Expression of AGPAT2, an enzyme involved in the glycerophospholipid/triacylglycerol biosynthesis pathway, is directly regulated by HIF-1 and promotes survival and etoposide resistance of cancer cells under hypoxia

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A B S T R A C T
Hypoxia inducible factor-1 (HIF-1) supports survival of normal cells under low oxygen concentration and cancer cells in the hypoxic tumor microenvironment. This involves metabolic reprogramming via upregulation of glycolysis, downregulation of oxidative phosphorylation and, less well documented, effects on lipid metabolism. To investigate the latter, we examined expression of relevant enzymes in cancer cells grown under hypoxia. We show that expression of acylglycerol-3-phosphate acyltransferase 2 (AGPAT2), also known as lyso phosphatic acid acyltransferase 2 (LPAATβ), was upregulated under hypoxia and this was impaired by siRNA-mediated knockdown of HIF-1α. Moreover, a sequence of the AGPAT2 gene promoter region, containing 6 putative Hypoxia Response Elements (HREs), activated transcription of a reporter gene under hypoxic conditions or in normoxic cells over-expressing HIF-1α. Chromatin immunoprecipitation experiments confirmed binding of HIF-1α to one of these HREs, mutation of which abolished hypoxic activation of the AGPAT2 promoter. Knockdown of AGPAT2 by siRNA reduced lipid droplet accumulation and cell viability under hypoxia and increased cancer cell sensitivity to the chemotherapeutic etoposide. In conclusion, our findings demonstrate that AGPAT2, which is mutared in patients with congenital generalized lipodystrophy and over-expressed in different types of cancer, is a direct transcriptional target of HIF-1, suggesting that upregulation of lipid storage by HIF-1 plays an important role in adaptation and survival of cancer cells under low oxygen conditions.

1. Introduction

Organisms and cells have evolved a series of adaptations critical for the survival in low oxygen concentrations (hypoxia) under physiological or pathological conditions, like embryogenesis, development, ischemia, inflammation and cancer. Exposure of cancer cells, in particular, to the hypoxic microenvironment of solid tumors, elicits induction of angiogenesis and changes in their metabolism that promote survival, proliferation, migration and invasiveness [1, 2]. This metabolic switch is largely achieved through changes in gene expression mediated by two members of the Hypoxia Inducible Factors (HIFs) family, HIF-1 and HIF-2 [3, 4].

Both HIF-1 and HIF-2 are heterodimeric transcription factors, consisting of unique, oxygen regulated HIFα subunits and the common, constitutively expressed HIFβ subunit (or aryl hydrocarbon receptor translocator; ARNT). The protein levels of the HIFα subunits are regulated mainly at the post-transcriptional level, through the action of specific prolyl hydroxylases (PHDs) [5]. Under physiological oxygen levels, PHDs modify HIFα that, as a consequence, is polyubiquitinated by the von Hippel-Lindau (VHL) protein and degraded at the proteasome. When oxygen levels become limited, PHDs become inactive, HIFα is stabilized, is imported into the nucleus, dimerizes with ARNT and the active HIF heterodimer binds to hypoxia-response elements (HREs) at the promoter or enhancer of HIF target genes and induces transcription [6]. HIF expression and activity are also controlled by oxygen-independent mechanisms. In this context, HIF-1 is regulated by many intracellular signaling pathways, including NF-κB, PKR-STAT3, PI3K-mTOR, ROS, MAPK and CK1δ [7–11].

Whereas the effect of HIFs on the expression of genes involved in carbohydrate metabolism has been extensively studied [3], the hypoxic regulation of lipid metabolism remained, until recently, poorly investigated. Recent findings demonstrate that HIF-1 is implicated in lipid...
metabolism through lipid droplet (LD) accumulation [12, 13], increase of fatty acid and lipid synthesis [13–15], decrease of fatty acid β-oxidation [16] and upregulation of fatty acid uptake [17, 18], while HIF-2 promotes lipid storage [19]. Importantly, HIF-mediated fatty acid accumulation under hypoxia may contribute to the development of metabolic disorders, such as liver steatosis, and hepatocellular carcinoma [20]. However, few direct targets of HFIs have been identified so far, among the many genes that code for lipogenic enzymes.

