Cytosolic Lipid Droplets Increase in Size by Microtubule-Dependent Complex Formation

Pontus Boström, Mikael Rutberg, Johanna Ericsson, Peter Holmdahl, Linda Andersson, Michael A. Frohman, Jan Borén, Sven-Olof Olofsson

Objectives—Adipocyte differentiation-related protein (ADRP)-containing lipid droplets have an essential role in the development of insulin resistance and atherosclerosis. Such droplets form in a cell-free system with a diameter of 0.1 to 0.4 μm, while the droplets present in cells vary in size, from small to very large, suggesting that the droplets can increase in size after being assembled. We have addressed this possibility.

Methods and Results—Experiments in NIH 3T3 cells demonstrated that the lipid droplets could increase in size independently of triglyceride biosynthesis. NIH 3T3 cells were either microinjected with ADRP–GFP (green fluorescent protein) or stained with Nile Red and followed by confocal microscopy and time-lapse recordings. The results showed that lipid droplets formed complexes with each other, with a volume equal to the sum of the merging particles. The formation of complexes could be inhibited by the nocodazole-induced depolymerization of the microtubules; thus, the process is dependent on microtubules. The presence of dynein on ADRP-containing droplets supports a role for this motor protein.

Conclusions—Lipid droplets can grow after they have been assembled. This increase in size is independent of triglyceride biosynthesis and involves formation of complexes, which requires intact microtubules. (Arterioscler Thromb Vasc Biol. 2005;25:1945-1951.)

Key Words: lipid droplets ■ lipid droplet fusion ■ adipocyte differentiation protein ■ microtubules

Cytosolic lipid droplets are organelles involved in the storage and turnover of triglycerides and cholesterol esters. Excessive accumulation of triglycerides, particularly in the liver and skeletal muscle, is associated with metabolic disorders such as insulin resistance and type 2 diabetes, with a strongly increased risk of cardiovascular diseases. Moreover, the accumulation of lipid droplets in macrophages is a key feature of both early and late stages of the atherosclerotic lesion. The PAT domain protein ADRP (adipocyte differentiation-related protein) both promotes the formation of lipid droplets and is an important constituent of the lipid droplets formed in liver, muscles, and macrophages. Thus, ADRP-containing lipid droplets are important in the pathogenesis of these diseases. In addition, lipid droplets have been proposed to have a central role in the inflammatory response, which may further link the structures to the development of both insulin resistance and atherosclerosis.

Phospholipase D (PLD), which catalyzes the conversion of phosphatidylcholine to phosphatidic acid, promotes the assembly of ADRP-containing lipid droplets from intracellular membranes (L.A., P.B., M.R., J.E., Marchesan D, Magnusson B, Ruiz M, P.H., Asp L, M.A.F., J.B., S.-O.O., unpublished data, 2005). The newly formed (“primordial”) droplets have a diameter of 0.1 to 0.4 μm, whereas the mature droplets present in a cell can be more than 10 to 50× larger (even larger in adipocytes) and vary considerable in size, indicating that the primordial lipid droplets increase in size after being formed. This increase appears to be an important step in the process leading to the accumulation of cytosolic lipid droplets in the cell.

In this article we have investigated the assembly of cytosolic lipid droplets, focusing on the mechanism by which they increase in size. We demonstrate that lipid droplets can grow in size by a process that is independent of triglyceride biosynthesis and involves the formation of complexes between the droplets.

Materials and Methods

For detailed Materials and Methods, please see the supplemental information available online at http://atvb.ahajournals.org.

Results

Newly Assembled ADRP-Containing Lipid Droplets Can Increase in Size

In the cell-free system, the newly formed lipid droplets were 0.1 to 0.4 μm in diameter. In NIH 3T3 cells, the smallest

Original article April 2005; final version accepted July 12, 2005.
From the Wallenberg Laboratory for Cardiovascular Research (P.B., M.R., J.E., L.A., J.B., S.-O.O.), Göteborg University, Sahlgrenska University Hospital and Holmstedt Biotech (P.H.), Göteborg, Sweden; and the Department of Pharmacological Science and the Center for Developmental Genetics (M.A.F.), Stony Brook University, Stony Brook, NY.
Correspondence to Sven-Olof Olofsson, Wallenberg Laboratory, Sahlgrenska University Hospital, SE-413 45 Göteborg, Sweden. E-mail Sven-Olof.Olofsson@wlab.gu.se

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Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org DOI: 10.1161/01.ATV.0000179676.41064.d4

