Original Article

MOLECULAR METABOLISM

Vacuolar protein sorting 13C is a novel lipid droplet protein that inhibits lipolysis in brown adipocytes

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ABSTRACT

Objective: Brown adipose tissue (BAT) thermogenesis depends on the mobilization and oxidation of fatty acids from intracellular lipid droplets (LD) within brown adipocytes (BAs); however, the identity and function of LD proteins that control BAT lipolysis remain incomplete. Proteomic analysis of mouse BAT subcellular fractions identified vacuolar protein sorting 13C (VPS13C) as a novel LD protein. The aim of this work was to investigate the role of VPS13C on BA LDs.

Methods: Biochemical fractionation and high resolution confocal and immuno-transmission electron microscopy (TEM) were used to determine the subcellular distribution of VPS13C in mouse BAT, white adipose tissue, and BA cell culture. Lentivirus-delivered shRNA was used to determine the role of VPS13C in regulating lipolysis and gene expression in cultured BA cells.

Results: We found that VPS13C is highly expressed in mouse BAT where it is targeted to multilocular LDs in a subspherical subdomain. In inguinal white adipocytes, VPS13C was mainly observed on small LDs and β3-adrenergic stimulation increased VPS13C in this depot. Silencing of VPS13C in cultured BAs decreased LD size and triglyceride content, increased basal free fatty acid release, augmented the expression of thermogenic genes, and enhanced the lipolytic potency and efficacy of isoproterenol. Mechanistically, we found that BA lipolysis required activation of adipose tissue triglyceride lipase (ATGL) and that loss of VPS13C greatly increased the association of ATGL to LDs.

Conclusions: VPS13C is present on BA LDs where is targeted to a distinct subdomain. VPS13C limits the access of ATGL to LD and loss of VPS13C elevates lipolysis and promotes oxidative gene expression.

Keywords Brown adipose tissue; Thermogenesis; Free fatty acids; ATGL; Perilipin 1; Oxidative genes

1. INTRODUCTION

Brown adipose tissue (BAT) plays a key role in nonshivering thermogenesis in rodents and certain newborn mammals, including humans, by mobilizing and oxidizing fatty acids. In adult humans, the prevalence of BAT correlates with improved glucose homeostasis and energy metabolism [1–5], suggesting that activation of BAT thermogenesis might be a therapeutic target for obesity and diabetes. A defining feature of brown adipocytes (BAs) is the presence of multiple small lipid droplets (LDs) that integrate triglyceride storage and mobilization [6]. LDs are often closely associated with other organelles including neighboring LDs, the endoplasmic reticulum (ER) and mitochondria [7,8], and it is thought that these contacts allow efficient coupling of lipid synthesis, mobilization, and oxidation.

The LD proteome is unique among organelles and can vary across tissues. For example, perilipin 1 is an LD scaffold protein in both brown and white adipocytes that suppresses lipolysis in the basal state and facilitates lipolysis during beta-adrenergic receptor activation [9–11]. In contrast, perilipin 5 is present in oxidative tissues like BAT, heart, and soleus muscle, where it links LDs to mitochondria and likely facilitates fatty acid trafficking for oxidation [12,13]. Thus, the LD proteome often reflects the specialized tissue function as it relates to fatty acid trafficking and LD interactions with other organelles.

Proteomic analysis of BAT subcellular fractions demonstrated that vacuolar protein sorting 13C (VPS13C) segregates with known LD proteins under basal and stimulated conditions, suggesting it is a *bona fide* LD protein. VPS13C is the mammalian ortholog of the yeast Vps13 and belongs to a family of genes involved in vacuolar function.

