Cardiac-specific overexpression of perilipin 5 provokes severe cardiac steatosis via the formation of a lipolytic barrier

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Abstract Cardiac triacylglycerol (TG) catabolism critically depends on the TG hydrolytic activity of adipose triglyceride lipase (ATGL). Perilipin 5 (Plin5) is expressed in cardiac muscle (CM) and has been shown to interact with ATGL and its coactivator comparative gene identification-58 (CGI-58). Furthermore, ectopic Plin5 expression increases cellular TG content and Plin5-deficient mice exhibit reduced cardiac TG levels. In this study we show that mice with cardiac muscle-specific overexpression of perilipin 5 (CM-Plin5) massively accumulate TG in CM, which is accompanied by moderately reduced fatty acid (FA) oxidizing gene expression levels. Cardiac lipid droplet (LD) preparations from CM of CM-Plin5 mice showed reduced ATGL- and hormone-sensitive lipase-mediated TG mobilization implying that Plin5 overexpression restricts cardiac lipolysis via the formation of a lipolytic barrier. To test this hypothesis, we analyzed TG hydrolytic activities in preparations of Plin5-, ATGL-, and CGI-58-transfected cells. In vitro ATGL-mediated TG hydrolysis of an artificial micellar TG substrate was not inhibited by the presence of Plin5, whereas Plin5-coated LDs were resistant toward ATGL-mediated TG catabolism. These findings strongly suggest that Plin5 functions as a lipolytic barrier to protect the cardiac TG pool from uncontrolled TG mobilization and the excessive release of free FAs.—Pollak, N. M., M. Schweiger, D. Jaeger, D. Kolb, M. Kumari, R. Schreiber, S. Kolleritsch, P. Markolin, G. F. Grabner, C. Heier, K. A. Zierler, T. Rülicke, R. Zimmermann, A. Lass, R. Zechner, and G. Haemmerle. Cardiac-specific overexpression of perilipin 5 provokes severe cardiac steatosis via the formation of a lipolytic barrier. J. Lipid Res. 2013. 54: 1092–1102.

Supplementary key words adipose triglyceride lipase • cardiac lipid • energy metabolism

This work was supported by the Austrian Ministry for Science and Research and the grants FWF project DK-MCD w1226, P20602-B05, and SFB Lipotox F30-B05, which are funded by the Austrian Science Fund (Fonds zur Förderung der wissenschaftlichen Forschung [FWF]).

Manuscript received 5 December 2012 and in revised form 22 January 2013.

Published, JLR Papers in Press, January 23, 2013.

DOI 10.1194/jlr.M034710

Abbreviations: Acadl, acyl-CoA dehydrogenase, long chain; Acadml, acyl-CoA dehydrogenase, medium chain; Acad3l, acyl-CoA dehydrogenase, very long chain; Acox1, acyl-CoA oxidase 1; ATGL, adipose triglyceride lipase; Cd36, cluster of differentiation 36; CGI-58, comparative gene identification-58; CM, cardiac muscle; CM-Plin5, cardiac muscle-specific overexpression of perilipin 5; COXIV, cytochrome c oxidase IV; CPT, carnitine palmitoyltransferase; Cpt1b, carnitine palmitoyltransferase 1B; FAO, fatty acid oxidation; G0S2, G0/G1 switch gene 2; HSL, hormone-sensitive lipase; LacZ, β-galactosidase; LD, lipid droplet; MHC, myosin heavy chain; PAT, perilipin, adipophilin, and TIP47; Pdhk4, pyruvate dehydrogenase kinase, isoenzyme 4; PGC, peroxisome proliferator-activated receptor γ coactivator; Pli1, perilipin 1; Plin5, perilipin 5; PPAR, peroxisome proliferator-activated receptor; Ppara, peroxisome proliferator-activated receptor α; Pparg1, peroxisome proliferator-activated receptor γ isoform 1; Ppargc1a, peroxisome proliferator-activated receptor γ coactivator 1α; Ppargc1b, peroxisome proliferator-activated receptor γ coactivator 1β; RT-qPCR, quantitative reverse transcriptase polymerase chain reaction; TG, triacylglycerol; Tg, transgenic line; wt, wild type.

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The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of two figures.

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TG mobilization from white and brown adipose tissue is a relatively well characterized process involving perilipin 1 (Plin1), adipose triglyceride lipase (ATGL), and hormone-sensitive lipase (HSL) among many other proteins and cofactors (11, 12). In contrast, much less is known about LD catabolism in nonadipose tissues, including muscle and liver, in which Plin1 is replaced by other PAT family members (7, 13). More recently, perilipin 5 (Plin5) was found to be highly present on LDs of oxidative tissues including cardiac, skeletal muscle, and liver (14, 15). Ectopic expression of Plin5 increased cellular TG levels and reduced FA oxidation (16) suggesting a role for Plin5 in energy catabolism (16–19). Interestingly, Plin5 colocalized with ATGL and its coactivator comparative gene indentification-58 (CGI-58) (17, 18), two critical players in the first and rate-limiting step of TG catabolism (20, 21). Furthermore, the interaction of Plin5 with ATGL decreased ATGL-mediated lipolysis implying that Plin5 participates in the regulation of ATGL activity (22). Mutations of both ATGL and CGI-58 are causative for the development of neutral lipid storage disease with a different clinical picture: ATGL mutations are exclusively linked to muscle TG accumulation and lethal cardiomyopathy in humans and mice (23, 24), whereas mutated CGI-58 (25) on heart lipid and energy metabolism. We found that Plin5 couples LD FA release to mitochondrial FAO. However, the global deletion of Plin5 provoked a relatively mild phenotype in mice (29), although LDs were virtually absent in cardiac muscle (CM), similar to the lack of LDs in transgenic mice with cardiac-specific ATGL overexpression (30). Thus, it appears feasible that Plin5 primarily shields LDs from uncontrolled TG mobilization but is not critical for ATGL-mediated lipolysis and FA channeling to mitochondria.

