A Regulatory Role for 1-Acylglycerol-3-phosphate-O-acyltransferase 2 in Adipocyte Differentiation*

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Received for publication, August 31, 2005, and in revised form, January 30, 2006 Published, JBC Papers in Press, February 22, 2006, DOI 10.1074/jbc.M509612200

Mutations in the 1-acylglycerol-3-phosphate-O-acyltransferase 2 (AGPAT2) gene have been identified in individuals affected with congenital generalized lipodystrophy (CGL). AGPAT2 catalyzes acylation of lysophosphatidic acid to phosphatidic acid, a precursor for both triacylglycerol (TAG) and phospholipid synthesis. Recent studies suggest that reduced AGPAT2 enzymatic activity may underlie the CGL clinical phenotype. To gain insight into how altered AGPAT2 activity causes lipodystrophy, we examined the effect of knockdown of AGPAT2 expression in preadipocytes on TAG synthesis and storage, and on adipocyte differentiation. We show that AGPAT2 mRNA expression is induced 30-fold during adipocyte differentiation and that AGPAT2 enzymatic activity is required for TAG mass accumulation in mature adipocytes. We demonstrate that small interference RNA-mediated knockdown of AGPAT2 expression prevents appropriate early induction of C/EBPβ and PPARγ, key transcriptional activators of the adipogenic program, and delays expression of multiple adipocyte-related genes. The unexpected finding, that levels of several phospholipid species, including phosphatidic acid (PA), are elevated in TAG-depleted adipocytes with AGPAT2 knockdown, suggests that impaired AGPAT2 activity affects availability of PA for TAG synthesis but not overall PA synthesis nor utilization of PA for phospholipid synthesis. These findings underscore the importance of an AGPAT2-mediated metabolic pathway in adipocyte differentiation.

Congenital generalized lipodystrophy (CGL),3 also referred to as Berardinelli-Seip syndrome, is an autosomal recessive disorder characterized by paucity of adipose tissue, severe insulin resistance, hypertriglyceridemia, and hepatic steatosis in childhood (1). Positional cloning strategies have identified mutations in the Berardinelli-Seip congenital lipodystrophy 2 gene, which encodes the seipin protein of unknown function, and in the 1-acylglycerol-3-phosphate-O-acyltransferase 2 (AGPAT2) gene in individuals affected with CGL (2, 3). AGPAT2 (also referred to as lysophosphatidic acid acyltransferase or LPAAT-β) encodes an enzyme that catalyzes the acylation of lysophosphatidic acid (LPA) to phosphatidic acid (PA), which serves as a precursor for triacylglycerol (TAG) and phospholipid synthesis (4, 5).

AGPAT2 is one of five murine lysophosphatidic acid acyltransferase isofoms that have been identified (AGPAT1–5), each of which contains a highly conserved catalytic (NHX,D) and substrate binding motif (EGTR) (6). Although there are differences in the tissue distribution of the AGPAT isofoms, the physiological significance of this diversity is not understood. AGPAT1 and AGPAT3 are ubiquitously expressed, whereas AGPAT2, AGPAT4, and AGPAT5 are expressed in a tissue-specific manner (6). In human tissues AGPAT2 is highly expressed in liver, pancreas, skeletal muscle, and small intestine (7) and is the most highly expressed AGPAT isofom in adipose tissue (3).

Structure-function studies of AGPAT2 mutations identified in CGL patients demonstrated reduced conversion of LPA to PA, suggesting that reduced AGPAT2 enzymatic activity may underlie the CGL clinical phenotype (8). Decreased availability of PA for TAG biosynthesis could cause lipodystrophy by resulting in reduced triglyceride synthesis and storage in adipocytes (9). On the other hand, individuals affected with CGL have reduced serum adipokines, such as leptin and adiponectin, suggesting that AGPAT2 function is critical for adipocyte growth and differentiation (9). It is possible that altered AGPAT2 activity causes lipodystrophy by affecting formation of intermediates in the TAG and phospholipid biosynthetic pathways, such as PA and diacylglycerol, rather than through disruption of TAG synthesis per se. PA serves as an important lipid second messenger that participates in intracellular signaling events, such as mediating the mitogenic action of growth factors and as a regulator of G protein-coupled signaling pathways (10, 11). PA can also be hydrolyzed to yield diacylglycerol, a key lipid-signaling molecule (12).