Fatty acids can be converted to triglycerides, neutral lipids that are stored in the form of LDs. The triglyceride (TG) synthesis pathway relies on the consecutive action of glycerol-3-phosphate acyltransferase (GPAT), acylglycerol-3-phosphate acyltransferase (AGPAT), phosphatidic acid phosphatase (PAP or lipin) and diacylglycerol acyltransferase (DGAT) enzymes, each one of which exists in multiple isoforms [21]. TG synthesis is important for metabolic homeostasis and cellular growth since LDs function as lipid stores providing both metabolic substrates and components for membrane synthesis [22]. Under hypoxia, storage of fatty acids as neutral lipids in LDs, protects hypoxic cancer cells from lipotoxicity and ROS production [23], while HIF-1 driven TG synthesis and LD accumulation is critical for cancer cell survival and proliferation [24]. In particular, HIF-1 mediates the hypoxic induction of the lipin 1 (but not lipin 2) isoform, the enzyme that catalyzes the penultimate step in TG biosynthesis [13].

AGPATs, also known as lysophosphatidic acid acyltransferases (LPATs), the enzymes preceding lipin-1, catalyze the conversion of lysophosphatidic acid (LPA) to phosphatidic acid (PA). Several AGPAT isoforms are known, that are encoded by different genes. Most but not all AGPAT isoforms reside in the ER, and cannot complement each other, possibly due to different substrate specificities [25]. In addition to their role in TG synthesis, generation of PA by AGPATs stimulates phospholipid production and regulates mTOR activity [26]. AGPAT1 and AGPAT2 are the earlier identified and better-studied isoforms. AGPAT1 is ubiquitously expressed, while AGPAT2 expression is mostly detected in adipose tissue, liver, heart and pancreas. AGPAT2 mutations are the cause of type 1 congenital generalized lipodystrophy (CGL) [27]. CGL patients lack adipose tissue, develop lipid accumulation in liver and muscle and exhibit severe metabolic complications [28, 29]. AGPAT2 expression is upregulated in different types of cancer including those of breast, pancreas, lung, bladder, ovary, melanoma, uterus, renal, prostate, central nervous system, lymphoma, colorectal, meiothelemia and leukemia [30–33]. Furthermore, AGPAT2 is highly expressed in osteosarcoma [34], pancreatic [26] and myeloma [35, 36] cancer cells and AGPAT2 silencing inhibits proliferation of cancer cells and enhances their sensitivity to chemotherapeutical drugs [26, 37–40].

We report here that hypoxia induces the expression of AGPAT2 gene in human hepatocellular carcinoma Huh7 and adenocarcinoma HeLa cells. We also show that AGPAT2 is a direct target gene of HIF-1 and that AGPAT2 induction is necessary for hypoxic accumulation of LDs and for sustaining viability and etoposide resistance under hypoxia.

2. Materials and methods

2.1. Materials

Tris-Base, Bromphenol Blue, etoposide, formaldehyde, Triton X-100, PMSF, Mowiol and Nile Red were purchased from Sigma-Aldrich (St Louis, MO, USA), SDS, DTT and glycerine from AppliChem (Darmstadt, Germany), glycerol, NaCl and MgCl₂ from Merck (Darmstadt, Germany), dimethylsulfoxide (DMSO) from Jena Bioscience (Jena, Germany). The human AGPAT2 promoter (region −1013 to −140) luciferase reporter plasmid

Table 1

Oligonucleotide sequence list.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
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<td>hAGPAT2 FOR</td>
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| hAGPAT2 REV | TTGAGTA

2.2. Cell culture and treatment

Human hepatocarcinoma (Huh7), cervical adenocarcinoma (HeLa) and embryonic kidney cells (HEK293) were cultured in Dulbecco's modified Eagle's medium (DMEM) High Glucose containing 10% fetal bovine serum (FBS)-South America Origin, 100 U/ml penicillin-streptomycin at 37 °C in a humidified incubator under 5% CO₂. For hypoxic treatment, cells were exposed to 1% O₂, 95% N₂ and 5% CO₂ in an IN VIVO 200 hypoxia workstation (RUSKINN Life Sciences/Baker Ruskinn, Sanford, Maine, USA). When required, cells were treated for the indicated time with DMOG (1 mM), etoposide (75 μM), or DMSO at the appropriate concentration as solvent control.