The aim of our study was to examine the consequences of cardiac muscle-specific overexpression of perilipin 5 (CM-Plin5) on heart lipid and energy metabolism. We found that Plin5 transgenic mice exhibit severe TG accumulation in CM and that Plin5-coated LDs are resistant to ATGL-mediated TG hydrolysis, suggesting that Plin5 acts as a lipolytic barrier to prevent uncontrolled TG mobilization.

**RESEARCH DESIGN AND METHODS**

**Animals**

Transgenic mouse expressing mouse *Plin5* cDNA under the control of the cardiomyocyte-specific α-myosin heavy chain (MHC) promoter (*Myh6, GenBank accession number U71441*) were generated by cloning full-length mouse *Plin5* cDNA (amplified from CM cDNA using the 5′-ACA GTG CGA CAT GGA GCA GAG AGG TGA AGA CAC-3′ forward and 5′-ACA GTG CGA CTC AAT GAT GAT GAT GAT GAG AGT GCA GCT CTC GGA TCA-3′ reverse primers) in the α-MHC promoter construct (31), kindly provided by J. Robbins as previously described (32). Characterized transgenic mice originated from a B6D2F2 background and were backcrossed four to five times on a C57BL6 background. Littermates were used for phenotyping and mice with CM-Plin5 were hemizygous with respect to the integrated transgene. Animals were housed in a specific pathogen-free facility and maintained on a regular light-dark cycle (14 h light, 10 h dark) with ad libitum access to a standard laboratory chow diet (4.5% fat; sniff Spezialdiäten, Germany) and water. For tissue collection, mice were euthanized by cervical dislocation and excised tissues were immediately snap-frozen. Maintenance, handling, and tissue collection from mice was approved by the Austrian Federal Ministry for Science and Research and by the ethics committee of the University of Graz.

**cDNA cloning and expression of recombinant proteins**

Mouse *Atgl*, HSL (*Lipe*), CGI-58, and G0/G1 switch gene 2 (*GOS2*) were cloned in the pcDNA4/HisMaxC expression vector. Full length mouse *Plin5* cDNA was amplified from CM cDNA (forward primer: 5′-AAG TCA CCA GAG CAG AGT GGT GAA GAC TCA GCC TCT GGG C-3′ and reverse primer: 5′-GA TCC CTC GGA GAT GAA GTC CAG CTC TGG CAT C-3′) and cloned into the pcDNA4/HisMaxC (Invitrogen Life Technologies) expression vector. COS-7 cells were grown in low glucose Dulbecco’s Modified Eagle Medium (GIBCO) supplemented with 10% fetal calf serum, 100 U/ml streptomycin, and 100 IU/ml penicillin at 37°C with 5% CO2 and 95% humidity. Cells were transfected with plasmids encoding respective cDNAs expressing plasmids using Metafectene reagent (Biontex) according to the manufacturer’s instructions.

**Quantitative analysis of mRNA expression levels**

Gene expression analysis was performed by quantitative reverse transcription polymerase chain reaction (RT-qPCR). Total RNA was extracted with the TRizol reagent (Invitrogen) and treated with DNaseI (Invitrogen). For first strand cDNA synthesis, 1 μg of total RNA was reverse transcribed at 37°C for 1 h using random hexamer primer (Applied Biosystems) and Superscript II reverse transcriptase (Invitrogen). Primers used for RT-qPCR were designed to span exon-intron boundaries with an amplicon length of more than 150 bp and a range of specificity. RT-qPCR reactions (20 μl) contained 8 ng of cDNA, 10 μM of each primer, and 10 μl of SYBR Green master mix (Fermentas) and were carried out using the ABIStepOnePlus™ detection system (Applied Biosystems). Relative mRNA levels were quantified using the comparative ΔΔCT method with β-actin as reference gene. The following primer sequences were used for RT-qPCR: β-actin forward, 5′-ACA GTG CGA CAT GGA GCA GAG AGG TGA AGA CAC-3′; reverse, 5′-TCT CCG GAG TCC ATC ACA ATG-3′; murine *Plin5* forward, 5′-AGG GGA CTA GAC AAA TTG G-3′; reverse, 5′-GCT TCT CCG AGC TGC C-3′; *Cpt1b* (carnitine palmitoyltransferase 1β) forward, 5′-GGC ACC TCT TCT GCC TTT AGC-3′; reverse, 5′-TTT GGG TGA AAC ATG CAG AT-3′; *Ppara* (peroxisome proliferator-activated receptor α) forward, 5′-GTA CCA GTA CCG AGT TCA GCC AT-3′; reverse, 5′-CCG CGA AAG AAG CCC TTA CCA-3′; *Acac1* (acyl-CoA oxidase 1) forward, 5′-AGA TTT GGA GAA ATT GCT GCA AAA-3′; reverse, 5′-AGG CCA AAT TTC TCT GGC TCT-3′; *Acadm* (acyl-CoA dehydrogenase, medium chain) forward, 5′-GAT GCA TCA CCC TCG TGT AAC-3′; reverse, 5′-AAG CCC TTT TCC OCT GAA-3′; *Acadl* (acyl-CoA dehydrogenase, long chain) forward, 5′-GTC TGC TGC TGG CAC C-3′; reverse, 5′-AAG CCA AAT TTC TCT GGC TCT-3′; *Acac2* (acyl-CoA dehydrogenase, long chain) forward, 5′-GAT GCA TCA CCC TCG TGT AAC-3′; reverse, 5′-AAG CCC TTT TCC OCT GAA-3′; *Acadl* (acyl-CoA dehydrogenase, long chain) forward,
5’T TTG GGA GAG TGT AAG GA3′, reverse, 5’-ACT TCT CCA GCT TCC TCC CA3′; Acetyl (acyl-CoA dehydrogenase, very long chain) forward, 5’-ACC TGT CCA GGG CCT GAT-3′, reverse, 5’-TGG CCT GGT CAC CCG TAA-3; Pikhkt (pyruvate dehydrogenase kinase, isoenzyme 4) forward, 5’-ATC TAA CAT CAG TAA ACC-3, reverse, 5’-GGA ACG TAC ACA ATG TGG ATT G-3; Pparγ1a (peroxisome proliferator-activated receptor γ coactivator 1α) forward, 5’-CCC TGC CAT TGT TTA GAC-3, reverse, 5’-GGC TGC TGT TCC TTT C-3; Pparγ1b (peroxisome proliferator-activated receptor γ coactivator 1β) forward, 5’-GGG TGC GGG ACT TCC-3, reverse, 5’-CCG ACT TGT TTT TCC CAG ATG-3; Lipf forward, 5’-TCC AGC CAG GAT GCA ACA-3, reverse, 5’-CCA CGT CTC CGA GTC CTC TCT-3; Cat36 (cluster of differentiation 36) forward, 5’-GAA CCA ATT GAA GGA TTA CAT-3; Pparγ1 (peroxisome proliferator-activated receptor γ isoform 1) forward, 5’-AAC AAG ACT CTT TAC TGA AAT TCC A-3, reverse, 5’-CAC AGA GCT GAT TCC GCA GTT G-3.

