PNPLA2 influences secretion of triglyceride-rich lipoproteins by human hepatoma cells \[\text{PNPLA2}\]

Apostolos Taxiarchis, Hovsep Mahdessian, Angela Silveira, Rachel M. Fisher, and Ferdinand M. van’t Hooft

Division of Cardiovascular Medicine, Department of Medicine Solna, and Center for Molecular Medicine, Karolinska Institutet, Stockholm, Sweden

Abstract Patatin-like phospholipase domain-containing proteins (PNPLAs) are involved in triglyceride hydrolysis and lipid-droplet homeostasis in mice, but the physiological significance of the PNPLAs for triglyceride metabolism in human hepatocytes is unclear. Here, we investigate the roles of PNPLA2, PNPLA3, and PNPLA4 in triglyceride metabolism of human Huh7 and HepG2 hepatoma cells using gene-specific inhibition methods. siRNA inhibition of PNPLA3 or PNPLA4 is not associated with changes in triglyceride homeostasis, secretion of triglyceride-rich lipoproteins (TRLs), or triglyceride accumulation. However, PNPLA2 siRNA inhibition, both in the absence and presence of oleate-containing medium, or treatment with the PNPLA2 inhibitor Atglistatin reduced intracellular triglyceride hydrolysis and decreased TRL secretion. In contrast, PNPLA2 inhibition showed no effects on lipid-droplet homeostasis, which is the primary physiological function of PNPLA2 in nonhepatic tissues. Moreover, confocal microscopy analysis found no clear evidence for the localization of PNPLA2 around lipid droplets. However, significant colocalization of PNPLA2 with the endoplasmic reticulum marker protein disulfide-isomerase was found in HepG2 and Huh7 cells with Rcoloc values of 0.61 ± 0.06 and 0.81 ± 0.05, respectively. In conclusion, PNPLA2 influences TRL secretion, but is not involved in lipid-droplet homeostasis in human hepatoma cells, a physiological role that is quite distinct from the metabolic function of PNPLA2 in nonhepatic tissues.—Taxiarchis, A., H. Mahdessian, A. Silveira, R. M. Fisher, and F. M. van’t Hooft. PNPLA2 influences secretion of triglyceride-rich lipoproteins by human hepatoma cells. J. Lipid Res. 2019, 60: 1069–1077.

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Hepatic steatosis, the most common form of liver disease, is a precursor to fibrosis, cirrhosis, and liver cancer (1) and is tightly linked to obesity, T2D, and CVD (2, 3). The hepatic triglyceride content defines steatosis, and it is generally assumed that disturbances in hepatic triglyceride metabolism are an integral part of the etiology of hepatic steatosis (4). A potential cause of steatosis is defects in the hydrolysis of triglycerides stored in hepatocytes, a metabolic process that is not only of importance for hepatic lipid-droplet homeostasis, but also influences the secretion of triglyceride-rich lipoproteins (TRLs) by the liver (5, 6). Unfortunately, little is known about the lipase(s) responsible for cellular triglyceride hydrolysis in human hepatocytes.

In 2004, three groups independently identified a member of the patatin-like phospholipase domain-containing protein (PNPLA) family, PNPLA2 (also known as adipose triglyceride lipase), as the enzyme responsible for the catalysis of the initial step of triglyceride lipolysis in adipocytes, converting triglycerides to diacylglycerols (7–9). PNPLA2 KO mice exhibited increased adipose tissue mass (9) as a consequence of impaired rates of adipocyte triglyceride hydrolysis (10). Because of its high expression and prominent role in adipose metabolism, subsequent research has largely focused on the role of PNPLA2 in adipose tissue (11). However, PNPLA2 is also expressed, albeit at lower levels, in nonadipose tissues, such as heart and liver. Cardiac muscle triglyceride content was markedly increased in PNPLA2 KO mice (9, 10). In contrast, only partial reductions in triglyceride-hydrolase activity and modest increases in triglyceride content were observed in the liver of PNPLA2 KO mice (9, 10). Subsequent studies evaluated the role of hepatic PNPLA2 in triglyceride metabolism using methods to modulate PNPLA2 activity specifically in the liver. Adenovirus-mediated knockdown of hepatic PNPLA2 with shRNA resulted in steatosis in mice and decreased triglyceride hydrolysis in primary murine hepatocyte cultures (12). In addition, mice with liver-specific inactivation of Pnpla2 developed a progressive form of

Abbreviations: PDI, protein disulfide-isomerase; PLIN2, perilipin2; PNPLA, patatin-like phospholipase domain-containing protein; TRL, triglyceride-rich lipoprotein.