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droplets observed after Oil Red O staining were 0.35 μm in diameter. However, when these cells were incubated with oleic acid for increasing periods, the smallest droplets decreased in number, and larger droplets (>3μm in diameter) formed (Figure 1A). To test the possibility that lipid droplets grow in size after being assembled, NIH 3T3 cells were pulsed with oleic acid for 2 hours and chased for 2 hours in the presence of 20μmol/L triacsin C, a potent inhibitor of the triglyceride biosynthesis. Under these conditions, insignificant amounts of radiolabeled fatty acids were incorporated into triglycerides (Figure I, available online at http://atvb.ahajournals.org). After the pulse, the greatest proportion of lipid droplets were of small size (<3μm) (Fraction I in Figure 1B and 1C). When the cells were chased in the presence of triacsin C, there was a significant decrease in the proportion of droplets present in Fraction I (Figure 1B and 1C, Chase), with a compensatory increase in larger particles present in Fraction II (Figure 1B and 1C, Fraction II). The total area of Oil Red O–stained lipid droplets per cell did not change during the chase. These results indicate that the assembled droplets can increase in size independently of triglyceride biosynthesis.

Imaging experiments with live NIH 3T3 cells after incubation with oleic acid showed that the droplets can form complexes with each other. This complex formation was observed in >20 experiments. The formation of typical complexes is shown in detail in two-dimensional (2D) images and in 3D reconstructions (Figures 2 and 3; Figure 1. Assembled lipid droplets increase in size with time. A, NIH 3T3 cells were incubated with oleic acid for the indicated periods and stained with Oil Red O. The size of the stained droplets was measured, and the percentages of droplets with a diameter <3 μm (squares) or >3 μm (triangles) were plotted. B, NIH 3T3 cells were pulsed with oleic acid for 2 hours (squares) and chased in the presence of 20 μmol/L triacsin C for 2 hours (triangles). The size distribution of the Oil Red O–stained lipid droplets was determined after the pulse and chase. Double arrows indicate the two fractions (I and II) analyzed in C (I, the small droplets present after the pulse; II, the larger droplets generated during the chase). C, Distribution of the total area of Oil Red O–stained lipid droplets between fraction I (major size droplets formed during the oleic acid pulse, see B) and fraction II (the larger droplets) after the oleic acid pulse (Pulse) and the triacsin C chase (Chase) (mean±SD, n=3; Fraction I, P=0.033; Fraction II, P=0.038; t test).

Figure 2. Complex formation and adhesion between lipid droplets. A, NIH 3T3 cells were microinjected with ADRP–GFP and imaged by confocal microscopy at intervals of 30 seconds. Shown are 2D images of a flattened z-stack. The green arrow shows a small droplet that moves toward the center of the cell to be adsorbed on and finally form a complex with a larger droplet (red arrow). The blue arrow shows a similar-sized droplet that also moves toward the center of the cell to adhere to the droplets marked with the yellow arrow. Bar=10 μm. B and C, Close-ups of the droplets indicated by red and green arrows in A. B shows the 2D images of the fattened z-stack, whereas C shows a 3D reconstruction. Bar=5 μm (For close-ups of the adhering droplets [blue and yellow arrows], see Figure II.)
Movies I through X, available online at http://atvb.ahajournals.org).

In Figure 2A, NIH 3T3 cells were microinjected with ADRP-GFP. After 2 hours, the fluorescent protein was associated with discrete droplets. The cells were then followed by time-lapse photography at 30-second intervals. The results show two events marked with blue-yellow and green-red arrows in Figure 2. The blue-yellow arrows show how a droplet moves toward the center of the cell, changes direction, and adheres to another droplet (Figure 2; see also Movie I). The close-up of the event (Figure IIA and Movie II, available online at http://atvb.ahajournals.org) as well as the 3D reconstruction (Figure IIB and Movie III) verifies the adhesion of the two droplets, but they did not form a complex with one fluorescent center during the time investigated.

The green-red arrows show an event in which a smaller droplet (Figure 2A, green arrow) moves toward the center of the cell to be adsorbed on the surface of a larger droplet (Figure 2A, red arrow) and disappears (see also Movie I). Figure 2B shows a close-up of the event (see also Movie IV). The 3D reconstruction (Figure 2C; Movie V) shows that the small droplet did not disappear behind the larger one, but instead interacted with it and was absorbed by it. Thus, the two droplets were replaced by one.

