G₀/G₁ Switch Gene 2 Regulates Cardiac Lipolysis*

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Background: Lipolysis of cardiac triacylglycerol stores requires the concerted action of lipases and accessory proteins.

Results: Overexpression and knock-out of G₀/G₁ switch gene 2 affects cardiac lipolysis and alters cardiac triacylglycerol levels.

Conclusion: G₀/G₁ switch gene 2 is a regulator of cardiac lipolysis affecting substrate utilization in cardiomyocytes.

Significance: G₀/G₁ switch gene 2 participates in cardiac energy homeostasis.

The anabolism and catabolism of myocardial triacylglycerol (TAG) stores are important processes for normal cardiac function. TAG synthesis detoxifies and stockpiles fatty acids to prevent lipotoxicity, whereas TAG hydrolysis (lipolysis) remodels fatty acids from endogenous storage pools as energy substrates, signaling molecules, or precursors for complex lipids. This study focused on the role of G₀/G₁ switch 2 (G₀S₂) protein, which was previously shown to inhibit the principal TAG hydrolase adipose triglyceride lipase (ATGL), in the regulation of cardiac lipolysis. Using wild-type and mutant mice, we show the following: (i) G₀S₂ is expressed in the heart and regulated by the nutritional status with highest expression levels after re-feeding. (ii) Cardiac-specific overexpression of G₀S₂ inhibits cardiac lipolysis by direct protein-protein interaction with ATGL. This leads to severe cardiac steatosis. The steatotic hearts caused by G₀S₂ overexpression are less prone to fibrotic remodeling or cardiac dysfunction than hearts with a lipolytic defect due to ATGL deficiency. (iii) Conversely to the phenotype of transgenic mice, G₀S₂ deficiency results in a de-repression of cardiac lipolysis and decreased cardiac TAG content. We conclude that G₀S₂ acts as a potent ATGL inhibitor in the heart modulating cardiac substrate utilization by regulating cardiac lipolysis.

The mammalian heart depends on a continuous energy supply for contraction. To guarantee unceasing provision of fuel upon rapidly changing metabolic situations, the heart utilizes multiple carbon sources for ATP production (1, 2). Despite this high metabolic flexibility, the healthy adult heart derives most of its energy from mitochondrial oxidation of fatty acids (FAs), which accounts for 50–70% of cardiac energy supply (2, 3). The heart receives exogenous FAs either from circulating triacylglycerol (TAG)-rich lipoproteins, which are hydrolyzed by lipoprotein lipase at the vascular endothelium, or as albumin-bound “free” FAs (4). Within cardiomyocytes, FAs become activated by coenzyme-A linkage and are subsequently either oxidized or esterified to TAG for transient storage in cytoplasmic lipid droplets (5, 6). The cardiac TAG pool is highly dynamic, and expands or decreases in response to FA delivery or utilization. Accordingly, it represents an important “cell-autonomous” endogenous source of FA supply (3, 7, 8). In addition to its function as energy store, the cardiac TAG pool also serves as transient “buffer” to prevent FA-induced lipotoxicity (9, 10). Cardiac lipotoxicity leading to impaired cardiac function is commonly observed in patients with diabetes, obesity, or neutral lipid storage disease (11–13).

The mobilization of FAs from stored TAGs requires the action of cytosolic lipases. Adipose triglyceride lipase (ATGL) initiates the intracellular catabolism of TAGs by selective hydrolysis of the first ester bond (14, 15). In humans, loss-of-function mutations in the gene coding for ATGL (PNPLA2, MIM*609059) cause a rare genetic disorder called neutral lipid storage disease with myopathy (NLSMD, MIM*610717) (16). Although the clinical manifestation of the disease is highly variable, many patients with NLSMD exhibit severe cardiac steatosis and heart dysfunction that can be lethal if they do not undergo heart transplantation (13, 17, 18). Many features of the NLSMD phenotype have been recapitulated in Atgl⁻/⁻ mice. As in humans, the most drastic consequence of ATGL deficiency in mice again concerns a severe cardiomyopathy that