1To whom correspondence should be addressed.

*E-mail: Ferdinand.vant.Hooft@ki.se

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hepatic steatosis (13). Moreover, liver overexpression of PNPLA2 in obese mice reduced hepatic steatosis (14, 15). Overall, these studies suggest that PNPLA2 plays an important role in hepatic lipid metabolism, at least in mouse models. To the best of our knowledge, no study has verified the physiological role of PNPLA2 in human hepatocytes.

PNPLA2 mRNA levels are low in human liver as compared with those of PNPLA3, an enzyme with in vitro triglyceride-hydrolase activity (7) and an established genetic risk factor for hepatic steatosis and its sequelae (16). Moreover, human hepatocytes express PNPLA4, another PNPLA family member with in vitro triglyceride-hydrolase activity that is not expressed in rodents (7). Here, we used siRNA inhibition methods to determine the physiological functions of PNPLA2, PNPLA3, and PNPLA4 in triglyceride metabolism in the human hepatoma Huh7 and HepG2 cell lines. We found that PNPLA2, but not PNPLA3 or PNPLA4, influences secretion of TRLs, but is not involved in lipid-droplet homeostasis of human hepatoma cells. Confocal microscopy analysis found no clear evidence for the localization of PNPLA2 around lipid droplets, whereas significant colocalization of PNPLA2 with the ER marker protein disulfide-isomerase (PDI) was observed.

MATERIALS AND METHODS

Cell culture and transfection

Huh7 and HepG2 cell lines were purchased from the Japanese Cancer Research Resources Bank and ATCC, respectively. Cells were maintained at 37°C and 5% CO2 and cultured in low-glucose DMEM containing l-glutamine, supplemented with 10% FBS, and 50 μg/ml penicillin/50 μg/ml streptomycin (PEST). The medium was changed twice a week, and cells were subcultured weekly. The universal mycoplasma detection kit (ATCC) was used at 3 month intervals to screen for mycoplasma infection of the cells.

For the siRNA silencing experiments, cells of 60–70% confluency were transfected with gene-specific or control siRNA oligonucleotides (supplemental Table S2) using Lipofectamine RNAiMax (Thermo Fisher Scientific) as transfection reagent. For each gene, two different siRNA probes were used for all experiments. The medium was replaced after 24 h with culture medium containing 14C-glycerol (PerkinElmer; see Assays below); 24 h later, the cell medium was collected, and the cells were harvested and analyzed.

For the Aglistatin experiments, cells were first incubated for 24 h with cell medium, alternatively transfected with gene-specific or control siRNA oligonucleotides as described above. After 24 h, the medium was replaced with culture medium containing 14C-glycerol supplemented with 50 μM Aglistatin (Gayman Chemical) or DMSO; 24 h later, the cells were harvested and analyzed.

For the oleic acid experiments, cells were first transfected with siRNA oligonucleotides as described above. The medium was replaced after 24 h with culture medium containing 14C-glycerol (PerkinElmer), 5% FBS, 50 U/ml PEST, and 0.2 mM oleic acid conjugated to FA-free BSA (Sigma). The cells were harvested after 24 h incubation.