**Immunoblot analysis**

Equal protein amounts (indicated in the corresponding figure legends) of tissue lysates, organellar preparations, and cell lysates were separated by SDS-PAGE and proteins were transferred onto a polyvinylidene fluoride membrane (Carl Roth, Karlsruhe, Germany). Blotted proteins were visualized using the following primary Abs: anti-ATGL (#21138; Cell Signaling Technology, Boston, MA), anti-Plin5 (#PAB12542; Abnova, Taipei City, Taiwan), anti-CGI-58 (#H00051099-M01; Abnova); anti-GAPDH (#2118; Cell Signaling Technology), anti-carminite palmitoyltransferase (CPT)-1 (#98834; Santa Cruz Biotechnology, Santa Cruz, CA), anti-cytochrome c oxidase IV (COXIV) (#4844; Cell Signaling Technology), and anti-His (#27-471001; GE Healthcare, Waukesha, WI). Specifically bound immunoglobulins were detected in a second reaction using a horseradish peroxidase-conjugated anti-rabbit IgG Ab (Vector Laboratories, Burlingame, CA) or anti-mouse IgG Ab (GE Healthcare). Immunoblots were developed using the ECL Plus Western Blotting Detection System (GE Healthcare). Densitometric analyses were performed using ImageJ software (National Institutes of Health, Bethesda, MD).

**Plasma parameters**

Blood samples were collected by retro-orbital puncture from isoflurane-anesthetized mice. Plasma parameters were analyzed with commercially available kits from Wako, Sigma, and Thermo Fisher Scientific and plasma glucose levels were determined using the Freestyle Freedom Lite® Blood Glucose Monitoring System (Abbott).

**Tissue homogenization and lipid analysis**

Snap-frozen hearts were homogenized in ice-cold lysis buffer A (0.25 M sucrose pH 7.0, 1 mM EDTA, 1 mM DTT, 20 μg/ml leupeptin, 2 μg/ml antipain, 1 μg/ml pepstatin) using an Ultra Turrax Homogenizer (IKA). The homogenates were centrifuged at 20,000 g for 30 min at 4°C and the supernatants were collected. Protein concentrations were determined using the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories GmbH) and lipid extractions were performed according to the method of Folch (33). Aliquots of the organic phase were evaporated and the lipid extracts were resuspended in ice-cold 1% Triton X-100 by brief sonication. TG concentrations were then measured using a colorimetric kit (Infinity TG reagent; Thermo Fisher Scientific).

**TG hydrolase assay**

TG hydrolase assays were performed as previously described (20). For the measurement of TG hydrolase activity cell lysates (1,000 g supernatant) or tissue extracts (10,000 g supernatant) were used. Stimulation of ATGL-mediated TG hydrolysis was performed by addition of purified murine GST-tagged CGI-58 (dissolved in 0.01% NP40) or COS7 cell lysates containing Histagged murine CGI-58 (20). Samples in a total volume of 100 μl buffer A were incubated with 100 μl substrate in a water bath at 37°C for 1 h. The micellar TG substrate contained 330 μM triolein, 3H-triolein as tracer, 45 μM phosphatidylcholine/phosphatidylinositol (PC:PI, 3:1), and was prepared by sonication (Virschow 475; Virtis, Gardiner, NJ).

**Labeling and preparation of LDs**

COS-7 cells were transfected with beta-galactosidase (LacZ)- or Plin5-expression vectors as described before. To promote LD formation, one day posttransfection cells were incubated overnight in medium supplemented with 0.4 mM oleic acid complexed to essentially FA-free BSA in a molar ratio of 3:1 together with 4 mCi 3H-9,10-oleic acid as radioactive tracer. For isolation of LDs, cells were trypsinized, centrifuged, and washed three times with phosphate-buffered saline. Thereafter, cells were suspended in buffer A and disrupted by sonication (Virschow 475; Virtis). Cell lysates were transferred to SW41 tubes, overlaid with buffer B (50 mM potassium phosphate pH 7.4, 100 mM KCl, 1 mM EDTA, 20 μg/ml leupeptin, 2 μg/ml antipain, 1 μg/ml pepstatin), and centrifuged in an SW41 rotor (Beckman, Fullerton, CA) (2 h, 40,000 rpm, 4°C). LDs (visible as a white layer on top of the tube) were collected, transferred to a new tube, and concentrated by centrifugation (20,000 g, 15 min, 4°C) and removal of the underlying fluid. Subsequently, LDs were resuspended in buffer B by brief sonication.