In the present study we examine the contribution of AGPAT2 to TAG synthesis in adipocytes and its role in adipogenesis. We demonstrate that AGPAT2 gene expression is induced during adipocyte differentiation and that AGPAT2 enzymatic activity is required for the TAG accumulation in mature adipocytes. We show that siRNA-mediated knockdown of AGPAT2 expression prevents appropriate early induction of the C/EBPβ and PPARγ transcription factors, providing evidence that AGPAT2 expression is essential for adipocyte differentiation. The unexpected finding that levels of multiple phospholipid species, including PA, are elevated in adipocytes with AGPAT2 knockdown suggests that other PA isofoms can partially compensate for decreased AGPAT2 activity by channeling metabolic intermediates to phospholipid, but not TAG, synthesis. These findings underscore the importance of an AGPAT2-mediated metabolic pathway in adipocyte differentiation.

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† Supported by National Institutes of Health Grant DK059577.

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§ These abbreviations are used: CGL, congenital generalized lipodystrophy; AGPAT, 1-acylglycerol-3-phosphate-O-acyltransferase; CHO, Chinese hamster ovary; ER, endoplasmic reticulum; ESI/MS, electrospray ionization/mass spectrometry; IBMK, isobutylmethylxanthine; LPA, lysophosphatidic acid; LPAAT, lysophosphatidic acid acyltransferase; PA, phosphatidic acid; PBS, phosphate-buffered saline; TAG, triacylglycerol; siRNA, small interference RNA; C/EBPα, C/EBPγ, peroxisome proliferator-activated receptor γ; HA, hemagglutinin; iPLA2, calcium-independent phospholipase A2; RT-PCR, reverse transcription quantitative PCR.
TABLE 1

| siRNA target sequences | Construct | Primes |
|------------------------|-----------|--------|
| siRNA-1 (control)      | 5'-CTACGTTGCTTATTAGTTG-3' | 5'-GCCTAGCTGCCTGGAA-3' |
| siRNA-2                | 5'-GGAGATTCACTGCTCC-3' | 5'-GGAGATTCACTGCTCC-3' |
| siRNA-3                | 5'-TTGACCAAGAAAGCTCTCC-3' | 5'-GAAGACTTCGCTTCGACA-3' |

EXPERIMENTAL PROCEDURES

Materials—The HA antibody was obtained from BD Biosciences. 16% paraformaldehyde solution was obtained from Electron Microscopy Sciences. Hematoxylin stain was obtained from Fischer Scientific. Dulbecco’s modified Eagle’s medium, minimal essential medium-α, Ham’s F-12 medium, trypsin-EDTA, Lipofectamine Plus, glutamine, penicillin/streptomycin, sodium pyruvate, and TRIzol were obtained from Invitrogen The periplin antibody was obtained from Research Diagnostics. Fetal bovine serum, insulin, LPA, PA, Oil Red O stain, propylene glycol, Triton X-100, insulin, dexamethasone, and isobutylmethylxanthine (IBMX) were obtained from Sigma-Aldrich. Hygromycin was purchased from VWR Scientific. All restriction enzymes were obtained from New England Biolabs. Complete Protease Inhibitor Mixture Tablets and phenylmethylsulfonyl fluoride were obtained from Roche Applied Science. [9,10-H]Lyso phosphatidic acid (48.6 Ci/mmole) and [glycerol-1-14C]phosphatidic acid (141 mCi/mmole) were obtained from PerkinElmer Life Sciences. Rabbit polyclonal antibody to ACSL1 has previously been described (13). Rabbit polyclonal antibodies to GLUT4, adiponectin, and AP2 were kindly provided by M. Meuckler (Washington University), P. Scherer (Albert Einstein College of Medicine), and J. Storch (Rutgers University), respectively.