2.3. Plasmid construction, site directed mutagenesis, transfection and luciferase assay

The human AGPAT2 promoter (region −1013 to −140, Acc. No. NG_008090.1) was amplified by PCR from genomic DNA isolated from Huh7 or HeLa cells using Quick-DNA Universal Kit (Zymo Research, Irvine, CA, USA), as template, specific primers (Table 1) and Taq DNA polymerase (Minotech Biotechnology, IMBB-FORTH, Heraklion, Crete, Greece). The PCR product was purified from agarose gel using Isolate II PCR and Gel Kit (Bioline, Luckenwalde, Germany), cloned into pminiT Vector (New England Biolabs. Inc., Hitchin, Hertfordshire, UK), subcloned into the XhoI sites of pGL3 basic Luciferase Reporter Vector (Promega Corporation, Madison, WI, USA), to construct pGL3-AGPAT2 promoter and sequenced. To mutagenize HRE4 of the AGPAT2 promoter in the pGL3-AGPAT2 plasmid, three nucleotide substitutions (CCGTG to CACAGC) were introduced by PCR using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) and specific primers (Table 1). The mutant construct was verified by sequencing (VBC-Biotech, Vienna, Austria).

HEK293 cells cultured in 24-well plates were transiently transfected by Polyethylenimine (PEI) reagent (Polysciences Inc., Hirschberg an der
Bergstrasse, Germany) with plasmids (1 or 0.25 μg) wild-type or mutant pGL3-AGPAT2 promoter, pGL3-Lipin-1 promoter [13] or empty pGL3 vector and the Renilla luciferase expressing plasmid pCI-Renilla (0.05 μg). When required, cells were also co-transfected with pEGFP-C1-HIF-1α (0.75 μg) [11] or pEGFP-N3 (0.75 μg) (BD Biosciences, San Jose, CA, USA). 24 h post transfection, cells were incubated under normoxia or hypoxia for the indicated times and luciferase activity was determined according to the Dual Luciferase Reporter Assay System protocol (Abnova, Neihu District. Taipei City, Taiwan).

2.4. siRNA-Mediated silencing

siRNA AGPAT2/LPAATβ (25 nM, Santa Cruz Biotechnology, Dallas, TX, USA), siRNA HIF-1α (20 nM, Qiagen, Venlo, Netherlands), or AllStars negative control siRNA (20 nM, Qiagen, Venlo, Netherlands) was introduced in cells using Viromer Blue (Lipocalyx, Halle-Saale, Germany).

2.5. RNA extraction and quantitative real-time PCR

Total RNA was isolated by NucleoZol Reagent (Macherey-Nagel, Düren, Deutschland) or by Direct-Zol RNA MiniPrep (Zymo Research, Irvine, CA, USA). Total RNA (0.5 μg) was reverse transcribed with PrimeScript RT Reagent Kit-Perfect Real-Time (Takara Clontech, Duren, Deutschland) or by Direct-Zol RNA MiniPrep (Zymo Research, Irvine, CA, USA) using the KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Wilmington, MA USA) using the KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Wilmington, MA, USA) and primers for human LPIN1, GPAT3, GPAT4, AGPAT1, AGPAT2, DGAT1 or DGAT2 (Table 1). mRNA levels were normalized to human β-actin mRNA. Relative gene expression was calculated using the ΔΔCt method.