**Determination of TG hydrolase activity using purified LDs as substrate**

LDs, prepared from COS-7 cells, were diluted to 0.05 μmol TG/100 μl (220 cpn/nmol) and 0.5% FA-free BSA in 100 mM potassium phosphate buffer (pH 7.0) was added. After incubation for 1 h, FA release from LDs was determined by extraction and determination of radioactivity essentially as described for the TG hydrolytic assay. LDs from cardiac tissue were isolated as described above and incubated with COS-7 cell lysates containing ATGL, HSL, CGI-58, or LacZ as control. LDs were diluted to a TG concentration of 0.4 μmol/100 μl and incubated in the presence of 0.5% FA-free BSA in 100 mM potassium phosphate buffer (pH 7.0) for 1 h at 37°C. The reaction was terminated by addition of 0.1% Triton X-100 followed by centrifugation at 20,000 g for 30 min. The lower phase was collected and FFAs were determined with a commercial kit (Wako Chemicals).

**Analysis of TG levels of COS-7 cells expressing recombinant proteins**

COS-7 cells were transfected with LacZ, Atgl or Plin5 expression plasmids or cotransfected with both Atgl and Plin5 expression plasmids. To induce LD formation, COS-7 cells were loaded with 0.4 mM oleic acid and 4 μCi 3H-9,10-oleic acid/nmol as tracer overnight (20 h). Total lipids were extracted and separated by thin-layer chromatography using hexane/diethyl ether/acetic acid (70:29:1) as solvent. TG-corresponding bands were excised and radioactivity was measured by liquid scintillation counting. For the analysis of the time-dependent incorporation of 3H-labeled oleic acid into TG, LacZ and Plin5 expressing COS-7 cells were loaded with 0.4 mM oleic acid and 4 μCi 3H-9,10-oleic acid/nmol for time periods of 2, 4, 8, and 16 h. Lipids were extracted and analyzed as described above at the indicated time periods.

**Preparation of mitochondria and determination of CPT-1 activity**

For isolation of mitochondria, cardiac tissues were minced and homogenized with a dounce homogenizer in ice-cold buffer.
C (0.25 M sucrose, 5 mM HEPES pH 7.7, 0.25 mM EDTA, 20 µg/ml leupeptide, 2 µg/ml antipain, 1 µg/ml pepstatin). After centrifugation (1,500 g, 4°C, 15 min), the infranatant was collected and mitochondria were pelleted (11,000 g, 4°C, 20 min) and resuspended in buffer C. CPT-1 activities were measured according to an established protocol (34, 35).

**Tissue LPL activity**

Tissue LPL activity was measured essentially as described in Ref. 36.

**Transmission electron microscopy**

Mice were euthanized at the age of 10 weeks by an overdose of anesthetic (xylazine-ketamine; Sigma) and immediately perfused with 4% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer pH 7.4, for 5 min. CM was dissected using a Zeiss OPI1 surgical microscope (Carl Zeiss). Small tissue fragments were fixed in 2.5% (wt/vol) glutaraldehyde and 2% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer pH 7.4, for 2 h, postfixed in 2% (wt/vol) osmium tetroxide for 2 h at room temperature, dehydrated in graded series of acetone and embedded in a TAAB epoxy resin. Thin sections (70 nm thick) were contrasted with uranyl acetate and lead citrate. Images were taken using an FEI Tecnai G² 20 transmission electron microscope (FEI Eindhoven) with a Gatan UltraScan 1000 charge-coupled device camera. Acceleration voltage was 120 kV.

**Statistical analysis**

Data are presented as mean ± SD. Statistical significance was determined by the Student’s unpaired two-tailed t-test. Group differences were considered significant for *P < 0.05, **P < 0.01, and ***P < 0.001.

**RESULTS**

**Cardiac-specific Plin5 overexpression provokes severe cardiac steatosis**

Cardiac-specific overexpression of Plin5 was achieved by cloning the murine Plin5 cDNA downstream of the α-MHC promoter and microinjection of the transgene DNA construct (Fig. 1A) into the pronucleus of mouse embryos. Measurement of Plin5 mRNA expression levels in CM of two founders showed a 14.8-fold and 50.6-fold increase in cardiac Plin5 mRNA levels of CM-Plin5 transgenic line (Tg)26 and Tg32, respectively, compared with wild type (wt) (Fig. 1B). CM-specific Plin5 overexpression caused massive cardiac steatosis (Fig. 1C, right) and the magnitude of TG accumulation (Fig. 1C, left) correlated with the degree of Plin5 mRNA expression levels in the transgenic lines. We then focused our characterization on Tg32 which is hereafter designated as CM-Plin5. The massive TG accumulation was also reflected by the relative increase of heart weight in relation to body weight of nonfasted and fasted CM-Plin5 mice (1.8- and 1.6-fold, respectively) compared with wt (Fig. 1D). Body weights were unchanged in CM-Plin5 mice compared with wt mice (22.1 ± 1.2 g vs. 21.8 ± 1.1 g in nonfasted mice and 20.8 ± 1.4 g vs. 20.2 ± 1.3 g in fasted mice, respectively). Next, we analyzed Plin5 protein levels in cytosolic, LD, and mitochondrial fractions of CM. Plin5 protein levels were similar in cytosolic preparations (Fig. 1E) of CM from CM-Plin5 and wt mice, respectively. After ultracentrifugation, an LD layer was visible only in CM homogenates of Plin5 transgenic mice which was withdrawn for Western blot analysis. Plin5 protein signals were abundant in these LD fractions of Plin5 transgenic mice (Fig. 1E, outermost panel, right). In contrast, Plin5 protein expression levels were similar in mitochondrial preparations of CM from both genotypes (Fig. 1F). As expected, Plin5 protein levels were unchanged in skeletal muscle (musculus quadriceps) of CM-Plin5 mice (supplementary Fig. 1) implying that expression of the Plin5 cDNA under the control of the α-MHC promoter was CM-specific.