Plasmids—Human AGPAT2 cDNA encoding a C-terminal HA tag was cloned into the XbaI and BamHI restriction sites of the ΔU3 construct (14). siRNA target sequences used for knockdown of murine AGPAT2 (siRNA-2 and siRNA-3) were selected using the Ambion Website (www.ambion.com), and complementary oligonucleotides were cloned into the pSilencer2.1-U6 hygromycin vector (Ambion). The siRNA target sequences are listed in Table 1.

Cell Lines—Chinese hamster ovary-K1 (CHO-K1) cells (CRL-9618) were obtained from ATCC, and OP9 cells were obtained from T. Nakano. To generate CHO/AGPAT2-HA cell lines, CHO-K1 cells were infected with retrovirus prepared by transient transfection of 293GPG packaging cells (14) with the ΔU3 AGPAT2-HA plasmid. Stable knockdown of AGPAT2 in OP9 cells was achieved by transient transfection with pSilencer2.1-U6 vectors encoding siRNA sequences and selection in media containing 0.3 mg/ml hygromycin. Populations of OP9 cells that stably expressed the siRNA sequences were obtained and were used for the siRNA-mediated knockdown experiments.

Cell Culture—Cells were maintained in monolayer culture at 37 °C with 5% CO₂. All CHO-derived cell lines were maintained in medium A (1:1 Dulbecco’s modified Eagle’s medium:Ham’s F-12, 5% v/v fetal calf serum, 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin). 293GPG cells were cultured as described previously (14). OP9 cells were cultured in medium B (minimal essential medium-α, 20% fetal calf serum, 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin). For differentiation, OP9 cells were fed medium C (minimal essential medium-α, 10% fetal calf serum inactivated at 65 °C for 30 min, 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, 0.5 mM IBMX, 0.25 μM dexamethasone, and 1 μg/ml insulin) on day 0 and then fed medium D (medium C without IBMX or dexamethasone) on day 2. On day 4, cells were re-fed and maintained in medium B.

Protein Preparation from Cells—For total post-nuclear supernatants, CHO cells were lysed in TN1 (1% Triton X-100, 0.15 M NaCl, 50 mM Tris, pH 8.0, 2 mM EDTA, 1X Complete Protease Inhibitor Mixture, and 1 mM phenylmethylsulfonyl fluoride), and nuclei were pelleted by centrifugation. For microsome isolation, CHO cells were homogenized in buffer containing 0.5 M sucrose, 1 mM EDTA, 20 mM Tris, pH 8.0, 1X Complete Protease Inhibitor Mixture, and 1 mM phenylmethylsulfonyl fluoride. Nuclei were pelleted by centrifugation, and microsomes were pelleted by centrifugation for 1 h at 356,000 × g. For alkaline extraction, CHO cells were homogenized in 100 mM Na₂CO₃, pH 11.5, with NaOH, and microsomes were prepared from post-nuclear supernatants as described. Detergent lysates were prepared from OP9 cells in radioimmunoprecipitation assay buffer (50 mM Tris HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA). Nuclei were pelleted by centrifugation at 1000 × g at 4 °C. For all protein preparations, proteins were quantified using the bicinchoninic acid (BCA) Protein Assay Kit (Pierce).

SDS-PAGE and Western Blot Analysis—All protein samples were resolved by SDS-PAGE and transferred onto nitrocellulose (0.45 μm, Schleicher & Schuell) with a semi-dry electroblotter (Owl Scientific). Western blot analysis was performed using primary antibodies to HA (1:1,000, murine monoclonal), ACSL1 (1:1,000, rabbit polyclonal), GLUT4 (1:500, rabbit polyclonal), perilipin (1:5,000, guinea pig polyclonal), adiponectin (1:1,000, rabbit polyclonal), β-actin (1:300, rabbit polyclonal), and AP2 (1:5,000, rabbit polyclonal). For secondary antibodies, horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:10,000, Jackson ImmunoResearch), horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:10,000, Jackson ImmunoResearch), and horseradish peroxidase-conjugated anti-guinea pig IgG (1:3,500, Jackson ImmunoResearch) were used. Detection was by chemiluminescence (Renaissance reagents, PerkinElmer Life Sciences).