Immunoblot analysis

Whole-cell protein lysates were extracted using RIPA buffer containing EDTA (Thermo Fisher Scientific), protease inhibitors (Halt Protease Inhibitor Cocktail, Thermo Fisher Scientific), and phosphatase inhibitors (Phosphatase Inhibitor Cocktail 2, Sigma). Ten micrograms of protein was loaded on 10% mini-Protein TGX gels (Bio-Rad) and transferred to activated PVDF membrane (Bio-Rad), blocked in 3% BSA, and incubated overnight with 1:1,000 diluted PNPLA2 (R&D) or PNPLA3 (Aviva) primary Ab, followed by incubation with HRP-conjugated secondary Abs against sheep and mouse (Bio-Rad). Proteins were visualized using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific) and analyzed with an LAS-1000 Imager (Fujifilm). The same blots were subsequently incubated with 1:3,500 diluted β-actin Ab (Bio-Rad), visualized, and analyzed as a loading control. See supplemental Table S1 for more details regarding the Abs used in this study.

Quantitative real-time PCR

RNA was isolated with the E.Z.N.A. Total RNA Kit 1 (Omega Bio-tek), and cDNA was synthesized with a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Taqman assays (supplemental Table S2) and an AB7500 sequence-detection system (Applied Biosystems) were used for relative mRNA expression analysis. Relative expression was assessed using the comparative ΔΔCT method and adjusted for the endogenous control RPLP0. The results of the gene-expression studies were verified using the relative standard curve method, according to the Applied Biosystems guidelines.

Assays

The triglyceride quantification colorimetric/fluorometric kit (Biovision) was used to measure the cellular triglyceride content. Cell-protein concentration was quantified using the Pierce BCA assay (Thermo Fisher Scientific). Triglyceride secretion was quantified in cell-culture medium following 24 h incubation of the cells with cell-culture medium containing 14C-glycerol (PerkinElmer) at a final concentration of 2.85 mCi/ml (18). The 14C-labeled lipids were extracted from the cell-culture medium and separated by TLC using hexane:diethylether:acetic acid (80:10:1). The radioactivity associated with the 14C-triglycerides was quantified using a scintillation counter. The APOB in the cell-culture medium was quantified by ELISA (ALerCHEK).

Triglyceride-hydrolase activity was measured essentially as described (19). Briefly, 24 h after siRNA transfection of the hepatoma cells, the cell-culture medium was switched to cell-culture medium containing 1.0 μCi of 14C-palmitic acid (PerkinElmer). After 24 h incubation, the cell-culture medium was changed to cell-culture medium containing 5 μM Triascin C (Sigma), an inhibitor of the long-chain fatty acyl CoA synthetase isoproteins 1, 3, 4, and 5. Cells were harvested at the indicated time intervals, and cellular 14C-triglyceride radioactivity was quantified as described above.

Confocal microscopy imaging

Hepatoma cells were cultured overnight on glass coverslips (Marienfeld) and fixed in 4% paraformaldehyde (Histolab) for 20 min at room temperature. For the lipid-droplet analysis, the hepatoma cells were stained with either 1:100 diluted BODIPY493/503 (Molecular Probes, Life Technologies) or HCS LipidTOX Red (Invitrogen) and mounted with Vectashield mounting medium containing DAPI (Vector Laboratories). Selected sample regions were imaged with a Leica SP5 confocal microscope, equipped with a 63× 1.4 lens and diode and argon lasers. Image stacks consisted of a Z-stack of 15–20 optical slices taken at 0.15–0.30 μm intervals to enhance the spatial signal allogration. Lipid-droplet area was quantified in every experiment by analyzing 10 randomly chosen fields, each of 30–50 cells. The number and area of the lipid droplets in each field were quantified using the ImageJ cell counter and Particle Analysis Plugin (Fiji), while the number of cells was calculated manually. The average lipid-droplet area per cell and lipid-droplet number per cell were calculated by dividing the overall lipid-droplet area and the
For the colocalization experiments, hepatoma cells were cultured on glass coverslips and fixed either with paraformaldehyde (for PDI) or with methanol [for perilipin2 (PLIN2)], followed by permeabilization and blocking with 10% goat serum (Vector Laboratories). The cells were subsequently incubated for 1 h with 1:50 diluted PNPLA2 mAb (Novus Biologicals) conjugated with Alexa Fluor 488 using the Zenon Alexa Fluor 488 Mouse IgG2b Labeling Kit (Thermo Fisher Scientific) at room temperature, followed by overnight incubation with either 1:100 diluted PDI (Enzo) or 1:100 diluted PLIN2 (R&D Systems) mAbs. The coverslips were then incubated with goat anti-mouse Alex Fluor 594 Ab, mounted with Vectashield (Vector Laboratories) medium containing DAPI, and stored at 4°C. Image stacks were obtained as described above. The average colocalization was calculated using the JACoP plug in of the ImageJ program in 10–15 images obtained for each condition. The relationships between the lipid droplets and PNPLA2 and PLIN2 were analyzed in the absence or presence of 0.4 mM oleic acid conjugated to FA-free BSA in cell-culture medium for 8 h, followed by fixation, permeabilization, blocking, and overnight incubation with 1:100 diluted PNPLA2 mAb. The cells were subsequently incubated with goat anti-mouse Alex Fluor 488 Ab and 1:1,000 HCS LipidTOX Red (Invitrogen) for 1 h, mounted, and analyzed as previously described.