**Plasma parameters of CM-Plin5 mice are virtually unchanged**

Next we examined the impact of cardiac Plin5 overexpression on plasma parameters (Table 1). Plasma lipid and blood glucose levels of CM-Plin5 mice were similar compared with wt while plasma TG levels were moderately reduced (−17%) in nonfasted animals. These data indicate that cardiac Plin5 overexpression does not significantly affect systemic energy homeostasis.

**Impaired ATGL- and HSL-mediated FA release of LD preparations from CM of CM-Plin5 mice**

To examine whether the marked TG accumulation in CM of Plin5 transgenic mice involves changes in cardiac lipolysis, we performed TG hydrolytic assays using an established micellar triolein substrate emulsified with phospholipids. Interestingly, TG hydrolytic activities were markedly increased in CM tissue extracts derived from nonfasted and fasted CM-Plin5 mice (1.4-fold in the nonfasted and 1.8-fold in the fasted state, respectively) compared with wt tissue extracts (Fig. 2A). Addition of the ATGL coactivator CGI-58 significantly increased TG hydrolytic activities in cardiac extracts of both genotypes independent of the nutritional state (Fig. 2B). Notably, the highest differences in TG hydrolytic activities were observed in cardiac tissue extracts of nonfasted CM-Plin5 mice compared with wt mice upon addition of recombinant CGI-58 (1.8-fold). Next, we examined whether the observed changes in cardiac TG hydrolytic activities of CM-Plin5 mice involve differences in ATGL and/or CGI-58 protein expression levels. Western blot analyses revealed a marked increase in ATGL and CGI-58 protein levels in cardiac homogenates of transgenic mice (Fig. 2C). This finding indicates that the increased TG hydrolytic activities measured in CM extracts of transgenic mice (Fig. 2A) are due to augmented protein expression of ATGL and its coactivator CGI-58. Because CM-Plin5 mice exhibited massive TG accumulation in CM, we assumed that the increased in vitro TG hydrolytic activities may not reflect the in vivo situation, where Plin5 is abundantly present on the LD surface. To address this issue, we prepared LDs from CM of transgenic mice and used these LD preparations as a substrate for TG hydrolysis and measured the FA release in the presence of ATGL, ATGL together with CGI-58, HSL, and LacZ as control. Because LDs are in extremely low abundance in CM of wt mice, we used LDs from CM of ATGL-knockout
mice instead. The comparison of the FA release of LD preparations from CM-Plin5 and ATGL-deficient mice is particularly intriguing with regard to the severe TG accumulation in CM of both genotypes. TG hydrolysis of LD preparations of both genotypes was induced by the addition of cell lysates containing ATGL, ATGL together with CGI-58, HSL, and LacZ which served as reference (Fig. 2D). Yet, FA release was in general markedly lower in LD preparations from CM of Plin5 mice upon addition of lysates containing ATGL (−81.4%), ATGL combined with CGI-58 (−63.5%), and HSL (−56.7%) compared with that of cardiac LD preparations from ATGL-deficient mice. These differences in LD TG mobilization upon addition of exogenous lipases are a strong indication that Plin5 could function as a lipolytic barrier and block the access of lipases to the TG substrate of Plin5-enriched LDs.

**Plin5 blocks lipase access to the LD TG moiety but does not specifically inhibit ATGL enzymatic activity**

To confirm our hypothesis that Plin5 functions as a lipolytic barrier to preserve the LD TG pool from unrestricted TG catabolism, we used a cell culture approach...
and examined TG hydrolysis and homeostasis under various conditions. First, we examined the in vitro impact of Plin5 on basal and ATGL-mediated TG hydrolysis using again the artificial 3H-labeled triolein substrate. TG hydrolytic activities were measured in a combination of cell lysates containing LacZ, ATGL, and Plin5. We also included COS-7 cell lysates containing G0S2, a known inhibitory protein of ATGL (37, 38). TG hydrolytic activities substantially increased in cell preparations containing ATGL (up to 11.6-fold compared with the LacZ control) independent of whether ATGL-containing cell lysates were mixed with lysates containing LacZ or Plin5 (Fig. 3A). In contrast, ATGL TG hydrolytic activity was markedly inhibited (−58.5%) by the addition of G0S2 cell lysates. These findings demonstrate that ATGL enzymatic activity per se is not affected by the presence of Plin5 in in vitro assays. To investigate the in vivo impact of Plin5 on ATGL-mediated TG hydrolysis, we cotransfected COS-7 cells with LacZ and ATGL- or Plin5-expressing plasmids, loaded cells with oleic acid and 3H-labeled oleic acid as tracer, and analyzed the incorporation of radioactivity into TG (Fig. 3B). COS-7 cells transfected with ATGL showed reduced incorporation of radioactivity into the TG pool (−33.0%) compared with LacZ, due to increased TG mobilization. In contrast, Plin5-transfected cells showed moderately increased incorporation of radioactivity into cellular TG (1.3-fold) and this effect was unchanged even if these cells were cotransfected with ATGL (1.4-fold). The increased radioactivity present in the TG fraction of Plin5-expressing cells could originate from increased lipogenesis or impaired TG catabolism. To address this question, we loaded LacZ- and Plin5-transfected COS-7 cells again with oleic acid and 3H-labeled oleic acid and measured radioactivity levels in TG at several time points over a period of 16 h. As shown in supplementary Fig. II, the incorporation of radioactivity constantly increased to a similar extent in LacZ- and Plin5-transfected cells indicating that Plin5 is not involved in the lipogenic pathway.