In the second experiment (Figure 3A), the neutral lipids in the cytosolic lipid droplets were stained with Nile Red and the cells were followed by time-lapse photography at 30-second intervals. The green and red arrows point to 2 closely localized droplets that are eventually replaced by a complex with one fluorescent center (Figure 3A; Movie VI). A close-up of the event is shown in Figure 3B and 3C. The 2 droplets adhered to each other (Figure 3B, recording 5) and were replaced by 1 droplet with a homogenous distribution of fluorescence (Figure 3B, recordings 7 to 9; Movie VII). The 3D reconstruction (Figure 3C; Movie VIII) shows that 1 droplet did not simply disappear under the other into a different confocal plane. Instead, the 2 droplets interacted (Figure 3C, recording 4); they clearly adhered to each other (Figure 3C, recordings 5 and 6) and were connected by a “waist-like” structure that disappeared when the 2 droplets were replaced by a single droplet (Figure 3C, recording 7).

The complexity of the complex-forming process is illustrated in Figure III (available online at http://atvb.ahajournals.org) showing a close-up of an event in cells microinjected with ADRP-GFP (time lapse started after 2 hours in culture). The 2D images (Figure IIIA and Movie IX) show one pair of droplets (2 centers of fluorescence and a clear border between the 2 droplets), which adhered to a third droplet (Figure IIIA, recordings 2 to 4), and all droplets were replaced by 1 droplet with homogenous fluorescence (Figure IIIA). The 3D reconstructions (Figure IIIB and Movie X) showed that 1 droplet adhered to the other (and did not simply drop out of the confocal plane), again resulting in the formation of a waist-like structure (Figure IIIB, recordings 2 and 3) that disappeared when a single larger droplet formed (Figure IIIB, recordings 5 to 10).

The volume of the droplets formed was almost identical to the expected volume (the sum of the volumes of the starting droplets). The difference was 6±19% (n=20 formed complexes) of the expected volume.

**The Formation of Lipid Droplet Complexes Is Microtubule-Dependent**

To begin to investigate the mechanism behind the formation of the complexes, we investigated whether the microtubule system is involved. The cells were incubated with oleic acid for 2 hours and chased for 0 or 2 hours in the presence of triacsin C with and without nocodazole, which at 2 μg/mL destroyed microtubules (Figure 4A). In the absence of nocodazole, the proportion of lipid droplets within Fraction I decreased (Figure 4B; compare with Figure 1B and 1C), and there was a compensatory increase in larger particles (Fraction II; Figure 4B; compare with Figure 1B and 1C). In the presence of nocodazole, however, the particle distribution was unchanged (Figure 4B and 4C). Cells chased in the presence of triacsin C differed from those chased in the
The formation of complexes between lipid droplets in NIH 3T3 cells is dependent on intact microtubules and their interaction with motor proteins. A, Cells were incubated with or without 2 μg/mL nocodazole for 1 hour and then subjected to immunohistochemistry with antibodies to α-tubulin. Bar=10 μm. B, Cells were incubated with oleic acid for 2 hours and chased for 0 hours (squares) or 2 hours in the presence of triacsin C (20 μmol/L; circles), or triacsin C and nocodazole (2 μg/mL; triangles). The size distribution of Oil Red O–stained lipid droplets was determined after each chase and plotted as a percentage of the total area of Oil Red O–stained lipid droplets between fraction I (major size droplets formed during the oleic acid pulse, see B) and fraction II (the larger droplets) after the oleic acid pulse (Pulse) and chase with triacsin C and nocodazole (Chase) (mean±SD; n=4). D, Cells were transfected with ADRP-GFP, homogenized, and immunoprecipitated with antibodies to GFP or a nonspecific Ig coupled to tosyl-activated Dynabeads. The retained fractions were immunoblotted against antibodies to dynein. E, Cells were incubated with oleic acid for 4 hours in the absence and presence of vanadate (10 μmol/L). The total area of Oil Red O–stained lipid droplets was measured in all cells in 20 randomly selected pictures. Values are mean±SD (P<0.001, Mann–Whitney rank-sum test). F, Cells were incubated with the oleic acid for 2 hours in the presence of triacsin C and in the presence (squares) or absence (triangles) of vanadate (10 μmol/L). The size distribution of Oil Red O–stained lipid droplets was determined after each chase and plotted as a percentage of the total area of Oil Red O–stained droplets in the cell.

We also carried out time-lapse studies after microinjection of ADRP–GFP or after staining of the cells with Nile Red and let the computer identify all fusions that occurred over 5 minutes. Treatment with nocodazole reduced formation of complexes by 88±5% (n=3) in the Nile Red–treated cells. The corresponding mean decrease in 2 ADRP–GFP experiments was 67%. Thus, nocodazole inhibited the formation of complexes between lipid droplets, indicating that both this process and the growth of lipid droplets are dependent on the microtubule system.