2.6. Total cellular protein extraction and western blot analysis

Cells were lysed in 20 mM Tris-CI pH 8.0, 150 mM NaCl, 1 mM MgCl2, 1% Triton X-100, 0.5 mM DTT and 0.2 mM PMSF, incubated on ice for 10 min and cell lysates were centrifuged at 10,000 g for 15 min at 4 °C. For AGPAT2 detection cells were lysed in 62.5 mM Tris-CI (pH 6.8), 2.3% SDS, 10% glycerol, 0.05% (w/v) Bromophenol Blue, 25 mM DTT (loading Buffer), incubated on ice for 10 min, sonicated, boiled for 5 min and centrifuged at 10,000 g for 10 min at 4 °C. Total protein extracts (40–80 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membranes and analyzed by Western blot. Membranes were incubated with anti-LPAATβ rabbit polyclonal antibody (1:500 dilution, Santa Cruz Biotechnology, Dallas, TX, USA), or anti-AGPAT2 (D8W9B) rabbit monoclonal antibody (1:500 dilution, Cell Signaling Technology, Leiden, The Netherlands), or anti-HIF-1α rabbit polyclonal antibody (1:1000 dilution in PBS-Tween20 containing 5% milk, antibody body, raised, prepared and purified as described in [41]) or anti-β-actin mouse monoclonal antibody (1:5000, Sigma-Aldrich, St Louis, MO, USA), followed by horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (1:3000 dilution, Cell Signaling Technology Inc., Danvers, MA, United States). Signals were visualized by the Enhanced Chemiluminescence kit Luminata™ Forte/Crescendo Western HRP Substrate (Millipore Corporation, Billerica, USA). Images and quantification of protein band intensities were obtained using an Uvitec Cambridge Chemiluminescence Imaging System with the help of Alliance Software (ver. 16.06).

2.7. Chromatin immunoprecipitation

HeLa cells seeded on glass coverslips in 24-well plates were transfected with siRNA against AGPAT2 or control siRNA, and 24 h later incubated under normoxia (21% O2) or hypoxia (1% O2) for another 24 h. When required, cells were treated with etoposide (75 μM) or with DMSO as solvent control for 5 h before the end of the incubation period, after which living cell number was determined using the “CellTiter 96 Aqueous One Solution Cell Proliferation Assay” kit (Promega Corporation, Madison, WI, USA).

2.10. Cell death assay

HeLa cells were seeded into 96-well plates (2.500 cells/well) and transfected with siRNAs targeting AGPAT2, HIF-1α or control siRNA. Cells were cultured in DMEM supplemented with 1% FBS for the duration of the experiment and 24 h post-transfection were incubated for the indicated periods of time under hypoxia (1% O2). To induce apoptosis cells were treated with 75 μM Etoposide for 5 h or with DMSO as solvent control, before the end of the incubation period. Cell death was determined using the “LDH Cytotoxicity Detection kit” (Takara Clontech, Mountain View, CA, USA).

2.11. Statistical analysis

Data are presented as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM), as indicated. Statistical differences between two groups of data were assessed using the unpaired t-test in the SigmaPlot v. 9.0 software (Systat); P < 0.05 was considered to be significant.
3. Results

3.1. AGPAT2 expression is induced in cancer cell lines under hypoxia

To investigate the effect of hypoxia on the expression of genes encoding for members of the enzyme families operating along the lipid biosynthesis pathway, we examined the mRNA levels of representative GPAT (GPAT3 and GPAT4), AGPAT (AGPAT1 and AGPAT2) and DGAT (DGAT1 and DGAT2) isoforms. As a positive control, we examined the mRNA levels of LPIN1, a known HIF-1 target gene [13]. To this end, human hepatocellular carcinoma Huh7 cells were incubated under normoxia (21% O2) or hypoxia (1% O2) for 8 or 24 h and mRNA levels of TG synthesis enzymes were analyzed by quantitative-RT-PCR. No significant effect was observed on the expression of GPAT or DGAT isoforms under hypoxic conditions (Fig. 1S). In contrast, the LPIN1 and AGPAT2, but not AGPAT1, mRNA levels significantly increased after incubation for 8 or 24 h under 1% O2 (Fig. 1A). Similar results were obtained when LPIN1, AGPAT1 and AGPAT2 mRNA levels were analyzed after incubation of Huh7 cells for 24 h with DMOG (1 mM), a prolyl hydroxylase inhibitor that mimics hypoxia (Fig. 1B). Furthermore, analysis by Western blot showed that, hypoxic treatment of Huh7 cells increased AGPAT2 protein levels in parallel to the expected induction of HIF-1α (Fig. 1C and Fig. 2SA). The induction of AGPAT2 expression under hypoxia is not limited in hepatocellular carcinoma cells, as it was also observed in human cervical adenocarcinoma HeLa cells (Fig. 1D-E and Fig. 2SB).

