Consequences of lipid droplet coat proteins down –regulation in liver cells: Abnormal lipid droplet metabolism and induction of insulin resistance

1,2Ming Bell M.D., 1,2Hong Wang PhD, 4Hui Chen, 3John C. McLenithan, 3Da-Wei Gong, 3Rong-Zee Yang PhD, 3Daozhan Yu MS, 3Susan K Fried PhD, 4Michael J. Quon MD, 5Constantine Londos PhD and 1,2Carole Sztalryd PhD.

From the 1Geriatric Research, Education and Clinical Center, Baltimore Veterans Affairs Health Care Center, the Divisions of 2Gerontology and 3Endocrinology, Department of Medicine, School of Medicine, University of Maryland, Baltimore, Maryland, 21201, the 4Diabetes Unit, National Center for Complementary and Alternative Medicine, National Institutes of Health, Bethesda, Maryland 20892-1632, and the 5Laboratory of Cellular and Developmental Biology, NIDDK, National Institutes of Health, Bethesda, Maryland 20892-8028

Corresponding author:
Carole Sztalryd, GRECC, Veterans Affairs Medical Center, 10 North Greene Street Baltimore, MD 21201, E-mail csztalry@grecc.umaryland.edu

Received 8 February 2008 and accepted 7 May 2008.

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org.
**Objective:** Accumulation of intracellular lipid droplets (LD) in non-adipose tissues is recognized as a strong prognostic factor for the development of insulin resistance in obesity. LD are coated with PAT proteins that are thought to regulate LD turnover by modulating lipolysis. Our hypothesis is that PAT proteins modulate LD metabolism and therefore insulin resistance.

**Research Design and Methods:** We used a cell culture model (murine AML12 loaded with oleic acid) and siRNA to directly assess the impact of PAT proteins on LD accumulation, lipid metabolism and insulin action. PAT proteins associated with excess fat deposited in livers of DIO mice were also measured.

**Results:** Cells lacking PAT proteins exhibited a dramatic increase in LD size and a decrease in LD number. Further, the lipolytic rate increased by ~2-2.5- fold in association with increased adipose triglyceride lipase (ATGL) at the LD surface. Down-regulation of PAT proteins also produced insulin resistance as indicated by decreased insulin stimulation of Akt phosphorylation (p<0.001). PDK-1 and PI3 kinase decreased; IRS-1 307 phosphorylation increased. Increased lipid in DIO mice livers was accompanied by changes in PAT composition, but also increased ATGL, suggesting a relative PAT deficiency.

**Conclusions:** These data establish an important role for PAT proteins as surfactant at the LD surface packaging lipids in smaller units and restricting access of lipases, preventing insulin resistance. We suggest that a deficiency of PAT proteins relative to the quantity of ectopic fat could contribute to cellular dysfunction in obesity and T2D.
The striking surge in obesity predicts further increase in associated complications, insulin resistance, diabetes and heart disease (1, 2). Increased fatty acid availability in obesity is associated with accumulation of ectopic fat, mainly in the form of triacylglycerol (TAG) (3). Although ectopic fat correlates with systemic and tissue insulin resistance (4-6), a number of circumstances are known in which high tissue lipid stores are not associated with insulin resistance. Endurance trained athletes have high intra-myo cellular lipid yet are highly insulin sensitive. Importantly, the size and intracellular distribution of cellular lipid droplets (LD) differs in muscle from insulin-sensitive athletes from that in patients from insulin resistant patients (7). Thus, the negative consequences of high cellular lipids may be related the ability of the cell to regulate lipid storage and utilization.

Lipid droplet (LD) is an energy storage organelle, but has a surprisingly complex function in lipid homeostasis. LD biogenesis is a fundamental cellular function; when exposed to NEFA, cells store it as TAG in LD (8). Such LD accumulation maintains low intracellular NEFA, avoiding its toxic effects on cellular physiology while supporting cellular needs by releasing NEFA for use in beta-oxidation and membrane synthesis. LD function to sequester and release NEFA is thus critical for proper cellular function. Non-adipogenic tissues in patients with metabolic syndrome are exposed to chronically elevated serum levels of NEFA and these tissues respond by LD accumulation. Such ectopic fat deposition protects from NEFA mediated lipotoxicity (9) but in patients with metabolic syndrome the LD is inadequate to prevent pathological consequences. An important question arises: what molecular mechanisms regulate lipid storage in non-adipogenic tissues?

To date, we have only limited information on non-adipose LD. Recent studies identified a proteomic “signature” consistently including at least one member of the “PAT” protein family: Perilipin, Adipose Tissue Related Protein (ADFP), Tail Interacting Protein of 47 kDa (Tip47), S3-12 and LSPD-5 (10,11). Despite tissue-dependence, the ubiquitous nature of the family suggests an important role in LD machinery. ADFP, Tip47 and LSPD-5 are broadly distributed, notably in non-adipogenic liver and muscle tissues which do not express perilipin (13,24).