Immunofluorescence—CHO cells expressing human AGPAT2-HA were plated on glass coverslips, fixed with 4% paraformaldehyde in PBS for 30 min, permeabilized in PBS/0.1% Triton X-100 for 10 min, and blocked in PBS/5% bovine serum albumin for 30 min. Detection of AGPAT2-HA and BiP was performed using an αHA monoclonal antibody (1:250 dilution) and an αBiP rabbit polyclonal antibody (1:50 dilution, Stressgen). Cells were incubated with goat anti-rabbit Alexa 568 and goat anti-mouse Alexa 488.


**Function of AGPAT2 in Adipocytes**

A. The Kyte-Doolittle plot of the amino acid sequence of human AGPAT2 was generated using a window of 19 amino acids. Hydrophobic amino acids are displayed above the zero line, and hydrophilic amino acids are below. B. Western analysis was performed on CHO cells expressing HA-tagged human AGPAT2 (hAGPAT2-HA, lanes 2–6) and parental cells (lane 1). Cellular proteins were harvested in buffer containing 1% Triton X-100 in all lanes 1 and 2. In lanes 3–6, cells were homogenized in sucrose buffer (lanes 3 and 4) or 0.1 M Na2CO3 (lanes 5 and 6), and soluble (S) and membrane (P) fractions were separated by centrifugation. For each lane, 15 μg of protein was separated by SDS-PAGE. Detection used αHA antibody, HRP-coupled secondary antibody and chemiluminescence. C. Immunofluorescence of fixed, permeabilized CHO cells expressing HA-tagged human AGPAT2 was performed using primary αHA or αBIP (as a marker for the ER), and Alexa-coupled secondary antibodies were used for detection, and confocal images were obtained. Bar = 10 μm. D. microsomes from parental and hAGPAT2-HA-expressing cells were assayed for the conversion of LPA to PA as described under “Experimental Procedures.” The graph displays activity normalized for micrograms of protein, and values are the mean of three independent determinations ± S.E. ∗, p < 0.001.

**Oil Red O Staining of OP9 Cells**—OP9 cells expressing siRNA-1 (control), siRNA2, or siRNA3 were differentiated to days 0, 2, 4, and 6. Cells were fixed with 5% formalin and stained with 0.7% Oil Red O solution for 15 min. Propylene glycol (85%) was placed on cells for 15 min with agitation. Cells were rinsed in TRizol reagent (Invitrogen). Northern blotting was performed as described (16), and blots were probed with a 0.84-kb [32P]-labeled probe complementary to hAGPAT2. 18 S RNA served as a loading control.

**Real-time Quantitative Reverse Transcription-PCR**—RNA was harvested above and was reverse-transcribed to cDNA using SuperScriptIII RNase H-reverse transcriptase and random hexamer primers (Invitrogen). cDNA was then amplified for 40 PCR cycles using SYBR Green PCR master mix (Applied Biosystems) and template-specific primers (50 nM) in an ABI Prism 7500 sequence detector. Primer sequences were selected using Primer express software (ABI) and are listed in Table 2. Relative quantification of gene expression was performed using the comparative threshold (Ct) method as described by the manufacturer. Changes in mRNA expression level were calculated following normalization to expression of 36B4, a housekeeping gene whose expression does not change during differentiation of OP9 cells (not shown).

**Oil Red O Staining of OP9 Cells**—OP9 cells expressing siRNA-1 (control), siRNA2, or siRNA3 were differentiated to days 0, 2, 4, and 6. Cells were fixed with 5% formalin and stained with 0.7% Oil Red O solution for 15 min. Propylene glycol (85%) was placed on cells for 15 min with agitation. Cells were rinsed in
distilled water and counterstained with hematoxylin for 2 min. A tap water rinse was followed by an ammonia water rinse. Cells were viewed with a Nikon Eclipse TS100 microscope, and images were captured with a Nikon COOLPIX5000 camera.