3.2. HIF-1 mediates the hypoxic upregulation of AGPAT2

We then tested the involvement of HIF-1 in the hypoxic induction of AGPAT2 mRNA expression, using siRNA-mediated repression of HIF-1α expression in Huh7 cells incubated under normoxia (21% O2) or hypoxia (1% O2). Successful knockdown of HIF-1α was confirmed by western blot analysis (Fig. 2A, upper panel and Fig. 2SC). Suppression of HIF-1α expression reduced the hypoxic induction of AGPAT2 protein (Fig. 2A, lower panel) and AGPAT2 mRNA levels (Fig. 2B) in hypoxia, while a control siRNA had no effect, suggesting that HIF-1 mediates the hypoxic induction of AGPAT2 gene transcription.

In silico analysis showed that 6 putative HREs, containing the consensus sequence NCGTG (where N is an A, G or C), are present within a 1 kb AGPAT2 promoter region (Fig. 3A). To test their role in the AGPAT2 promoter induction under hypoxia, an 873 bp AGPAT2 cells increased AGPAT2 protein levels in parallel to the expected induction of HIF-1α (Fig. 1C and Fig. 2SA). The induction of AGPAT2 expression under hypoxia is not limited in hepatocellular carcinoma cells, as it was also observed in human cervical adenocarcinoma HeLa cells (Fig. 1D-E and Fig. 2SB).

Fig. 1. The expression of AGPAT2 is induced under hypoxic conditions in cancer cells.
(A) & (B) Quantitative real-time PCR determination of AGPAT1, AGPAT2 and LPIN1 mRNA levels in Huh7 cells incubated under hypoxia (1% O2) for 0-24 h (A) or in the presence of DMOG (1 mM) for 24 h (B), as indicated.
(C) Western blotting analysis of Huh7 cells incubated at 1% O2 for 0-24 h using antibodies against the indicated proteins.
(D) Quantitative real-time PCR determination of AGPAT1 and AGPAT2 mRNA levels in HeLa cells incubated under hypoxia (1% O2) for 24 h.
(E) Western blotting analysis of HeLa cells incubated at 1% O2 for 24 h using antibodies against the indicated proteins.
In (A), (B) and (D) values represent the mean ± SD of 3 experiments performed in duplicates (*P < 0.05, **P < 0.01 and ***P < 0.001 compared to cells incubated for 0 h at 1% O2).
promoter region (corresponding to bases −1013 to −140) including the potential HREs was cloned to the pGL3 luciferase reporter plasmid. HEK293 cells were transfected with the AGPAT2 promoter reporter plasmid, a reporter plasmid harboring the LPIN1 promoter region, a known target of HIF-1 [13], or the empty pGL3 plasmid as a negative control (Fig. 2C). In normoxia, the AGPAT2 promoter plasmid showed higher transcriptional activity compared to the empty vector. Moreover, incubation of cells under hypoxia increased significantly the activity of the AGPAT2 promoter compared to normoxia. This increase was comparable to that of the LPIN1 promoter, which was used as a positive control (Fig. 2C), suggesting the HRE containing AGPAT2 promoter is indeed stimulated under hypoxic conditions. Importantly, a similar significant increase of AGPAT2 promoter activity was also achieved after overexpression of HIF-1α in HEK293 cells under normoxia (Fig. 2D), suggesting that AGPAT2 is a direct HIF-1 target.

