Original article

MOLECULAR METABOLISM

AGPAT2 is essential for postnatal development and maintenance of white and brown adipose tissue

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ABSTRACT

Objective: Characterize the cellular and molecular events responsible for lipodystrophy in AGPAT2 deficient mice.
Methods: Adipose tissue and differentiated MEF were assessed using light and electron microscopy, followed by protein (immunoblots) and mRNA analysis (qPCR). Phospholipid profiling was determined by electrospray ionization tandem mass spectrometry (ESI-MS/MS).
Results: In contrast to adult *Agpat2*$^{-/-}$ mice, fetuses and newborn *Agpat2*$^{-/-}$ mice have normal mass of white and brown adipose tissue. Loss of both the adipose tissue depots occurs during the first week of postnatal life as a consequence of adipocyte death and inflammatory infiltration of the adipose tissue. At the ultrastructural level, adipose tissue of newborn *Agpat2*$^{-/-}$ mice is virtually devoid of caveolae and has abnormal mitochondria and lipid droplets. Autophagic structures are also abundant. Consistent with these findings, differentiated *Agpat2*$^{-/-}$ mouse embryonic fibroblasts (MEFs) also have impaired adipogenesis, characterized by a lower number of lipid-laden cells and ultrastructural abnormalities in lipid droplets, mitochondria and plasma membrane. Overexpression of PPARγ, the master regulator of adipogenesis, increased the number of *Agpat2*$^{-/-}$ MEFs that differentiated into adipocyte-like cells but did not prevent morphological abnormalities and cell death. Furthermore, differentiated *Agpat2*$^{-/-}$ MEFs have abnormal phospholipid compositions with 3-fold increased levels of phosphatidic acid.
Conclusion: We conclude that lipodystrophy in *Agpat2*$^{-/-}$ mice results from postnatal cell death of adipose tissue in association with acute local inflammation. It is possible that AGPAT2 deficient adipocytes have an altered lipid filling or a reduced capacity to adapt the massive lipid availability associated with postnatal feeding.
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Keywords AGPAT2; Adipose tissue; Adipogenesis; Phospholipid; Lipodystrophy

1. INTRODUCTION

Congenital generalized lipodystrophy (CGL, Berardinelli-Seip syndrome) is a rare autosomal recessive disorder characterized by a lack of adipose tissue (AT). Affected individuals are prone to metabolic complications including insulin resistance, diabetes mellitus, hepatic steatosis and hypertriglyceridemia [1,2].
Mutations in the *AGPAT2* gene, encoding 1-acylglycerol-3-phosphate O-acyltransferase 2, cause the most common form of CGL, designated CGL-1 [3]. Patients with CGL-1 have total absence of metabolically active adipose tissue, which is present in most subcutaneous regions, intra-abdominal and intra-thoracic regions and bone marrow; mechanical adipose tissue, which is present in the palms, soles, scalp, peri-articular regions and orbits, is completely preserved [4]. Similarly, mice lacking AGPAT2 (*Agpat2*$^{-/-}$mice) have complete loss of both white and brown adipose tissue and manifest severe insulin resistance and hyperglycemia [5,6]. The mechanisms underlying the loss of adipose tissue in *Agpat2*$^{-/-}$ mice remain unknown.
AGPAT2 belongs to a family of enzymes catalyzing the *sn*-2 acylation of the glycerol-3-phosphate backbone. This reaction converts lysophosphatidic acid (LPA) to phosphatidic acid (PA) in the *de novo* glycerolipid synthesis pathway [7]. In humans and mice, AGPAT2 is a ~31 KDa protein with four predicted transmembrane domains [8] and specificity for LPA and acyl-CoA substrates [9,10]. Epitope tagged AGPAT2 localizes to the endoplasmic reticulum (ER) of CHO cells and primary mouse hepatocytes [10].
Lipodystrophy can result from either insufficient generation of mature adipocytes, i.e. defective adipogenesis, or accelerated adipocytes loss,

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i.e., defective adipose maintenance. Previous work with cell lines [11,12] and muscle-derived multipotent cells isolated from subjects harboring AGPAT2 mutations [12] has suggested that AGPAT2 is required for adipogenic differentiation; however, the roles of AGPAT2 for *in vivo* adipose tissue dynamics is unknown.

Herein, we characterized morphological, ultrastructural, and molecular changes of AT from *Agpat2*$^{-/-}$ mice and assessed adipogenic differentiation in *Agpat2*$^{-/-}$ mouse embryonic fibroblasts (MEFs).

2. MATERIALS AND METHODS

2.1. Mice

Agpat2$^{-/-}$ mice were generated as described previously [5]. *Agpat2*$^{-/-}$ and *Agpat2*$^{+/+}$ mice were obtained by mating of *Agpat2*$^{+/-}$ mice. Genotyping was performed by the PCR protocol as described [5]. Adult mice were fed chow ad libitum. All mouse procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Pontificia Universidad Católica de Chile and University of Texas Southwestern Medical Center (UTSW).

2.2. Mouse embryonic fibroblasts (MEFs)

Agpat2$^{+/-}$ pregnant females (14.5 d.p.c.) were sacrificed by isoflurane overdose. The embryos were removed, washed with ice cold sterile PBS, and sacrificed by decapitation. Maternal tissues, placental membranes, and internal organs were removed, and carcasses were rinsed with PBS and manually minced. The resulting material was digested with 0.25% trypsin/1 mM EDTA for 3 h at 4 °C and centrifuged. Cells were resuspended in DMEM 4.5 g/L glucose, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 μg/ml penicillin/streptomycin, and 0.1 mM 2-β-mercaptoethanol and seeded onto 150 mm plastic dishes. Because of *Agpat2*$^{+/-}$ mice mating, the embyros are of all three genotypes: *Agpat2*$^{+/+}$; *Agpat2*$^{+/-}$ and *Agpat2*$^{-/-}$. Genomic DNA was isolated from tail biopsies of each embryo and only those of *Agpat2*$^{+/+}$ and *Agpat2*$^{-/-}$ genotypes were further processed. Genotyping PCR conditions are similar as mentioned before [5]. MEFs were cultured until 100% confluence and frozen in liquid nitrogen at passage 1. All the experiments were performed at passage 3.