Statistical analysis

Statistical analysis and graphic design was conducted using GraphPad Prism (Version 6.01) software. Data are presented as mean ± SD unless stated otherwise. Statistical significance was evaluated by Student’s t test with a threshold value of \( P = 0.05 \). Bonferroni correction was used to adjust the threshold \( p \)-values for multiple testing in the gene-expression studies.

RESULTS

siRNA inhibition of PNPLA2, PNPLA3, and PNPLA4

Functional analysis of the roles of PNPLA2, PNPLA3, and PNPLA4 in hepatic triglyceride metabolism was performed in human hepatoma Huh7 and HepG2 cell lines using transient transfection techniques with gene-specific siRNA probes. For each gene, two different siRNA probes were used for all experiments. Comparable results were obtained for both probes for each of the three PNPLA genes, and most data are presented as the average results of the two gene-specific siRNA probes, but, occasionally, results of the two probes are shown separately. As shown in Fig. 1A, substantial reductions in mRNA levels of PNPLA2, PNPLA3, and PNPLA4 are achieved, in the absence of compensatory increases in the expression of nontargeted PNPLA genes. Corresponding decreases in PNPLA2 and PNPLA3 protein concentrations after respective siRNA inhibition, evaluated using Western blot analysis, are shown in Fig. 1B, C. Unfortunately, no suitable PNPLA4 Ab for Western blot analysis could be identified. No consistent changes in mRNA levels are noted for genes involved in triglyceride synthesis, lipid-droplet, or TRL metabolism after gene-specific siRNA inhibition in Huh7 or HepG2 cells (Fig. 1D and supplemental Fig. S1).

PNPLA2 siRNA inhibition reduces triglyceride hydrolysis

We were unable to quantify the low triglyceride-hydrolase activity in Huh7 or HepG2 cells using an established technique for the quantification of triglyceride hydrolysis in

