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Adipose Triglyceride Lipase Regulates Basal Lipolysis and Lipid Droplet Size in Adipocytes

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ABSTRACT

In adipocytes, lipid droplet (LD) size reflects a balance of triglyceride synthesis (lipogenesis) and hydrolysis (lipolysis). Perilipin A (Peri A) is the most abundant phosphoprotein on the surface of adipocyte LDs and has a crucial role in lipid storage and lipolysis. Adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) are the major rate-determining enzymes for lipolysis in adipocytes. Each of these proteins (Peri A, ATGL, and HSL) has been demonstrated to regulate lipid storage and release in the adipocyte. However, in the absence of protein kinase A (PKA) stimulation (basal state), the lipases (ATGL and HSL) are located mainly in the cytoplasm, and their contribution to basal rates of lipolysis and influence on LD size are poorly understood. In this study, we utilize an adenoviral system to knockdown or overexpress ATGL and HSL in an engineered model system of adipocytes in the presence or absence of Peri A. We are able to demonstrate in our experimental model system that in the basal state, LD size, triglyceride storage, and fatty acid release are mainly influenced by the expression of ATGL. These results demonstrate for the first time the relative contributions of ATGL, HSL, and Peri A on determination of LD size in the absence of PKA stimulation. J. Cell. Biochem. 105: 1430–1436, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: PERILIPIN; ATGL; HSL; LIPID DROPLET; ADIPOCYTE; LIPOLYSIS

Obesity is characterized by increased adipose tissue mass due to an increase in the size of individual adipocytes and the generation of new adipocytes [Crossno et al., 2006]. The size of individual adipocytes is mainly defined by lipid droplet (LD) size because the adipocyte consists of >95% triglyceride (TG) that is hydrolyzed during lipolysis to glycerol and fatty acids (FAs) [Arner, 2005].

Peri A (the predominant perilipin isoform in adipocytes) is the most prevalent protein kinase A (PKA) substrate in adipocytes and is a key regulator of both lipid storage and lipolysis [Greenberg et al., 1991; Martinez-Botas et al., 2000; Brasaemle et al., 2000b; Tansey et al., 2001]. The TG-rich core is surrounded by a single layer of phospholipids with their hydrophobic acyl-chains dissolved in the TG core and the hydrophilic head groups interfacing with the aqueous cytosol [Tauchi-Sato et al., 2002]. Peri A anchors to the surface layer of the LD with its central domain and forms a "scaffold" acting as an organizing center for lipid metabolic enzymes and transporters that is altered by metabolic signals [Subramanian et al., 2004]. Although the mechanism(s) by which Peri A phosphorylation facilitates TG/lipase interaction in adipocytes is not fully understood, recent studies have continued to reveal important aspects of the mechanism [Brasaemle, 2007; Granneman et al., 2007; Miyoshi et al., 2007].

Adipose triglyceride lipase (ATGL; Unigene name, PNPLA 2 (patatin-like phospholipase domain)) [Jenkins et al., 2004; Villena et al., 2004; Zimmermann et al., 2004] and hormone-sensitive lipase (HSL) [Fredrikson et al., 1981] are the major rate-determining enzymes in adipocyte lipolysis. It is well established that following PKA-stimulation, HSL translocates from the cytoplasm to the LD surface and interacts with Peri A in stimulated lipolysis [Brasaemle et al., 2000a; Miyoshi et al., 2006; Granneman et al., 2007]. ATGL also interacts with Peri A following PKA stimulation although this

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most likely occurs indirectly via the co-activator, comparative gene identification (CGI)-58 (UniGene name, ABHD5) [Subramanian et al., 2004; Yamaguchi et al., 2004; Granneman and Moore, 2008]. Recent studies have focused on the interactions occurring between Peri A and these two lipases during PKA-stimulated lipolysis [Miyoshi et al., 2007]; however, the role of these proteins in the maintenance of basal lipolysis rates in adipocytes has not been fully characterized [Zimmermann et al., 2004; Langin et al., 2005; Schweiger et al., 2006; Ryden et al., 2007]. It is important that we understand the factors regulating basal lipolysis as it may influence adipocyte cell size. Importantly, increasing fat cell size is correlated with an increased secretion of pro-inflammatory adipokines and metabolic dysfunction [Arita et al., 1999; Yatagai et al., 2003; Kadowaki et al., 2006; Skurk et al., 2007].