Next, we examined ADRP-containing droplets for the motor protein dynein. Cells were transfected with ADRP–GFP, and the chimeric protein was isolated by immunoprecipitation in the absence of detergents (to preserve droplet structure). As shown by immunoblotting, ADRP coprecipitated with dynein (Figure 4D), suggesting that the ADRP-containing droplets could be transported on microtubules by this motor protein.

Finally, we assessed the effect of vanadate, which blocks ATPase activity among that of dynein, on the assembly and fusion of lipid droplets in NIH 3T3 cells. Vanadate reduced the amount of Oil Red O–stained lipid droplets, supporting the possibility that the interaction between motor proteins and microtubules has a central role in the formation of the droplets (Figure 4E). In the experiment shown in Figure 4F, NIH 3T3 cells were incubated with oleic acid in the presence of triacsin C (to prevent the biosynthesis of triglycerides; compare with Figure 4B) and in the absence or presence of vanadate, and the sizes of the lipid droplets were measured. The results indicate that vanadate prevented the increase in size of the droplets.

To study the adhesion process in greater depth, we established a cell-free system. NIH 3T3 cells in separate dishes were transfected with ADRP–GFP or incubated with [3H]-palmitic acid to label triglycerides. Homogenates from both dishes were then coincubated and immunoprecipitated with antibodies to GFP using Dynabeads, and labeled triglycerides were recovered. ADRP–GFP precipitated ≈30% of the [3H]-labeled triglycerides from the second cell homogenate; nonspecific binding (to a nonimmune Ig) was 6% (Figure 5A). This indicates that droplets from ADRP–GFP–labeled cells had formed complexes with droplets from [3H]-labeled cells. When the incubation was carried out in the presence of 1 mmol/L calcium, 63% of the radioactivity could be precipitated with the GFP antibody, but nonspecific precipitation was unchanged (Figure 5A). Thus, complex formation was calcium-dependent. There was also an increase in the amount of immunoprecipitable radiolabeled triglycerides when the cell-free system was treated with Mg2+; however, the increase was twice as high when Ca2+ was used. This points to a specific effect of Ca2+. 
The formation of complexes between lipid droplets is dependent on microtubules in a cell-free system. A, Cells in one culture dish were transfected with ADRP–GFP, and cells in a second dish were incubated with [3H]-palmitic acid. Homogenates of cells from both dishes were co-incubated for 1 hour with or without 1 mmol/L Ca2+. The sample was first precipitated with a nonspecific Ig bound to Dynabeads (control for nonspecific precipitation), and the bound and unbound fractions were analyzed for radiolabeled triglycerides. The supernatant (unbound fraction) was then immunoprecipitated with antibodies to GFP (coupled to Dynabeads), and the bound and unbound fractions were analyzed for radiolabeled triglycerides. Results are expressed as the percentage of total radiolabeled triglycerides precipitated; values are mean±SD (nonimmune Ig, n=9; anti-GFP, n=8; nonimmune Ig+Ca2+, n=3; anti-GFP+Ca2+, n=11). *P<0.003 vs nonimmune Ig, †P<0.001 vs Ca2+ nonimmune Ig; 1-way ANOVA. B, The same experiment as in A, but the cell culture transfected with ADRP–GFP was also incubated with [14C]-palmitic acid; whereas the other cell-culture dish was labeled with [3H]-palmitic acid. The anti-GFP precipitate was analyzed for the recovery of [14C]- and [3H]-labeled triglycerides. (mean±SD, n=4). ‡P<0.001 vs nonimmune Ig [14C]; †P<0.001 vs nonimmune Ig [3H]; ‡P=0.063 vs anti-GFP [14C]. One-way ANOVA. C, Experiment as in A in the absence (control) or presence of Ca2+ and nocodazole as indicated. Results are expressed as recovery of [3H]-triglycerides after immunoprecipitation with antibodies to GFP. Values are mean±SD (Control, n=4; 1 mmol/L Ca2+, n=7; 1 mmol/L Ca2+-nocodazole, n=4). *P=0.018 vs control, †P=0.019 vs control, ‡P<0.001 vs Ca2+-nocodazole; 1-way ANOVA. D, Immunofluorescence of tubulin in the cell-free system. E, Immunofluorescence of tubulin after treatment of the cell-free system with nocodazole. F, The influence of Ca2+ on the amount of dynein on ADRP-containing lipid droplets in the cell-free system. The system was incubated in the presence or absence of 1 mmol/L Ca2+; ADRP–GFP-containing lipid structures were recovered by immunoprecipitation as described in A, and the retained fraction was blotted against antibodies to dynein.