Taken together, these data suggest that Plin5 protects TG from ATGL-mediated hydrolysis in vivo presumably by limiting the access of lipases to TG stores. To validate this hypothesis, we tested whether Plin5-coated LDs are resistant toward in vitro ATGL-mediated TG hydrolysis. We also included HSL in the experiment to address whether Plin5 may represent a general lipolytic barrier or if it is specifically shielding/inhibiting ATGL. Therefore, we incubated LacZ- and Plin5-transfected COS-7 cells with oleic acid and 3H-labeled oleic acid to induce TG synthesis and LD formation. Western blot experiments confirmed that the recombinant proteins were present in the investigated cell lysates and that LDs isolated from Plin5-transfected COS-7 cells contained large amounts of Plin5 (Fig. 3C). Then, in vitro assays were performed using isolated control LDS or LDs coated with Plin5 as substrate after the addition of lysates containing various recombinant proteins. FAs were efficiently released from control LDS (prepared from LacZ-transfected COS-7 cells incubated with 3H-labeled oleic acid) when incubated with an ATGL-containing lysate (7.3-fold), lysates containing ATGL and CGI-58 (70.4-fold), and a lysate containing HSL (40.9-fold) as compared with FA release upon addition of LacZ-containing lysates (Fig. 3D). Notably, FA release from Plin5-coated LDS was markedly reduced compared with that of control LDS when incubated with lysates containing ATGL (−16.6%), ATGL and CGI-58 (−62.4%), or HSL (−65.9%) strongly suggesting that Plin5 represents a lipolytic barrier that hinders lipase access to the TG substrate.

To investigate whether Plin5 interferes with ATGL-mediated TG hydrolysis per se, we performed TG hydrolytic activity assays using again the artificial micellar 3H-labeled triolein substrate. TG hydrolytic activities of lysates containing ATGL or ATGL and CGI-58 by about 50% (Fig. 3E). In contrast, TG hydrolytic activities of lysates containing ATGL or ATGL and CGI-58 were mildly affected upon addition of Plin5-coated LDS, indicating that Plin5 in fact substantially limits the access to the TG substrate.

Moderately decreased FAO gene expression levels and reduced mitochondrial CPT-I activity in CM of CM-Plin5 mice

Because mice lacking ATGL exhibit severe cardiac TG accumulation linked to defective PPARα-activated FAO gene expression, we hypothesized that cardiac Plin5 overexpression may similarly affect FAO gene expression levels. To address this hypothesis, we measured mRNA

|          | Nonfasted            | Fasted             |
|----------|----------------------|--------------------|
|          | wt       | CM-Plin5  | wt       | CM-Plin5  |
| Glucose (mg/dl) | 181.2 ± 21.5 | 168.4 ± 25.3 | 103.2 ± 4.1 | 98.2 ± 9.0 |
| FFA (mmol/l)    | 0.60 ± 0.06 | 0.69 ± 0.12 | 1.32 ± 0.36 | 1.15 ± 0.32 |
| Glycerol (mmol/l) | 0.41 ± 0.08 | 0.40 ± 0.14 | 0.43 ± 0.07 | 0.46 ± 0.10 |
| TG (mg/dl)      | 102.2 ± 13.6 | 81.2 ± 3.4* | 65.6 ± 11.0 | 72.8 ± 11.2 |
| TC (mg/dl)      | 102.6 ± 18.6 | 91.4 ± 7.4  | 92.1 ± 16.5 | 84.0 ± 17.6 |

Various parameters were assayed with a glucometer and commercial kits in plasma samples obtained from nonfasted and fasted 12-week-old female mice (n = 4, *P < 0.05). Comparable values were obtained from male mice (data not shown). TC, total cholesterol.
Fig. 2. Measurement of tissue TG hydrolytic activities and FAs released from cardiac LDs. A: Cardiac TG hydrolase activities were increased in nonfasted and fasted 12-week-old Plin5 transgenic mice compared with the activity of wt tissues (n = 5; **P < 0.01 versus wt; ***P < 0.001 versus nonfasted). B: Addition of recombinant GST-tagged CGI-58 significantly increased TG hydrolytic activities in both genotypes (n = 5; **P < 0.01; ***P < 0.001 versus wt; ***P < 0.001 fasted versus nonfasted). C: Protein expression levels of ATGL and its coactivator CGI-58 were markedly elevated in CM of CM-Plin5 mice compared with wt (12-week-old mice). Analyzed samples contained 30 μg protein. Specific signals for ATGL and CGI-58 are indicated as arrows. GAPDH served as protein loading control. D: FA release of LD preparations isolated from CM of Plin5 transgenic and ATGL-deficient mice when incubated with COS-7 cell lysates containing LacZ, ATGL, ATGL + CGI-58, and HSL. The ATGL- and HSL-mediated FA release was significantly reduced in LD preparations of Plin5 transgenic mice compared with that of LDs from ATGL-deficient mice (n = 3). Data are shown as mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001 versus LDs incubated with LacZ containing lysates.

expression levels of PPARα, PPARγ (and PPARβ/δ) target genes, and peroxisome proliferator-activated receptor γ coactivator (PGC)-1α and PGC-1β in CM of overnight fasted mice (Fig. 4A). Cardiac mRNA expression levels of Ppara (−22%) and several established PPARα and PPARβ/δ target genes including Cpt1b (−32%), Acox1 (−23%), Acat1 (−32%), and Acat2 (−18%) were moderately reduced in CM of CM-Plin5 mice compared with levels found in wt mice. Furthermore, expression levels of Ppargc1a and Ppargc1b were significantly reduced (−52% and −54%, respectively) (Fig. 4A) in CM of CM-Plin5 mice compared with controls. Nonetheless, mRNA levels of Acoxl and Pdhk4 (+55%) were similar, or even increased, in transgenic mice compared with controls (Fig. 4A). To further examine the impact of cardiac Plin5 overexpression on mitochondrial energy metabolism, we measured CPT-1 protein levels (Fig. 4B) and CPT-1 activity (Fig. 4C) in mitochondrial preparations derived from CM-Plin5 transgenic and wt mice, respectively. Mitochondrial CPT-1 protein signals and activity levels were decreased (−28.4% and −28.5%, respectively) in CM of transgenic mice suggesting decreased mitochondrial FA uptake.