Smirnova et al. [2006] showed that expression level of ATGL is important to define LD size in cultured COS-7 and HeLa cells which are non-adipocyte cell lines and do not express Peri A or HSL. The importance of ATGL in defining LD size in adipocytes has not been examined, and it is possible that its effects may differ between non-adipocyte and adipocyte cell lines. Recently, it was reported that knockdown of CGI-58, a binding partner of Peri A, effected LD accumulation differently in non-adipocytes and adipocytes [Yamaguchi et al., 2007]. Therefore, our objective was to determine the relative abilities of Peri A, HSL, and ATGL to influence TG storage, basal lipolysis, and ultimately LD size in a model adipocyte system. The present study provides the first empirical comparison of regulatory factors for LD size in adipocytes. Results demonstrate that among the three major proteins previously demonstrated to regulate adipocyte lipolysis, ATGL had the strongest effect on LD size independent of Peri A expression. These observations define the relative role of these factors involved in the regulation of adipocyte size and provide further insight into this complicated mechanism, which could potentially be targeted to control obesity or metabolic disorders.

MATERIALS AND METHODS

ANTIBODIES

A polyclonal anti-perilipin antibody [Souza et al., 2002; Zhang et al., 2003], a polyclonal anti-HSL antibody [Wang et al., 2005], and a polyclonal anti-ATGL antibody [Miyoshi et al., 2007] were generated as previously described. Guinea pig anti-ADRP was purchased from Research Diagnostics (Concord, MA). Horseradish-linked anti-rabbit IgG was purchased from Amersham Biosciences (Piscataway, NJ). Alexa Fluor 647-conjugated goat anti-rabbit or anti-guinea pig IgG was purchased from Molecular Probes (Eugene, OR).

GENERATION AND DIFFERENTIATION OF STABLE LINES OF PERI−/− MEF ADIPOCYTES

Stable lines of mouse embryonic fibroblasts (MEF) were generated from embryos of Peri−/− mice as described [Rosen et al., 2002; Miyoshi et al., 2006]. MEF adipocytes were generated by retroviral expression of PPARγ [Rosen et al., 2002] followed by selection, expansion, and differentiation using a standard differentiation medium [Miyoshi et al., 2006]. MEF adipocytes attained a differentiated adipocyte phenotype within 7 days of culturing in differentiation medium [Miyoshi et al., 2006].

ADENOVIRAL EXPRESSION IN PERI−/− MEF ADIPOCYTES

Recombinant adenovirus was transduced into Peri−/− MEFs with LipofectAMINE Plus^{TM} (Invitrogen, Carlsbad, CA) on day 2 (adenovirus small hairpin RNAs) [Miyoshi et al., 2007], day 3 (adenovirus Peri A) [Souza et al., 2002], or day 4 (adenovirus HSL [Zhang et al., 2003] and newly generated adenovirus myc tagged ATGL) after induction of differentiation. Adenovirus expressing Aequoria Victoria green fluorescent protein (GFP) [Souza et al., 2002] was used as a control for non-specific adenoviral effects. A "scrambled" version of the shRNAi [Miyoshi et al., 2007] was used as a control for non-specific effects of shRNAi on the assays. The amount of each adenovirus used was selected to assure ideal levels of expression, which was confirmed by Western blots and densitometry [Zhang et al., 2003; Miyoshi et al., 2006].