Our hypothesis is that saturation of non-adipogenic tissue’s capacity to appropriately regulate storage and release of NEFA via the LD results from variations in the expression and/or activity of PAT proteins. To study functional consequences of down-regulating two major PAT proteins, ADFP and Tip47 on insulin resistance and lipid metabolism, we used small interfering ribonucleic acid (siRNA) in a cell culture model. To assess the in vivo relevance of this finding, we measured the expression of PAT proteins associated with excess lipid accumulated in the livers of high fat fed obese mice.

RESEARCH DESIGN AND METHODS

Cell culture - AML12 cells ((Dr Steven Farmer Boston University) were grown with the standard protocol (American Type Culture Collection, Manassas, VA). For siRNA experiments, cells were plated in 24 multi-well dishes (Costar, Thermo Fisher Scientific, PA) at a density of 1x 10⁴ cells/well, transfected the next day with HyPerfect (Qiagen, Valencia, CA) according to the manufacturer’s instructions. For immunocytochemistry, cells were plated in 4-well chamber slide (Labtek, Thermo Fisher Scientific, Pittsburg, PA). For insulin signaling assays, cells were deprived of insulin for 24 hour and for the last 12 hours incubated in DMEM/F-12 media (1:1)
PAT Proteins and insulin resistance

containing 1% defatted BSA (Sigma Aldrich, St Louis, MO) and supplemented with 400 µM oleic acid complexed to 0.4% bovine serum albumin to promote triacylglycerol deposition. The next day cells were incubated in DMEM/F-12 media (1:1) containing 1% defatted BSA (Sigma Aldrich, St Louis, MO) for 6 hours prior to a 10 min incubation with insulin at the concentration indicated. Cells were harvested in cold lysis buffer (150 mmol/l NaCl, 50 mmol/l Tris-HCl, pH 7.5, 1% Triton X-100, 0.5% NP-40, 0.25% sodium deoxycholate, 1 mmol/l EDTA, 1 mmol/l EGTA, 0.2 mmol/l ortho-vanadate, 1 mmol/l NaF, and protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN).

Oligos- siRNA were purchased Qiagen (HP guaranteed siRNA). Positive siRNA for Map Kinase 1 and fluorescent oligos (Qiagen, Valencia, CA) were used to establish efficient conditions for transfection to obtain 80% inhibition by western blot (12) vs All Star (Ctl). For off-target toxicity, we used siRNA negative for insulin signaling: a negative siRNA targeting ADFP with 2 base mismatch (Ctl1), sense sequence 5’AACGTCTGCTTGAGCCGAATA-3’, (Fig.S-9), and a negative - siRNA targeting Tip47 (Ctl2), sense sequence 5’-GCGUGUCCCAAUCAGUCAU-3’ (12). Sense sequences for Tip47 (5’-AACAGCACAGAGAAUGAGGAG-3’) and ADFP (5’-AACGTCTGCTTGAGCCGAATA-3’) were selected by potency. Total siRNA per well was 10nM for all transfections, for double transfection using 1/3 Tip47 and 2/3 ADFP siRNA, for triple using 1/4 Tip47, 1/2 ADFP and 1/4 ATGL siRNA, and down regulation confirmed (Fig S-4, Fig S-7). siRNA for ATGL was reported (35) and confirmed by immunoblot (Fig.S-3). SiRNA efficiencies were quantified by Western Blot (Un-scan Silk Scientific Corporation, Orem, UT) and results expressed as percentage of control.

Liver tissues from DIO and ad libitum mice: C57BL/6 (8 weeks old) mice were ad libitum fed either a high fat (60 kcal % fat, Research Diets, New Brunswick, NJ) diet containing primarily lard or a low fat (10 kcal % fat, Research Diets, New Brunswick, NJ) diet for 12 weeks. Mice on high fat diet exhibited diet-induced obesity (1.52 fold increase in body weight over low fat control mice) and impaired glucose tolerance (data not shown). On the day of the experiments, animals were sacrificed by cervical dislocation and tissues were immediately frozen in liquid nitrogen. Tissue lipid was extracted (40) and expressed in %lipid weight / total sample weigh (mg/mg).

Antibodies and dyes - Rabbit Anti-Tip47, LSPD-5 and goat anti-ADFP were used (12,13). Neutral lipid dye Bodipy 558/568, alexa fluor 488, hoescht, alexa fluor 598 species specific secondary antibodies were from Molecular Probes (Invitrogen, Carlsbad, CA). Rabbit antibodies against human ATGL (Rockland Immunochemicals for Research, Gilbertsville, PA) were tested in human pre-adipocytes and 3T3-L1 adipocytes and in human and mice adipose tissues (unpublished data) or purchased as were Akt, Phosph Akt, PDK-1, GS3-Ka, phospho GS3-K α, phospho-specific antibodies to PKCβ, ERK, ERK Phosp antibodies (Cell Signaling technology, Beverly, MA), phospho-IRS-1 (Ser307) (Upstate Biotechnology, Lake Placid, NY), IRS-1 and Foxo-1 (Santa Cruz Biotechnology Santa Cruz, CA), β-Actin (Abcam, Cambridge, UK). CGI- 58 was a gift (Dr. Brasaemle, Rudgers U. (31)). Phospho-specific antibodies to PDK-1 were a gift from Dr. Quon. IP for IRS-1 were performed as described (36).