To verify that ADRP–GFP complexed with the droplets from the second cell homogenate carried triglycerides, we performed double labeling experiments (Figure 5B). Cells in the first dish were transfected with ADRP–GFP and labeled with [14C]-palmitic acid; cells in the second dish were labeled with [3H]-palmitic acid. Both [14C]-labeled and [3H]-labeled triglycerides were recovered after immunoprecipitation of ADRP–GFP, indicating that triglyceride-containing structures had formed complexes with each other. Recovery of [14C]-labeled triglycerides tended to be higher, probably because the GFP antibody also precipitated noncomplexed droplets (ie, those containing only [14C]-labeled triglycerides).

Next, we addressed the importance of microtubules and motor proteins in the cell-free system. Incubation of cell homogenates in the presence of nocodazole completely inhibited complex formation (Figure 5C; compare with the nonspecific binding in Figure 5A). To investigate whether the cell-free system contained organized microtubules, we analyzed the tubulin in the homogenate by immunofluorescence. The results (Figure 5D) demonstrated the presence of polymerized microtubules. Treatment of the system with nocodazole depolymerized the microtubules (Figure 5E). These results clearly demonstrated that intact microtubules were essential for the merging of droplets in the cell-free system.

To determine whether microtubules were important for complex formation or for keeping the complex together, we investigated the effect of conditions that depolymerized microtubules before and during the immunoprecipitation (ie, after the incubation). Decreasing the temperature to 4°C, which rapidly depolymerizes microtubules, failed to influence the recovery of the droplet complex; the ratio between treated and control cells yielded a similar ratio between treated and control cells (0.93±0.26; n=3). These results, together with the observation that the complex formation was completely inhibited when nocodazole was added during the incubation, indicate that microtubules participate in the formation, but not in the stabilization, of the lipid droplet complexes.

Next, we assessed the effect of calcium and vanadate on the interaction between droplets and microtubules in the cell-free system. Ca2+ (1 mmol/L) increased the amount of dynein that coprecipitated with ADRP (Figure 5F), suggesting that the calcium dependence reflects the importance of the interaction between lipid droplets and microtubules. Vana-
date (10 μmol/L, with 1 mmol/L Ca^{2+}) reduced the recovery of labeled triglycerides during the immunoprecipitation from 64.5±10.5% (n=11) to 44.6±13.4% (n=3; P<0.017, t test), indicating decreased formation of lipid droplet complexes.

The Rate of Complex Formation
To assess the quantitative importance of the formation of lipid droplet complexes, we used the computer program to identify all complexes that were formed in four 5-minute time-lapse experiments and that met the following criteria: (1) the volume of the droplet formed was at least equal to, but did not exceed by more than 50%, the sum of the volumes of the two substrate droplets; (2) the substrate droplets were clearly identifiable and not more than 3.5 μm apart at time points before the complex formation; and (3) the droplet formed was present at the next time point and had not changed in volume. All formation of complexes identified by the program was confirmed manually by analysis of 2D and 3D images.

The formation of complexes was more frequent when the droplets were smaller and more abundant and became less frequent as the droplets become larger and less abundant (Figure IV, available online at http://atvb.ahajournals.org). Thus, the number of lipid droplets was linearly related to the frequency of complex formation (r=0.76). The sizes of the droplets that formed complexes with each other were not correlated (r=0.24), indicating that droplets of any size can form complexes with each other.

Discussion
In this article, we present for the first time results indicating that lipid droplets increase in size by a process that is independent of triglyceride biosynthesis. Time-lapse recordings and confocal microscopy of NIH 3T3 cells microinjected with ADRP-GFP or stained with Nile Red indicated that fusion between the droplets could explain this increase in size. The process is dependent on intact microtubules.

An understanding of the mechanism behind the assembly of cytosolic lipid droplets is of utmost importance for our understanding of the metabolic diseases of the twenty-first century: insulin resistance, type II diabetes, and cardiovascular diseases. The droplets of most direct importance for these diseases are formed in liver, muscles, and macrophages. Such droplets contain the PAT protein4,5 ADRP,8,9 which has a central role in the assembly of lipid droplets; another key factor in the assembly of lipid droplets,12 results that were confirmed recently in intact cells (L.A., P.B., M.R., J.E., Marchesan D, Magnusson B, Ruiz M, P.H., Asp L, M.A.F., J.B., S.-O.O., unpublished data, 2005). Thus, without any doubt, information on how ADRP-containing lipid droplets are assembled is essential for the understanding of our most important metabolic diseases.