LIPOLYSIS ASSAYS

Glycerol and FA release were quantified after 2 h of treatment with 200 nM PIA to repress adenyl cyclase activity (basal condition) as described [Zhang et al., 2003; Miyoshi et al., 2006].

TRIGLYCERIDE STORAGE

Lipid was extracted from cells plated in 6-well plates using 250 μl hexane:isopropanol (3:2) at 4°C for 15 min, and repeated after collecting the first organic extract. The second extract was added to the first and the solvent allowed to evaporate at room temperature. Lipids were then dissolved in 100 μl of isopropanol and TG content was measured using Triglyceride reagent (Sigma). TG storage was adjusted by cell lysate protein concentration and expressed in μmol/mg protein. Measurements were performed in triplicates in three independent experiments [Souza et al., 2007].

IMMUNOFLUORESCENCE MICROSCOPY

Differentiated Peri−/− MEF adipocytes were fixed and incubated with perilipin or ADRP antibody [Gross et al., 2006]. Images were acquired with a Leica TCS SP2 confocal microscope equipped with an acoustico-optical beam splitter.

LD DIAMETER

Digital images of minimum 100 adipocytes per treatment were obtained from three separate experiments. LDs were estimated based on Peri A or ADRP immunoflourescence and diameter of 300 LDs was measured on the screen.

STATISTICAL ANALYSIS

Data are reported as mean ± SEM. Treatment effects were analyzed by ANOVA using Tukey's procedure for multiple comparisons (Systat v10 for Macintosh, SAS Institute). Significance was set at $P < 0.05$.

RESULTS

ATGL IS THE MOST INFLUENTIAL FACTOR DETERMINING TRIGLYCERIDE STORAGE AND LD SIZE IN ADIPOCYTES

We have previously shown that Peri−/− MEFs, despite a lack of perilipin, differentiate and exhibit typical molecular and physiological characteristics of adipocytes including expression of ATGL and HSL [Gross et al., 2006; Miyoshi et al., 2006, 2007]. To determine the relative role of Peri A, HSL, and ATGL in the regulation of TG storage in adipocytes, we used adenoviruses expressing Peri A, HSL, myc-tagged ATGL, and GFP (control protein), and transduced them to Peri−/− MEF on day 3 (Peri A and GFP) or day 4 (HSL, myc-tagged ATGL, and GFP) of differentiation. Overexpression of ATGL significantly reduced TG storage compared with control, while overexpression of HSL failed to alter TG storage significantly (Fig. 1A). Expression of Peri A resulted in a trend for increased TG storage ($P = 0.082$), but did not affect the ability of overexpressed ATGL to decrease adipocyte TG accumulation.

We next utilized adenoviruses expressing HSL-directed shRNAi, ATGL-directed shRNAi, and scramble (SC, control adenovirus), and transduced them into Peri−/− MEFs on day 2 after induction of differentiation. To determine the efficacy of adenoviral shRNAi mediated knockdown, Western blots were performed using cell lysates. HSL-directed shRNAi reduced HSL protein expression by 95% and ATGL-directed shRNAi reduced ATGL protein expression by ~100% (Fig. 1C). Knockdown of ATGL dramatically increased TG storage compared with control (SC) both in the absence or presence of Peri A ($P < 0.001$ or $P = 0.005$, respectively). In contrast, knockdown of HSL did not alter TG storage significantly regardless of Peri A expression (Fig. 1B). These results indicate that in the basal state ATGL is a major regulator of TG storage in adipocytes and its effects are independent of Peri A expression in our adipocyte model system.