Marked divergences in cardiomyocyte morphology and LD size in cardiac tissue of CM-Plin5 mice compared with that of ATGL-deficient mice

The severe TG accumulation in CM of CM-Plin5 and ATGL-deficient mice together with impaired FAO gene expression in both mouse models prompted us to examine cardiomyocyte morphology in WT, CM-Plin5, and ATGL-deficient mice, respectively. Therefore, mice were euthanized with anesthetic and immediately perfused with parafomaldehyde. Thin sections from fixed cardiac tissue were stained with uranylacetate and lead citrate and examined by transmission electron microscopy. At first view, there were obvious differences in cardiomyocyte morphology of all examined genotypes. While LDs were not present in CM sections of wt tissue (Fig. 5A–C), there was a pronounced increase of uniform and hypertrophied LDs which were homogenously dispersed in cardiomyocytes of CM-Plin5 mice (Fig. 5D–F). Notably, ATGL-deficient cardiomyocytes contained LDs of all sizes including giant LDs, and the cellular architecture appeared atrophic (Fig. 5G–I). A higher resolution revealed that cardiac mitochondria of CM-Plin5 mice were tightly attached to LDs (Fig. 5E, F) and the increased number of LDs seemed not to affect cellular integrity as assumed for ATGL-deficient cardiomyocytes. Furthermore, mitochondrial appearance and cristae structure were similar in CM-Plin5 tissue sections compared with WT mice. In contrast, ATGL-deficient cardiomyocytes exhibited changes in mitochondrial shape and cristae structure (see Fig. 5H, I). The marked differences in cardiomyocyte morphology of CM-Plin5 mice compared with ATGL-deficient mice suggest that the cardiac phenotype of CM-Plin5 mice is not as severe as in the hearts of mice lacking ATGL.

Reduced mRNA expression levels of lipoprotein lipase, CD36, and PPARγ in CM of Plin5 transgenic mice

Finally, we examined whether changes in cardiac FA uptake and lipogenesis contribute to TG accumulation in CM of CM-Plin5 mice. Therefore, we measured mRNA expression levels of genes implicated in FA uptake and lipogenesis. LPL and the FA transporter CD36 are critical players in cardiac FA uptake (39) and the expression of both genes is regulated by PPARγ. As shown in Fig. 6, mRNA expression levels of lpl (−51%), cd36 (−33%) and pparg1 (−68%) are significantly reduced in CM of CM-Plin5 mice compared with levels found in wt mice. In contrast, LPL activities were similar in CM of fasted Plin5 transgenic mice compared with wt indicating that the marked increase in cardiac TG levels of CM-Plin5 mice does not involve changes in cardiac FA uptake and lipogenic pathways.
Perilipin 5 acts as a lipolytic barrier

22, 40) suggesting a role for Plin5 in the regulation of cardiac lipolysis. This assumption was also corroborated by the phenotype of Plin5-deficient mice which exhibited an almost complete depletion of cardiac LDs (29).

Here we show that mice with cardiac-specific overexpression of Plin5 showed massive cardiac TG accumulation, similar to the cardiac phenotype of ATGL-deficient
lipoysis on the LD surface, because:

i) ATGL- and HSL-mediated TG catabolism was impaired in Plin5-enriched LDs; ii) ATGL TG hydrolytic activity, as determined in in vitro assays, was not affected by addition of Plin5 mice. This phenotype suggests that ATGL-mediated lipoysis is impaired by Plin5 overexpression and inspired us to carefully examine cardiac TG hydrolysis in this transgenic mouse model. Surprisingly, in in vitro assays, we measured increased TG hydrolytic activities in heart extracts of Plin5 transgenic mice using an artificial triolein substrate (41). This finding was even more challenging with respect to the report that mice with cardiac-specific ATGL overexpression exhibit a severe decline in cardiac TG levels (30) suggesting that results of our in vitro assay may not reflect in vivo cardiac lipolysis of CM-Plin5 mice. In accordance with this assumption, we found that isolated LDs from CM of Plin5 transgenic mice are a less effective substrate for exogenously added ATGL as compared with LDs isolated from CM of ATGL-deficient mice. This reduced ability of ATGL to hydrolyze TG of LDs derived from CM of Plin5 transgenic mice could be a consequence of Plin5 directly inhibiting ATGL enzymatic activity, or from the more general formation of a lipolytic barrier that hinders lipase access to the TG substrate.

Employing recombinant proteins and cell experiments, we examined the direct effect of Plin5 and Plin5-coated LDs on ATGL activity in vitro assays as well as in living cells. We found that Plin5 apparently acts as a general lipolytic barrier on the LD surface, because: i) ATGL- and HSL-mediated TG catabolism was impaired in Plin5-enriched LDs; ii) ATGL TG hydrolytic activity, as determined in in vitro assays, was not affected by addition of Plin5

![Graph A](image)

**Fig. 4.** Measurement of cardiac mRNA levels of established PPARα and PPARβ/δ target genes and mitochondrial CPT-1 activity. A: mRNA expression levels of PPARα and PPARβ/δ target genes, PGC-1α and PGC-1β were determined by RT-qPCR in CM RNA prepared from 12-week-old fasted Plin5 transgenic and wt mice, respectively (n ≥ 5). *P < 0.05 and **P < 0.01 versus wt samples. B: Western blot and densitometric analysis of CPT-1 protein expression levels in mitochondria preparations of 15-week-old fasted mice. COXIV served as loading control (**P < 0.01). Ten micrograms of mitochondrial protein were separated by SDS-PAGE prior to blotting. C: CM-specific Plin5 overexpression significantly decreased CPT-1 activity in mitochondria preparations from CM of CM-Plin5 mice compared with that of controls (n ≥ 5; **P < 0.01 versus WT mice).

