Active involvement of micro-lipid droplets and lipid-droplet-associated proteins in hormone-stimulated lipolysis in adipocytes

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Summary

The regulation of lipolysis in adipocytes involves coordinated actions of many lipid droplet (LD)-associated proteins such as perilipin, hormone sensitive lipase (HSL), adipose triglyceride lipase (ATGL), and its activator protein, CGI-58. Here, we describe the cellular origin and physiological significance of micro LDs (mLDs) that emerge in the cytoplasm during active lipolysis, as well as the roles of key lipolytic proteins on mLDs in differentiated 3T3-L1 adipocytes. Multiplex coherent anti-Stokes Raman scattering (CARS) microscopy demonstrated that mLDs receive the fatty acid (FA) moiety of triglyceride from pre-existing LDs during lipolysis. However, when FA re-esterification was blocked, mLDs did not emerge. Time-lapse imaging of GFP-tagged LD-associated proteins and immunocytochemical analyses showed that particulate structures carrying LD-associated proteins emerged throughout the cells upon lipolytic stimulation, but not when FA re-esterification was blocked. Overall lipolysis, as estimated by glycerol release, was significantly lowered by blocking re-esterification, whereas release of free FAs was enhanced. ATGL was co-immunoprecipitated with CGI-58 from the homogenates of lipolytically stimulated cells. Following CGI-58 knockdown or ATGL inhibition with bromoenol lactone, release of both glycerol and FA was significantly lowered. AICAR, an activator of AMP-activated protein kinase, significantly increased FA release, in accordance with increased expression of ATGL, even in the absence of CGI-58. These results suggest that, besides on the surface of pre-existing central LDs, LD-associated proteins are actively involved in lipolysis on mLDs that are formed by FA re-esterification. Regulation of mLDs and LD-associated proteins may be an attractive therapeutic target against lipid-associated metabolic diseases.

Key words: Lipid droplet, Lipolysis, Lipid metabolism, Fatty acid, CARS microscopy

Introduction

Excessive lipid accumulation in adipocytes is a central feature of obesity and metabolic syndrome. Lipid droplets (LDs) were long regarded as metabolically inactive lipid storage depots lacking an active role in cellular lipid metabolism. However, because of the recent discovery of LD-associated proteins, they are now considered as active organelles involved in diverse cellular processes, such as membrane traffic and lipid metabolism (Farese and Walther, 2009). LDs are composed of a central core of triacylglycerol (TAG) or cholesterol ester (CE) and a surrounding phospholipid monolayer. Excess energy is primarily stored as TAG in LDs of adipose tissue, and the TAG reserves are hydrolyzed by a process called lipolysis, to supply fatty acids (FAs) to various tissues in cases of energy demand such as starvation and exercise. The prevailing pathway of regulated lipolysis involving several lipases and LD-associated proteins is summarized as follows (Brasaemle, 2007; Granneman and Moore, 2008; Granneman et al., 2009; Yamaguchi, 2010; Zechner et al., 2009). In quiescent adipocytes, perilipin binds to comparative gene identification (CGI)-58 (also called α,β-hydrolase domain-containing [ABHD] 5), a co-activator of adipose triacylglycerol lipase (ATGL), on the surface of LDs, hence preventing CGI-58 from interacting with ATGL. Perilipin also blocks hormone-sensitive lipase (HSL) from accessing LDs. Upon lipolytic stimulation by catecholamines, protein kinase A (PKA) phosphorylates perilipin, and phosphorylated perilipin releases CGI-58. CGI-58 is now free to interact with ATGL, and resulting ATGL/CGI-58 complex efficiently hydrolyzes TAG to diacylglycerol (DAG) and FA. DAG is then hydrolyzed to monoacylglycerol (MAG) and FA by phosphorylated HSL that is now freed from blockage by perilipin to be translocated to LD-surfaces. MAG is further decomposed to glycerol and FA by MAG lipase.
Using coherent anti-Stokes Raman Scattering (CARS) microscopy, we previously demonstrated that numerous micro-lipid droplets (mLDs) appeared in all areas of the cytoplasm (Yamaguchi et al., 2007). We suggested that they are formed from organelles, e.g. endoplasmic reticulum (ER), but not from pre-existing central LDs. A very recent study demonstrated, also by CARS microscopy, that mLDs are formed de novo during lipolysis when cellular FAs are overloaded, and suggested that mLDs protect cells from the toxicity of excess FAs (Paar et al., 2012). On the other hand, it was suggested that mLDs are derived from large central LD coated with perilipin, based on the observations that mLDs are coated with perilipin and their formation required phosphorylation of perilipin on Ser492 (Marcinkiewicz et al., 2006). It was also reported that LD-associated proteins including ATGL, HSL, perilipin and CGI-58 are distributed on small particulate structures (Granneman et al., 2007). Hence, the cellular origin and physiological significance of mLDs are to be established. It is also critical to discover where the LD-associated proteins functionally cooperate during active lipolysis, given the observation that CGI-58 is dissociated from LDs in response to PKA activation (Yamaguchi et al., 2007). Finally, the roles of central LDs, mLDs, and other cellular structures in active lipolysis should individually be defined.

In the present study, we performed microscopy and biochemical studies to examine the cellular origin and physiological significance of mLDs as well as the functional interplay of LD-associated proteins during lipolysis in differentiated 3T3-L1 adipocytes. We show that mLDs are formed by packaging of TAG that was produced by FA re-esterification. They are engaged in active rehydrolysis of TAG to increase the proportion of FAs to be released rather than re-stored.

**Results**

**Time-lapse imaging of LD-associated proteins during lipolysis**

We first performed microscopy studies of the trafficking of key lipolytic proteins in differentiated 3T3-L1 adipocytes. During lipolysis, GFP-perilipin was retained on the surface of large LDs, and also appeared on particulate structures in the cytoplasm (Fig. 1A; supplementary material Movie 1). While some GFP-CGI-58 was also retained on the surface of large LDs, it was dissociated from large LDs to the cytoplasm upon lipolytic stimulation, and appeared on particulate structures in the cytoplasm (Fig. 1B; supplementary material Movie 2). On the other hand, both HSL and ATGL showed cytosolic distribution in the basal state (Fig. 1C,D). During lipolysis, HSL was rapidly redistributed to the surface of large LDs, and later exhibited particulate structures in the cytoplasm (Fig. 1C; supplementary material Movie 3). While ATGL was mostly retained in the cytoplasm during lipolysis, this protein was also redistributed to the surface of large LDs, and exhibited particulate structures in the cytoplasm, though the images were blurred by intense cytosolic fluorescence (Fig. 1D; supplementary material Movie 4). Overall, these lipid-associated proteins showed numerous particulate structures in the cytoplasm of 3T3-L1 adipocytes upon lipolytic stimulation.