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¶This abbreviations used are: FA, fatty acid; G₀S₂, G₀/G₁ switch 2; ATGL, adipose triglyceride lipase; CGI-58, comparative gene identification-58; MGL, monoacylglycerol lipase; HSL, hormone-sensitive lipase; TAG, triacylglycerol; NLSMD, neutral lipid storage disease with myopathy; WAT, white adipose tissue; BAT, brown adipose tissue; PPARα, peroxisome proliferator-activated receptor α; RT-qPCR, RT-quantitative PCR; DMSO, dimethyl sulfoxide; LZ, β-galactosidase.
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results in premature death of Atgl−/− mice around 12 weeks after birth (15). The lethal cardiomyopathy is characterized by a metabolic and structural remodeling of the myocardium and defective peroxisome proliferator-activated receptor α (PPARα) signaling leading to impaired mitochondrial and respiratory function (15, 19, 20). Thus, efficient cardiac TAG catabolism is an important metabolic requirement for normal cardiac metabolism and function.

ATGL action is regulated by effector proteins that directly interact with the enzyme and either stimulate or inhibit its enzymatic activity. Comparative gene identification 58 (CGI-58) increases ATGL activity and is essential for efficient TAG turnover in the heart (21–23). Muscle-specific loss of CGI-58 in mice results in cardiac steatosis and cardiomyopathy similar to that observed upon cardiac-specific ATGL deficiency (23). Conversely, G0/G1 switch 2 (G0S2) protein inhibits the TAG hydrolyase activity of ATGL (24). G0S2 was initially identified in mononuclear blood cells as a gene whose expression was induced following a similar strategy as described previously (31).

The genomic sequence of a 2.1-kb DNA fragment encompassing the complete G0s2 genomic sequence was amplified by PCR from genomic murine HM-1 embryonic stem cell DNA using the forward primer 5′-TCA AGC TTC TAG GCA AGC ACT CCA CCA C-3′ and the reverse primer 5′-GGA CTA GGT TGT GTG AGT AGA ACT GTC TTG TGC-3′. The PCR product was digested with HindIII and Spel and ligated into a pBK-CMV plasmid harboring a neomycin resistance gene cassette flanked byloxP recombination sites. The resulting plasmid was digested with XbaI and Spel and the DNA fragment encompassing the selection cassette, and the G0s2 genomic sequence was ligated upstream of a single loxP site into a pBluescript KS (−) vector. Next, a 2.2-kb DNA fragment upstream of the G0s2 genomic sequence was amplified using the forward primer 5′-AGC CTG TCT GGT AGA ATT GGT G-3′ and the reverse primer 5′-GCT CTA GAT GGA GTG CCT GCC TAG TAT GTG-3′, and ligated into a pBluescript KS (−) vector upstream of a floxed neomycin cassette using the Xbal restriction site. Finally, a 4.0-kb DNA fragment downstream of the G0s2 genomic sequence was amplified using the forward primer 5′-TTG ATA TCG GCACAA GAC AGT TCT ACAG CAC-3′ and the reverse primer 5′-CCA TCG ATA CAA GCA GTG TGC CTC TGA AG-3′, and was ligated into the pBluescript KS (−) vector downstream of the single loxP site next to the G0s2 genomic sequence using EcoRV and ClaI. From the resulting plasmid, a 9.5-kb fragment encompassing the G0s2 gene targeting construct was released by digesting with NotI and ClaI, and was ligated into a pUC plasmid harboring two Dta gene cassettes. The vector was linearized by restriction digest with Sall and introduced into HM-1 embryonic stem cells by electroporation. Geneticin (G418)-resistant clones were picked and expanded. Cell clones that underwent homologous recombination were identified by PCR. For Cre-mediated recombination of theloxP sites, ES cell clones were electroporated as described above with pCre-Pac plasmid DNA and cultivated in the presence of 1.5 μg/ml puromycin (Clontech) for 36 h. To identify ES cells harboring the knock-out allele, cells were tested for G418 sensitivity and screened by PCR using specific primer sets. ES cells heterozygous for the knock-out allele were injected into 3.5-day-old C57BL/6J blastocysts, which were transferred into pseudopregnant recipient mice. Offspring with a high degree of coat color chimerism were bred with C57BL/6J mice, and germline transmission of the targeted allele was verified by coat color and PCR using specific primers. Heterozygous mice were backcrossed at least five times with C57BL/6J (i.e. 96.9% C57BL/6J background) prior to any further analysis.

Mice were bred and maintained at regular housing temperatures and a 12-h light/12-h dark cycle. Animals had ad libitum access to water and chow diet (4.5% fat, 34% starch, 5.0% sugar, and 22.0% protein; sniff Spezialdiäten GmbH, Soest, Germany) unless otherwise indicated. Animals were anesthetized with isoflurane (IsoFlo®, Abbott, Abbott Park, IL) and euthanized by cervical dislocation.