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Abbreviations: VPS13C, Vacuolar Protein Sorting 13C; BAT, brown adipose tissue; BAs, brown adipocytes; LDs, lipid droplets; ATGL, adipose triglyceride lipase; ABHD5, α-β hydrolase domain-containing protein 5; PLIN1, perilipin 1; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; HSL, hormone sensitive lipase; *Cidea*, cell death-inducing DNA fragmentation factor, alpha subunit-like effector A; *Cox8b*, Cytochrome c oxidase subunit 8B; *Ucp1*, uncoupling protein 1; *Ppara*, Peroxisome proliferator-activated receptor alpha; *Ppargc1a*, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; *Hadhb*, hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein); beta subunit, *Acadl*; Long-chain specific acyl-CoA dehydrogenase, *Ebf2*; early B cell factor 2, *Esrra*; estrogen related receptor, alpha

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Relatively little is known about mammalian VPS13C, but recent studies found that mutations in VPS13C cause early-onset Parkinson's disease in humans [14]. Interestingly, gene wide association studies have also linked VPS13C with diabetes, glucose levels and pro-insulin release [15–17]. In the study described below, we used a combination of mass spectrometry analysis, biochemical fractionation, confocal and transmission electron microscopy as well as metabolic assays to examine the role of VPS13C in BAs.

2. MATERIALS AND METHODS

2.1. Animals

All protocols involving animals were approved by the Institutional Animal Care and Use Committee of Wayne State University and followed the National Institutes of Health Guide for the care and use of Laboratory Animals (NIH Publications No. 8023, revised 1978). Male and female mice 129S1/SvImJ (129S1; stock number 002448), Tg(Ucp1-cre)1Evdr (stock number 024670), C57BL/6J (C57B/6; stock number 000664), and td-tomato (R26-LSL-tdTomato; stock number 007909) were purchased from Jackson Laboratory and bred in house. Mice were fed regular chow and kept at a 12 h light/dark cycle.

For β3-adrenergic receptor agonist studies, vehicle (phosphate-buffered saline, PBS) or CL 316,243 (Sigma–Aldrich) was infused at a dose of 0.75 nmol/h using osmotic minipumps (Durect-ALZET; Cupertino, CA) for 3 days. For mass spectrometry analysis, CL 316,243 was infused at the same dose for 1 day.

2.2. Cell culture

Unless specified, chemicals used were obtained from Sigma–Aldrich (St. Louis, MO). BAs were grown as pre-adipocytes in Dulbecco's Modified Eagles Medium (DMEM) (HyClone GE Lifesciences; Pittsburgh, PA) containing 10% fetal bovine serum (FBS) (Atlanta Biologicals; Flowery Branch, GA), penicillin/streptomycin (Hyclone, GE Lifesciences), 20 nM insulin and 1 nM 3,3′,5-triiodo-L-thyronine (T3; differentiation media) for 3 days until confluent [18,19]. Adipocyte differentiation was induced by incubating cells in differentiation media containing 2 ug/mL dexamethasone, 0.5 mM isobutylmethylxanthine (IBMX) and 0.25 mM indomethacin for 1 day. The following day, medium was replaced with differentiation media (day 1) and after that media was changed every day (days 2–6). Unless specified, cells were harvested at day 4 after induction.

In experiments where free fatty acids were measured, cells were incubated in Krebs–Ringer Bicarbonate Buffer containing HEPES (H-KRBB) or Hanks Balanced Salt Solution (HBSS) (Gibco, Thermo Fisher Scientific) supplemented with 1% Bovine Serum Albumin (BSA) (Alkali Scientific Inc. Pompano Beach, FL). Cells were treated for 1 or 2 h and media was collected. Non-esterified fatty acids were measured using a fluorescence kit (WAKO Diagnostics; Richmond, VA) using the fluorogenic substrate amplex red (Cayman Chemical; Ann Arbor, MI) and glycerol was measured by colorimetry using free glycerol reagent (Sigma; St. Louis, MO). For pharmacological inhibition of ATGL, cells were pre-incubated with 50 μM atglistatin (N′-[4′-(dimethylamino)[1,1′-biphenyl]-3-yl]-N,N-dimethyl-urea; (Cayman Chemical; Ann Arbor, MI) or vehicle (dimethyl sulfoxide, DMSO) for 30 min before treatment with vehicle (PBS) or isoproterenol.