To determine the influence of Peri A, HSL, and ATGL expression on LD size in adipocytes, we overexpressed HSL, ATGL, or GFP (control) in MEF adipocytes as described above and measured the diameter of LDs. Consistent with the TG data, MEF adipocytes overexpressing ATGL showed a marked reduction in LD size compared with the other groups and independent of Peri A expression ($P < 0.01$, Figs. 2A and 3). The average diameter of LDs in adipocytes transfected with ATGL was 2.86 ± 0.21 and 3.43 ± 0.51 μm without/with Peri A, respectively, whereas LD diameter was 4.70 ± 0.40 and 5.06 ± 0.36 μm without/with Peri A, respectively, in control adipocytes. This change in diameter corresponds to a three- to five-fold decrease in the volume of LDs in adipocytes overexpressing ATGL. Overexpressing HSL did not alter LD diameter as compared to control adipocytes (4.57 ± 0.25 and 4.94 ± 0.39 μm without/with Peri A, respectively). Expression of Peri A resulted in an increase in LD size in control and HSL transfected adipocytes ($P < 0.05$), whereas LD size was not effected by Peri A expression in ATGL transfected adipocytes.

We next investigated LD size in the absence of HSL or ATGL using HSL-directed shRNAi, or ATGL-directed shRNAi adenoviruses transduced in MEF adipocytes in the absence or presence of Peri A. LD diameter was not significantly altered in adipocytes transduced with HSL-directed shRNAi, while LDs of adipocytes transfected with ATGL-directed shRNAi were significantly larger than those of control (6.95 ± 0.55 μm vs. 5.06 ± 0.36 μm) independent of Peri A expression ($P < 0.01$, Figs. 2B and 3). This change in diameter associated with ATGL knockdown corresponds to a 2.6-fold increase in LD volume. This result is consistent with our previous

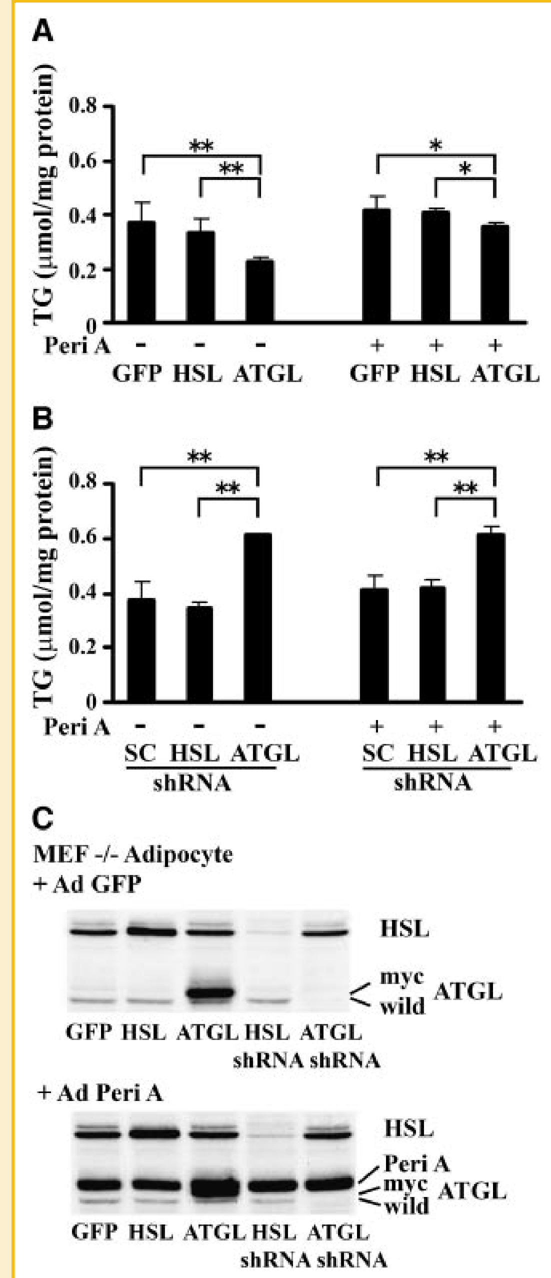

Fig. 1. Triglyceride storage in Peri−/− MEF adipocytes transduced (A) with adenovirus HSL/myc-tagged ATGL or (B) with adenovirus HSL-directed shRNAi/ATGL-directed shRNAi in the absence or presence of adenovirus Peri A. Adenovirus GFP or adenovirus SC (scrambled) is used as a control. Measurements were performed in triplicates and are the average for three independent experiments. C: Western blots with anti-perilipin IgG [Souza et al., 2002], anti-HSL IgG [Miyoshi et al., 2006], and anti-ATGL IgG [Miyoshi et al., 2007] are shown in a representative experiment. There are two ATGL bands that show endogenous ATGL (lower band) and myc-tagged ATGL (upper band). $^{*}P < 0.05$ and $^{**}P < 0.01$.