One could conceivably argue that the above dynamic trafficking of LD-associated proteins were an artifact observed in the cells grown on two-dimensional (2D) flat surfaces. Such cells are morphologically different from the unilocular fat cells in vivo. We therefore cultured 3T3-L1 adipocytes in 3D collagen matrix and examined the trafficking of GFP-perilipin upon lipolytic stimulation (supplementary material Fig. S1). The mature adipocytes contained 1–3 giant central LDs (>20 μm in diameter) and peripheral small LDs in the cytoplasm in the basal state, and GFP-perilipin was abundant in the peripheral LDs as previously reported (Moore et al., 2005). In response to 1 hr lipolytic stimulation, GFP-perilipin became observed on particulate structures in the cytoplasm, besides retained on the surface of large LDs. The appearance of these particulate structures was similar to that in 2D-cultured cells, suggesting the physiological relevance of the trafficking of LD-associated proteins.

**Multiplex CARS imaging of mLDs during lipolysis**

To examine where mLDs are derived from, we employed multiplex CARS microscopy (for details, see Okuno et al., 2010). We asked whether mLDs contain FAs derived from pre-existing LDs or synthesized de novo. For this purpose, we pre-loaded 3T3-L1 adipocytes with deuterium-labeled palmitate (D-palmitate), and then subjected the cells to lipolysis in the absence of D-palmitate (Fig. 2A). In the basal state, D-palmitate was detected in the pre-existing large LDs (Fig. 2B). During lipolytic stimulation, mLDs containing both C-H and C-D bonds appeared in the cytoplasm, and increased with time (Fig. 2B). This result suggests that mLDs receive FAs from pre-existing LDs, directly by fragmentation of large LDs and/or indirectly by re-esterification of FAs. The latter possibility seems particularly significant, because a considerable portion of FA produced by lipolysis is usually re-esterified in adipocytes (Reshef et al., 2003).

Hence, we next asked whether mLDs are formed as a consequence of the fragmentation of large LDs or formed by FA re-esterification. To address this issue, we inhibited the FA
re-esterification and TAG synthesis by an acyl-CoA synthetase (ACS) inhibitor, triacsin C, and a DAG acyltransferase (DGAT) inhibitor, 2-bromooctanoate, and examined the appearance of mLDs by multiplex CARS microscopy. mLDs did not appear even after 32 min or 64 min of lipolytic stimulation, when re-esterification of FAs was inhibited (Fig. 2C,D). This result indicates that mLDs are formed by re-esterification of FAs derived from central LDs and possibly in part from the media.

**Distribution of LD-associated proteins under inhibition of FA re-esterification**

We examined whether the particulate structures containing perilipin, CGI-58, HSL, and ATGL were generated during lipolysis, when FA re-esterification was inhibited. In the basal state, similar to the results on GFP-tagged proteins (Fig. 1), the localization of HSL was cytoplasmic, whereas that of perilipin was on large central LDs (Fig. 3A). Upon lipolytic stimulation, HSL was redistributed to the surface of large central LDs and coexisted with perilipin. Furthermore, HSL and perilipin were distributed on small particulate structures throughout the cytoplasm. When re-esterification of FAs was inhibited, the structures coated with both HSL and perilipin did not emerge, both proteins coexisting solely on the surface of large central LDs. This distribution pattern lasted even during 6 hrs after lipolytic stimulation. These results indicate that the microstructures carrying perilipin and HSL are consistent with and likely to be the mLDS that are visualized by CARS microscopy.

Distribution of CGI-58 and ATGL during lipolysis was examined under the same conditions (Fig. 3B). In the basal state, the localization of ATGL was cytoplasmic, while that of CGI-58 was on large LDs. Upon lipolytic stimulation, CGI-58 was translocated to the cytoplasm with a speckle-like pattern, where being co-localized at least partially with ATGL. Even when re-esterification of FAs was inhibited, CGI-58 was translocated to the cytoplasm with a speckle-like pattern in response to lipolytic stimulation, being co-localized at least partially with ATGL, whose cytoplasmic distribution was not altered during lipolysis as in Fig. 1D and supplementary material Movie 4.

On the other hand, when cells were permeabilized with 0.01% digitonin before fixation with parafomaldehyde to decrease the cytosolic staining (see Materials and Methods), CGI-58 was retained on large LDs, while ATGL was depleted in the basal state (Fig. 3C). Upon lipolytic stimulation, ATGL was observed on
In accordance with this idea, we also observed that the particulate structures observed in double-immunostaining with the combinations of perilipin-HSL, perilipin-ATGL, and CGI-58-HSL (Fig. 3C).

Previously, Yamaguchi et al. observed that mLDs emerged upon lipolytic stimulation in 3T3-L1 cells in which CGI-58 was knocked down with siRNA (Yamaguchi et al., 2007), and hence suggested that CGI-58 is not required for the formation of mLDs. In accordance with this idea, we also observed that the particulate distribution of HSL and ATGL in the CGI-58 knockdown cells responding to lipolytic stimulation was not different from that in normal 3T3-L1 cells (data not shown).

A previous study showed that mLDs are not formed in lipolytically stimulated 3T3-L1 adipocytes, when 2% BSA (Paar et al., 2012) was added to the culture medium. We found, however, that the particulate structures containing perilipin and HSL appeared upon lipolytic stimulation even in the presence of 2% BSA (data not shown). Again, when re-esterification of FAs was inhibited, these particulate structures did not appear. Although the reason for the inconsistency between the previous and present studies is not clear, a possibility is that the lipolytic stimulation in the present study (0.5 mM IBMX and 10 μM isoproterenol) was strong enough to induce overflow of cellular FA.