RNA Extraction and Northern Blotting Analysis—Animal tissues were surgically removed and snap-frozen in liquid nitrogen. RNA was extracted using the TRIzol® reagent (Invitrogen™, Life Technologies), and Northern blotting analysis was performed as described previously using DNA probes covering...
the coding sequence of the G0s2 and the Atg1 gene, respectively (22).

Analysis of mRNA Expression by RT-qPCR—RT-qPCR was performed as described previously using the StepOnePlus™ real-time PCR system (Applied Biosystems, Life Technologies) and the Maxima™ SYBR Green/ROX PCR reaction mix (Thermo Scientific Fermentas) (23).

The following primer pairs were used: β-actin forward 5′-AGC CAT GTA CGT AGC CAT CCA-3′, β-actin reverse 5′-TCT CCG GAG TCC ATC ACA ATG-3′; Uxt forward 5′-CTC ACA GAG CTC AGC GAC AGC-3′, Uxt reverse 5′-AAA TTC TGC AGG CCT TGT AGT TCT C-3′; Col1a1 forward 5′-CCG CCT CCT GCT CCT CTA-3′, Col1a1 reverse 5′-CCA TTG TTG ATG CTT ACT ACT-3′; Col3a1 forward 5′-CTG TAA CAT GGA AAC TGG GGA AA-3′, Col3a1 reverse 5′-CCA TAG CTG AAG TCA AAA CCA CC-3′; Myh7 forward 5′-GAT GTT TTG GTG CCC GAT GA-3′, Myh7 reverse 5′-TGG CCA ACT TGG GTG T-3′; Nppb forward 5′-GCT TTG TGG GCC ACA AGA TAG-3′, Nppb reverse 5′-GCA GCC AGG AGG AGT TCA TCA-3′; Tgf-β forward 5′-ACT GAG GTT GTA CGG CAG TGG-3′, Tgf-β reverse 5′-GCA GTG AGC GCT GAA TCG A-3′; Mcp-1 forward 5′-GGG TGG AGA GCT ACA AGA GG-3′, Mcp-1 reverse 5′-ATG TCT GGA CCC ATT CT-3′; Tnf-α forward 5′-TGG GAG TAG TAC AGG TAC AAC CC-3′, Tnf-α reverse 5′-CAT CTC CTC AAA ATT CTG GGA ACA-3′; G0s2 forward 5′-TAG TGA AGT ACG TGC TGG GC-3′, G0s2 reverse 5′-GGG TGG CGG CTG TGA AAG GTG-3′; Ppara forward 5′-GTGACCTACCGAGTGTCCAGCAT-3′, Ppara reverse 5′-CCG CAA GAG ACC CCC TTA C-3′; Pparγ1a forward 5′-CCC TGC CAT TGA TAA GAC C-3′, Pparγ1a reverse 5′-TGC TGC TCT TCG TTT TCT C-3′; Acadm forward 5′-GCA ACT GCC CGC AAG TTT T-3′, Acadm reverse 5′-GAT TCC CCT CTT TTG TTG T-3′; Cpt1b forward 5′-GAG AAA ATA CTG GCG ATC TGA-3′, Cpt1b reverse 5′-CTC CTG GGT GTA GCA CAC AT-3′; Cpt1b forward 5′-CGA GGA TCT TCT GGA ACT GC-3′, Cpt1b reverse 5′-GGT CGC TTC TAC GTC TG-3′; Aoxl forward 5′-AGA TTT GTA GAA ATT GCT GCA AAA-3′, Aoxl reverse 5′-ACG CCA CTT CTT TGC TGC TCT C-3′; Pdk4 forward 5′-ATC TAA CAT CGC CAG AAT TAA ACC-3′, Pdk4 reverse 5′-GGA TCT TAC TAC ATG TGG TTG ATG-3′. Relative mRNA levels were quantified according to the ΔΔCt method using β-actin or ubiquitously expressed transcript (Luxt) as references.

