 3D fluorescence imaging data, CARS microscopy data can be analyzed in order to determine the volume of LDs and, from repeated imaging, their volume change as a function of time. This analysis gives some direct insights into the growth mechanisms of LDs. Most obviously, it shows that during adipogenic differentiation, LDs grow by a slow exchange of material between adjacent LDs. This process has recently been reported in a number of publications (16–18). Our data show that its result is the growth of one LD, while the other one is completely
vanishing simultaneously (Figs. 2A, 3). Therefore, we refer to this process as LD fusion. These LD fusions are observed in nearly all cells. One can thus state that under these conditions, LD growth is a commonly observed phenomenon and not a rare event for differentiating adipocytes. Our data not only show that LDs can grow by fusion, they also give insight into the importance of this process with respect to other growth mechanisms. Thus, Fig. 2C shows that, within our measurement accuracy, the complete volume growth of a LD, which has undergone nine separate fusion events with other LDs over the course of altogether more than 90 h, originates from fusion. LD fusion therefore seems to be by far the most important growth mechanism of large LDs during adipogenic differentiation.

**Pressure difference as driving force of LD fusion**

As has been reported previously (16), all fusion events observed in our experiments take place such that net lipid transfer always occurs from the smaller to the larger LD. The directionality and the rate of lipid transfer during LD fusion therefore seem to be determined by the size difference between the fusing LDs. The difference $\Delta P$ between the internal pressure $p_{\text{in}}$ of an LD and the pressure of its environment $p_{\text{out}}$ can be calculated using Laplace’s equation (16)

$$\Delta P = p_{\text{in}} - p_{\text{out}} = \frac{2\gamma}{r}$$

Here, $r$ is the radius of the vesicle and $\gamma$ denotes its surface tension. Assuming that the fusing LDs 1 and 2 have the same surface tension, the difference of internal pressure $\Delta P$ between the LDs is then given by

$$\Delta P = p_{\text{in1}} - p_{\text{in2}} = 2\gamma \left(\frac{1}{r_1} - \frac{1}{r_2}\right)$$

Three different models for the lipid transfer during the fusion process need to be discussed, a purely physical model with the pressure difference as the driving force, a model with active transport by a dedicated protein machinery, and a passive diffusion model with specific proteins maintaining an appropriate fusion structure.

Several arguments make it improbable that LD fusion is a process driven solely by minimization of surface energy, such as Ostwald ripening. Under this condition, closely packed LDs, as they are always found at intermediate and later stages of adipogenic differentiation (e.g., Fig. 4B), would have to undergo spontaneous fusion. Fusion, however, always only takes place between a few of the LDs, while many other LDs can be in close contact without fusing. In addition, the recent finding that Fsp27 is recruited to the contact point (16) hints at the fusion process being dependent on specific proteins. By contrast, a model based on the transport of LD material by proteins is unlikely because it is difficult to reconcile with the directionality of the transport being dependent on the LD diameter. Also, the finding that LDs can, in some fusions, be the acceptors of material from other LDs and later on become donors of LD material in another fusion event (17) is difficult to explain in an active transport model. For these reasons, we favor a model in which specific proteins first establish a contact point between two fusing LDs, and eventually generate a pore between both LDs through which flow driven by the pressure difference between the differently sized...
LDs leads to a net transport of material from the smaller to the larger LD. This net transport is atypically a slow process. Figure 2B shows that complete fusion events mostly take place on an hour timescale. Only if the size difference between the fusing LDs becomes very large, can one observe fusion events which are completed faster. This is in agreement with the assumption that the pressure difference determines the transfer rate. This assumption, however, leads to the conclusion that the material transfer should also accelerate during a single fusion process, because the difference in diameters of the fusing LDs increases. Yet, we observe that the lipid transfer rate frequently slows down until the fusion is completed. This behavior is especially pronounced when the pressure difference between the fusing LDs is comparatively small, and hints to the point that the lipid transfer rate cannot be determined by the pressure difference alone.