The direct binding of HIF-1α to the AGPAT2 promoter was examined by chromatin immunoprecipitation (ChIP). Huh7 cells were cultured under hypoxia or normoxia for 8 h, cross-linked and DNA was immunoprecipitated with anti-HIF-1α or rabbit IgG antibodies. The immunoprecipitated DNA was amplified by PCR with primers specific for the AGPAT2 promoter regions harboring HREs 1–3, HRE 4 or HREs 5–6 (Fig. 3A). Only the AGPAT2 promoter region containing HRE 4 (−685 to −492) was specifically enriched in immunoprecipitates of DNA-protein complexes isolated from cells grown under hypoxia, using an anti-HIF-1α antibody, in comparison to rabbit IgG immunoprecipitates or anti-HIF-1α immunoprecipitates from normoxic cells (Fig. 3B). This result was corroborated, quantitatively, by RT-PCR using specific primers for the HRE 4 region (Fig. 3C).

To examine whether HRE 4 present within the [−685 to −492] AGPAT2 promoter region is indeed responsible for HIF-1 mediated induction of AGPAT2, the consensus CGTG HRE sequence was mutated to AACG in plasmid pGL3-AGPAT2 (Fig. 3D). HEK293 cells were transfected with wild type or mutant AGPAT2 promoter reporter plasmids or the empty pGL3 plasmid as a negative control. In normoxia, the mutant AGPAT2 promoter showed comparable transcriptional activity to the wild type AGPAT2 promoter. However, when cells were incubated under hypoxia, the activity of the mutant AGPAT2 promoter did not change compared to normoxia and was, subsequently, significantly lower than the activity of the wild type AGPAT2 promoter (Fig. 3D). Therefore, HIF-1α not only activates but also physically associates with the AGPAT2 promoter by binding to HRE 4. Overall our data show that AGPAT2 is a novel direct transcriptional target of HIF-1.

3.3. Knockdown of AGPAT2 impairs LD formation, viability and etoposide resistance of cancer cells under hypoxia

Hypoxia has been previously shown to increase LD formation in a HIF-1-dependent manner [13]. To investigate the contribution of
AGPAT2 upregulation in this metabolic adaptation, we measured LD accumulation in HeLa cells treated with siRNA to AGPAT2 and incubated under normoxia (21% O2) or hypoxia (1% O2). To confirm suppression of AGPAT2 expression, AGPAT2 mRNA levels were determined by quantitative-RT-PCR (Fig. 4A) and AGPAT2 protein levels by western blot analysis (Fig. 4B), respectively. Under the same conditions, we did not detect any changes of the AGPAT1 mRNA levels, both under normoxia or hypoxia (Fig. 3S), suggesting that regulation of AGPAT1 expression is independent of AGPAT2 expression, at least in HeLa cells. As expected, fluorescence microscopy of cells stained with Nile Red to visualize neutral lipids, showed a profound accumulation of LDs under hypoxic treatment in cells transfected with a control siRNA (Fig. 4C). However, LD accumulation under hypoxia was significantly impaired after AGPAT2 knockdown. Quantitative analysis showed that silencing of AGPAT2 significantly reduced LD number per cell (Fig. 4D, upper panel), the average cell area covered by LDs (Fig. 4D, middle panel) and mean LD size under hypoxia (Fig. 4D, lower panel). These findings show that AGPAT2 is required for the hypoxic induction of LD accumulation.

We then examined whether this function of AGPAT2 is important
Fig. 4. AGPAT2 silencing impairs hypoxic induction of LD accumulation and growth.

HeLa cells were transfected with siRNAs against AGPAT2 (siAGPAT2) or control (siControl) and incubated 24 h post-transfection for another 24 h under normoxia (21% O₂) or hypoxia (1% O₂).

(A) Quantitative real-time PCR determination of AGPAT2 mRNA levels. Values represent the mean ± SD of 4 independent experiments performed in duplicates (*P < 0.05, ***P < 0.001).

(B) Western blotting analysis of HIF-1α and AGPAT2 protein levels in cells treated as above.

(C) Fluorescence microscope images of cells treated as above and stained with Nile Red to visualize LDs. Cell nuclei were stained with DAPI.