Immunoblotting Analysis—Tissues were homogenized in a 40 mmol/liter HEPES buffer, pH 7.8, containing 1% Nonidet P-40, 120 mmol/liter NaCl, 1 mmol/liter EDTA, and protease inhibitor cocktail (Complete™ EDTA-free, Roche Diagnostics) using an Ultra-Turrax™ tissue homogenizer (IKA). Cell debris was removed by centrifugation at 13,000 × g for 1 min, and the infranatant was collected. 8 µl rabbit anti-ATG5 antibody (2138, Cell Signaling) or 15 µl rabbit anti-G0s2 antibody (provided by Sander Kersten) were mixed with 500 µl tissue homogenate and incubated for 2 h at 4 °C on a rotating wheel. Immunoprecipitation was performed by incubating the infranatants with 25 µl of PureProteome™ G-protein coupled magnetic beads (Merck Millipore, Darmstadt, Germany) for 1 h on a rotating wheel. The beads were washed three times with 500 µl of 40 mmol/liter HEPES buffer, pH 7.8, containing 1% Nonidet P-40, 120 mmol/liter NaCl, and 1 mmol/liter EDTA to remove nonspecifically bound proteins. Immunoprecipitates were recovered by incubating the beads for 5 min at 99 °C with a denaturing SDS sample buffer and subjected to SDS-PAGE and immunoblotting as described above. A HRP-coupled rabbit TrueBlot® anti-rabbit IgG antibody was used to minimize detection of immunoglobulin heavy and light chains.

Lipid Analysis—Total acylglycerol content was determined in crude heart homogenates using a commercially available kit (Infinity™ triglycerides kit, Thermo Fisher Scientific). For the specific determination of TAG levels, total lipids were extracted by the method of Folch et al. (32). After extraction, protein pellets were dried and dissolved in 0.3 mol/liter NaOH containing 0.1% SDS, and the protein content was determined using the Pierce™ BCA protein assay kit (Thermo Fisher Scientific) according to the manufacturer’s instructions with bovine serum albumin as standard. Aliquots of the lipid extracts were analyzed by HPLC using an Agilent HP 1100 HPLC system (Agilent Technologies, Santa Clara, CA) equipped with a BETASIL Diol-100 column (150 × 4.6 mm; Thermo Fisher Scientific) and a Dionex evaporative light-scattering detector (Dionex, Sunnyvale, CA). Lipid extracts were separated using a ternary gradient of solvent A (iso-octane/ethyl acetate 99:0.2, v/v), solvent B (acetone/ethyl acetate/acetic acid 20:10:0.006, v/v/v), and solvent C (2-propanol/water/acetic acid/ammonium acetate 85:15:0.05:0.3, v/v/v/v). Total levels of triacylglycerol were determined using trioleoylglycerol (Sigma-Aldrich) as standard.
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Purification of Recombinant CGI-58—Recombinant murine CGI-58 was expressed in Escherichia coli and purified as described previously (33).

TAG Hydrolyase Assay—Tissue samples were homogenized in 0.25 mol/liter sucrose containing 1 mmol/liter EDTA, 20 mg/liter leupeptin, 2 mg/liter antipain, and 1 mg/liter pepstatin using a Dounce homogenizer, and TAG hydrolyase activity was measured as described previously (34).

Cultivation and Infection of H9c2 Cells—Adenovirus expressing murine G0S2 or β-galactosidase (LZ), respectively, was prepared as described previously (34). H9c2 cells were maintained in DMEM containing 4.5 g/liter glucose (InvitrogenTM, Life Technologies), 10% FBS, 100 IU/liter penicillin, and 0.1 mg/liter streptomycin. Cells were differentiated with DMEM containing 1% FBS, antibiotics, and 10 nmol/liter retinoic acid for 8 days. Cells were infected with adenovirus at a multiplicity of infection of 1000. 24 h later, infected cells were used for further experiments.

Measurement of Lipid Turnover in H9c2 Cells—Cells were incubated for 16 h in medium containing 400 μmol/liter oleic acid complexed to bovine serum albumin.3H-labeled oleic acid (Moravek Biochemicals, Brea, CA) was added to yield a final specific activity of ~0.25 mCi/μmol FA. Atglistatin (kindly provided by Rolf Breinbauer, Technical University Graz) was dissolved in DMSO and added to the incubation medium to yield a final concentration of 40 μmol/liter (35). Cellular lipids were extracted with hexane/isopropanol (3:2, v/v) and separated by thin layer chromatography using Silica G and hexane/diethylether/acetic acid (70:30:1, v/v/v) as solvent system. To monitor the release of FA into the cell culture medium, cells were pre-labeled for 16 h and switched to DMEM containing 2% bovine serum albumin (essentially fatty acid-free, Sigma-Aldrich). Aliquots of the medium were withdrawn, and lipids were identified by co-migrating standards, and the associated radioactivity was determined by liquid scintillation counting.