**Hagen-Poiseuille model for LD fusion through a pore**

Mainly, the finding that Fsp27 localizes to the contact point between two fusing LDs has led to the assumption that material exchange between the fusing LDs might take place via a fusion pore between the two LDs (32). Pore formation between the LDs would require the interaction of several proteins. During the fusion process, it is necessary that the pore is kept open and that its shape is stabilized, such that the membranes of the two LDs do not instantly fuse. FRAP experiments have been used earlier to demonstrate that the membrane between two fusing LDs is not continuous (16). A possible function of Fsp27 in this scenario, is to provide a tether at the contact site between two LDs (31). Such a tethering function has already been shown in other processes where two membranes generate a contact site, which reduces the distance between the membranes to less than 30 nm (52). This facilitates the exchange of a variety of substances, including lipids, between the two participating organelles. Meanwhile, a more detailed model describing the potential mechanism by which Fsp27 stabilizes the membrane contact point has been published (32). It is postulated that at the contact point, Fsp27 can exist in an active and an inactive conformation, with the active form being a Fsp27 homodimer. This homodimer, which is believed to facilitate the lipid transfer between the LDs, might be stabilized by the interaction of Plin1 with the CIDE-N domain of Fsp27.

The simplest model for the calculation of the fusion pore size assumes laminar flow through a narrow and, in comparison to its diameter, long channel. This gives rise to a laminar flow which is described by the Hagen-Poiseuille equation. The assumptions just described do not hold true here, and one would thus expect turbulent flow. Theoretical investigations of water transport through carbon nanotubes, however, have shown a no-slip Hagen-Poiseuille type behavior also for pores with a radius on the order of the pore length (53). Because the viscosity of the lipids under consideration, in our case, is generally higher than that of water, we therefore expect a Hagen-Poiseuille type behavior also in the case of LD fusion. The resulting pore radius \( r \) is then given by

\[
r = \sqrt{\frac{dV}{8nlt}} \sqrt{\frac{\pi \Delta \rho}{l}}
\]

where \( V \) is the transported volume, \( l \) the fusion pore length for which we estimate a value of 10 nm for a channel crossing two membrane layers, and \( \eta \) the viscosity of the transported LD content. The pressure difference \( \Delta \rho \) between two fusing LDs depends on their respective radii and the surface tension \( \gamma \). The surface tension of the LD will be determined by the exact composition of the phospholipid monolayer and the nature of the interior and the exterior of the LD, as well as by its size. Recent measurements on phosphatidylcholine adsorption onto triolein bubbles yield a value of \( \gamma = 20.8 \text{ mN/m} \) (54). At the same time, the radius of the LD only seems to change the surface tension significantly at very strong curvatures, i.e., radii at around 5 nm (55). We therefore adopt this value for the further calculation. LD radii for the calculation of \( \Delta \rho \) as well as the transfer rates, \( dV/dt \), are directly retrieved from the CARS measurements by rendering of the LD volumes from the 3D CARS imaging data. The only remaining missing value for the calculation of the pore sizes then is the viscosity \( \eta \), which we determine from the FLIM experiments with BODIPY-C12 to be 57 cP.

Using the Hagen-Poiseuille model for the analysis of our experimental data, we find that while the pressure difference between the LDs clearly determines the directionality of the net lipid transport, it does not seem to be the only factor determining the lipid transfer rate. A calculation of the lipid transfer rate for each time point from a total amount of 44 individual fusion events is shown in Fig. 6A. As can be seen in the figure, the lipid transfer rate lies in the range between approximately 5 \( \mu \text{m}^3/\text{h} \) and more than 140 \( \mu \text{m}^3/\text{h} \) depending on the size of the two participating LDs. Sun et al. (32) have reported similar values of \( \sim 14 \mu \text{m}^3/\text{h} \) for the lipid transfer rates in cells expressing Fsp27 and Plin1 and \( \sim 5.4 \mu \text{m}^3/\text{h} \) for cells expressing Fsp27 alone. In both cases, the diameter of the acceptor was \( \sim 5 \mu \text{m} \) and the diameter of the donor was \( \sim 3 \mu \text{m} \).