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**Fig. 1.** Effect of gene-specific siRNA silencing on mRNA levels of selected genes. A: Effect of gene-specific siRNA silencing on the PNPLA2, PNPLA3, and PNPLA4 mRNA levels in human hepatoma Huh7 (left) and HepG2 (right) cells (n = 4–7). B: Representative Western blot images of PNPLA2 from HepG2 cells treated with either control or PNPLA2 siRNA probe obtained after 24 h (left) and 48 h (right) incubations. C: Representative Western blot images of PNPLA3 from HepG2 cells treated with either control siRNA or the independent PNPLA3 siRNA probes A and B. D: Effect of gene-specific siRNA silencing on mRNA levels of selected genes involved in lipid metabolism in human hepatoma cells (n = 3–6). The values in A and D are expressed as percent of control experiments, indicated with a dotted line. Values represent mean ± SD (A) or mean ± SEM (D). Differences were determined using unpaired Student’s t-test, followed by Bonferroni post hoc analysis (D). * \( P < 0.05 \); ** * \( P < 0.001 \).
adipocytes in vitro (17). We therefore developed a semi-quantitative in vivo triglyceride-hydrolysis assay using the approach of Hobbs and coworkers (19). In this assay, cellular triglycerides were first labeled with 14C-palmitate for 24 h, followed by inhibition of de novo triglyceride synthesis with Triascin C and subsequent quantification of cellular 14C-labeled triglycerides after 4 and 8 h. We found that PNPLA2 siRNA inhibition decreased triglyceride hydrolysis at both time points in Huh7 and HepG2 cells (Fig. 2A). A similar effect was observed when 3H-oleate instead of 14C-palmitate was used for the analysis of triglyceride hydrolysis in Huh7 cells (supplemental Fig. S2). We subsequently quantified triglyceride hydrolysis using only the 8 h time point and found that PNPLA2 inhibition, but not gene-specific inhibition of PNPLA3 or PNPLA4, led to significant reductions of cellular triglyceride hydrolysis (Fig. 2B). Finally, we found no evidence that gene-specific inhibition of either PNPLA2, PNPLA3, or PNPLA4 influenced the cellular uptake of 14C-palmitate or the incorporation of 14C-palmitate in triglycerides (a measure of triglyceride synthesis) in the hepatoma cell lines (supplemental Fig. S3), two factors that can, in theory, influence the triglyceride hydrolysis assay. Overall, these results indicate that PNPLA2, but not PNPLA3 or PNPLA4, influences cellular triglyceride hydrolysis in human hepatoma cells. In agreement with the data from PNPLA2-KO mice (9, 10), we found that PNPLA2 siRNA inhibition decreased the overall cellular triglyceride hydrolysis by only around 50%, despite effective inhibition of PNPLA2. This suggests that a substantial proportion of the triglyceride hydrolyase activity in human hepatoma cells cannot be accounted for by PNPLA2 and must instead be attributed to the action of other triglyceride hydrolyase enzyme(s).

PNPLA2 siRNA inhibition reduces TRL secretion without effects on lipid-droplet content

The effects of gene-specific inhibition of PNPLA2, PNPLA3, or PNPLA4 on the secretion of TRLs and cellular triglyceride accumulation by Huh7 and HepG2 cells were analyzed using methods described previously (18). Significant reductions in the secretion of triglycerides and APOB were observed in Huh7 and HepG2 cells following siRNA inhibition of PNPLA2, whereas no effects of PNPLA3 or PNPLA4 inhibition on TRL secretion were found (Fig. 2C). However, no effects of gene-specific inhibition of PNPLA2, PNPLA3, or PNPLA4 on cellular triglyceride content were observed in the hepatoma cell lines (Fig. 2D). We performed confocal microscopy studies to verify this observation. We did not identify significant effects of PNPLA2 inhibition on lipid-droplet area or the overall lipid-droplet size distribution in either Huh7 (Fig. 3A–C) or HepG2 (Fig. 3D–F) cells. Thus, PNPLA2 inhibition reduced TRL secretion, whereas no evidence was found for a significant role of PNPLA2 in lipid-droplet homeostasis in human hepatoma Huh7 and HepG2 cells.

Olate treatment does not influence the effects of PNPLA2 siRNA inhibition on triglyceride metabolism

Incubation of Huh7 and HepG2 cells with 0.2 mM oleate-supplemented cell medium increased, as expected, the secretion of triglyceride and APOB and the cellular triglyceride content of the hepatoma cells as compared with cells incubated with cell medium without oleate supplement (Fig. 4A, B). Nevertheless, Huh7 and HepG2 cells cultured in 0.2 mM oleate-supplemented cell medium showed similar effects of PNPLA2 siRNA inhibition on PNPLA2 mRNA levels (Fig. 4C), triglyceride secretion (Fig. 4D), and cellular triglycerides content (Fig. 4E), as compared with human hepatoma cells cultured without oleate supplement (Figs. 1A, 2C, 2D).