Histology—Sudan III staining, H&E staining, and Sirius Red staining of tissue sections were performed as described previously (36).

Echocardiography—Transthoracic echocardiography was performed in 16–20-week-old male mice that were mildly anesthetized with 0.75% isoflurane as described previously (19).

Statistical Analysis—Results are expressed as means ± S.D. Comparisons between groups were made by unpaired two-tailed Student’s t test. p values of less than 0.05 were considered statistically significant.

Results

Cardiac G0S2 Expression Is Dynamically Regulated by the Feeding Status—Previous studies showed that G0S2 expression is high in liver and adipose tissue and tightly regulated by the feeding status (24, 29, 37). To compare the expression and regulation of cardiac G0S2 with that of other tissues, we first performed Northern blotting analysis of samples obtained from ad libitum fed or fasted C57BL/6J mice. In the ad libitum fed state, G0s2 mRNA expression was highest in white adipose tissue (WAT) and brown adipose tissue (BAT), intermediate in liver, skeletal muscle, heart, lung, and tongue, and low in all other tissues examined (Fig. 1A). Overnight fasting resulted in a marked induction of hepatic G0s2 mRNA expression as compared with the ad libitum fed state (Fig. 1B). Conversely, G0s2 mRNA expression in WAT decreased upon fasting, suggesting contrary regulation of G0S2 expression in liver and WAT. Cardiac G0s2 mRNA levels also decreased upon fasting, indicating a similar nutritional response as in WAT. Next, we determined the expression of cardiac G0S2 during the fasting/re-feeding transition. Re-feeding for 4 and 8 h resulted in a marked induction of cardiac G0S2 mRNA and protein expression as compared with the fasted state (Fig. 1C). G0S2 expression levels after re-feeding were much higher than in the ad libitum fed state, indicating a transient peak of G0S2 expression soon after food ingestion. In contrast, ATGL mRNA and protein expression declined during the transition from the fasted to the ad libitum fed state. Thus, food ingestion suppresses the lipolytic system in cardiomyocytes by decreasing ATGL expression and transiently increasing G0S2 expression.

Heart-specific Overexpression of G0S2 Leads to Severe Cardiac Steatosis—To investigate the consequences of increased G0S2 expression on cardiac TAG metabolism, we generated transgenic mice that overexpress G0S2 under the control of the α-MyHC promoter specifically in cardiac muscle (G0s2Tg; Fig. 2A). Northern blotting analysis confirmed a strong overexpres-
The cardiac steatosis observed in Tg mice was associated with statistically inhibiting ATGL-catalyzed hydrolysis of TAG. As shown in Fig. 4C, G0S2 was co-immunoprecipitated from G0S2Tg heart homogenates with an antibody against ATGL. Similarly, ATGL was co-immunoprecipitated from the same sample with an antibody against G0S2 arguing for a direct interaction between ATGL and G0S2 in G0S2Tg hearts.

To further establish the impact of G0S2 on TAG turnover in myocytes, we overexpressed G0S2 in H9c2 cells by adenoviral gene transfer (Ad-G0S2). After infection, cells were loaded with FAs, and TAG turnover was monitored using radioactive tracers. Overexpression of G0S2 in Ad-G0S2 cells (Fig. 4D) decreased FA release into the medium by ~50% as compared with LZ-infected control cells (Fig. 4E). This defect in TAG hydrolysis was associated with a 3-fold increase in intracellular TAG levels (Fig. 4F). A similar increase in cellular TAG was observed when H9c2 cells were incubated with the ATGL-specific inhibitor Atglistatin (35), whereas incubation of G0S2-infected H9c2 cells with Atglistatin did not result in an additive increase of cellular TAG content (Fig. 4F). Thus, overexpression of G0S2 in cardiomyocytes elevates TAG levels by specifically inhibiting ATGL-catalyzed hydrolysis of TAG.