While the pressure difference is expected to increase with an increase in the difference in diameters between the two fusing LDs, we find that the maximum lipid transfer is obtained when both LDs, the acceptor and the donor, are as large as possible (Fig. 6A). An explanation for this observation is a dependence of the size of the putative fusion pore on the size of the two LDs. Because the lipid transfer rate depends on \( r \), any change in the pore radius \( r \) will have a drastic effect. Interestingly, it has already been shown for pores of exocytotic vesicles which are fusing with the plasma membrane that their sizes depend on the size of the vesicle (56). Equally, it has been demonstrated for supramolecular SNARE complexes between two liposomes that the size of the SNARE aggregates depends on the size of the two participating vesicles (57). If there is a dependence of the fusion pore diameter on the size of the two participating LDs because of their limited contact area, one would assume...
that this should be mainly influenced by the smaller donor LD. Therefore, we calculated the pore size with the aforementioned equation for the 44 observed fusion events and plotted the fusion pore size as a function of the diameters of the two LDs (see Fig. 6B). Indeed, we observe a clear dependence of the fusion pore size on the diameter of the donor LD, whereas there seems to be no direct influence of the diameter of the acceptor LD. The calculated fusion pore sizes range from 2 to 25 nm. Their derived size dependence on the LD diameter agrees very well with that reported for pores on exocytotic vesicles (56).

Another possible contribution to the observed decrease of the lipid transfer rate can be derived from the observation that the volume of the fusing LDs is maintained during the fusion process (Fig. 2G, right), whereas the net surface increase is only a third of the sum of the surface areas given by the donor LDs (Fig. 2C, left). This means that during the fusion process, superfluous phospholipid membrane must be removed. There are several possibilities how this can be accomplished. Compression of the membrane is not a relevant process for membrane area reduction because, for a modest compaction of phospholipids in the membrane by a few percent, the energetic cost was found to rise exponentially (58). This would lead to the emergence of a strong force counteracting the pressure difference driven fusion. Also, the fact that the membranes of the fusing LDs were found to remain discontinuous at the fusion site (16) excludes membrane compaction as a reason for the lipid transfer rate deceleration. While compared with the volume of a LD, the absolute amount of phospholipids which need to be removed is small (59), other mechanisms for membrane removal must be present. The phospholipids could be inserted into either the lipids of the LD or expelled into the cytoplasm after metabolic transfer into significantly more water-soluble lysophospholipids. Several mechanisms for the accumulation and removal of phospholipids in LD membranes are under discussion (59). Protein-mediated transport with different transport proteins has been shown for phosphatidylcholine in vivo as well as in vitro (60). Members of the STARD family as well as of the PITP and SEC 14 families seem to be capable of transporting phospholipids, even if the starting- and end-point of this transport are not yet identified (61, 62). Phospholipid transport could also occur toward the ER, either by contact points established by the protein seipin (59, 63), or by a permanent association of the LDs with the ER (64). One could thus assume that the lipid transfer rate is reduced by the removal of the excess LD membrane phospholipids through one of the above mentioned processes.

CONCLUSION

In conclusion, our work confirms recent reports that the growth of LDs during adipogenic differentiation is due to LD fusion processes. We have followed such fusion events with label-free CARS microscopy on the same cells over several days. The quantitative observation of these fusion events, together with the earlier finding that Fsp27...
locates to the contact site between the fusing LDs, points to a fusion model in which passive diffusion driven by a pressure difference is transferring the LD content through a fusion pore defined by a specific protein complex. Based on this assumption, we have expanded the established Laplace model in which the directionality of the lipid transfer during fusion is determined by the size of the respective LDs by a simple model of laminar flow through a fusion pore. In order to calculate the size of the putative fusion pore, we have measured the viscosity of the LD content in living adipocytes using a FLIM-based approach. In contrast to the expectation from the Hagen-Poiseuille model, we find that the volume transfer during fusion decreases with time. At the same time, the transfer rate shows a clear dependence on the diameter of the donor LD. We discuss several possible reasons for this apparent deviation and propose that the diameter of the fusion pore, which is calculated to range from 2 to 25 nm, directly depends on the size of the donor LD.

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