A Murine Model of Isolated Cardiac Steatosis Leads to Cardiomyopathy

Denis J. Glenn, Feng Wang, Minobu Nishimoto, Michelle C. Cruz, Yoshikazu Uchida, Walter M. Holleran, Yan Zhang, Yerem Yeghiazarians, David G. Gardner

See Editorial Commentary, pp 148–150

Abstract—Lipid accumulation in the heart is associated with obesity and diabetes mellitus and may play an important role in the pathogenesis of heart failure seen in this patient population. Stored triglycerides are synthesized by the enzyme diacylglycerol acyl transferase (DGAT). We hypothesized that forced expression of DGAT1 in the cardiac myocyte would result in increased lipid accumulation and heart dysfunction. A cardiac myocyte–selective DGAT1 transgenic mouse was created and demonstrated increased lipid accumulation in the absence of hyperglycemia, plasma dyslipidemia or differences in body weight. Over time, expression of DGAT1 in the heart resulted in the development of a significant cardiomyopathy. Echocardiography revealed diastolic dysfunction with increased early mitral inflow velocity to late mitral inflow velocity ratio and decreased deceleration time, suggesting a restrictive pattern in the transgenic mice. Moderate systolic dysfunction was also seen at 52 weeks. Histological analysis showed increased cardiac fibrosis and increased expression of procollagen type 1A, matrix metalloproteinase 2, and tissue inhibitor of matrix metalloproteinase 2 in the transgenic mice. Mitochondrial biogenesis was reduced in the transgenic hearts, as was expression of cytochrome c oxidase 1 and cytochrome c. Expression of key transcription factors important in the regulation of mitochondrial biogenesis were reduced. These findings suggest that triglyceride accumulation, in the absence of systemic metabolic derangement, results in cardiac dysfunction and decreased mitochondrial biogenesis. (Hypertension. 2011;57:216-222.) ● Online Data Supplement

Key Words: diacylglycerol acyltransferase ▪ lipoxicity ▪ cardiomyopathy ▪ cardiac fibrosis ▪ mitochondrial biogenesis

Obesity is associated with the development of cardiomyopathy and in some cases overt heart failure that seems to be independent of comorbid conditions such as diabetes mellitus, hypertension, coronary artery disease, and dyslipidemia. Remarkably, obesity-related cardiomyopathy has been estimated to cause up to 11% of all cases of heart failure in men and up to 14% in women.¹ A frequent finding in the hearts of obese and diabetic patients is the presence of lipid inclusions within myocardial cells, a condition referred to as cardiac steatosis.²–⁴ Some have suggested that this steatosis is toxic to the cardiac myocyte⁵ while others have argued that it may serve a protective function to neutralize the effects of fatty acids and their metabolites⁶ through esterification to neutral lipids.

In rodents, high-fat diet has been shown to result in both increased deposition of lipid within myocytes and cardiac dysfunction.⁷,⁸ Myocardial lipid accumulation has also been observed in genetic models of obesity and insulin resistance.⁹–¹¹ Studies carried out using transgenic mice or mice with targeted gene deletions that selectively promote fatty acid uptake (eg, overexpression of acyl CoA synthase [ACS],¹² fatty acid binding protein,¹³ or lipoprotein lipase¹⁴) or that prevent lipid turnover (eg, deletion of the adipose triglyceride lipase gene¹⁵) each demonstrate increased myocyte lipid deposition and cardiac dysfunction. Collectively, these findings support the notion that increased lipid or fatty acid accumulation within the myocyte leads to structural and mechanical changes in the heart that compromise pump function.

Excess lipid in the heart is stored in droplets as neutral triglyceride,¹⁶ which is synthesized from fatty-CoA and diacylglycerol (DAG) in a reaction that is catalyzed by diacylglycerol acyl transferase (DGAT).¹⁷ Two gene products, DGAT1 and DGAT2, have been identified,¹⁸,¹⁹ and tissue-specific transgenic expression of either DGAT1 or DGAT2 results in enhanced lipid accumulation.²⁰–²³ To explore the effect of intracellular triglyceride on cardiac structure and function, we generated a murine model of cardiac steatosis by overexpressing DGAT1 selectively...
in the cardiac myocyte. This mouse displays both myocyte steatosis and progressive deterioration of myocardial structure and function over time.

Methods

An expanded Methods section is available in the online Supplement at http://hyper.ahajournals.org.

Results

DGAT1 Expression in the Heart

Expression of DGAT1 in rat neonatal cardiomyocytes resulted in lipid accumulation and activation of a B-type natriuretic peptide (BNP)-luciferase reporter gene, implying that enhanced DGAT1-mediated triglyceride synthesis and storage is associated with activation of the hypertrophic gene program (Figure S1 in the online Supplement). To extend these findings to an in vivo model, we created a transgenic mouse that selectively expresses DGAT1 in the cardiac myocyte. A line which showed moderate expression of the transgene was selected for further analysis (Figure S2). Expression of DGAT1 was quantified in the transgenic hearts and compared to nontransgenic (NTg) littermates (mice aged 12 weeks) by Q-PCR and Western Blot (Figure 1A and 1B). The latter demonstrated an approximately 2.5 fold increase in cardiac DGAT1 expression versus NTg controls. Selectivity of transgene expression was confirmed by Western blot using an antibody to the FLAG epitope. (Figure S2).