Triglyceride was extracted from BAs using a modified method from Barkley et al. [20]. Briefly, BAs were collected and homogenized in PBS. Neutral lipids were extracted three times with isooctane: ethyl-acetate (1:9) (Fisher Scientific). The organic layer containing neutral lipids was collected and dried for 2 h under N_2. Triglycerides were resuspended in 5% NP-40 by sonication. Triglyceride content was measured using triglyceride determination kit (Sigma; St. Louis, MO). BA cells grown in parallel were used to measure protein content using bicinchoninic acid assay (BCA; Pierce, Thermo Fisher Scientific Grand Island, NY).

2.3. Lentivirus production

Lentiviruses were produced by transfecting 293T cells using VPS13C shRNA-containing vector (Sigma cat# TRCN0000246772) or control (Sigma cat# SHC216), packaging psPAX2 plasmid (from Didier Trono to Addgene plasmid# 12260) and envelope plasmid pMD2.G (from Didier Trono to Addgene plasmid# 12259) at a ratio 1:0.75:0.25 respectively. Media containing lentiviruses was collected 40 h and 64 h after transfection, combined, filtered and centrifuged at 35,000×*g* for 2 h. Lentiviruses were resuspended in OptiMEM (Gibco). BAs were infected with lentiviruses, in the presence of OptiMEM and Polybrene (Millipore). Thirty hours later, media was replaced for regular media (DMEM) containing 10% FBS.

2.4. Subcellular fractionation

Cells were washed with PBS (Gibco; Thermo Fisher Scientific Grand Island, NY) and scraped in 20% sucrose solution containing 20 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES), 1 mM ethylenediaminetetraacetic acid (EDTA) and EDTA-free proteases inhibitor cocktail (Thermo Fisher Scientific). Cells were homogenized by 10 passages through a 26 1/2G needle. Homogenates were spun 5 min at 1000×*g* and the supernatant or post-nuclear fraction (PN) was recovered. The PN fraction was layered with 10 and 0% sucrose solution respectively (containing HEPES, EDTA and proteases inhibitors) and centrifuged at 100,000×*g* (Beckman TL-100 ultracentrifuge) for 30 min. Floating LDs were transferred to another tube and proteins were precipitated using acetone (Fisher Scientific). Precipitated proteins were resuspended in 2% sodium dodecyl sulfate (SDS) and heated at 70 °C for 10 min. The cytoplasmic fraction was recovered from the upper portion of 20% sucrose layer. Proteins were measured by bicinchoninic acid assay (BCA; Pierce, Thermo Fisher Scientific Grand Island, NY) in all the fractions.

In experiments where nuclei, mitochondria and membrane fractions were collected, PN fraction was spun at 10,000×*g* to pellet mitochondria. The resultant supernatant was centrifuged at 100,000×*g* for 30 min. LD and cytosol fractions were collected as described above and pellet was collected as membrane fraction. Nuclei, mitochondria and membrane pellets were then resuspended in Buffer A (20 mM HEPES, 100 mM KCl, 2 mM MgCl, pH = 7.4), passed through a 26G needle 10 times and spun again at their corresponding speed. Pellets were resuspended in 2% SDS and protein measured in each fraction by BCA assay.

2.5. Mass spectrometry

For mass spectrometry analysis BAT LD fractions from sham-operated or CL 316,243 treated mice were obtained as described above. LD fractions and total lysate were run on PAGE and gel was stained with Sypro Ruby. The whole lane was cut and submitted for mass spectrometry analysis to the Proteomics Core of Wayne State University.

2.5.1. In-gel digestion

Washed gel slices were reduced with Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), alkylated with iodoacetamide and digested with sequencing-grade trypsin (Promega) overnight at 37 °C. Peptides were eluted from the gel slices using 0.1% formic acid and acetonitrile and dried. Peptides were separated by reverse phase chromatography by HPLC using a C18 column and analyzed with an LTQ-XL mass