### Lipolytic activity under inhibition of mLD generation

Next, we explored the significance of mLDs in lipolysis. To compare lipolytic activities in the presence and absence of mLDs, release of FA and glycerol into the media during lipolytic stimulation was measured with (2Br-TriaC) or without (Control) triacsin C and 2-bromooctanoate.

**Fig. 3. Distribution of endogenous perilipin, CGI-58, HSL, and ATGL during lipolysis in 3T3-L1 adipocytes.** (A) Differentiated 3T3-L1 cells were treated with 0.5 mM IBMX and 10 μM isoproterenol for the time indicated and permeabilized with 0.2% Triton X-100/PBS after fixation with paraformaldehyde, and distribution of endogenous HSL (green) and perilipin (red) during lipolysis was obtained by immunofluorescence microscopy. (A-a) In the basal state, the localization of HSL was cytoplasmic, while that of perilipin was on large LDs. (A-b) Upon lipolytic stimulation, HSL was redistributed to the surface of large central LDs and coexisted with perilipin. Furthermore, HSL and perilipin were distributed on small particulate structures throughout the cytoplasm. (A-c) When re-esterification of FAs was inhibited by the treatment of triacsin C and 2-bromooctanoate, mLDs coated with both HSL and perilipin did not appear, although HSL and perilipin were co-localized on the surface of large LDs. (A-d) Under inhibition of FA re-esterification, HSL/perilipin-coated mLDs did not appear even 6 hrs after lipolytic stimulation. Arrows indicate representative of mLDs. Scale bar: 20 μm. (B) Differentiated 3T3-L1 cells were treated with 0.5 mM IBMX and 10 μM isoproterenol for the time indicated and permeabilized with 0.2% Triton X-100/PBS after fixation with paraformaldehyde, and distribution of Myc-CGI-58 (green) and ATGL (red) during lipolysis was obtained by immunofluorescence microscopy. (B-a) In the basal state, ATGL was located throughout the cytoplasm, while CGI-58 was on large LDs. (B-b) Upon lipolytic stimulation, CGI-58 was translocated to the cytoplasm with a speckle-like pattern, where being co-localized at least partially with ATGL. (B-c) Even when re-esterification of FAs was inhibited with triacsin C and 2-bromooctanoate, CGI-58 was translocated to the cytoplasm and co-localized with ATGL. (B-d) A similar pattern of distribution was observed after 6 hrs of lipolytic stimulation in the presence of triacsin C and 2-bromooctanoate. Bar, 20 μm. (C) Differentiated 3T3-L1 cells were treated with 0.5 mM IBMX and 10 μM isoproterenol for 1 hr and permeabilized with 0.01% digitonin before fixation with paraformaldehyde, and lipid-associated proteins were detected by confocal microscopy. (C-a) In the basal state, perilipin (red) and CGI-58 (red) were found on large LDs, while HSL (green) and ATGL (green) were not detected. (C-b) Upon lipolytic stimulation, HSL (green) and ATGL (green) were distributed on the surface of large central LDs and coexisted with either perilipin (red) or CGI-58 (red). Furthermore, these proteins were also distributed on small particulate structures throughout the cytoplasm. (C-e) Note that when re-esterification of FAs was inhibited by the treatment with triacsin C and 2-bromooctanoate, microstructures coated with perilipin, CGI-58, HSL and ATGL did not appear, while the pairs of perilipin-HSL, perilipin-ATGL, CGI-58-HSL, and CGI-58-ATGL were co-localized on the surface of large LDs. Arrows indicate representative mLDs. Scale bar: 20 μm.
2-bromo-octanoate and triacsin C (Fig. 4). As expected, FA release was significantly increased with the inhibitors (2Br-TriaC) as compared with Control, indicating that FAs are in a large part re-esterified under usual lipolytic conditions, which were released into media when re-esterification was blocked (Fig. 4A). Consistent with this, total TAG levels and the size of large central LDs were reduced to a larger extent in the presence of the inhibitors of FAs re-esterification than those under usual lipolytic conditions (supplementary material Fig. S2). On the other hand, the inhibitors significantly reduced glycerol release (Fig. 4B).

Adipocytes have poor activity of glycerol kinase that is essential for the production of glycerol 3-phosphate, a key substrate for TAG synthesis (Leroyer et al., 2006; Reshef et al., 2003). Thus, glycerol produced by lipolysis in adipocytes is not efficiently re-used as the partner of FA re-esterification, but mostly released from the cells immediately. Hence, the glycerol moiety of TAG stored in mLDs, and released from there by lipolysis, is continuously supplied de novo (mostly by glycolysis in the present experimental setting), but not by glycerol at central LDs. In Control, glycerol release represents the sum of lipolytic activities at central LDs and mLDs, whereas in 2Br-TriaC, only lipolysis at central LDs because of the absence of mLDs. Thus, the decrease in glycerol release in 2Br-TriaC as compared with that in Control most conceivably represents the loss of lipolysis at mLDs, suggesting the significant contribution of mLDs to active lipolysis.

As both central LDs and mLDs release FAs by lipolysis, assuming a similar relative proportion of those FAs from either pool being re-esterified, the contribution of mLDs to FA release would be estimated (mLDs contribution); this would be achieved by applying the fraction for relative contribution of mLDs to FA release from Control cells (Fig. 4A, dotted line). Thus, mLDs contributes to nearly 50% of total FA release during lipolysis, indicating that a large amount of FAs generated by lipolysis is re-esterified into TAG, which is actively re-hydrolyzed on mLDs. Additionally, even in DMEM/10% FBS, glycerol release was significantly decreased, whereas FA release was significantly increased with the inhibitors (2Br-TriaC) (data not shown). These results mean that mLDs are involved in active lipolysis, contributing to increase in FA release.

Subcellular distribution and interaction of CGI-58 and ATGL

To exactly locate where CGI-58 and ATGL interacts, we analyzed their intracellular distribution before and after lipolytic stimulation by subcellular fractionation (Fig. 5A). In the basal state, ATGL was mainly cytosolic (Cyt), and also found in LD (LD) and pellet (Pel) fractions. On the other hand, CGI-58 was mainly found in the LD fraction. Upon lipolytic stimulation, the contents of ATGL in the LD fraction and CGI-58 in the Pel fraction increased, where ATGL and CGI-58 now abundantly co-existed.