Lipid Determination by ESI/MS—siRNA1 (control) and siRNA2 cells were washed twice with PBS and scraped into 8 ml of medium B. Cells were pelleted and washed in PBS twice. Lipids of the pelleted cells were extracted by a modified Bligh and Dyer procedure after performing protein assay on the cell homogenates as described previously (17). Lipid analyses were quantitatively performed by shotgun lipidomics based on intrasource separation and multidimensional ESI mass spectrometric array analysis as previously described (17–19).

Statistics—All results are expressed as mean ± S.E. The statistical significance of differences in mean values was determined by single-factor analysis of variance. Data shown are representative of at least two similar experiments.

RESULTS

Characterization of Human AGPAT2—Human AGPAT2 is a 278-amino acid protein that belongs to a family of eight acyltransferase proteins, identified on the basis of enzymatic activity or sequence homology (5–7, 20). Hydrophobicity analysis of the primary amino acid sequence following the Kyte-Doolittle algorithm (21) using a window of 19 suggests 4 potential membrane-spanning regions (Fig. 1A). The predicted membrane topology for human AGPAT2 is similar to that for the murine ortholog (not shown), with which human AGPAT2 shares 77% amino acid identity (6). To further characterize the AGPAT2 protein, we expressed HA epitope-tagged AGPAT2 in wild-type CHO cells to determine whether the protein is membrane-associated. Western blot analysis shows that AGPAT2 associates with the membrane fraction in the absence of detergent and is associated with the alkaline-extractable microsomal fraction (Fig. 1B), suggesting that AGPAT2 is an integral membrane protein. Immunofluorescence studies show a high degree of co-localization with the resident ER luminal protein BiP (Fig. 1C), consistent with the previously reported association with the microsomal fraction (22). To demonstrate the lysosphatidic acid acyltransferase activity of AGPAT2, AGPAT2-HA was expressed in CHO cells and the transacylation of LPA was assayed by monitoring conversion LPA to PA. We found that acyltransferase activity was increased 5-fold in CHO cells overexpressing AGPAT2 as compared with wild-type cells (Fig. 1D). Thus, AGPAT2 is a resident ER protein that catalyzes the conversion of LPA to PA.

Induction of AGPAT2 during Adipocyte Differentiation—Because mutations in AGPAT2 are associated with human lipodystrophy, we hypothesized that AGPAT2 may play a central role either in regulation or maintenance of adipocyte differentiation. To test this hypothesis, we first examined AGPAT2 expression during adipocyte differentiation. For these studies we used the murine OP9 cell line (23, 24), which differentiates into mature adipocytes in response to IBMX, dexamethasone, and insulin (25). Northern blot analysis in parental OP9 cells and adipocytes stably transfected with control (i.e. scrambled) siRNA (siRNA1) showed that AGPAT2 expression was induced early in differentiation (day 2) and persisted in the mature adipocyte (Fig. 2A). By contrast, stable siRNA expression (siRNA-2 and siRNA-3) prevented the early induction of AGPAT2 expression and markedly reduced expression throughout the differentiation period. Quantification of the AGPAT2 gene expression in siRNA-1-transfected adipocytes demonstrated a nearly 30-fold induction in mRNA expression, which was suppressed by up to 91% (day 2) and 63% (day 4) in adipocytes with stable knockdown of AGPAT2 (siRNA-2 and siRNA-3) (Fig. 2B). Acyltransferase activity, which was increased 2-fold in the adipocytes, was significantly reduced in cells with knockdown of AGPAT2 expression (siRNA-2 and siRNA-3), as compared with control siRNA (siRNA-1) (Fig. 2C).