Effects of the PNPLA2 inhibitor Atglistatin on hepatic triglyceride metabolism

Atglistatin is an inhibitor of PNPLA2 in mouse cells/tissues (20), but a recent study noted that Atglistatin is not an effective inhibitor of PNPLA2 in human Simpson-Golabi-Behmel syndrome adipocytes (21). Nevertheless, we found that incubation of human hepatoma Huh7 cells
with 50 μM Atglistatin resulted in 30–40% reductions of total cellular triglyceride-hydrolysis activity (supplemental Fig. S4). We subsequently compared the effects of PNPLA2 siRNA inhibition and PNPLA2 inhibition with 50 μM Atglistatin on triglyceride-hydrolysis activity in both Huh7 and HepG2 cells (Fig. 5A). It was found that both PNPLA2 siRNA inhibition and PNPLA2 inhibition with Atglistatin are associated with partial reductions in overall triglyceride-hydrolysis activity in both hepatoma cell lines. However, PNPLA2 siRNA inhibition was a slightly more effective method to reduce triglyceride-hydrolysis activity in Huh7 and HepG2 cells, as compared with PNPLA2 inhibition with Atglistatin (Fig. 5A). We subsequently evaluated the effect of a combination of Atglistatin and PNPLA2 siRNA inhibition on triglyceride-hydrolysis activity in the hepatoma cells. As shown in Fig. 5A, no evidence was found that a combination of the two PNPLA2-inhibition methods led to a greater inhibition of total cellular triglyceride-hydrolysis activity as compared with PNPLA2 siRNA inhibition alone. These observations indicate that both PNPLA2-inhibition techniques are able to inhibit PNPLA2-related triglyceride-hydrolysis activity in the two human hepatoma cell lines.

No consistent changes in mRNA levels for genes involved in triglyceride synthesis, lipid-droplet, or TRL metabolism after Atglistatin inhibition or a combination of Atglistatin and PNPLA2 siRNA inhibition were observed (Fig. 5B and supplemental Fig. S5). As shown in Fig. 5C, the two PNPLA2-inhibition techniques reduced the secretion of triglycerides by the Huh7 and HepG2 cells by approximately 45% and 35%, respectively. Again, no additive effects of the combined PNPLA2 siRNA and Atglistatin inhibition on triglyceride secretion were observed when compared with PNPLA2 siRNA inhibition alone. The effects of the two inhibition methods on APOB secretion showed a similar pattern (Fig. 5C): a modest decrease of APOB secretion following Atglistatin inhibition, but no significant differences in the reduction of APOB secretion when the PNPLA2 siRNA inhibition and the combined PNPLA2 siRNA and Atglistatin inhibitions are compared in the two hepatoma cell lines. In contrast, the two PNPLA2-inhibition strategies did not influence cellular triglyceride levels (Fig. 5D). Confocal microscopy confirmed that neither Atglistatin inhibition alone nor the combination of PNPLA2 siRNA and Atglistatin inhibition influenced lipid-droplet area or size distribution in Huh7 cells (supplemental Fig. S5). Overall, we found comparable qualitative effects of PNPLA2 siRNA and Atglistatin inhibition on TRL secretion, whereas no effects of these inhibition strategies on lipid-droplet homeostasis were detectable in either Huh7 or HepG2 cells.

**No clear evidence for preferential localization of PNPLA2 around lipid droplets**

Subcellular localization studies in 3T3-L1 adipocytes demonstrated that PNPLA2 is located predominantly around lipid droplets (4, 22). Here, we used confocal microscopy to determine the subcellular localization of PNPLA2 in human hepatoma Huh7 and HepG2 cells. PNPLA2 was visualized using a mAb that showed specificity for PNPLA2 and no cross-reactivity with other proteins in Huh7 and HepG2 cells (supplemental Fig. S6). Immunofluorescence staining of PNPLA2 showed a patchy/mottled distribution of PNPLA2 in the cytoplasm of the hepatoma cells (Fig. 6). However, no evidence was found for the enrichment of PNPLA2 around lipid droplets of hepatoma cells cultured in either 10% FBS-supplemented or oleate-supplemented cell medium (Fig. 6). Nevertheless, in agreement with previous reports (23, 24), immunofluorescence staining of PLIN2 showed a preferential localization of PLIN2 around lipid droplets in human hepatoma cells cultured in 10% FBS medium and an enhanced accumulation of PLIN2 around lipid droplets in human hepatoma cells cultured in oleate-supplemented cell medium (supplemental Fig. S7). Overall, these subcellular localization studies provided no clear evidence for the preferential localization of PNPLA2 around lipid droplets. Of note, we did not study the subcellular localization of PLIN1, whereas we found that the PLIN1 mRNA levels were below 2% of the PLIN2 mRNA concentration in Huh7 and HepG2 cells. Furthermore, we are not aware that anyone has been able to detect PLIN1 protein in human hepatoma cell lines.
Partial colocalization of PNPLA2 with PLIN2