We next examined the association of CGI-58 and ATGL in response to lipolytic stimulation. Immunoprecipitation of exogenously expressed Flag-CGI-58 was performed in the lysates from 3T3-L1 adipocytes that were lipolytically stimulated or not (Fig. 5B). Endogenous ATGL was co-immunoprecipitated with Flag-CGI-58 upon 2 hrs of lipolytic stimulation.

Significance of ATGL and CGI-58 in lipolysis

Given with the interaction of CGI-58 and ATGL upon lipolytic stimulation, release of glycerol and FAs was measured during lipolytic stimulation, in the presence (BEL (+)) or absence (BEL (−)) of bromoeno lactone (BEL), an irreversible inhibitor of the iPLA2 family of phospholipases/lipases including ATGL.
Experiments were performed with 3T3-L1 adipocytes treated with 2-bromo-octanoate and triacsin C (2-Br-triaC) or not (Control) with 2-bromo-octanoate and triacsin C (Fig. 6A,B). BEL reduced the activity of PKA-stimulated lipolysis in both Control and 2-Br-TriaC conditions, suggesting that ATGL is indeed involved in active lipolysis.

We also examined the significance of CGI-58 in lipolysis with 3T3-L1 adipocytes in which CGI-58 was knocked down with siRNA (CGI-58 KD). Glycerol release in response to lipolytic stimulation was significantly decreased in CGI-58 KD cells as compared to that in control cells (mismatch) (P<0.01) (Fig. 6C), consistent with our previous report (Yamaguchi et al., 2007). Notably, FA release was dramatically more decreased in CGI-58 KD cells as compared to that in mismatch cells (P<0.01) (Fig. 6D,E). When 3T3-L1 adipocytes were treated with 2-bromo-octanoate and triacsin C (2Br-triaC), CGI-58 knockdown had little effect on glycerol or FA release as compared to control cells.

We examined whether the apparent effect of CGI-58 RNAi was exactly due to knockdown of CGI-58 itself, not an off-target. For this purpose, a rescue experiment was performed by expressing a mutant CGI-58 mRNA that is not targeted with the siRNA, still encoding the wild-type amino acid sequence, in the CGI-58 KD cells (supplementary material Fig. S3). The rescued cells exhibited significant increase in the release of both glycerol and FA, and importantly, more prominently in FA release. This result supports the specificity of CGI-58 knockdown in the present study (Fig. 6F,G).

Effects of AICAR on lipolytic activity and the traffic of LD-associated proteins
Previously, Gaidhu et al. demonstrated that activation of AMP-activated protein kinase (AMPK) with N’-(β-D-Ribofuranosyl)-5-aminimidazole-4-carboxamide (AICAR) increased ATGL activity and FA release in response to lipolytic stimulation (Gaidhu et al., 2009). However, AICAR inhibited HSL activity, glycerol release upon lipolytic stimulation, and incorporation of glucose, FA, and glycerol into lipids. Accordingly, the authors suggested that FA re-esterification and lipogenesis were reduced by AICAR treatment (Gaidhu et al., 2009). Their suggestion prompted us to examine whether AICAR treatment decreased the generation of mLDs, thereby increasing FA release whereas decreasing glycerol release in response to lipolytic stimulation. We found that AICAR treatment significantly increased FA release, whereas it did not affect glycerol release (Fig. 7A). Furthermore, emergence of mLDs as assessed by immunostaining of perilipin, HSL, CGI-58, and ATGL was not influenced by AICAR treatment (data not shown). AICAR treatment significantly increased the amounts of phospho-acetyl-CoA carboxylase (Ser79) (p-ACC), phospho-AMPK (Thr172) (p-AMPK), and ATGL. No change, however, was observed in the amounts of perilipin, phospho-HSL (Ser565) (p-HSL), HSL, DGAT1, and CGI-58 (Fig. 7B,C).

Finally, we examined whether the effects of AICAR treatment are still observed even when CGI-58 was knocked down. AICAR treatment significantly increased FA release, although no change was observed in glycerol release, in CGI-58-KD cells (supplementary material Fig. S4). We also found that AICAR treatment significantly increased the amounts of p-ACC, p-AMPK, and ATGL, whereas no change was observed in perilipin, p-HSL, and HSL in these cells (supplementary material Figs S5, S6).

Discussion
The present study provides important findings in understanding molecular mechanisms of lipolysis in adipocytes: during lipolysis, significant amount of FAs generated by TAG hydrolysis are re-esterified, leading to formation of mLDs. These mLDs are coated with perilipin, HSL, CGI-58, and ATGL, representing active sites of lipolysis.

mLDs were already noted by other researchers, and purported to be derived from central large LDs (Braasemle et al., 2004; Guo et al., 2008; Marcinkiewicz et al., 2006; Miyoshi et al., 2007). On the other hand, a very recent study revealed, by using real-time quantitative imaging and electron tomography, that mLDs are formed throughout the cytoplasm during lipolysis, but are not formed by the fission from large LDs (Ariotti et al., 2012). In addition, another very recent study revealed, by using CARS microscopy, that mLDs are formed de novo during lipolysis when excess FAs are accumulated in the cells (Paar et al., 2012). In the present study, we used multiplex CARS microspectroscopy (CARS spectral imaging method) in order to elucidate the origin of the TAG contents in mLDs. CARS microscopy has high sensitivity to lipids abundant in long-chain hydrocarbons by...
tuning the laser wavelengths to CH stretch vibrational mode, and has proven to be an excellent tool for LD tracking (for a review, see Evans and Xie, 2008). The deuterium in a CD bond is heavier than hydrogen, placing CD vibrational stretching frequencies into near 2100 cm\(^{-1}\), which is an otherwise silent region of the biological Raman spectra. By incubating differentiated adipocytes with deuterium-labeled palmitate and replacing some CH bonds with CD bonds, it is possible to selectively image a specific chemical bond (i.e. CD bond) using CARS contrast. The multiplex CARS microspectroscopy enabled us to obtain simultaneous imaging of CD and CH stretch vibrational modes, and revealed that newly appearing mLDs are formed as a consequence of the fragmentation of large LDs and/or the re-esterification of intracellular FAs. On the other hand, with ACS and DGAT inhibitors, emergence of mLDs was suppressed. These results indicate that mLDs are formed by re-esterification of FAs derived from central LDs, presumably occurring at the ER, because ACS and DGAT are localized primarily in ER, and ER membranes are often found close to LDs (Blanchette-Mackie et al., 1995; Brasaele and Wolins, 2012). Although the precise mechanism that newly synthesized TAG is packaged in mLDs is uncertain, the prevailing model postulates that TAG is accumulated between the leaflets of phospholipid bilayer, and resulting lens-shaped structures are then pinched off (Brasaele, 2011; Brasaele and Wolins, 2012; Farese and Walther, 2009). It seems reasonable that mLDs are formed at ER, because ER can serve to provide adequate amounts of phospholipid to cover the large surface areas of the newly synthesized mLDs (Farese and Walther, 2009; Guo et al., 2008; Ohsaki et al., 2009).