Immunocytochemistry and Immunoblotting - Fixation and staining were performed as described (12). Cells were viewed with a confocal laser microscope using a 63x or 40x oil objective lens. LD size was determined (LSM510; Carl Zeiss MicroImaging, Inc.).
Cellular extracts for immunoblot were obtained by scraping cells in Laemmli sample buffer. Each SDS-PAGE lane was loaded with protein from a single well of a 24-multiwell dish. Four wells were pooled for each data point for insulin signaling immunoblots. Quantitative analysis was performed using Un-scan (Silk Scientific Corporation). $^{32}$PI3P bands were revealed by PhosphorImager (Storm 860, GE Healthcare), and band intensities quantified (ImageQuant 5.0 GE Healthcare). Live cell imaging was performed using an axiovert 200 microscope equipped with a camera (Zeiss).

**Cellular TG turnover**— TG synthesis and lipolysis were performed (21, 39). Briefly, cells were incubated for 12 hour with growth medium supplemented with 400 µM oleic acid complexed to 0.4% bovine serum albumin to promote triacylglycerol deposition. $[^3]$H]Oleic acid, at $1 \times 10^6$ dpm/well, was included as a tracer. In lipolysis experiments, reesterification of fatty acids in AML12 cells was prevented by inclusion of 10 µM Triacsin C (Biomol, Plymouth Meeting, PA), an inhibitor of acyl co-enzyme A synthetase, in the medium.(21,38,39), Quadruplicate wells were tested for each condition. Lipolysis was determined by measuring radioactivity release (12,21,39).

**Lipid extraction and thin layer chromatography** - Cell monolayer was washed with ice-cold PBS and scraped into 1 ml of PBS. For total TAG, lipids were extracted by Dole method (40) from five wells of a 24-multiwell dish. Total upper phase was dried down, resuspended in isopropanol and assayed with triglyceride kit (Wako chemicals USA, Richmond, VA). Protein was measured by kit (PierceBiotechnology Inc, Rockford, IL). For TG synthesis, lipids were extracted by Bligh-Dyer method (41) and 10 % of the total lipid were analyzed with thin layer chromatography extractions performed as reported (37,38), see supplementary. Intracellular DAG, NEFA and ceramide were measured by analytical service (Avanti Polar lipids, Albaster, AL) (43)

**Glucose output** - Measurements were performed according to Berusi et al (48). Briefly, two days following siRNA transfection, cells were incubated in DMEM/F12 medium containing 1% bovine serum albumin, 1 µM dexamethasone (Sigma-Aldrich Corp, St Louis, MO) and 1 µM cAMP (EMD Chemicals, Inc, Gibbstown, NJ) for 24 hours. Cells were incubated in 0.35ml (per well) of phenol red-free, glucose-free DMEM containing 2 mM pyruvate and 20mM lactate containing dexamethasone and cAMP. Some wells also contained insulin (100nM concentration). Media was collected 5 h later for glucose measurement (48) with a fluorimeter (Molecular Devices, CA) in triplicate. Glucose output rate was normalized by cellular protein concentration and expressed as nmol of glucose /mg of protein /5hr. Two separate experiments were performed.

**Fat Cake Preparation** - Four 24-multiwell dishes for each condition were treated with siRNA negative or ADFP and Tip47. LD isolation was as reported (25). Total homogenate protein was determined and adjusted to 1 µg/µl. The lipid fat cake was isolated and suspended in 200 µl of PBS containing 5% SDS.

**Statistical analysis** - Statistical significance was by one-way ANOVA or two-tailed Student's $t$ test. (GraphPad software Inc, San Diego, CA)

**RESULTS**

AML12 cell LD contains mostly ADFP at the surface. After incubation of AML12 liver cells with 400 µM oleic acid, numerous LD appear, most coated with ADFP and fewer with Tip47 (Fig.1A.). Relatively few exhibit co-localized ADFP and Tip47 at the surface. ADFP was found exclusively at LD surfaces while Tip47 was found in both cytosolic and
PAT Proteins and insulin resistance

LD compartments. Other PAT proteins were not detected by immunocytochemistry or immunoblot, (results not shown). Hence AML12 cells express two PAT proteins, ADFP and Tip47.