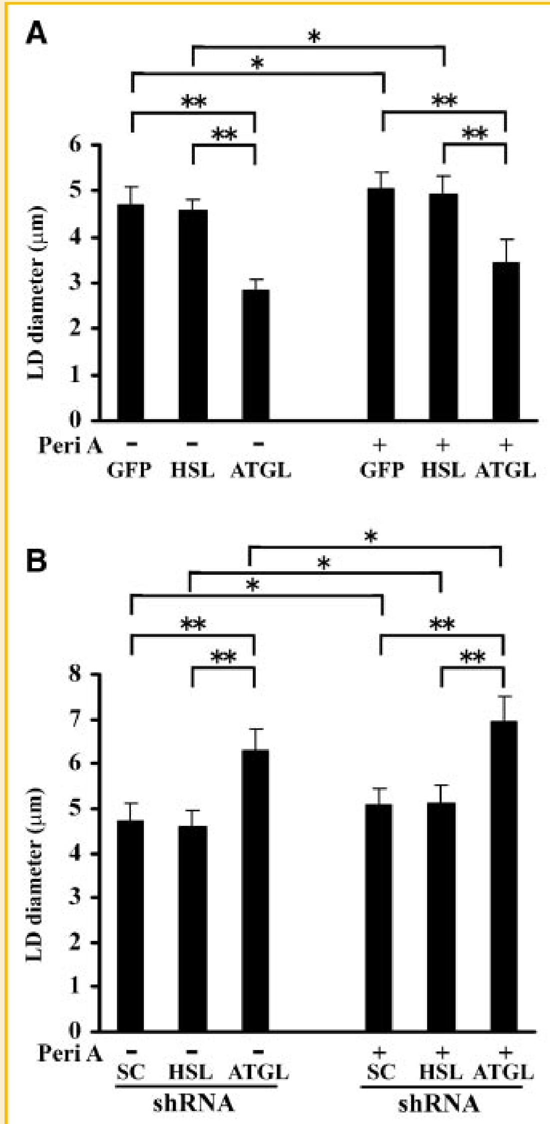

Fig. 2. ATGL regulates LD size in Peri−/− MEF adipocytes independent of Peri A expression in the basal state. Peri−/− adipocytes were transfected with adenovirus HSL/myc-ATGL/HSL shRNAi/ATGL shRNAi in the absence or presence of Peri A. Adenovirus GFP or adenovirus SC (scrambled) was used as controls. LDs were stained by BODIPY and analyzed by confocal microscopy. The diameters of the largest three LDs in each 100 adipocytes (total 300 LDs) from each group were measured on the screen. An average of the LDs was calculated. $^{*}P<0.05$ and $^{**}P<0.01$.