Next, we examined the significance of mLDs as well as the functional interplay of LD-associated proteins in lipolysis. The significance of mLDs might be explained by the increase in the surface area available for lipolysis. Indeed, by a genome-wide RNAi screen in Drosophila Schneider 2 cells, Guo et al. demonstrated a defect in lipolysis in cells containing only a few large central LDs due to the lack of Cct1, which encodes an enzyme catalyzing the rate-limiting step in phosphatidylcholine synthesis (Guo et al., 2008). In addition, a previous study suggested a higher lipolytic activity on mLDs by the observation that smaller LDs shrunk faster than larger LDs (Paar et al., 2012). In the present study, overall lipolysis as estimated by glycerol release was significantly lowered by blocking FA re-esterification and thus mLD formation. During TAG synthesis on mLDs, glycerol produced by lipolysis was virtually all derived from glucose through glycolysis, not from free glycerol (Fig. 8); therefore, it is likely that the lowered glycerol release by re-esterification inhibitors represents loss of contribution of mLDs in total lipolysis rather than any change in intracellular utilization of free glycerol (see Results). Thus, mLDs are most probably active sites of lipolysis. In accordance with this, HSL and ATGL, pivotal lipases in adipocytes, co-existed with perilipin and CGI-58 on mLDs, which disappeared upon blocking FA re-esterification. The present study shows substantial rates of FA re-esterification, implicating a "futile metabolic cycle". What is the significance of mLDs generated on the basis of a futile metabolic cycle? The net efflux of FAs from the adipocytes,

**Fig. 7. Effect of AICAR on lipolytic activity.** (A) Release of glycerol and FA. (B) Estimation by densitometric scan of immunoblots for p-ACC, p-HSL, HSL, p-AMPK, perilipin, ATGL, CGI-58, and DGAT1, normalized with GAPDH. (C) Representative immunoblots examining the effect of AICAR treatment. Extracts from 3T3-L1 cells treated with (AICAR) or without (Control) AICAR were immunoblotted for p-ACC, p-HSL, HSL, p-AMPK, perilipin, ATGL, CGI-58, DGAT1, and GAPDH. *P<0.05, and **P<0.01 versus Control.
determined by the balance between lipolysis and FA re-
esterification, can at most be equal to the total lipolytic rate
and is typically less because of an active FA re-esterification
process. Increased lipolysis assisted by mLDs would be a
significant way to enhance the net efflux of FAs even without
a change in re-esterification rate. The fractional turnover rate
of mLDs is much higher than that of large LDs, so a relative shift
in the site of TAG turnover from large LDs to mLDs would greatly
increase the probability of re-stored FAs being rapidly
re-esterified and thus mobilized from the cells. Overall, the
appearance of rapidly turning-over mLDs may have a
physiological relevance to enhance the lipolytic activity,
thereby preventing an abundance of FAs recycling back to
slowly turning-over pools of glycerolipids and phospholipids.
Additionally, if there is any means of direct transfer of TAG from
central LDs to mLDs, not via hydrolysis and re-esterification,
mLDs would also be able to support the increase in net lipolytic
capacity. Protein-mediated transfer or vesicular transport would
be the possible means, though neither of them has been identified
as a mechanism of TAG transfer.

Again, the present study showed the elaborated cooperation of
perilipin, CGI-58, HSL, and ATGL on mLDs during lipolysis. In
addition to previously reported functional interaction of HSL with
perilipin on LDs (Granneman et al., 2007; Miyoshi et al., 2006; Shen
et al., 2009), interaction of exogenously expressed CGI-58 and
ATGL on the surface of LDs was also confirmed by biomolecular
fluorescence complementation (BiFC) or co-immunoprecipitation
studies in COS7 and CHO-K1 cells (Granneman et al., 2009; Wang
et al., 2011). In the present study, we demonstrated in differentiated
3T3-L1 adipocytes that endogenous CGI-58 and ATGL are co-
localized on the surface of LDs including mLDs, and Flag-tagged
CGI-58 expressed at a physiological level is co-immunoprecipitable
with ATGL upon lipolytic stimulation. Furthermore, the present
study demonstrates that BEL, an irreversible inhibitor of the iPLA2
family of phospholipases/lipases including ATGL, reduced the
activity of PKA-stimulated lipolysis. These results suggest that
ATGL is involved in active lipolysis interacting with CGI-58, at
least in part on the surface of LDs including mLDs. This finding
corroborates the previous report showing in a fluorescence
resonance energy transfer (FRET) experiment that CGI-58 and
ATGL exhibit interaction on particulate structures upon PKA
activation (Granneman et al., 2007).