**PAT protein down-regulation induces change in LD morphology and surface profile.** The effect from down-regulation of Tip47, ADFP or both with siRNA are shown in Fig.1B,C. SiRNA transfection produced minimal cellular toxicity, from unchanged levels of β actin. Efficiency of Tip47 inhibition was greater than 95% and of ADFP was 80%. SiRNA treatments effectively produced droplets coated with only one of the two PAT proteins or none (Fig.1B). ADFP down-regulation led to a dramatic increase in Tip47 protein at the LD surface, although total Tip47 protein in homogenates was not affected by increased exogenous oleic acid (Fig.S-1). Thus increased Tip47 at the LD surface is recruited from preexisting cytosolic Tip47. Behavior of PAT proteins in control cell homogenates in response to increased exogenous NEFA is also different (Fig.S-1): while ADFP increased proportionally to the exogenous lipid, Tip47 was barely affected. Importantly, knockdown of one or more PAT protein resulted in substantial changes in LD size and number, observed by Bodipy staining (Fig.2, Fig.S-5A-D, Fig.S-7), phase contrast microscopy (Fig.S-2) or quantify (Fig.3). Absence of PAT proteins induced a marked increase in LD size and decrease in LD number (28±2.6 vs 48.09±6.06 for control, p<0.05). When Tip47 was predominant, the LD morphology appeared similar to control treatment cells. When ADFP was predominant, lipid droplet number increased -251.7±22.6 vs 48.09±6.06 for control p<0.01) but lipid droplet size was smaller. Despite changes in LD morphology, TAG content in cellular extracts of oleic acid loaded cells was unchanged (Fig.4B). However, total TAG decreased in PAT protein deprived cells when grown without oleic loading. (Fig.4A). This difference prompted examination of PAT protein effects on lipid metabolism.

**Metabolic consequences of altered PAT protein profiles.**

- **Lipid metabolism:** In cells lacking either ADFP or Tip47, no significant alteration in lipolysis was observed (Fig.5A). However, lipolysis significantly increased up to 2-fold (Fig. 5A) (one-way ANOVA; p<0.02) in AML12 cells lacking both proteins. A recent phenotype reported for ATGL null mice suggests that ATGL is operative in liver (14). Presence of ATGL in AML12 cells was revealed by ATGL targeted siRNA treatment (Fig.S-3), which increased LD, expected from decreased lipolytic activity from loss of ATGL. In cells lacking PAT proteins, ATGL increased at the LD surface (Fig.5B, Fig.S-4). Immunoblot analysis revealed increase in both ATGL and CGI-58 in fat cakes from these cells (Fig.5C). CGI-58 is known to increase ATGL activity and to interact with PAT proteins (30). Increase of ATGL at LD in cells lacking ADFP and Tip47 supports the hypothesis that these proteins limit access of endogenous lipase to the LD. In contrast, lack of PAT proteins did not affect uptake of 3H exogenous oleic acid and had little influence on ability of cells to utilize exogenous NEFA for TAG or phospholipids synthesis (Fig.S-4). HPLC analysis did not reveal significant differences in intracellular content of DAG (1.45% ± 0.25 (w/w) for Ctl vs 1.4 % ± 0.2 (w/w)) for cells lacking PAT proteins or ceramide (1.1% in both conditions) but 1.25% (w/w) intracellular NEFA was found in cells lacking PAT proteins while NEFA remains undetected in control cells (n=2 experiments).

- **Insulin signaling:** Because dysregulation of cellular lipid metabolism has been linked to insulin resistance, we measured the effect of PAT siRNA treatment on insulin activation of protein kinase B (Akt) in AML12 cells by immunoblot analysis of cellular protein extracts using phosphospecific antibodies. In
both control and siRNA treated cells, insulin stimulated phosphorylation Akt phosphorylation at Ser-473, an effect previously shown to correlate with the extent of Akt activation in hepatocytes (16). However, absence of PAT proteins resulted in significant decrease in insulin responsiveness and sensitivity compared to the control (Fig.6A,B). Akt phosphorylation was not affected when only one PAT protein was down regulated (results not shown). Effect on Akt phosphorylation was also observed with decreasing concentration of oleic acid in media (Fig.S-8). To test if increased lipolysis contributed to the observed decrease in insulin responsiveness, cells lacking all PAT proteins were additionally treated with ATGL siRNA. Fig.6C, shows that the triple inhibition normalized insulin stimulation of Akt to control levels. Total amount of Akt did not differ among the various treatments. Upstream regulation of Akt was also affected by lack of PAT proteins: phosphorylations of PDK-1 (Fig.7A), IRS-1/PI3 kinase activity were decreased in cells lacking PAT proteins (Fig.7B). IRS-1-Ser 307 phosphorylation, which promotes general inhibition of IRS-1 signaling (17), was increased in cells lacking PAT proteins (Fig. 7C). However, this did not result in a detectable decrease in tyrosine phosphorylation of IRS-1 (results not shown). Downstream targets of Akt, Foxo-1 and GS3-Kα/β were affected (Fig.7E-F). Activation of PKC theta was previously shown to be involved in insulin resistance induced by fatty acids (18). Upon insulin stimulation, cells lacking PAT proteins also increased PKC theta protein and phosphorylation in the total cellular membrane fraction (Fig.6D). ERK1/2 phosphorylation was increased in both basal and stimulated conditions in cells lacking PAT proteins (Fig.7G). Overall glucose output was very low in AML112 cells, however, 10^{-8}M insulin was able to suppress glucose output in cells transfected with control siRNA to a greater extent than in cells lacking ADFP and Tip47, confirming a defect in these latter cells in insulin signaling (supplementary table 1).