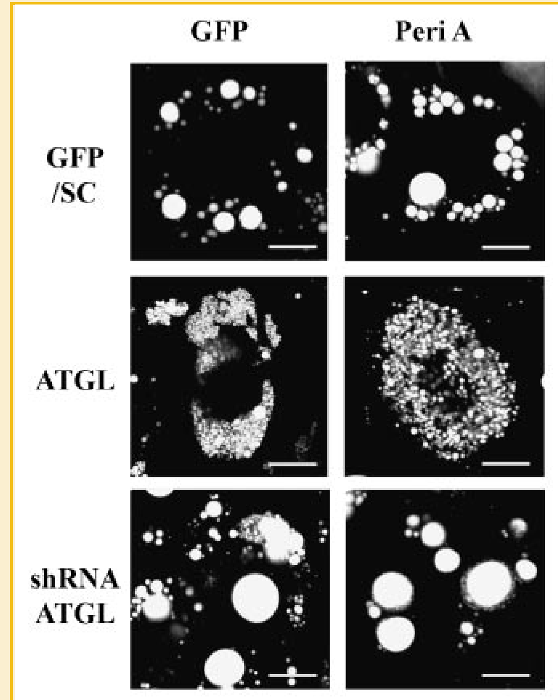

Fig. 3. The representative picture of adipocytes transduced with each adenovirus is shown in the figure. Adipocytes transduced with HSL and HSL-directed shRNAi are not shown in this figure because those are similar to those with control adenovirus. LDs were stained by BODIPY and analyzed by confocal microscopy. The scale bar = 10 μm.

observations that overexpression of ATGL results in reduced LD size. In the presence of Peri A, the largest LD in each cell was measured and an average diameter of 6.94, 6.93, and 9.78 μm was observed for control, HSL-directed shRNAi, and ATGL-directed shRNAi, respectively ($P<0.01$, control/HSL-directed shRNAi vs. ATGL-directed shRNAi). This tendency was similar in the absence of Peri A (Fig. 3).

ATGL PARTICIPATES IN BASAL LIPOLYSIS IN ADIPOCYTES, WHEREAS THE ROLE OF HSL IS MINOR

Several studies have already revealed the importance of both ATGL and HSL for stimulated lipolysis in adipocytes. Both are necessary to promote maximum stimulated lipolysis. To understand the relationship between LD size and basal lipolysis, we determined glycerol and FA release in media of each experimental condition during 2 h of culture. Among the three proteins (Peri A, HSL, and ATGL), basal lipolysis was mainly influenced by Peri A expression (Fig. 4). Both glycerol and FA release were dramatically decreased by expressing Peri A (78.8% in glycerol release). HSL-directed shRNAi did not significantly alter basal glycerol or FA release when compared with control. ATGL-directed shRNAi significantly decreased both glycerol and FA release in the absence of Peri A ($P<0.05$; Fig. 4). In the presence of Peri A, ATGL-directed shRNAi significantly decreased glycerol release ($P<0.05$), but a significant reduction in FA release was not observed. This lack of an effect on FA release could be attributed to the large reduction in FA release caused by Peri A expression or may simply be due to limitations in the sensitivity of detection.

DISCUSSION

Adipocytes provide the largest storage depot for energy in the form of esterified FAs that are mobilized to skeletal muscle and other tissues of the body for metabolism through β-oxidation to support ATP production. Non-adipocytes are poorly adapted to store excess TG and may sustain lipotoxic disruption of cellar function by mechanisms that are being actively investigated [Schaffer, 2003]. Adipocyte size is an important determinant of adipokine secretion.