We also observed in immunocytochemical analysis that upon
lipolytic stimulation, CGI-58 is translocated to the cytoplasm
with a speckle-like pattern, where being co-localized at least
partially with ATGL. In addition, a subcellular fractionation
study indicated that ATGL and CGI-58 co-existed in not only LD
fraction, but also cytosolic and pellet fractions, the latter of which
contained membranous organelles. Although the precise entity
and physiological significance of their cytoplasmic location
remain to be elucidated, previous reports suggested that CGI-58
is a coenzyme A-dependent lysophosphatidic acid acyltransferase
(LPAAT) (Ghosh et al., 2008; Montero-Moran et al., 2010), and
LPAAT enzymes are located in both ER and Golgi complex
(Schmidt and Brown, 2009). Hence, CGI-58 may act as LPAAT
at organelles such as ER and Golgi. On the other hand, we found
in the present study that CGI-58-KD cells suffered a drastic
decrease in FA release as compared with the reduction in the
glycerol release, suggesting an unidentified role of CGI-58 to
promote FA release. The enhanced decrease in FA release as
compared with glycerol release by CGI-58 knockdown was
diminished, if FA re-esterification was inhibited and thus mLDs
were not generated. Thus, we conclude that the lipolytic function
of CGI-58 is predominant on the surface of mLDs, and is related
to recruiting FA to be secreted rather than re-esterified.

AMPK is a sensor of cellular energy state that responds to
metabolic stress and other regulatory signals, and the
administration of AMPK activator AICAR to rats diminished
adiposity (Ruderman et al., 2003b). Given the previous study
suggesting the effect of AICAR on reduced re-esterification of FA
and lipogenesis (Gaidhu et al., 2009), we hypothesized that
AICAR treatment may have a similar effect to that by the
inhibition of FA re-esterification with 2-bromooctanoate and
triacsin C. In the present study, we found that AICAR treatment
significantly increased FA release, but without decreasing glycerol
release, contrary to the previous observation (Gaidhu et al., 2009).
As the appearance of mLDs decorated by perilipin, HSL, CGI-58,
and ATGL was not influenced by AICAR treatment, it is not
surprising that the glycerol release, representing the total lipolytic
activity, was not decreased. Because AICAR treatment
significantly increased the expression of ATGL, and its
co-activator CGI-58 seemed to have a role to increase FA release,
ATGL may also increase the ratio of FAs to be released. Hence,
increasing the AMPK activity in adipose tissue, for instance by
endurance exercise (Ruderman et al., 2003a), could be an attractive
target against obesity-associated metabolic disorders.

In summary, we conclude that significant amount of FAs
produced by TAG hydrolysis are re-esterified into TAG at ER
during lipolysis to form mLDs (Fig. 8). These mLDs are coated
with perilipin, HSL, CGI-58 and ATGL, and are active sites of
lipolysis, probably devoted to increasing total TAG turnover and
thus potentially net FA release (Fig. 8). CGI-58 is also possibly
dispersed to structures including organelles such as ER and/or
Golgi apparatus upon lipolytic stimulation, the physiological
meaning of which is to be elucidated.

Materials and Methods

Construction of recombinant lentiviruses
cDNAs of mouse CGI-58, HSL and ATGL were obtained as described previously
(Yamaguchi et al., 2004; Yamaguchi et al., 2007). Mouse perilipin cDNA was
obtained from adipocyte total RNA by reverse transcription (RT)-PCR. GFP-fusion
constructs were prepared using pGFP(105), a GFP mutant that has an increased
brightness (Yamazaki et al., 1999). From a lentiviral expression vector, CSII-
MiTHIA(AGRE)-MCS-IRE2-S-Venus (Fumoto et al., 2007), the IRES2-Venus
region was removed using appropriate restriction sites, and GFP(105)-coding
sequence was inserted into the multimeric site, yielding CSII-MT-GFP. cDNAs of
the LD-associated proteins were then inserted into CSII-MT-GFP, cDNA of CGI-58
was also subcloned into CSII-MT-myc or CSII-MT-Flag plasmid, in which the
GFP(105)-coding sequence was replaced with that of myc or Flag tag.

Recombinant lentiviruses were prepared with these plasmid constructs as described
previously (Yamashita et al., 2007; Yamashita et al., 2004). A lentivirus vector
expressing short hairpin RNA (shRNA) against mouse CGI-58 was prepared as
reported previously (Yamaguchi et al., 2007). For a rescue experiment, a mutant CGI-
58 cDNA in which the siRNA target sequence, 5'-AAGAAGATGTGACACGTCT-
AGTTT-3', was changed to 5'-AAGAAGATGTGACACGTCTTTT-3', but still
encoding the wild-type amino acid sequence, was inserted into CSII-MT-flag vector
containing a flag tag sequence instead of myc.

Cell culture
All reagents for cell culture were obtained from Wako (Osaka, Japan) unless
otherwise mentioned. 3T3-L1 cells were cultured and differentiated as described
previously (Yamaguchi et al., 2007). Three-dimensional culture of 3T3-L1
adipocytes was prepared as described previously (Yamaguchi et al., 2007).
DMEM, 50 mM NaOH, 260 mM NaHCO3, and
200 mM HEPES. Suspension of 2×105 cells was placed in a 35-mm glass bottom
dish and allowed to solidify at 37°C for 30 min. Growth medium (DMEM/10%
FBS (Nichirei Biosciences, Tokyo, Japan) supplemented with 5 μg/ml insulin) was
dispersed to structures including organelles such as ER and/or
Golgi apparatus upon lipolytic stimulation, the physiological
meaning of which is to be elucidated.
(Sigma-Aldrich, St. Louis, MO, USA) was added to differentiated 3T3-L1 adipocytes at 2 mM for 12 hrs prior to the assay for lipolytic activity and western blotting.

**Lipolytic stimulation**

For microscopy studies, lipolytic stimulation was applied to differentiated 3T3-L1 adipocytes as follows: After washing twice with Hank’s buffer, cells were incubated with DMEM containing 10% FBS/4 mM L-glutamine (Gibco, Grand Island, NY, USA)/25 mM HEPES (pH 7.4)/0.5 mM IBMX/10 mM isoproterenol (Tocris bioscience, Ellisville, MO, USA) at 37°C. When lipolytic activity was analyzed, 2% fatty acid-free BSA was used instead of 10% FBS.