To determine whether attenuation of acyltransferase activity resulted directly from knockdown of AGPAT2, or indirectly by modulating
expression of other AGPAT isoforms, we measured during differentiation mRNA expression of AGPAT isoforms 1, 3, 4, and 5 in control adipocytes (siRNA-1) and adipocytes with stable knockdown of AGPAT2 (siRNA-2 and siRNA-3). Among the AGPAT isoforms, we observed that AGPAT2 shows the greatest -fold induction during differentiation. We further found that AGPAT2 knockdown blunted expression of the other AGPAT isoforms (Fig. 3), suggesting that AGPAT2 activity is required for induction of these isoforms during differentiation. The decreased expression of these AGPATs is unlikely due to “off-target” effects, because neither of the siRNA sequences directed against AGPAT2 share greater than a 53% identity with the nucleotide sequences of any of the other AGPAT isoforms. These knockdown studies demonstrate that AGPAT2 is responsible for a significant portion of the acyltransferase activity present in mature adipocytes, either directly or indirectly through regulation of expression of other AGPAT isoforms.

Role of AGPAT2 in TAG Accumulation in OP9 Adipocytes—The AGPAT family of proteins participates in the biosynthetic pathway that converts glycerol 3-phosphate to TAG (4, 5). In light of the finding that AGPAT2 expression is markedly induced during adipocyte differentiation, we examined whether AGPAT2 contributes to TAG accumulation in mature adipocytes. Oil Red O staining of adipocytes stably transfected with control siRNA (siRNA-1) or siRNAs that target AGPAT2 (siRNA2 and siRNA3) at days 0, 2, and 4 of differentiation were fixed, stained with Oil Red O, and counterstained with hematoxylin. Micrographs show representative images viewed at 400× magnification. Scale bar, 100 μm. B, lipids were extracted from siRNA1 (control)- and siRNA2 (knockdown)-treated cells and TAG analyzed by ESI/MS. The graph displays TAG normalized per microgram of protein, and values reported are the mean ± S.E. for three independent determinations. *, p < 0.01; **, p < 0.001 for siRNA2 versus control.

FIGURE 4. siRNA targeting of AGPAT2 in OP9 cells inhibits TAG accumulation during adipogenesis. A, OP9 cells stably transfected with control siRNA (siRNA1) or siRNAs that target AGPAT2 (siRNA2 and siRNA3) at days 0, 2, and 4 of differentiation were fixed, stained with Oil Red O, and counterstained with hematoxylin. Micrographs show representative images viewed at 400× magnification. Scale bar, 100 μm. B, lipids were extracted from siRNA1 (control)- and siRNA2 (knockdown)-treated cells and TAG analyzed by ESI/MS. The graph displays TAG normalized per microgram of protein, and values reported are the mean ± S.E. for three independent determinations. *, p < 0.01; **, p < 0.001 for siRNA2 versus control.

Knockdown of AGPAT2 Alters Phospholipid Metabolism in OP9 Adipocytes—Because PA is an important lipid intermediate for both TAG and phospholipid biosynthesis, we investigated whether knockdown of AGPAT2 may affect these biosynthetic pathways through reduced PA availability. Cellular lipid species were quantified using ESI tandem mass spectrometry. Despite marked reduction in TAG synthesis, we found increased levels of all phospholipid species examined, including up to 3-fold elevation of PA, in the differentiated knockdown cells as compared with control cells (Fig. 6A). Analysis of the acyl chain composition of PA sub-species revealed a shift from medium-chain

FIGURE 5. siRNA targeting of AGPAT2 in OP9 cells inhibits adipogenic program of gene expression. A, on days 0, 2, and 4 of differentiation, RNA was harvested from OP9 cells stably transfected with control siRNA (siRNA1) or with siRNA to target AGPAT2 (siRNA2 and siRNA3). RTQ-PCR was performed using primers for C/EBPβ and PPARγ. Values are means ± S.E. *, p < 0.05 for siRNA2 or siRNA3 versus control (siRNA1). B, Western analysis of radioimmune precipitation assay lysates of siRNA-treated OP9 cells as in A.
(14:0–14:1) to long-chain (16:0–18:2 and 16:0–18:1) fatty acid incorporation into PA in the AGPAT2 knockdown cells (Fig. 6B). Incorporation of arachidonate (20:4) was not detected in PA or phosphatidylglycerol in either the control or knockdown cells (not shown). In summary, AGPAT2 knockdown appears to affect availability of PA for TAG synthesis but not overall PA synthesis or utilization of PA for phospholipid synthesis.