(D) Quantitative analysis of fluorescence microscope images of cells presented in (C) showing mean number of LDs per cell (upper panel), average percentage of cell area occupied by LDs (middle panel) and mean LD size (lower panel). Data represent the mean ± SEM of 100 cells. (**P < 0.01, ***P < 0.001, NS non-significant).
AGPAT2 silencing significantly impaired the expression of AGPAT2 protein both under normoxia and hypoxia (Fig. 5A). Analysis of HeLa cell numbers after AGPAT2 silencing showed that suppression of AGPAT2 expression reduced cell viability under hypoxia compared to control siRNA-treated cells, although viability of normoxic cells under the same conditions was not significantly affected (Fig. 5B and Fig. 4S). Moreover, silencing of AGPAT2 increased the sensitivity of cancer cells to the apoptotic agent etoposide, an effect that was much more pronounced under hypoxic conditions (Fig. 5B and Fig. 4S). To evaluate the contribution of AGPAT2 to HIF-1-dependent survival under hypoxia, we measured cell death after AGPAT2 or HIF-1α knockdown (Fig. 5C). Silencing of AGPAT2 increased cell death under normoxia as well as under hypoxia in both the absence and presence of etoposide. As expected, knockdown of HIF-1α for cancer cell survival under hypoxia. AGPAT2 silencing significantly impaired the expression of AGPAT2 protein both under normoxia and hypoxia (Fig. 5A). Analysis of HeLa cell numbers after AGPAT2 silencing showed that suppression of AGPAT2 expression reduced cell viability under hypoxia compared to control siRNA-treated cells, although viability of normoxic cells under the same conditions was not significantly affected (Fig. 5B and Fig. 4S). Moreover, silencing of AGPAT2 increased the sensitivity of cancer cells to the apoptotic agent etoposide, an effect that was much more pronounced under hypoxic conditions (Fig. 5B and Fig. 4S). To evaluate the contribution of AGPAT2 to HIF-1-dependent survival under hypoxia, we measured cell death after AGPAT2 or HIF-1α knockdown (Fig. 5C). Silencing of AGPAT2 increased cell death under normoxia as well as under hypoxia in both the absence and presence of etoposide. As expected, knockdown of HIF-1α
increased cell death only under hypoxia. The effects of HIF-1α knockdown under hypoxia were more pronounced than those of AGPAT2 knockdown, as also to be expected by the much wider role of HIF-1 in cellular adaptation to hypoxia. Nevertheless, comparison of the data suggests that AGPAT2 contributes substantially to HIF-1-dependent survival under low oxygen conditions.

Taken together, our data suggest that HIF-1-mediated induction of AGPAT2 facilitates metabolic adaptation, survival and chemoresistance of cancer cells under hypoxic conditions (Fig. 6).

4. Discussion

Several studies have previously reported that AGPAT2 expression is elevated in cancer cell lines or human tumors and is associated with increased cancer cell proliferation and migration, tumor growth or aggressiveness and worse prognosis [30, 32, 34]. However, despite the important role of AGPAT2 in TG biosynthesis and its link to pathological conditions, the mechanisms responsible for regulation of AGPAT2 gene transcription have so far remained largely unknown. We now report for the first time that AGPAT2 is a direct target of HIF-1 and AGPAT2 induction under hypoxia is required for cancer cell survival and chemoresistance. HIF-1α is often upregulated in solid tumors as a result of the hypoxic microenvironment. In addition, tumor HIF-1α levels can also be increased by oxygen independent mechanisms under the influence of growth factors and cell signaling pathways that are stimulated in cancer cells [9]. Therefore, our finding that HIF-1 is responsible for the activation of AGPAT2 gene transcription, offers a plausible explanation for the observed upregulation of AGPAT2 expression in tumors and cancer cells. Our in vitro data are also in agreement with previous reports showing strong correlation of AGPAT2 expression with the expression of heme oxygenase 1 (HO-1), a known HIF-1 target gene, in a comparative analysis of human tumors [33]. Moreover, kaempferol, a flavonoid that was previously shown to inhibit HIF-1 activity under hypoxia [43], was also found to decrease AGPAT2 expression in 3 T3-L1 preadipocytes [44].