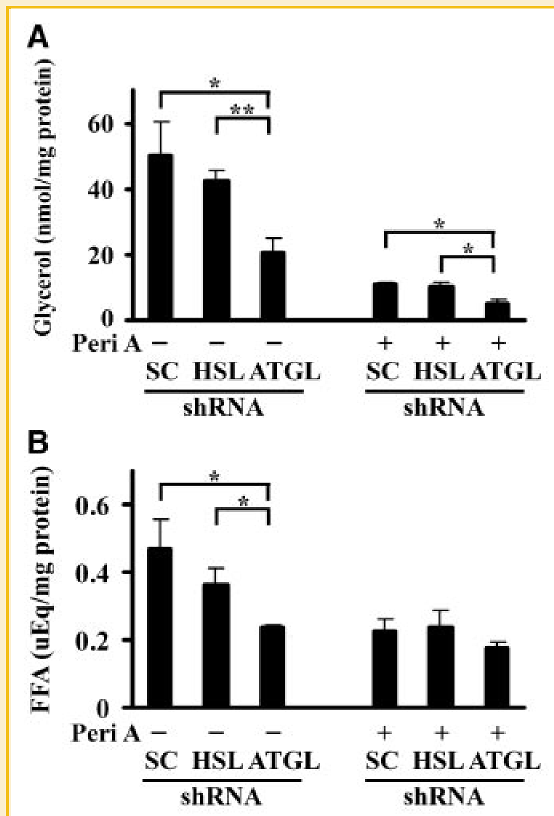

Fig. 4. Constitutive (basal) lipolysis is mainly influenced by Peri A expression. Overexpression of Peri A decreases both glycerol (A) and fatty acid release (B). Knockdown of ATGL decreases the release of glycerol and fatty acids independent of Peri A expression. HSL or ATGL was reduced by adenoviral HSL-directed or ATGL-directed shRNAi in Peri−/− MEF adipocytes in the absence or presence of adenoviral Peri A. Culture media including 200 nM PIA were collected after a 2 h incubation, and concentrations of glycerol (A) and NEFA (B) measured. Data are adjusted for protein content of cell lysates. Measurements were performed in duplicate and are the average of three independent experiments. $^{*}P<0.05$ and $^{**}P<0.01$.

There is a differential expression of pro- and anti-inflammatory factors with increasing adipocyte size resulting in a shift toward dominance of pro-inflammatory adipokines largely as a result of a dysregulation of hypertrophic, very large cells [Arita et al., 1999; Yatagai et al., 2003; Kadowaki et al., 2006; Skurk et al., 2007]. The ability to regulate adipocyte size and presumably the release of FAs and/or adipokines from adipocytes provides a potentially important tool for preventing various kinds of metabolic disorders. Therefore, in the present study, we investigated the relative abilities of Peri A, an adipocyte "scaffold" protein and the lipases HSL and ATGL to regulate LD size in adipocytes in the basal state. Peri A is the most abundant protein expressed on the surface of adipocyte LDs, and the major substrate for cAMP-dependent protein kinase (PKA) in lipolytically stimulated adipocytes [Greenberg et al., 1991]. Peri A functions as a "barrier" between stored neutral lipid and lipases in the basal state and conversely in the stimulated state acts to enhance PKA-stimulated lipolysis [Martinez-Botas et al., 2000; Tansey et al., 2001]. Peri A expression in adipocytes derived from perilipin null mice caused an increase in LD size ($P<0.05$) in the present study. These data are consistent with the previously reported results demonstrating that the fat cell size of perilipin null mice was much smaller than that of wild type mice [Martinez-Botas et al., 2000; Tansey et al., 2001].

HSL and ATGL are the major rate-determining enzymes in TG catabolism in adipocytes, although the relative contributions of these lipases in basal and stimulated lipolysis is still controversial [Zimmermann et al., 2004; Langin et al., 2005; Schweiger et al., 2006; Ryden et al., 2007]. Under basal conditions, ATGL is located in the cytoplasm [Villena et al., 2004; Granneman et al., 2007] and on LDs [Zimmermann et al., 2004; Bartz et al., 2007] of adipocytes, while HSL is predominantly found in the cytoplasm [Granneman et al., 2007]. Following catecholamine stimulation, activated PKA phosphorylates Peri A and HSL. Phosphorylated HSL requires Peri A to translocate from cytoplasm to LDs and interacts with phosphorylated Peri A to gain access to lipid substrates [Sztalryd et al., 2003; Miyoshi et al., 2006].