**In vivo studies.** Lack of information on PAT protein and lipase content of liver fat cake in established models of insulin resistance prompted an examination of the DIO mice. Liver fat cakes isolated from DIO mice have 4-fold increased lipid content (11.7%±2.2 (w/w) vs 3.4%±1 (w/w) for ad libitum fed (p<0.05). Importantly, ATGL content also increased (Fig.8). Despite the large increase in lipid, a matching increase was observed only for LSPD-5 while a significant change in Tip47 was not found and the increase in ADFP appears modest.

**DISCUSSION**

Using siRNA technology, we were able to develop evidence supporting the role of the PAT proteins in the regulation of ectopic fat deposition and demonstrate the importance of defect in lipid droplet utilization in the development of cellular insulin resistance. First, the composition of PAT proteins at the LD surface dictates their size and number. Second, the affinity of ADFP may be greater than Tip47 for binding to the LD. Third, the PAT proteins help maintain intracellular NEFA homeostasis by protecting the LD against lipolysis by decreasing the recruitment of ATGL at the LD surface. Fourth, lack of PAT proteins induces cellular insulin resistance and affects multiple steps in the insulin signaling pathway. Fifth, DIO liver fat cakes differ in their content of PAT proteins and ATGL.

In this study, a loss of function approach was developed using siRNA targeting PAT proteins in a liver cell line since such a system provides a model for one of the most important tissues where ectopic fat develops. Value of a “loss of function” approach for PAT proteins has been proven with the perilipin null mice which exhibit a lean phenotype and enabled identification of its
regulatory role of lipolysis (19, 20). However, the ADFP null mice exhibit few phenotypic alterations (22), attributed tentatively to a compensatory replacement by Tip47 (12). Further analyses of ADFP null fibroblast cells treated with siRNA against Tip47, supported the importance of the PAT proteins role in lipid metabolism (12). AML12 cells are attractive for studies of LD and insulin signaling (15, 23).

Only two PAT proteins, ADFP and Tip47 were found in AML12 cells; predominately ADFP at the liver LD surface (25), whereas Tip47 was observed on some droplets but mainly in the cytosol. This corroborates previous findings that Tip47 and ADFP behave differently in most cells in that Tip47 can be present both in the cytosol and at the LD while ADFP is seen primarily at the LD upon lipid loaded conditions (25). Inhibition of ADFP expression resulted in increased Tip47 at the LD surface in AML12 cells, similar to our earlier report with cells from ADFP null mice, suggesting that ADFP has a greater affinity for the LD than Tip47. Thus, when ADFP expression is inhibited, Tip47 moves to the LD surface without major change in LD size and number. However, if Tip47 is repressed, ADFP is then the only PAT protein present and the LD number increases while the sizes decrease. This morphological change may reflect ADFP’s greater ability to bind to LD and greater surfactant function. Finally, absence of both PAT proteins generated fewer but larger LD. Since PAT proteins have surfactant properties, in their absence LD probably fuse to minimize surface area contact with the surrounding aqueous cytosol. These studies demonstrate that the PAT protein composition in liver cells is a critical determinand of LD size and number.

Interestingly, varying the LD coat protein did not affect the ability of cells to take up exogenous NEFA and to esterify it into TAG. Thus, ADFP and Tip47 are not important factors in determining the amount of TAG produced, but they are important factors in determining how it is packaged. However, without PAT proteins, TAG lipolysis increased, demonstrating a function of these two PAT proteins to inhibit LD hydrolysis by endogenous lipase(s). This property is a feature shared for most PAT proteins. Perilipin is known to regulate lipid storage in adipose cells (21,42) and published studies support that ADFP (34), Tip47 (12), and recently LSDP-5 (13) protects LD TAG against lipolysis.

Endogenous lipases in liver cells responsible for LD turnover have not yet been catalogued to the same extent as in adipose tissue (26). The phenotype of ATGL null mouse indicates that it may be an important lipase not only in adipose tissue but also in non-adipose tissues. Notably, hepatic fat content was doubled in ATGL null mice compared to wild type (14). Interestingly, by immunocytochemistry and immunoblot analysis we found an ATGL increase at the LD surface when all PAT proteins were absent, confirming a recent report that lack of ADFP increases ATGL presence in fibroblasts (44). Furthermore, by down-regulating ATGL expression we could show an active role of ATGL in LD turnover, as judged by increased LD in cells lacking this lipase. These results led us to hypothesize that ATGL is one of the enzymes participating in LD hydrolysis in liver cells and importantly, that non adipose tissue PAT proteins also have a role inhibiting LD hydrolysis.