To inhibit FA re-esterification, differentiated 3T3-L1 adipocytes were pre-incubated with growth medium (DMEM/10% FBS supplemented with 5 μg/ml insulin) containing 1.2 mM 2-bromooctanoate (TCL, Tokyo, Japan) and 10 μM tracin C (Biomol, Plymouth Meeting, PA, USA) for 1 hr. Lipolytic stimulation was applied with DMEM containing 2% fatty acid-free BSA, 20 mM HEPES (pH 7.4), 0.5 mM IBMX, 10 μM isoproterenol, 1.2 mM 2-bromooctanoate, and 10 μM tracin C.

To inhibit ATGL activity, the cells were treated as above, except that 25 μM bromo-1-lactone (BEL) (Cayman, Ann Arbor, MI, USA) was added to the medium instead of 2-bromooctanoate and Tracin C.

**Time-lapse imaging of LD-associated proteins**

3T3-L1 adipocytes expressing GFP-fused perilipin, CGI-58, GFP-HSL, and GFP-ATGL was differentiated on 35 mm glass-bottom culture dishes. Time-lapse images during lipolysis were obtained through an oil-immersion objective lens (U-Apo 40/NA1.35, Olympus, Tokyo, Japan) in the DeltaVision microscope system placed in a temperature-controlled room (37°C), as described previously (Haraguchi et al., 1999).

**CARS microscopy**

3T3-L1 adipocytes were differentiated on 35 mm glass-bottom culture dishes, and 200 μM deuterium-labeled palmitate (D1_{15})-palmitate (Isotec, St. Louis, MO, USA) was added to DMEM and allowed to be incorporated into large LDs by incubation for 24 hrs. During stimulation of lipolysis, no exogenous FA was added in the media. CARS imaging experiments were performed by multiplex CARS microscopy, on which the details have been described elsewhere (Okuno et al., 2010). All experiments were performed at room temperature.

**Immunofluorescence microscopy**

All reagents for microscopy were obtained from Wako (Osaka, Japan) unless otherwise mentioned. For immunostaining of LD-associated proteins, differentiated 3T3-L1 cells were fixed with PBS containing 3.7% paraformaldehyde (PFA) for 15 min at room temperature. Fixed cells were permeabilized in 0.2% Triton X-100/PBS for 10 min and blocked with 1% BSA/PBS. In the case of digitonin treatment, cells were treated with PBS containing 0.01% digitonin for 5 min on ice and followed by fixation with 3.7% PFA/PBS. Cells were then incubated with primary antibodies against HSL (Cell Signaling, Danvers, MA, USA), ATGL (Cell Signaling, Danvers, MA, USA), perilipin (Progen, Heidelberg, Germany), CGI-58/ABHD-5 (Santa Cruz biotechnology, Santa Cruz, CA, USA), and Myc (MBL, Nagoya, Japan) overnight, washed with PBS, and incubated with Alexa 488-, 546-, and 633- (Molecular Probes, Grand Island, NY, USA), Cy3- or fluorescein isothiocyanate (Jackson ImmunoResearch, West Grove, PA, USA)-conjugated secondary antibody for 1 hr. After washing with PBS, cells were mounted and observed under a fluorescence microscope (Biozero, Keyence, Osaka, Japan) (Yamaguchi et al., 2007) or a confocal microscope (FV 1000, Olympus, Tokyo, Japan).

**Subcellular fractionation**

All reagents for subcellular fractionation were obtained from Wako (Osaka, Japan) unless otherwise mentioned. Subcellular fractionation was performed as described by Liu et al. (Liu et al., 2004), with some modifications. Differentiated 3T3-L1 cells from five 10-cm dishes were washed with PBS and harvested in 2 ml of ice-cold buffer A: 25 mM tricine- HCl, pH 7.6, 250 mM sucrose, 10 mM sodium fluoride, containing 1 mM PMSF (Sigma-Aldrich, St. Louis, MO, USA), a protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO, USA), and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), and incubated on ice for 20 min. Cells were then homogenized using Potter-Elvehjem tissue homogenizer (AS ONE, Osaka, Japan) on ice with ten gentle strokes with the motor-driven pestle at 2500 rpm. Postnuclear supernatant (PNS) fraction was obtained by centrifugation at 40,000 g for 5 min at 4°C (Himac CS 15RXII, Hitachi, Tokyo, Japan). Lipid droplet (LD) fraction, concentrated in a white band at the top of the gradient, and cytosolic (Cyt) fraction were collected, and mixed with cold 100% acetone, and incubated at −30°C overnight. The pellet (Pel) fraction was rinsed with PBS and resuspended in buffer C (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% Triton X-100). These fractions were subjected to SDS-PAGE.

**Immunoprecipitation**

All reagents for immunoprecipitation were obtained from Wako (Osaka, Japan) unless otherwise mentioned. Differentiated 3T3-L1 cells containing exogenously expressed Flag-CGI-58 from five 10-cm dishes with or without 2 hrs lipolytic stimulation were washed with PBS and harvested in 2 ml of ice-cold lysis buffer: 25 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% digitonin containing 1 mM PMSF (Sigma-Aldrich, St. Louis, MO, USA), a protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO, USA), and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), and incubated on ice for 20 min. Cells were then gently pipetted and centrifuged at 10000 g for 5 min at 4°C (CF 15RXII, Hitachi, Tokyo, Japan). Immunoprecipitation of Flag-CGI-58 was performed by using Dynabeads Protein G (Life Technologies, Carlsbad, CA, USA) for the cell lysates by following manufacturer’s instructions. Precipitants performed with an anti-Flag antibody (Wako, Osaka, Japan) were eluted by Flag peptide (Wako, Osaka, Japan) at a concentration of 0.5 mg/ml in TBS, and endogenous ATGL in the immunoprecipitate was analyzed. Normal IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a negative control.

**Western blotting**

3T3-L1 cells were washed with PBS and directly dissolved in the heated SDS-PAGE sample buffer. Aliquots of the extracts were SDS-PAGE and protein was transferred to a nitrocellulose membrane. The blots were probed with antibodies to CGI-58 (Santa Cruz or self-made (Yamaguchi et al., 2007), p-ACC (Ser79) (Cell Signaling, Danvers, MA, USA), p-HSL (Ser565) (Cell Signaling, Danvers, MA, USA), HSL (Cell Signaling, Danvers, MA, USA), p-AMPK (Thr172) (Cell Signaling, Danvers, MA, USA), ATGL (Cell Signaling, Danvers, MA, USA), perilipin (Progen, Heidelberg, Germany), and DGAT1 (abcam, Cambridge, MA, USA). Signals were detected by the ECL method (GE Healthcare Life Science, Fairfield, CT, USA).