ATGL is believed to catalyze the initial step in TG hydrolysis in the adipocyte [Zimmermann et al., 2004; Schweiger et al., 2006] and HSL hydrolyzes diglyceride (DG) and minor amounts of TG [Cook et al., 1981; Fredrikson et al., 1981; Wei et al., 1997]. With PKA stimulation, the activity of ATGL is increased as much as 20-fold via the co-activator CGI-58 [Lass et al., 2006; Schweiger et al., 2006]. Peri A acts as a sink for CGI-58 and is reported to sequester CGI-58 from ATGL under basal (non-PKA-stimulated) conditions [Granneman et al., 2007]. We recently identified phosphorylation of serine 517 of Peri A as essential for ATGL-mediated adipocyte lipolysis in response to PKA activation [Miyoshi et al., 2007]. While Peri A and ATGL do not interact directly with LDs in adipocytes [Granneman et al., 2007], we hypothesize that PKA-induced phosphorylation of serine 517 alters the conformation of Peri A causing a release of sequestered CGI-58 and indirectly regulating ATGL activity [Granneman et al., 2007; Miyoshi et al., 2007].

In the present study, HSL expression did not affect LD size regardless of Peri A expression. While HSL is located both in the cytoplasm and on the LD in the basal state, it is likely that HSL does not associate with Peri A in the basal state and can act as a lipase only with the help of phosphorylated Peri A [Miyoshi et al., 2006]. This mechanism may explain why HSL null mice were not obese and their basal lipolysis was unaffected [Osuga et al., 2000; Wang et al., 2001; Haemmerle et al., 2002].

ATGL regulates both basal [Langin et al., 2005; Kershaw et al., 2006; Ryden et al., 2007] and stimulated lipolysis [Zimmermann et al., 2004; Haemmerle et al., 2006; Lass et al., 2006; Schweiger et al., 2006; Miyoshi et al., 2007] in adipocytes. One of the most dramatic results in the present study was the change in LD size (Fig. 3). Visual differences between control adipocytes and those transduced with adenoviral ATGL or ATGL shRNAi were apparent after only 3–4 days. These differences occurred both in the absence and presence of Peri A. The apparent lack of interaction between Peri A and ATGL in the basal state is not entirely surprising. Peri A exists only in adipocytes, whereas ATGL is present and active not only in adipocytes but also in other tissues in the body (heart, muscle, testis, adrenal grand, and colon) [Villena et al., 2004; Zimmermann et al., 2004] indicating that the ability of ATGL to function is not dependent on the presence of Peri A. In support of this, Smirnova

et al. [2006] showed that expression level of ATGL is an important determinant of LD size in cultured non-adipocytes, which lack Peri A and HSL. ATGL is not a direct PKA target, however, in PKA-stimulated states CGI-58 acts to dramatically increase the TG hydrolyase activity of ATGL. The present data demonstrate that ATGL can also act as a TG hydrolyase and regulate LD size to some extent without the interaction of CGI-58 which has been shown to be sequestered by Peri A in the basal state [Granneman et al., 2007]. However, one caveat of these studies is that ATGL was overexpressed in our model system of differentiating adipocytes while perilipin expression was increasing. It is unclear if lipase overexpression and knockdown in differentiated adipocytes that endogenously express perilipin would result in similar effects and will be the focus of future studies.

In the present study, Peri A expression was able to regulate LD size to a lesser extent than that of ATGL; however, the ability of Peri A expression to regulate basal lipolysis was greater than that of ATGL. These results indicate that the magnitude of basal lipolysis does not always correlate with LD size and suggest that the mechanism(s) regulating LD size are complex. It appears that in the basal state of adipocytes, the major role of ATGL is the breakdown of LD, while Peri A acts to block lipolysis. The rate at which these two processes occur will ultimately define the LD size.

The present study determined the impact of ATGL, HSL, and Peri A expression on LD size in the absence of PKA stimulation. Overall, our study of a model adipocyte system in the basal state indicated that: (1) Peri A expression is positively correlated with LD size and (2) independent of Peri A, expression of ATGL, but not HSL, dramatically alters LD size, TG storage, and FA release. These results provide a new perspective on the role of ATGL in regulating intracellular lipid metabolism and its function in regulating adipocyte cell size.

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