A recent finding on ATGL regulation is that this enzyme appears to require CGI-58 for full activity in cytosolic extracts (27). CGI-58 is a protein that when mutated or truncated has been found to be responsible for LD accumulation in most tissues (Chanarin-Dorfman syndrome) (28,29). Most recently CGI-58 was identified to bind to Perilipin and ADFP (30, 31). In our studies, fat cake extract from cells lacking PAT proteins was
enriched in CGI-58 protein. This led us to hypothesize that common mechanisms exist among PAT proteins to regulate ATGL access to the lipid droplet surface, both in adipose and non-adipose tissues.

We found that PAT protein down-regulation is sufficient to induce insulin resistance in liver cells. These results led us to conclude that the mechanism underlying the defect in insulin resistance in the absence of PAT proteins is due at least in part to an increase in lipolytic rate and/or uncoupling to NEFA utilization. Absence of PAT proteins at the LD surface facilitates access of endogenous triglyceride lipases such as ATGL to the TAG substrate, releasing NEFA that in turn affects the signaling cascade at multiple levels. These findings support our working hypothesis that ADFP and Tip47 play important roles in regulation of ectopic fat, similar to the key role of perilipin in adipose tissue, and that their function can explain at least partially the connection between ectopic fat and development of insulin resistance. If true, then alteration in the composition and/or activity of PAT proteins will result in dysfunctional lipolysis triggering a cascade of deleterious events in cellular function. Hence, a strong connection should be visible in non-adipogenic tissues between PAT protein composition, surface protein content, lipolysis and insulin signaling. However, in vivo study of ADFP anti-sense down-regulation in liver (45) reported improved insulin sensitivity, no change in VLDL secretion – contrary to cell culture study results (47) and no compensation by tip47, at least in the whole cellular extract. However, no attempt was reported to assess LSPD-5 presence/activity while its expression has been shown to correlate with increased β-oxidation and is present in liver (46). Knowing the redundancy with the PAT protein family, LSPD-5 may be responsible for the apparent discrepancy existing between these in vivo results and cell culture (47). The presence of LSPD-5 in liver LD brings another level of complexity to the regulation of lipid droplet utilization. To date, little knowledge exists of lipid droplet PAT protein composition in insulin resistance models. We show here liver LD from the DIO disease model differ in ratio of PAT proteins at the lipid droplet surface and importantly that ATGL is increased at the lipid droplet surface. Since ATGL null mice have increased insulin sensitivity, we hypothesize that defects increasing ATGL access to the lipid surface and/or activity will have the opposite effect, i.e. promote insulin resistance. Thus, we hypothesize that DIO alters liver lipid turnover contributing to insulin resistance.

It is widely accepted that excessive FFA induced insulin resistance involves intramyocellular and intrahepatocellular accumulation of TAG, activation of several serine/threonine kinases, reduction in tyrosine phosphorylation of the insulin receptor substrate (IRS)-1/2, and impairment of IRS/PI3-kinase pathway (32) and ERK1/2 pathway (32). Attention has been given in the literature so far for a failure of mitochondrial oxidative function but little thought has been given to the failure of the LD compartment to store appropriately the excess NEFA (34). The results from these studies indicate that defects in LD lipolysis/utilization contribute to loss of ability of non-adipose tissue to maintain NEFA homeostasis and thus are responsible for deleterious consequences observed in the signaling pathway. These defects likely occur in the function/activity of the PAT family LD surface proteins regulating lipolysis. Future research focusing on LD utilization and its regulation will provide us with important clues for understanding regulation of cellular energy homeostasis.
ACKNOWLEDGEMENTS

We thank Dr. MC Woodle for his constant help and support and Dr AK Kimmel for his helpful criticisms. This work was supported by a career development award 1-05-CD-17 from the American Diabetes Association (to C.S.), by a grant from NIH 1RO1 DK 075017-01A2 (to C.S.), by the Geriatric Research, Education and Clinical Center, Baltimore Veterans Affairs Health Care Center, by the Clinical Nutrition Research Unit of Maryland (SKF, DK072488), and Intramural Research Programs of NIDDK and NCCAM, National Institutes of Health.
REFERENCES