As mentioned, ADRP, which is a fatty acid binding protein,20 has a central role in the assembly of lipid droplets; thus, overexpression of ADRP gives rise to an increase in the formation of droplets8 (L.A., P.B., M.R., J.E., Marchesan D, Magnusson B, Ruiz M, P.H., Asp L, M.A.F., J.B., S.-O.O., unpublished data, 2005). However, the mechanism remains to be clarified. We observed that PLD and the production of phosphatidic acid were essential for the assembly of lipid droplets,12 results that were confirmed recently in intact cells (L.A., P.B., M.R., J.E., Marchesan D, Magnusson B, Ruiz M, P.H., Asp L, M.A.F., J.B., S.-O.O., unpublished data, 2005). The results from the experiments in intact cells also indicated that part of the effect of PLD1 could be explained by regulation of the intracellular concentration of ADRP (L.A., P.B., M.R., J.E., Marchesan D, Magnusson B, Ruiz M, P.H., Asp L, M.A.F., J.B., S.-O.O., unpublished data, 2005). Thus, an increase in PLD1 gives rise to a large increase in ADRP, resulting in increased formation of lipid droplets.

Another key factor in the assembly of lipid droplets, originally found to be a cytosolic activator of lipid droplet assembly,12 was recently identified as extracellular signal regulated kinase (ERK)2 (L.A., P.B., M.R., J.E., Marchesan D, Magnusson B, Ruiz M, P.H., Asp L, M.A.F., J.B., S.-O.O., unpublished data, 2005). The results from studies in a cell-free system indicated that the newly assembled lipid droplets had a size between 0.1 and 0.4 μm.12 Small droplets were also detected in cells both after subcellular fractionation12 and by microscopy after Oil Red O staining (this study); however, it is well known that the cells also contain much larger droplets, suggesting that the process by which lipid droplets are formed is complex and involves a second step in which the ‘primordial’ droplets can grow in size. In this article, we have demonstrated that such growth can occur without ongoing triglyceride biosynthesis. One reason for this was elucidated by in vivo imaging studies, which demonstrated that 2 droplets could form a complex with each other, giving rise to a larger droplet with homogenous fluorescence. The 3D reconstructions excluded the possibility that 1 droplet simply disappeared from the confocal plane, revealing instead an intermediate waist-like struc-
ture that connected the droplets and disappeared when the 2 droplets were replaced by a single droplet. Moreover, the volume of the droplet formed was very close to the sum of the volumes of the substrate droplets. Together, these results indicate that two droplets are replaced by one, ie, they fuse with each other, to form a larger droplet.

Nocodazole inhibited the fusion of the droplets, indicating a dependence on microtubules. In several cases, we observed that lipid droplets are transported toward the center of the cell, suggesting dynein-mediated transport on microtubules. Consistent with this notion, ADRP-containing droplets also contained the motor protein dynein. An involvement of dynein is also supported by the observation that vanadate, which inhibits the ATPase of the motor proteins, prevented the droplets from growing in size. However, when evaluating these results it should be kept in mind that vanadate has a rather unspecific effect. Moreover, in many cases we could not observe an unequivocal central transport of the droplets before the complex was formed. Instead, they seemed to be localized close to each other before they fused. This suggests that the microtubules are not only important for long-distance transport, but also for the process by which the droplets are brought so close together that the fusion machinery can start working. This is supported by the observations in the cell-free system, which verifies the importance of microtubules in the formation of complexxes but argues against the idea that they keep the droplets together after the complex has been formed.

The importance of microtubules and motor proteins for the transport of cytosolic lipid droplets is in agreement with observations in Drosophila.”21

Our results indicate that the fusion process seen in intact cells can be reconstructed in the cell-free system. Such a system will be very useful when evaluating the molecular background to the fusion process. Indeed, the lipid droplets contain proteins that may affect the process through their involvement in sorting processes (the Rab proteins)22,23 and fusion events (α-SNAP).22

The rate of merging events varied, and droplets of any size could form complexes with each other. This makes it difficult to estimate how long it would take for a large droplet to form. A conservative estimate based on our results would suggest that a lipid droplet with a diameter of 4 μm could be assembled in <10 minutes. Thus, the fusion between droplets is a quantitatively important mechanism in the formation of larger lipid droplets.

In summary, lipid droplets can increase in size by a mechanism that does not involve triglyceride biosynthesis but is based of the fusion of the droplets with each other. The process requires microtubules and, most likely, the motor protein dynein.

Acknowledgments

This study was supported financially by the Swedish Medical Research Council (grant 7142) and by the Swedish Heart and Lung Foundation, Novo Nordic Foundation, Swedish Strategic Funds (National Network and Graduate School for Cardiovascular Research), and the Söderberg Foundation. The imaging was carried out at the Swegene center for cellular imaging in Göteborg. L.A. holds a Swegene postdoctoral position in proteomics.