Comparison of the Myocardial Phenotype of G0S2Tg and Atgl−/− Mice—Previous studies suggested that defective cardiac lipolysis in ATGL-deficient mice results in a fibrotic and inflammatory remodeling of the myocardium, ultimately leading to cardiomyopathy (15, 20). To elucidate whether inhibition of ATGL by G0S2 results in a similar cardiac phenotype, we compared various cardiac parameters of the two mouse lines. ATGL deficiency caused a 2.6-fold higher cardiac TAG mass as compared with inhibition of ATGL by G0S2 in G0S2Tg mice (Fig. 5A). The myocardial structure was also different. Atgl−/− hearts exhibited a dramatic vacuolization of the myocardium, which was more pronounced than in G0S2Tg and WT mice (Fig. 5B). Moreover, Sirius Red staining of heart tissue sections revealed severe interstitial cardiac fibrosis in Atgl−/− mice that was not observed in age-matched G0S2Tg or WT animals. In

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FIGURE 2. Generation of mice with heart-specific overexpression of G0S2 (G0s2Tg). A, scheme of the transgenic construct used to generate G0S2Tg. The coding sequence (cds) and the 3′-UTR of the G0S2 gene were placed downstream of the α-MyHC promoter and upstream of a noncoding sequence of human HBB. B, Northern blotting analysis of G0s2 mRNA expression in multiple tissues of WT (−) and G0s2Tg (+) mice (Tg). Tissue samples of three mice were pooled prior to RNA extraction. C, quantification of cardiac G0s2 mRNA levels by RT-qPCR. Values are expressed as relative mRNA levels and are normalized to Actb. Data are presented as mean ± S.D. (n = 4). Statistical significance was determined using Student’s unpaired t test. *, p < 0.05. D, Northern blotting analysis of cardiac G0s2 mRNA levels in WT and two independent G0S2Tg founder lines (Tg03 and Tg58). E, immunoblotting analysis of cardiac G0S2 protein levels in WT, Tg03, and Tg58. β-Tubulin was used as a loading control. All samples were obtained from 16–20-week-old male mice fed ad libitum.

FIGURE 3. Morphology and neutral lipid content of G0S2Tg hearts. A, upper panel, representative image of isolated hearts obtained from WT and Tg58 mice. Lower panel, Sudan III staining of cardiac cryosections. B, acylglycerol content of cardiac homogenates obtained from WT, Tg58, and Tg03 mice. Total acylglycerols were determined enzymatically using a commercially available kit and are expressed as μg of TAG. C, heart wet weight of WT and Tg58 mice. Data are presented as mean ± S.D. (n = 4–6). Statistical significance was determined using Student’s unpaired t test. *, p < 0.05. All samples were obtained from 16-week-old male mice fed ad libitum.

The cardiac steatosis observed in G0S2Tg mice resembled the cardiac phenotype of ATGL- or CGI-58-deficient mice (15, 23). Therefore, we assessed whether overexpression of G0S2 affects cardiac lipolysis. ATGL and CGI-58 protein concentrations were higher in G0S2Tg than in WT hearts, whereas hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL) were similarly expressed in both genotypes (Fig. 4A). Despite increased lipase expression, TAG hydrolyase activities were 35% lower in G0S2Tg than in WT heart lysates. The addition of purified CGI-58, which specifically stimulates ATGL activity, increased the TAG hydrolase activity in WT heart lysates in a dose-dependent manner up to 40% but failed to increase the TAG hydrolase activity in G0S2Tg heart homogenates (Fig. 4B). To confirm that G0S2 directly interacts with ATGL, co-immunoprecipitation experiments were performed. As shown in Fig. 4C, G0S2 was co-immunoprecipitated from G0S2Tg heart homogenates with an antibody against ATGL.

Heart-size and a yellowish color (Fig. 3A). Cardiac steatosis in G0S2Tg mice (15, 23). Therefore, we assessed whether overexpression of G0S2 affects cardiac lipolysis. ATGL and CGI-58 protein concentrations were higher in G0S2Tg than in WT hearts, whereas hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL) were similarly expressed in both genotypes (Fig. 4A). Despite increased lipase expression, TAG hydrolyase activities were 35% lower in G0S2Tg than in WT heart lysates. The addition of purified CGI-58, which specifically stimulates ATGL activity, increased the TAG hydrolase activity in WT heart lysates in a dose-dependent manner up to 40% but failed to increase the TAG hydrolase activity in G0S2Tg heart homogenates (Fig. 4B). To confirm that G0S2 directly interacts with ATGL, co-immunoprecipitation experiments were performed. As shown in Fig. 4C, G0S2 was co-immunoprecipitated from G0S2Tg heart homogenates with an antibody against ATGL. Similarly, ATGL was co-immunoprecipitated from the same sample with an antibody against G0S2 arguing for a direct interaction between ATGL and G0S2 in G0S2Tg hearts.