**Release of glycerol and FA**

3T3-L1 cells were grown in 12-well dishes. Differentiated cells were washed twice with Hank’s buffer and incubated with DMEM containing 2% fatty acid-free BSA/20 mM HEPES-NaOH (pH 7.4) with or without 0.5 mM IBMX and 10 μM isoproterenol at 37°C. After incubation, aliquots of the medium were collected at appropriate times, and assayed for glycerol and FA contents using TG E-test kit and NEFA C-test kit, respectively (Wako, Osaka, Japan).

**Measurements of TAG storage and LD diameter**

3T3-L1 cells were grown in 12-well dishes. For TAG measurements, differentiated cells were washed twice with Hank’s buffer and treated with 0.5 mM IBMX and 10 μM isoproterenol for 6 hrs with or without 2-bromooctanoate and triacsin C, washed with PBS and harvested in a lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100). TAG was measured using a TG E-test kit (Wako, Osaka, Japan). For LD diameter measurements, differentiated cells were washed twice with Hank’s buffer and treated with 0.5 mM IBMX and 10 μM isoproterenol for 6 hrs with or without 2-bromooctanoate and triacsin C, washed with PBS and fixed with PBS containing 3.7% PFA for 15 min at room temperature. LD diameters were measured for phase-contrast microscopic images under each condition. The diameters of the three largest LDs in at least 100 each adipocytes (more than 300 LDs in total) under each condition were measured (Miyoshi et al., 2008).

**Statistical analysis**

Statistical analysis was performed by unpaired t-tests or using either one- or two-way analysis of variance, as appropriate. Bonferroni/Dunn post-hoc test was used in the event of a significant (p<0.05) ratio. All results are presented as means ± s.e.m.

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Lipid droplets finally get a little
Evans, C. L. and Xie, X. S.

References


References
**Fig. S1. Trafficking of GFP-perilipin of 3T3-L1 adipocytes grown in 3D collagen matrix.** In the basal state, mature adipocytes contained 1-3 major large central LDs (>20 μm in diameter) and peripheral small LDs in the cytoplasm, and GFP-perilipin was intensely localized in the latter. In response to 1 hr lipolytic stimulation, GFP-perilipin was retained on the surface of large LDs, and also appeared on the particulate structures in the cytoplasm. Arrows indicate representative of mLDs. Bar, 20 μm.

**Fig. S2. Changes in TAG content and LD size during lipolysis.** Differentiated 3T3-L1 cells were treated with 0.5 mM IBMX and 10 μM isoproterenol for 6 hr with (2-Br-TriaC lipolysis) or without (Normal lipolysis) 2-bromooctanoate and triacsin C and (A) TAG content and (B) LD diameter were compared with those in the basal state (Basal). LD diameters were measured for phase-contrast microscopic images under each condition. The diameters of the three largest LDs in at least 100 each adipocytes (more than 300 LDs in total) from each condition were measured, and the average of the LDs was calculated. **p<0.01 vs. Basal; ***p<0.01 vs. Normal lipolysis.
**Fig. S3. Rescue of CGI-58 expression with a mutant construct that is not targeted by siRNA.** Extracts from 3T3-L1 cells in which CGI-58 was knocked down with a CGI-58 siRNA (KD), mismatch control cells (mismatch), CGI-58 KD cells infected with a lentivirus vector containing siRNA-resistant CGI-58 mutant cDNA (mutant), or CGI-58 KD cells infected with a lentivirus vector lacking CGI-58 cDNA (empty) were immunoblotted for CGI-58. Endogenous CGI-58 was drastically reduced in KD cells, whereas exogenously expressed flag-tagged CGI-58 was detected in mutant.

**Fig. S4. Effect of AICAR on the release of glycerol and FAs in CGI-58-KD 3T3-L1 adipocytes.** Lipolysis was stimulated in the presence (AICAR (+), closed bar) or absence (AICAR (-), open bar) AICAR in differentiated CGI-58-KD 3T3-L1 cells (KD) or mismatch control cells (mismatch). **p<0.01 vs. AICAR (-); ***p<0.01 vs. mismatch.
**Fig. S5. Effect of AICAR treatment in CGI-58-KD cells.** Differentiated CGI-58-KD (KD) or control (mismatch) 3T3-L1 cells were treated with (*AICAR* (+), closed bar) or without (*AICAR* (-), open bar) AICAR. Densitometric analysis of immunoblots for p-ACC, p-HSL, HSL, p-AMPK, perilipin, ATGL, and CGI-58, normalized with GAPDH, are shown. **p<0.01 vs. *AICAR* (-); ^p<0.05, ^^p<0.01 vs. mismatch.

**Fig. S6. Representative immunoblots examining the effect of AICAR in CGI-58-KD cells.** Differentiated 3T3-L1 cells, treated with (KD) or without CGI-58 RNAi (mismatch), were treated with (*AICAR*) or without (Control) AICAR. Cell extracts were immunoblotted for p-ACC, p-HSL, HSL, p-AMPK, perilipin, ATGL, CGI-58, and GAPDH.
Movie 1. Time-lapse image of GFP-perilipin during 2 hrs lipolysis was obtained by the DeltaVision microscope system placed in a temperature-controlled room (37°C). Bar, 20 μm.

Movie 2. Time-lapse image of GFP-CGI-58 during 2.5 hrs lipolysis was obtained by the DeltaVision microscope system placed in a temperature-controlled room (37°C). Bar, 20 μm.
**Movie 3.** Time-lapse image of GFP-HSL during 2 hrs lipolysis was obtained by the DeltaVision microscope system placed in a temperature-controlled room (37°C). Bar, 20 μm.

**Movie 4.** Time-lapse image of GFP-ATGL during 2.5 hrs lipolysis was obtained by the DeltaVision microscope system placed in a temperature-controlled room (37°C). Bar, 20 μm.