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Arterioscler Thromb Vasc Biol. 2005;25:1945-1951; originally published online July 28, 2005; doi: 10.1161/01.ATV.0000179676.41064.d4
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/25/9/1945

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SUPPLEMENTARY INFORMATION

MATERIAL AND METHODS

Sodium pyruvate, L-glutamine, nonessential amino acids, calf serum, penicillin, and streptomycin were obtained from PAA Laboratories (Linz, Austria). Trasylol (aprotinin) was from Bayer Leverkusen (Leverkusen, Germany). Fatty acid-free bovine serum albumin, phenylmethylsulfonyl fluoride, pepstatin A, ALLN, and oleic acid were from Sigma. Leupeptin was from Chemicon (Temecula, CA). Calf serum was from Invitrogen.

Antibodies—Antibodies to ADRP were obtained from Research Diagnostics (Flanders, NJ). Horseradish peroxidase-linked anti-guinea pig and anti-rabbit immunoglobulin (Ig) were from Dako (Glostrup, Denmark) and Amersham Biosciences (UK), respectively. Fluorescein-conjugated anti-guinea pig Ig, antibodies to dynein and antibodies to α-tubulin were obtained from Abcam (Cambridge, UK).

Cell Culture—NIH 3T3 cells were cultured as recommended by the American Type Culture Collection. Incubation with oleic acid (360 µM in 0.3% BSA) was carried out as described previously.

Quantification of Lipid Droplets—To quantify the amount of triglycerides stored in lipid droplets, we determined the total area of lipid droplets in the cell. Cells were grown on coverslips and stained with Oil Red O as described previously and the total area of red pixels in the Oil Red O stained droplets was calculated as described previously. As shown, this measure of the amount of neutral lipids in cytosolic lipid droplets showed an excellent correlation with the amount of triglycerides in the cell.
The BioPix software (www.biopix.se) used to estimate the total area of Oil Red O-stained lipid droplets could also be used to measure the size of the individual droplets. 

**Microinjection and imaging of living cells.** Purified ADRP-GFP plasmids (0.11 mg/ml) were microinjected into the nuclei of NIH 3T3 cells grown in 35-mm glass-bottomed culture dishes (MatTek, Ashland, MA). Injections were done at room temperature with a semi-automatic system consisting of Microinjector 5242 and Micromanipulator 5171 (Eppendorf, Germany) connected to an inverted Axiovert 100TV microscope (Carl Zeiss, Germany). Needles were pulled from 1.2-mm I.D. and 0.94-mm I.D glass capillaries GC12OTF-10 (Harvard, UK) with a P-97 needle puller (Sutter Instruments, Novato, CA). After injection, the cells were incubated for 1–2 h at 37°C in a 5% CO₂ atmosphere before transfer, or they were transferred directly to a 37°C stage chamber, equipped with a flow of moist CO₂, on a Nikon eclipse TE 2000-U microscope. The microscope was equipped with a QLC100 Dual Disc confocal laser, with two XR/MEGA-10 ICCD cameras using ITT model 9910C GenIV Intensifier Tubes (Visitech International, Alexandria, VA). Pictures of 20–40 confocal planes through the cell (z-stack) with a step size of 0.2–0.3 μm were taken with a 63× objective, every 30 sec for 5–10 min using Voxcell 3.61 software (Visitech). Images were converted to TIF format in Voxcell and further converted to JPEG format using Image J, 1.32j (freeware from NIH, MD).

To obtain a three-dimensional model of droplets during the time-lapse studies, we integrated the confocal planes, 20 slices (0.3 μm) per time point, with a previously described algorithm. Under these conditions, the pixel is either black or white, and the software uses the intensity to determine whether the pixel belongs to the droplet or not. Adjacent pixels were identified and used to construct the three-dimensional image as described previously. The volume of the objects was determined as the sum of pixels in the object.
A cell-free system to investigate the complexes between droplets. NIH 3T3 cells in two cell culture dishes (10 cm²) were used. The cells in one dish were transfected with ADRP-GFP and both cell cultures were incubated with oleic acid (see above) overnight. The nontransfected cells were also incubated overnight with [³²H]-palmitic acid to label the triglycerides formed. The cells were harvested, suspended in 1 ml PBS and homogenized by 30 strokes in a Dounce homogenizer. Non-homogenized cells were removed by centrifugation (500~g for 1 min). 50 µl from each homogenate was mixed, an additional 50 µl of PBS was added, and the mixture was incubated at 37°C for 1 h. After this incubation, the homogenate was first precipitated with unspecific immunoglobulins coupled to Dynabeads (as recommended by the manufacturer). The retained fraction from this precipitation was used as a control of the unspecific binding to the precipitation system. The unretained fraction was immunoprecipitated with antibodies to GFP (bound to Dynabeads). Triglycerides were isolated from unretained and retained fractions and the radioactivity determined. In some cases (as indicated in the text), the ADRP-GFP transfected cell culture was also labeled with [¹⁴C]-palmitic acid. The second cell culture was incubated with [³²H]-palmitic acid under these conditions.