2.3. MEFs adipogenic differentiation

4×10^5 MEFs/well were seeded on 12-well plates and cultured in DMEM 4.5 g/L glucose, supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 μg/ml penicillin/streptomycin, and 0.1 mM 2-β-mercaptoethanol. Adipogenesis was induced 2 days after confluency with induction medium (standard medium plus 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 10 μg/ml insulin) for 3 days. After induction, MEFs were fed an adipogenic differentiation medium (standard medium plus 10 μg/ml insulin and 10 μM rosiglitazone) for 2 additional days and then cultured in adipogenic maintenance medium (standard medium supplemented with 10 μg/ml insulin).

2.4. Histology and immunofluorescence

E18.5 embryos and newborn mice were euthanized by CO_2 anesthesia following cervical decapitation. Immediately, they were rinsed with 1X PBS and fixed overnight in 4% PFA/PBS and then transferred into 30—18% sucrose/PBS gradient. For histological studies in dorsal skin and interscapular BAT (iBAT) of P0-P6.5 mice, tissues were fixed in 4% PFA/PBS and then embedded in paraffin. Cryo and paraffin embedding, sectioning, H&E and Oil Red O staining were performed at UTSW Molecular Pathology Core. For Perilipin-1 and MAC-2 immunofluorescence in AT, sections were deparaffinized in xylene and rehydrated in a graded series of ethanol followed by dH_2O. Antigen unmasking was carried out by heating slices in 10 mM sodium citrate buffer (pH 6.0) at 95—99 °C for 10 min. Tissue sections were blocked and then incubated overnight at 4 °C with primary antibodies. After the washing steps, fluorochrome-conjugated secondary antibodies were incubated for 1 h at room temperature. For immunofluorescence detection in cultured cells, MEFs were seeded on glass coverslips and adipogenic differentiation was induced as described above. At the indicated days, differentiated MEFs were fixed in 4% PFA, washed with PBS and permeabilizated/blocked in 0.3% Triton X-100; 3% BSA/PBS. Primary and secondary antibody incubation steps were performed as described above. Finally, all stained slides and coverslips were mounted with ProLong® Gold Antifade Reagent with 4′,6′-diamidino-2-phenylindole (DAPI) (Molecular Probes). Images were captured with Leica SP5 Tandem Scanner Spectral 2-photon confocal microscope (Leica Microsystems, Inc., Buffalo Grove, IL) and processed with ImageJ (NIH, Bethesda, MD, US) and Bitplane Imaris software v. 7.3.1 (Andor Technology PLC, Belfast, N. Ireland). The following antibodies and dilutions were used: rabbit anti-Perilipin-1 (1:300, Cell Signalling), rabbit anti-Caveolin-1 (1:100, Cell Signalling), rabbit anti-PPARγ (1:100, Cell Signalling) and rat anti-MAC-2 (1:200, Cedarlane). Alexa Fluor® 488 or 594 goat anti-rabbit IgG (H + L) and Alexa Fluor® 488 goat anti-rat IgG (Molecular Probes) were diluted 1:300 in blocking buffer. F-actin was stained with rhodamine phalloidin (1:30 in PBS, Molecular Probes). For neutral lipid staining in MEFs, samples were incubated with 1 μg/ml BODIPY 493/503 (Molecular Probes).

2.5. TUNEL assay

Apoptotic cells were detected by the terminal deoxynucleotidyl-transferase-mediated deoxyuridine-triphosphate-biotin nick-end labeling (TUNEL) method using DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) according the manufacturer's protocol. For TUNEL/Perilipin-1 double-labeled assay, paraffin sections of adipose tissue were first treated for TUNEL staining followed by immunofluorescent detection of Perilipin-1. Nuclei were counterstained with propidium iodide or DAPI as indicated. Stained samples were examined by confocal scanning laser microscopy.

2.6. Transmission electron microscopy

Dissected tissues from anterior subcutaneous regions and differentiated MEFs were fixed in 2% glutaraldehyde; 0.1 M cacodylate buffer and processed at the UTSW Electron Microscopy Core Facility. Sections were examined with a TEM Tecnai Spirit electron microscope and photographed with a Morada CCD camera.

2.7. Immunoblot analysis and antibodies

MEF protein extracts were prepared in RIPA buffer (50 mM Tris pH 8.0, 180 mM NaCl, 1% NP-40, 1% sodium deoxycholic acid, 0.1 mM EGTA and 0.1% sodium dodecyl sulfate (SDS)) supplemented with protease and phosphatase inhibitor cocktails (Roche) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Proteins (50 μg) were denaturated in Laemmli's sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, and 5% β-mercaptoethanol), separated in a SDS-polyacrylamide gel and electrotransferred onto a nitrocellulose membrane. Membranes were blocked with 5% bovine serum albumin (BSA)/tris phosphate buffer 0.1% Tween 20 (TBS-T) and incubated overnight at 4 °C with primary antibodies diluted in 5% BSA/TBS-T solution. Rabbit antibodies against PPARγ, C/EBPα, C/EBPβ, C/EBPγ, β-actin, ATG12, Beclin, Akt (pan) and anti-rabbit IgG,

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