46. Wolins NE, Quaynor BK, Skinner JR, Tzekov A, Croce MA, Gropler MC, Varma V,


FIGURE LEGENDS

Fig. 1: Identification and down-regulation of all PAT proteins (ADFP and Tip47) in AML12 cells. A. Cells were incubated with 400 µM oleic acid for 12 hr prior to staining. Cells were co-stained with a polyclonal goat anti-ADFP antibody and with a rabbit polyclonal anti-Tip47 antibody and respectively alexa fluor 488 or 594 conjugated secondary antibodies. Fluorescent and phase images were generated by LSM 510 confocal laser microscope. Bar 50 μm. B. Co-immunostaining with ADFP and Tip47 of AML12 cells treated for 4 days with either siRNA ADFP or SiRNA Tip47 or both combined or control. Cells were treated as above. C. Immunoblots of total cellular protein extract from AML12 cells treated with control siRNA (Qiagen) (lane 1) or with siRNA ADFP (lane 2) or with siRNA Tip47 (lane 3) or with both combined (lane 4); Rabbit polyclonal anti-Tip47, anti ADFP, anti-beta-actin antibodies were used as loading control.
Fig. 2: Morphological differences in AML12 cells following down-regulation of PAT proteins. Cells were stained with Bodipy fl568 which preferentially stained neutral lipid. Fluorescent and phase images were generated by LSM 510 confocal laser microscopy. Bar 50 μm.
Fig. 3: Composition of the PAT proteins affects the lipid droplet size distribution. Number and diameter of lipid droplets were measured following imaging cells treated with siRNA control (Ctl) (Qiagen) (empty bar), both siRNAs Tip47 and ADFP (Both) (filled bar), siRNA ADFP (right hatched) and siRNA Tip47 (left hatched). Data are mean ± SEM from 11 separate experiments for Ctl and ADFP, from 3 separate experiments for Tip47, from 27 separate experiments for both ( ***p<0.0001 , *p<0.05 vs Ctl) (t-test).
**Fig. 4:** Absence of exogenous NEFA reveals differences in accumulating lipids in cells lacking ADFP and Tip47. 

A- Triglycerides were measured in cells grown in culture media treated with siRNA control (Qiagen) (empty bar), both siRNAs Tip47 and ADFP (filled bar), siRNA ADFP (right hatched) and siRNA Tip47 (left hatched). 

B- Triglycerides were measured in cells grown in culture media supplemented overnight with 400μM of oleic acid in cells treated as described above. Data are mean ± SEM from 3 separate experiments and p<0.001 (one-way ANOVA) for cells lacking both ADFP and Tip47 grown without exogenous NEFA addition.
Fig. 5: Increased lipolysis in AML-12 cells lacking ADFP and Tip47.
A- Cells were loaded overnight with 2 106 dpm [3H]oleic acid and 400 μM cold oleic acid, the efflux of [3H]oleic acid was tracked over 180 minutes in the presence of 10 μM of triacsin C. Values represent the mean ± SEM of triplicate determinations of nmoles released per ug of protein at time 0, 15, 30, 60, 120, and 180 min. Data are mean ± SEM from 16 separate experiments for siRNA control and ADFP and Tip47 combined. Data are mean ± SEM from 8 separate experiments for single siRNA transfection with Tip 47 or ADFP. p<0.0001 (one-way ANOVA) for time points 1, 2 and 3 hr efflux and p<0.002 (one-way ANOVA) for time point 15 min. B. Increase of ATGL and CGI-58 at the lipid droplet surface in cells lacking ADFP and Tip47. Immuno-staining with ATGL of AML12 cells treated for 4 days with siRNA control or with both combined siRNA ADFP and Tip47. Cells were stained with a polyclonal rabbit ATGL anti- antibody. Alexa fluor 488 conjugated secondary antibodies were used. C. Fat cakes resulting from crude fractionation of AML12 cellular extract treated with siRNA: control (Ctl) (lane 1) or combined ADFP and Tip47 (lane 2). Rabbit polyclonal anti-ATGL and anti-CGI-58 antibodies were used.
A. 

\[ ^{3}H \text{Oleic acid released (nmoles/mg protein)} \]

Control
Both
Tip47
ADFP

\[ \begin{align*} 
&15 \\
&60 \\
&120 \\
&180 
\end{align*} \]

Time (min.)

B. 

<table>
<thead>
<tr>
<th>SiRNA</th>
<th>ATGL</th>
<th>Nomarski</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Both</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C. 