Other methods. Immunofluorescence was carried out as described in ⁵. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting and separation of radioactive triglycerides were carried out as described ⁶. Immunoprecipitation was carried out with antibodies bound to tosyl-activated Dynabeads using the protocol recommended by the manufacturer (Dynal Biotech, Oslo, Norway). The Mann-Whitney rank sum test, t-test or one-way ANOVA were used for statistical analysis as indicated.
Fig I. Triacsin C blocks the incorporation of [3H]-palmitic acid into triglycerides. NIH 3T3 cells were treated with 20µM triacsin C (Triacsin C) or without (Control) for 2 hours and then incubated with [3H]-palmitic acid for 15 or 60 min as indicated. The cells were subjected to solvent extraction, the triglycerides were recovered by thin-layer chromatography and the radioactivity was determined (Mean ± SD; n = 3).

Fig II. Two droplets adhere to each other, but do not merge. A shows a close up of the event marked with blue and yellow arrows in Fig. 2A, while B shows a 3D reconstruction of this event. (Bar, 5 µm).

Fig III. The merging of lipid droplets. NIH 3T3 cells were microinjected with a plasmid encoding the fusion protein ADRP-GFP. After 2 hours, the cells were followed by confocal microscopy and time-lapse recordings. A, (2D) shows a close-up of an event in which one of two closely localized droplets starts a fusion process with a third droplet. After the fusion has been completed, all droplets have been replaced by one large droplet with one fluorescence center. B shows the 3D reconstruction. The recordings demonstrate that none of the droplets involved disappear out of the confocal plane. Moreover, the waist-like connection between the merging droplets can be seen clearly. (Bar, 1µm).

Fig IV. The rate of fusion of lipid droplets is dependent on the amount of droplets in the cell. NIH 3T3 cells were stained with Nile Red or microinjected with a vector encoding ADRP-GFP, and the fusions that appeared over 5 min were recorded (after 3D reconstruction) and correlated with the quantity of droplets present in the cell before the recording period. To vary the quantity of lipid droplets, the cells were cultured in the absence or presence of oleic acid for different times.
**Movie 1.** Corresponding to Fig. 2A. NIH 3T3 cells were microinjected with ADRP-GFP and investigated with confocal microscopy and time-lapse recording. One small droplet (at 12 o’clock) moves toward the center of the cell and is adsorbed on a larger droplet and merges with this droplet. Another droplet (at 8 o’clock) also moves toward the center of the cell, stops and changes direction to adhere to another droplet. In this case, no merging occurs.

**Movie 2** Corresponding to Supplementary Fig. II A. A close-up of the 8 o’clock event seen in Movie 1. One droplet moves towards another and adheres to it, but does not fuse with it.

**Movie 3.** Corresponding to Supplementary Fig. II. A close-up of the 8 o’clock event also seen in Movie 1 (3D reconstruction).

**Movie 4** Corresponding to Fig. 2B. A close-up of the 12 o’clock event in Movie 1. One droplet moves toward the center of the cell. The droplet is adsorbed to a larger droplet and merges with this droplet.

**Movie 5.** Corresponding to Fig. 2C. A close-up of the 12 o’clock event in Movie 1 (3D reconstruction).

**Movie 6.** Corresponding to Fig. 3A. The lipid droplets of NIH 3T3 cells were stained with Nile Red and the cells were investigated with confocal microscopy and time-lapse recordings. Two droplets located close to each other at 3 o’clock merge with each other to form one fluorescence center.
**Movie 7.** Corresponding to Fig. 3B. A close-up of the event seen in Movie 6. Two droplets at 3 o’clock merge with each other.

**Movie 8.** Corresponding to Fig. 3C. A close-up of the event seen at 3 o’clock in Movie 6 (3D reconstruction)

**Movie 9.** Corresponding to Supplementary Fig. IIIA. One of two closely located droplets starts a fusion process with a third droplet. After the fusion, all droplets have been replaced by one large droplet with one fluorescence center.

**Movie 10.** Corresponding to Supplementary Fig. IIIB. A 3D reconstruction of Movie 9.

**REFERENCES**

Formed triglycerides (dpm)

Fig I
Supplementary Fig III
Supplementary Fig IV