The subcellular localization studies of PNPLA2 and PLIN2 shown in Fig. 6 and supplemental Fig. S6 demonstrated that essentially all PNPLA2 and a substantial fraction of PLIN2 are found in the cytoplasm of the hepatoma cells cultured in 10% FBS cell medium. In view of the lipophilic nature of PNPLA2 and the preferential association of PLIN2 with lipid droplets, we hypothesized that PNPLA2 and PLIN2 are both associated with small lipid droplets present in the cytoplasm of the hepatoma cells. These lipid droplets, because of their small size, are not detectable by conventional light microscopy as distinct lipid droplets, but are expected to show a diffuse staining pattern when performing confocal microscopy, explaining the patchy/mottled appearance of the PNPLA2-stained hepatoma cells shown in Fig. 6. Indeed, PNPLA2 and PLIN2 exhibited comparable patchy/mottled distribution patterns in the cytoplasm of both hepatoma cell lines (Fig. 7A). Moreover, partial colocalization was observed between PNPLA2 and PLIN2, with Rcoloc values (mean ± SD) of 0.49 ± 0.07 (n = 10) and 0.66 ± 0.02 (n = 13) for Huh7 and HepG2 cells, respectively. Note that these calculations are based on the overall distribution of PNPLA2 and PLIN2 in the hepatoma cells. Because a proportion of PLIN2, but not PNPLA2, is preferentially localized around large lipid droplets (Fig. 6 and supplemental Fig. S7), it can be expected that the Rcoloc values are higher for the cytoplasmic regions devoid of large lipid droplets.

Colocalization of PNPLA2 with PDI

The involvement of PNPLA2 in the regulation of TRL secretion suggests that PNPLA2 resides in a subcellular compartment that is part of, or in close proximity to, the TRL-synthetic machinery present in the ER. We therefore analyzed the colocalization of PNPLA2 with PDI, a subunit of the microsomal triglyceride-transfer complex involved in the assembly of TRLs and commonly used as an ER marker. As shown in Fig. 7B, we found that both PNPLA2 and PDI exhibited a patchy/mottled pattern in the cytoplasm of the hepatoma cells. Moreover, a substantial colocalization of PNPLA2 with PDI was observed, with Rcoloc values (mean ± SD) of 0.61 ± 0.06 (n = 10) and 0.81 ± 0.05 (n = 11) for Huh7 and HepG2 cells, respectively.

DISCUSSION

This study evaluated the biological significance of PNPLA2, PNPLA3, and PNPLA4 for hepatic triglyceride metabolism in the human hepatoma Huh7 and HepG2 cell lines, with particular emphasis on the roles of these proteins in triglyceride hydrolysis, TRL secretion, and cellular triglyceride accumulation. Gene-specific inhibition of PNPLA3 or PNPLA4 was not associated with changes in triglyceride hydrolysis, TRL secretion, or cellular triglyceride accumulation, indicating that PNPLA3 and PNPLA4 do not play major roles in these aspects of hepatic lipid metabolism. In contrast, PNPLA2 siRNA inhibition, both in the absence or presence of oleate-containing medium, or treatment with the PNPLA2 inhibitor Aglistatin reduced intracellular triglyceride hydrolysis and decreased TRL secretion. However, no effects of PNPLA2 inhibition were observed on cellular triglyceride concentration or the number and size of the lipid droplets. Subcellular localization studies found no clear evidence for associations between PNPLA2 and large lipid droplets, whereas colocalization studies revealed that PNPLA2 is primarily found in close proximity to the TRL-synthetic machinery present in the ER of human hepatoma cells. Together, these observations suggest that the triglyceride-hydrolase activity of
PNPLA2 contributes to the regulation of TRL secretion by human hepatocytes, but does not influence lipid-droplet homeostasis of human hepatoma cells.