AGPAT2 belongs to a family of several isoforms that catalyze the conversion of LPA to PA in the triglyceride and phospholipid biosynthesis pathway [25]. We have shown that, unlike AGPAT2, the expression of its closest homologue AGPAT1 is not increased under hypoxia or after treatment with the PHD inhibitor DMOG. Similar selectivity has also been shown regarding the hypoxic induction of lipins, the enzymes acting downstream of AGPAT2 in the TG biosynthesis pathway, as only lipin 1, but not its isof orm lipin 2, is upregulated by HIF-1 and plays a role in hypoxic adaptation [13, 24]. AGPAT1 and AGPAT2 exhibit diverse expression levels in different tissues, show distinct knockout phenotypes and cannot complement each other [28]. Importantly, AGPAT1, which is more ubiquitously expressed than AGPAT2 and appears to serve important functions in the physiology of many organ systems [45–47], is not overexpressed in tumors and cancer cell lines [48], emphasizing the specificity of the HIF-1-mediated upregulation of AGPAT2 and its significance for cancer cell adaptation under hypoxia. The importance of AGPAT2 for cancer cells is further underlined by the fact that AGPAT2 inhibitors and miRNAs targeting AGPAT2 inhibit cell proliferation and migration and increase cell sensitivity to cytotoxic drugs [31, 35–40, 49, 50]. This is consistent with our data showing that AGPAT2 induction under hypoxia sustains cancer cell survival and increases their chemoresistance.

An interesting question is how induction of AGPAT2 supports cancer cell adaptation to low oxygen conditions. We have shown that upregulation of AGPAT2 is required for LD accumulation under hypoxia. It is well established that regulation of lipid metabolism and storage are very important for cancer cells and the related pathways represent valid therapeutic targets [51]. Moreover, HIF-1 driven TG synthesis and LD accumulation is critical for cancer cell survival and proliferation under hypoxia [24], as storage of fatty acids as neutral lipids in LDs can protect hypoxic cancer cells from lipotoxicity and ROS production [23]. Indeed, hypoxia induces additional genes directly involved in lipid storage and LD formation. These include, besides lipin 1 [13], the non-enzymatic proteins HIG2 [12] and fatty acid binding proteins FABP3 and FABP7 [17], which are regulated by HIF-1, as well as perilipin 2, which is induced by HIF-2 [19]. Uptake of fatty acids, synthesis of neutral lipids and their storage in LDs under hypoxia are processes required for avoiding endoplasmic reticulum stress [19] and damage by ROS [17] or for providing energy after reoxygenation [17], and, as such, they may be essential for cancer cell proliferation in the hostile microenvironment of solid tumors.

An alternative role of AGPAT2 induction under hypoxia may be related to the production of PA. PA, apart from its role as an intermediate in TG and phospholipid synthesis, is also involved in cell signaling via the activation of the mTOR pathway [52]. The mTOR signaling pathway affects the cell response to hypoxia as active mTORC1 increases synthesis of HIF-1α. On the other hand, hypoxia inhibits mTORC1 activation [4]. It is, thus, also possible that the effect of AGPAT2 silencing on cell survival under hypoxia reflects a reduction of
cellular PA concentration and, subsequently, mTOR activity, as was previously shown in pancreatic cancer cells under normoxia [26]. However, PA can be produced in cells through the action of two additional enzymes, phospholipase D (PLD) and diacylglycerol kinase-ζ (DGKζ), which can compensate for the downregulation of AGPAT2 [52].

5. Conclusion

In conclusion, AGPAT2 is specifically and directly upregulated by HIF-1 in cancer cells and promotes their survival and chemoresistance under hypoxia. Its identification, following that of lipin 1, as a second TG synthesis enzyme under HIF-1 transcriptional control, further establishes the strong and multifaceted involvement of HIF-1 in lipid synthesis and storage and their connection to cancer cell physiology (Fig. 6). Additionally, it suggests that these lipogenic enzymes may be used as prognostic markers and/or novel therapeutic targets of hypoxic tumors.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbalip.2018.06.015.

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Transparency document

The Transparency document associated with this article can be found, in online version.

References