There was also a sharp reduction in lysophosphatidylcholine (LPC) in the knockdown cells, which correlated with decreased expression of phospholipases iPLA$_2$-$\beta$ and iPLA$_2$-$\gamma$ in the knockdown cells (Fig. 7). Reduced activity of these phospholipases, which are members of a large family of enzymes (phospholipase A$_2$) that catalyze the hydrolysis of the sn-2 bond of phospholipids producing fatty acids and lysophospholipids (29), could contribute to both reduced LPC levels and elevated phospholipid levels in the AGPAT2 knockdown cells. In light of previous studies demonstrating that iPLA$_2$-$\beta$ selectively catalyzes the release of arachidonate from phospholipids (30), we examined whether reduced iPLA$_2$-$\beta$ expression influenced the phospholipid acyl chain composition in the knockdown cells. ESI tandem MS analysis demonstrated a significant increase in the representation of phospholipids with 20:4 acyl chains among phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine species, as well in TAG, in the AGPAT2 knockdown cells. Moreover, lipid analysis demonstrated a 1.6-fold increase in 1-palmitoyl-2-arachidonoylsn-glycero-3-phosphatidylcholine and a 2-fold reduction in 2-arachidonoyl-LPC (not shown). The latter results are consistent with reduced iPLA$_2$-$\gamma$ activity in the knockdown cells.

**DISCUSSION**

In this study we examined the role of AGPAT2 in adipogenesis. AGPAT2 gene expression is markedly induced during adipocyte differentiation and is accompanied by increased lysophosphatidic acid acyltransferase activity. Knockdown of AGPAT2 gene expression delayed adipocyte differentiation and impaired TAG synthesis and storage. Moreover, levels of several phospholipid species, including PA, are unexpectedly increased in the TAG-depleted cells with AGPAT2 knockdown.
Function of AGPAT2 in Adipocytes

FIGURE 7. Lysophospholipid analysis and phospholipase expression during adipocyte differentiation. A, siRNA1 (control) and siRNA2 cells were harvested and pelleted, and lipids were extracted. ESI/MS was performed, and lipid species were analyzed. Values are means ± S.E. and are representative of two independent experiments. *, p < 0.05; **, p < 0.005 for siRNA2 versus control (siRNA1). B, on days 0, 2, and 4 of differentiation, RNA was harvested from OP9 cells stably transfected with control siRNA (siRNA1) or with siRNA to target AGPAT2 (siRNA2 and siRNA3). RTQ-PCR was performed using primers for iPLA2-β and iPLA2-γ. Values are means ± S.E. and are representative of two independent experiments. *, p < 0.001; **, p < 0.002 for siRNA2 or siRNA3 versus control (siRNA1). C, lipids from siRNA1 (control) and siRNA2 cells were analyzed by ESI/MS, and 20:4 acyl chain composition was determined for phospholipid and TAG species. Values are means ± S.E. and are representative of two independent experiments. *, p < 0.05; **, p < 0.005 for siRNA2 versus control (siRNA1).

TAG synthesis and/or storage in adipose tissue. The modest increase in Oil red O staining and TAG accumulation in the day 4 siRNA2 adipocytes is accompanied by a 10-fold increase in AGPAT2 mRNA expression between days 2 and 4. This suggests that the stable siRNA-mediated knockdown of AGPAT2 driven by an RNA polymerase III promoter can be partially overcome by the marked induction (>30-fold) of AGPAT2 expression during differentiation. Thus, knockdown of AGPAT2 may directly affect TAG accumulation by limiting precursors for TAG synthesis, or indirectly through inhibition of adipogenesis and consequent failure to induce adipocyte proteins required for TAG synthesis (e.g., other AGPAT isoforms) and storage (e.g., perilipin).