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FIGURE 4. Effects of G0S2 overexpression on cardiac lipolysis. A, immunoblotting analysis of ATGL, HSL, MGL, and CGI-58 protein levels in hearts obtained from WT and G0s2Tg mice. α-Tubulin was used as loading control. B, TAG hydrolysis activities of heart homogenates obtained from 16-week-old male WT and G0s2Tg mice (n = 3). Protein samples were mixed with increasing amounts of purified recombinant CGI-58 and incubated with a 3H-labeled TAG substrate. C, co-immunoprecipitation of ATGL and G0S2 in G0s2Tg hearts. ATGL and G0S2 were immunoprecipitated (IP) using specific antibodies and detected by immunoblotting. Rabbit immunoglobulin was used as a negative control. D, overexpression of G0S2 in H9c2 myocytes. Cells were infected with an adenovirus encoding for His6 (6xHis)-tagged G0S2 (Ad-G0S2) or β-galactosidase (Ad-LZ). G0S2 was detected by immunoblotting using an antibody against the N-terminal His6 tag. E and F, the release of 3H-labeled FAs into the medium (E) and the incorporation of 3H-labeled FAs into intracellular TAG (F) were monitored after infecting cells with Ad-G0S2 or Ad-LZ using [3H]oleic acid as tracer (n = 3). Cells were treated with either DMSO or 40 μM Atglistatin. Lipids were extracted with organic solvents and separated by thin-layer chromatography, and radioactivity was quantified by liquid scintillation counting. Data are presented as mean ± S.D. Statistical significance was determined using Student’s unpaired t test. *, p < 0.05.
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G0S2 has been shown to inhibit ATGL-mediated lipolysis in liver and adipose tissue (24, 29). However, its role in cardiac TAG metabolism remained elusive. Here we show that cardiac muscle expresses considerable amounts of G0S2 and that cardiac G0S2 expression is under tight nutritional control. G0S2 mRNA and protein levels are lower in fasted than in ad libitum fed mice. This nutritional response in cardiac muscle resembles G0S2 expression in adipose tissue. Conversely, hepatic G0S2 mRNA levels increase upon fasting, indicating opposing feeding/fasting regulation of lipolysis in hepatic and non-hepatic tissues. Notably, cardiac G0S2 expression peaks between 4 and 8 h after re-feeding, greatly exceeding the levels observed in ad libitum fed and fasted conditions. The regulatory mechanism underlying G0S2 induction during re-feeding remains to be elucidated, but it seems plausible that elevated plasma insulin or glucose concentrations contribute to this response. Insulin has previously been shown to potently induce G0S2 expression in adipocytes, and the G0S2 promoter contains a carbohydrate response element known to mediate the effect of glucose on G0s2 expression (24, 38). However, most of these studies on the regulation of G0s2 expression were performed in vitro and require in vivo confirmation. Taken together, the expression profile of cardiac G0S2 during fasting/refeeding suggests that it

discussion

The myocardial TAG pool is a dynamic, short-term FA store preventing lipotoxicity at times when FAs delivery to the heart exceeds FA oxidation rates. Conversely, it can provide FAs as energy substrates when exogenous FA supply diminishes (3, 7, 8). As a consequence, imbalanced cardiac TAG metabolism is expected to impact both cardiac energy metabolism and cardiac performance. Transgenic mouse models overexpressing or lacking members of the lipolytic machinery in cardiomyocytes exhibit severe phenotypes. For example, deficiency of ATGL or its co-activator CGI-58 results in dramatic alterations in cardiac TAG homeostasis and cardiac performance, providing compelling evidence that cardiac lipolysis is essential for normal heart function (15, 20, 23).