![Graph B](image)

![Graph C](image)

**Fig. 5.** Transmission electron microscopy of CM sections revealed marked differences in cardiac morphology of Plin5 transgenic and ATGL-deficient mice. Cardiac tissue was prepared from mice after perfusion with paraformaldehyde. Fixed tissue sections were stained and examined by transmission electron microscopy. Cardiac sections from wt (A–C) showed a typical intermyofilibrillar network and clusters of mitochondria. CM-Plin5 cardiac sections (D–F) showed hypertrophied LDs of similar size homogenously dispersed throughout the cytoplasm which seems not to interfere with the intermyofilibrillar network. Most obviously, LDs were tightly associated with mitochondria. In contrast, cardiac sections obtained from ATGL-deficient mice [ATGL-knockout (ko) (G–I)] showed the accumulation of LDs of varying sizes including giant droplets. Overall, the cardiomyocyte architecture as well as the shape of mitochondria seemed to be markedly hampered by the giant droplets. Scale bars: 1 μm upper panel, 0.5 μm middle panel, 0.2 μm lower panel. m, mitochondria; mf, intermyofilibrillar network.

![Graph D](image)

**Fig. 6.** mRNA expression levels of LPL, CD36, and PPARγ were reduced in CM of Plin5 transgenic mice whereas LPL activities were comparable to that of wt mice. A: mRNA expression levels were measured by RT-qPCR in cardiac tissue RNA derived from 12-week-old Plin5 transgenic and wt mice, respectively (n ≥ 4). **P < 0.01 and ***P < 0.001 versus WT mice. B: For the measurement of cardiac LPL-activities, hearts from overnight-fasted mice were surgically removed and minced in a medium containing heparin. LPL-activity of the supernatant was measured in duplicates. Values are shown as mean ± SD of tissue samples from 4 mice of each genotype.
containing lysates alone; iii) ATGL-mediated TG hydrolysis of an artificial triolein substrate was not affected by the addition of Plin5-coated LDs whereas control LDs competed with the hydrolysis of the artificial substrate; and iv) in living cells, overexpression of Plin5 led to increased TG accumulation even if ATGL was coexpressed. To summarize, these data suggest that Plin5 forms a lipolytic barrier at the LD surface thereby impairing the access of ATGL and HSL to the TG substrate. Whether this Plin5-mediated barrier function mechanistically involves the inhibition of ATGL enzymatic activity per se when Plin5 and ATGL co-localize and interact on the LD surface needs further experimental clarification.

Cardiac steatosis of ATGL-deficient mice was mainly caused by a severe defect in PPARα-activated gene expression and the TG accumulation could be reversed by treatment with a PPARα agonist (32). Interestingly, the mRNA expression levels of selected PPARα and PPARγ target genes which have been implicated in mitochondrial FA uptake and oxidation, were moderately decreased in CM of CM-Plin5 mice when compared with the pronounced defect in PPARα-activated gene expression in CM of ATGL-deficient mice (32). Furthermore, cardiomyocyte morphology strongly differed in CM of Plin5 transgenic mice compared with the cellular architecture of ATGL-deficient cardiomyocytes. Cardiac Plin5 overexpression provoked the accumulation of hypertrophied LDs of similar sizes which were homogenously distributed throughout the cytosol in tight association with mitochondria. In contrast, ATGL-deficient cardiomyocytes appeared atrophic and showed a severe accumulation of heterogenous LDs, including giant droplets which strongly interfered with the myofibrillar architecture and mitochondrial shape. These divergences in cardiac morphology strongly imply that Plin5 overexpression does not severely impair ATGL-mediated TG catabolism and hence may moderately affect cardiac function. In accordance with this assumption, we actually have no evidence that Plin5 overexpression provokes an early lethal heart dysfunction reported for mice lacking ATGL (data not shown).

Ectopic Plin5 expression variably increased TG levels in several cell lines and affected mRNA expression levels of genes implicated in FAO (16, 19). Notably, the induction of Plin5 expression particularly impaired TG mobilization under nonstimulated conditions and this effect could be partially reversed via forskolin-induced protein kinase A stimulation which was paralleled by increased Plin5 phosphorylation (22). Accordingly, stimulation of cardiac lipolysis may lead to Plin5 phosphorylation/modification and a relaxation of the lipolytic barrier. Given that cardiac Plin5 overexpression mainly impairs lipolysis under non-stimulated conditions, a more moderate cardiac phenotype would be predicted compared with the severe cardiac dysfunction of mice globally lacking ATGL. Along that line, we cannot exclude that changes in LD protein composition (42) and/or Plin5 interaction partners including CGI-58 and ATGL in response to increased energy demands possibly affect Plin5 modification and its binding to interaction partners thereby allowing lipase access to the TG substrate. Finally, Plin5 protein concentrations may generally differ among LDs and thus variably affect TG mobilization leading to increased TG accumulation of a distinct LD population thereby not generally impairing TG catabolism. In line with such an assumption, Plin5-coated LDs did not affect the TG hydrolytic activity of the micellar TG substrate.

Plin5 protein is also present on mitochondria where the protein promotes the tight association of mitochondria and LDs thereby spatially connecting LD TG mobilization and FA release to mitochondrial FA uptake and oxidation (15, 17, 43). Notably, virtually every LD present in CM sections of Plin5 transgenic mice was in close proximity to one or more mitochondria strongly supporting the assumption that Plin5 recruits and tightly attaches mitochondria to the LD surface. Whether this observed phenomenon has an adverse or even beneficial impact on TG mobilization and/or mitochondrial function in cardiomyocytes of Plin5 transgenic mice is currently unknown and an important question to be addressed in further studies.

In summary, the present study reveals an important role of Plin5 in cardiac TG homeostasis via the formation of a lipolytic barrier. Accordingly, mutations linked to increased Plin5 expression may also be involved in the development of cardiac steatosis and dysfunction in humans.  

The authors thank B. Juritsch, B. Seisser, and A. Steiner for their excellent technical assistance.

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