In a recent report, the enzymatic activity of AGPAT2 mutations found in CGL patients was examined. Overexpression of these AGPAT2 mutants in CHO cells revealed moderate to severe reduction in enzymatic activity in eight of nine mutants examined, suggesting that decreased conversion of LPA to PA underlies the lipodystrophic phenotype (8). In CGL patients with defects in AGPAT2, failure to synthesize and store TAGs may lead to diversion of serum fatty acids from adipose tissue to liver and skeletal muscle tissue, causing the severe insulin resistance that is the hallmark of lipodystrophy. A central question with respect to the pathophysiology of CGL is whether impaired AGPAT2 activity results in mature but TAG-depleted adipocytes or affects adipogenesis itself. In the present study, we demonstrate that AGPAT2 is required not only for adipocyte TAG synthesis but for appropriate temporal expression of the key transcriptional activators C/EBPβ and PPARγ and multiple adipocyte-related proteins, including adiponectin. Moreover, knockdown of AGPAT2 expression inhibited induction of phospholipases iPLA2-β and iPLA2-γ, whose activity is required for adipogenesis (33). Together with the observation that serum levels of leptin and adiponectin are reduced in individuals with CGL (8, 34), our findings lend support to the hypothesis that CGL is primarily a disorder of adipocyte differentiation and not simply a defect in TAG accumulation.

It is possible that reduced AGPAT2 activity affects adipocyte differentiation principally through modulation of phospholipid synthesis. Altered phospholipid levels could have profound effects on the composition and organization of cellular membranes, as well as on intracellular signaling events. In the AGPAT2 knockdown cells, levels of PA, an important lipid second messenger, were unexpectedly increased 3-fold. Because PA serves as a precursor for all other phospholipids and TAG, flux through this lipid pool, which represents only 1–2% of total ER membrane lipids, is extremely rapid (31). Accordingly, modest changes in the rates of PA synthesis or utilization could result in large shifts in the steady-state PA levels. Thus, in the knockdown cells reduced incorporation of PA into TAG provides a possible explanation for the observed increase in PA levels.

In the adipocytes with reduced AGPAT2 expression, it is likely that other AGPAT isoforms are responsible for bulk of the PA synthesis. Although AGPAT2 is the predominant isofrom expressed in OP9 cells, all five murine AGPATs are expressed during adipocyte differentiation, including AGPAT3 and AGPAT-4, which are induced 7.5- and 5-fold, respectively. The altered acyl chain distribution among the PA species in the AGPAT2 knockdown cells, indicative of differing substrate specificity between AGPAT2 and the other isoforms, further suggests that the PA is non-AGPAT2-derived. Curiously, despite the elevated PA levels in the AGPAT2 knockdown cells, TAG synthesis was reduced 60%, suggesting that the non-AGPAT2-derived PA present in other cellular pools is unavailable to serve as substrate for TAG synthesis. The latter findings are consistent with either compartmentalization of enzymatic activity of AGPAT isoforms or decreased expression of TAG synthetic enzymes due to inhibition of adipocyte differentiation.
The etiology of the overall increase in other phospholipid levels is less clear. One possibility is that reduced conversion of LPA to PA by AGPAT2 could result in shunting of the LPA precursor from the TAG synthetic pathway to pathways involving other AGPAT isoforms whose cellular localization or substrate specificity may favor phospholipid formation. Alternatively, in AGPAT2 knockdown cells, the blunted induction of phospholipases iPLA2-β and iPLA2-γ may limit phospholipid turnover resulting in increased steady-state levels. This hypothesis is supported by the marked reduction in LPC levels in the knockdown cells, along with the increased representation of 20:4 acyl chains among phospholipid species. In the AGPAT2-knockdown adipocytes, an important implication of altered phospholipase activity is reduced phospholipid species. In the AGPAT2-knockdown adipocytes, along with the increased representation of 20:4 acyl chains among phospholipid species. In the AGPAT2-knockdown adipocytes, along with the increased representation of 20:4 acyl chains among phospholipid species.

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