Table 1: Cardiac function in G0s2Tg mice

| Parameter                  | WT         | G0s2Tg    |
|----------------------------|------------|-----------|
| IVSd (mm)                  | 0.82 ± 0.07| 1.19 ± 0.03*|
| LVPWd (mm)                 | 0.80 ± 0.04| 0.89 ± 0.02*|
| LV mass/BW (mg/g)          | 4.63 ± 0.45| 6.06 ± 0.75*|
| LVIdDm (mm)                | 4.04 ± 0.36| 4.21 ± 0.18|
| LVIdDs (mm)                | 2.52 ± 0.47| 2.81 ± 0.16|
| LVF5 (%)                   | 38.0 ± 6.5 | 33.1 ± 5.2 |

Statistical significance was determined using Student’s unpaired t-test. *, p < 0.05.
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participates in the instant down-regulation of lipolysis in cardiomyocytes upon food intake. This may prevent cardiac “lipotoxicity” at times of postprandial substrate overload.

To assess whether overexpression or absence of G0S2 affects cardiac lipid metabolism and function, we characterized “extreme” G0s2 genotypes, i.e. heart-specific transgenic as well as global knock-out mouse lines. Consistent with a functional role as ATGL inhibitor, heart-specific overexpression of G0S2 resulted in massive cardiac steatosis. The extent of TAG accumulation resembled the one observed in mouse models lacking G0S2 as ATGL inhibitor, heart-specific overexpression of G0S2 or CGI-58 (15, 23). The increase in cardiac TAG stores resulted in a much more benign phenotype.

severe alterations of cardiac TAG metabolism, loss of G0S2 overexpression hearts, confirming previous findings of an interaction between the enzyme and its inhibitor (24, 29). Finally, the findings in mice were recapitulated in H9c2 myocytes, which displayed decreased TAG turnover in response to G0S2 overexpression.

Although overexpression of G0S2 in the heart of mice caused severe alterations of cardiac TAG metabolism, loss of G0S2 resulted in a much more benign phenotype. G0s2-/- mice exhibited increased cardiac TAG hydrolase activity and decreased cardiac TAG content. Notably, the difference between G0s2-/- and WT mice was more pronounced in the re-fed than in the fasted period. This finding is consistent with the concept that the induction of cardiac G0S2 expression during the postprandial period represses cardiac lipolysis. This in turn may contribute to a substrate utilization switch from lipid droplet-associated FAs to exogenous FAs or glucose when excessive nutrients become available during the postprandial phase. Consistent with this conclusion, loss of G0S2 in the liver leads to unrestrained lipolysis of hepatic TAG stores and increases hepatic FA utilization (29). Alternatively, it is possible that the lack of G0S2 in WAT, BAT, or other tissues of G0s2-/- mice provokes changes in cardiac TAG levels. Clarification of the tissue-specific contribution of G0S2 to cardiac TAG metabolism will require the characterization of conditional knock-out mice lacking G0S2 specifically in cardiomyocytes. However, our finding that the differences in cardiac TAG levels are highest in re-fed G0s2-/- as compared with WT mice when WAT lipolysis is shut down in both genotypes argues for a cardiac-specific action of G0S2.

Recent studies closely linked defects in cardiac lipolysis and the resultant accumulation of myocardial TAG to an impairment of cardiac function and a decrease in life span. For example, global or cardiomyocyte-specific loss of ATGL or muscle-specific loss of its co-activator CGI-58 triggers a metabolic and structural remodeling of the myocardium, ultimately resulting in severe cardiomyopathy (15, 20, 23). In contrast, G0s2Tg mice developed essentially no signs of pathologic myocardial remodeling or cardiac dysfunction as reflected by a similar expression of genes commonly associated with cardiac hypertrophy, inflammation, and cardiac stress in G0s2Tg as compared with WT hearts. Importantly, cardiac fibrosis, a hallmark of the myocardial remodeling process in Agtl-/- mice, was not observed in G0s2Tg mice. Extensive fibrotic remodeling is associated with increased cardiac stiffness and decreased electro-
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chemical coupling. Consequently, the lack of cardiac fibrosis in G0s2Tg mice provides a reasonable framework underlying the preserved left ventricular function, which was observed in these animals. It is noteworthy that G0s2Tg hearts exhibited a lower degree of steatosis than Atgl−/− mice, suggesting different rates of cardiac TAG turnover in both mouse models. A reasonable explanation might be that residual ATGL activity permits a minimal TAG turnover in G0s2Tg hearts but not in Atgl−/−.

Taken together, our data identify G0S2 as a potent modulator of lipolysis in cardiomyocytes. Its transient up-regulation following re-feeding suggests that G0S2 inhibits FA release from endogenous lipid stores. Accordingly, we conclude that G0S2 modulates cardiac fuel selection in response to different nutritional, (patho)physiological, or developmental conditions.

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