Previous studies demonstrated that PNPLA2 plays a major role in the hydrolysis of triglycerides in adipocytes and other nonhepatic tissues (11). Correspondingly, PNPLA2 is preferentially localized around lipid droplets in 3T3-L1 adipocytes (9, 22). However, the current study provides two lines of evidence against a role of PNPLA2 in the hydrolysis of triglycerides stored in lipid droplets of human hepatoma cells. First, PNPLA2 siRNA inhibition, both in the absence and presence of oleate-containing medium, or treatment with the PNPLA2 inhibitor Atglistatin, was found to have no effect on cellular triglyceride concentrations or lipid-droplet size and distribution of the hepatoma cells. Second, colocalization studies found no clear evidence for the localization of PNPLA2 on the surface of the large lipid droplets visualized by confocal microscopy. These observations largely exclude a role for PNPLA2 in the hydrolysis of triglyceride molecules in large lipid droplets in human hepatoma cells and thereby indicate that yet-unidentified enzyme(s) are involved in this process.

To the best of our knowledge, this is the first report demonstrating that PNPLA2 influences TRL secretion by human hepatoma cells. There is long-standing evidence that approximately half of the FAs used for TRL synthesis are derived from triglycerides stored in hepatocytes (5, 6). It is therefore conceivable that the triglyceride-hydrolase activity of PNPLA2 is responsible for mobilizing FAs from stored triglycerides, which are subsequently used for TRL synthesis, but the nature of these temporary triglyceride storage sites is elusive. Our colocalization studies in human hepatoma cells demonstrated that PNPLA2 is predominantly localized near the ER marker PDI. We also observed colocalization of PNPLA2 with PLIN2, a protein that is best known as a lipid-droplet-associated protein. However, only a minor fraction of PLIN2 was actually associated with the large lipid droplets in our colocalization studies. Most of the PLIN2 protein was located in the cytoplasm of the hepatoma cells, and the cytoplasmic PLIN2...
was largely responsible for the observed colocalization with PNPLA2. Together, these colocalization studies suggest that PNPLA2 and cytoplasmic PLIN2 are both located near the ER. It is tempting to speculate that PNPLA2 and cytoplasmic PLIN2 are constituents of lipid droplets that are loosely associated with the ER, but are too small to be visualized as distinct lipid droplets by confocal microscopy. Small lipid droplets, designated initial lipid droplets (iLDs), were recently identified as a specific lipid-droplet subclass that is not detectable by conventional light microscopy and is metabolically distinct from so-called expanding lipid droplets, which are large lipid droplets that can be visualized with confocal microscopy (4, 25). An attractive hypothesis is that the small iLDs in human hepatoma cells act as a temporary storage site for triglycerides synthesized in the ER and subsequently acquire/activate PNPLA2 for the generation of FAs for TRL formation, but more detailed studies are required to substantiate this hypothesis.

The strength of our study is that various aspects of hepatic triglyceride metabolism are analyzed using complementary methods. For example, two different hepatoma cell lines (HepG2 and Huh7) are used, different siRNA inhibitors and Atglistatin inhibition are used, TRL secretion is analyzed by two different parameters (APOB and 14C-triglyceride measurements), and cellular triglyceride accumulation is estimated by triglyceride concentration measurement and confocal microscopy analysis. A limitation of our study is the use of human hepatoma cell lines, given their secretion of TRLs as compared with the VLDL lipoproteins secreted in vivo by mammalian liver (26), although it is generally thought that the overall mechanism of triglyceride metabolism and TRL secretion is retained in these cells (reviewed in Ref. 27).

In summary, this study demonstrates that PNPLA2 inhibition of human hepatoma Huh7 and HepG2 cells is associated with decreased triglyceride hydrolysis and reduced TRL secretion, whereas no changes in the cellular triglyceride content are observed. In contrast, the primary physiological function of PNPLA2 in nonhepatic tissues, such as adipose tissue, is the hydrolysis of triglycerides stored in lipid droplets. This suggests that PNPLA2 plays a metabolic role in human hepatoma cells that is quite distinct from its function in nonhepatic tissues.

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PNPLA2 influences TRL secretion of human hepatoma cells

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