<table>
<thead>
<tr>
<th>Size (kd)</th>
<th>ATGL</th>
<th>CGI-58</th>
</tr>
</thead>
<tbody>
<tr>
<td>75 kd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 kd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 kd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37 kd</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 2
Fig. 6: Effect of lack of PAT proteins on Akt. **A. left** - Decrease insulin sensitivity and responsiveness in AML12 cells lacking both ADFP and Tip47, measured by Akt phosphorylation and **A. right** - Inhibition of lipolytic activity by ATGL siRNA, returns the Akt phosphorylation to control levels. AML 12 cells were treated with siRNA control 1 or combined siRNAs ADFP and Tip47 for 4 days or combined siRNAs ADFP, Tip47 and ATGL (triple siRNA). Akt phosphorylation was determined in the whole cell lysate by immunoblotting with rabbit polyclonal antibody against Akt or against an Akt phospho specific antibody. **A. (left and right)** - Representative Western Blots. **B.** An insulin dose-response curve was generated by quantifying Western Blot ECL signals, with the help of an image software analysis UN-scan-it (Silk Scientific Corporation). Each data point is expressed in arbitrary units and represents the average of calculated ratio of the amount of phosphorylated Akt versus the total amount of Akt protein. All experiments were repeated at least three times. Each bar represents mean ± SE. (*, P < 0.02); (**, P<0.01).
Fig. 7: Insulin signaling steps upstream and downstream from Akt are also perturbed in cells lacking both Tip47 and ADFP. AML 12 cells were cultured as above. The day of the experiment, cells were treated with no or $10^{-8}$ M insulin for 15 min prior to being scraped. A. PDK-1 phosphorylation is decreased in AML12 cells lacking ADFP and Tip47. PDK-1 phosphorylation was determined in the whole cell lysate by immunoblotting with rabbit polyclonal antibody against PDK-1 or against a PDK-1 phospho-specific antibody. B. Cellular extracts were immunoprecipitated with an anti–IRS-1 antibody, and PI 3-kinase activity was measured. Results are means ± SE arbitrary units. *$P < 0.05$ vs. basal of each group, PIP3, phosphatidylinositol 3,4,5,-triphosphate. C. IRS-1 serine 307 phosphorylation was determined in the whole cell lysate by immunoblotting with rabbit polyclonal antibody against IRS-1 or against an IRS-1 phospho-specific antibody (Upstate cell signaling solutions). D. Activation of PKCθ in AML12 cells lacking both ADFP and Tip47. Activation of PKCθ was determined with phospho-specific antibodies to PKCθ (Thr538). Pellets were obtained by centrifuging the whole cell lysate at 10,000g. The experiment was repeated three times with consistent results. E. Activation of Foxo-1 in AML12 cells lacking both ADFP and Tip47. F. Activation of GS3 alpha kinase in AML12 cells lacking both ADFP and Tip47. G. Activation of ERK1/2 in AML12 cells lacking both ADFP and Tip47.
PAT Proteins and insulin resistance

**A.**

<table>
<thead>
<tr>
<th></th>
<th>PDK-1-P</th>
<th>PDK-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiRNA</td>
<td>Insulin (M) 0</td>
<td>10^{-8}</td>
</tr>
<tr>
<td></td>
<td>Ctl1</td>
<td>Both</td>
</tr>
</tbody>
</table>

**C.**

<table>
<thead>
<tr>
<th></th>
<th>IRS-1 serine 307-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiRNA</td>
<td>Insulin (M) 0</td>
</tr>
<tr>
<td></td>
<td>Ctl1</td>
</tr>
</tbody>
</table>

**B.**

<table>
<thead>
<tr>
<th></th>
<th>3PI3P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiRNA</td>
<td>Insulin (M) 0</td>
</tr>
<tr>
<td></td>
<td>Ctl1</td>
</tr>
</tbody>
</table>

**D.**

<table>
<thead>
<tr>
<th></th>
<th>PKC theta</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiRNA</td>
<td>Insulin (M) 0</td>
</tr>
<tr>
<td></td>
<td>Ctl1</td>
</tr>
</tbody>
</table>

**E.**

<table>
<thead>
<tr>
<th></th>
<th>FOXO1</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiRNA</td>
<td>insulin (M) 0</td>
</tr>
<tr>
<td></td>
<td>Ctl1</td>
</tr>
</tbody>
</table>

**F.**

<table>
<thead>
<tr>
<th></th>
<th>GS3Kα/β</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiRNA</td>
<td>Insulin (M) 0</td>
</tr>
<tr>
<td></td>
<td>Ctl1</td>
</tr>
</tbody>
</table>

**G.**

<table>
<thead>
<tr>
<th></th>
<th>ERK1/2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiRNA</td>
<td>Insulin (M) 0</td>
</tr>
<tr>
<td></td>
<td>Ctl1</td>
</tr>
</tbody>
</table>
**Fig. 8:** Content of PAT proteins in DIO mice liver fat cake appears insufficient to protect the lipid droplet against the presence of increased ATGL. Fat cakes extracted from 3 pooled liver tissues from 8 mice fed ad libitum or 9 DIO mice were loaded in lanes 1,2,3 or lanes 4,5,6 respectively. Fat cakes were isolated by ultracentrifugation, 10ul aliquots used for protein determination following cold acetone precipitation overnight and the proteins pellets washed. Equal amounts of total protein were loaded but for ADFP, 1/20 dilution was performed before loading the samples to avoid over-exposed blots.
PAT Proteins and insulin resistance

A. PAT proteins
- ADFP
- Tip47
- LSPD-5

B. Lipase and Co-lipase
- ATGL
- CGI-58

C. Gel Loading

1 2 3 4 5 6
Ad Libitum DIO