DGAT1-mediated lipid accumulation in mice 12 weeks of age was demonstrated by enhanced oil red O staining in mice subjected to an overnight fast (Figure 1C). Triglycerides were quantified and the MHC-DGAT1 Tg hearts demonstrated a 1.8-fold increase over NTg littermates (Figure 1D). Cardiac free fatty acid and DAG levels were quantified in a similar fashion; however, there were no significant differences in these levels (Figure 1E and 1F) in extracts from NTg versus DGAT1 Tg hearts. The serum lipid profile, fasting blood glucose, and the response to glucose or insulin challenge were unaltered in the 12-week-old MHC-DGAT1 Tg mice (Figure S3). Levels of phospho-Akt, a marker of insulin sensitivity in heart, were comparable in NTg versus MHC-DGAT1 Tg mice at baseline and following an insulin challenge (Figure S3). Collectively, these results suggest that systemic lipid and glucose homeostasis and cardiac insulin sensitivity are unchanged by the enhanced expression of DGAT1 in the heart.

DGAT1 Overexpression Is Associated With the Development of a Cardiomyopathy and Activation of the Hypertrophic Gene Program

DGAT1 Tg mice at 52 weeks of age demonstrated cardiac wall thickening and chamber dilation (Figure 2A and the Table). A subset of DGAT1 Tg mice aged 52 to 60 weeks displayed a gross increase in heart size and marked wall thinning and chamber dilation in H&E stained cross sections compared to littermate controls (Figure 2A). These mice demonstrated overt signs of heart failure (ie, piloerection, tachypnea, lethargy and ascites). Gravimetric analysis demonstrated no significant increase in biventricular weight, normalized for total body weight or tibial length, in MHC-DGAT1 Tg versus NTg mice suggesting wall thickening is limited in scope or transient as the hearts progress to dilated cardiomyopathy (Figure S4). Individual

Figure 1. A cardiac myocyte selective MHC-DGAT1 Tg mouse. A, Q-PCR analysis of DGAT1 mRNA expression in heart and liver normalized for GAPDH. N=4 each group. B, Western blot analysis of DGAT1 and GAPDH expression. Representative blot and pooled data are shown, N=4. C, Photomicrographs of frozen ventricular sections stained with ORO, where red stain indicates lipid accumulation, from 16 hour fasted NTg or DGAT Tg hearts. Original magnification ×400. Quantification of extracted cardiac (D) triglycerides (TG), (E) free fatty acid (FFA), (F) and diacylglycerol (DAG) in NTg and MHC-DGAT1 Tg heart tissue extracts. N=5–6 each group. Values normalized for wet weight heart tissue (mg). *P<0.05, ***P<0.001. NTg and MHC-DGAT1 Tg mice aged 12 weeks.
myocyte cross sectional area was quantified in cardiac sections stained with Wheat Germ Agglutinin-Fluorescein Iso-thiocyanate (FITC-WGA) (Figure 2B). Compared to littermate controls the MHC-DGAT1 Tg hearts showed increased myocyte cross sectional area at 12 and 52 weeks compatible with hypertrophy of the individual myocytes (Figure 2C). Expression of atrial natriuretic peptide (ANP), BNP, and αSA, was enhanced in the MHC-DGAT1 Tg mouse hearts at 12 weeks of age (Figure 2D). Similar results were found at 32 and 52 weeks of age (data not shown).

Cardiac Function Is Impaired in the MHC-DGAT1 Tg Mouse

Echocardiography was used to examine function in the MHC-DGAT1 Tg versus NTg mice (the Table and Figure S5). In mice aged 52 weeks (prior to the onset of external signs of heart failure, as described above) end diastolic (EDV) and systolic volumes (ESV) increased in both groups at 52 weeks, albeit to a larger degree in the transgenic mice. This increase in volume and presumably the accompanying increase in wall stress likely contributed to the heart dysfunction seen in the transgenic animals. There was a significant decrease in left ventricular ejection fraction (LVEF) in the MHC-DGAT1 Tg mice compared to NTg littermates.

Diastolic function was reduced at 12 weeks and continued to show impairment at 32 and 52 weeks in the MHC-DGAT1 Tg mice compared to the NTg mice. At each time point, MHC-DGAT1 Tg mice demonstrated a significant increase in the ratio of early mitral inflow filling velocity to late filling velocity (E/A ratio) and decreased deceleration time (DT), suggesting a restrictive pattern of diastolic dysfunction.

MHC-DGAT1 Hearts Demonstrate Increased Cardiac Fibrosis

Masson trichrome stained sections demonstrated cardiac fibrosis in the MHC-DGAT1 Tg mouse compared to NTg controls at 12 and 52 weeks of age (Figure 3A). Quantification of collagen volume showed a significant increase in the 12- and 52-week-old transgenic mice (Figure 3B). Procollagen type I was increased in left ventricular tissue from 12-week old MHC-DGAT1 Tg mice (Figure 3C). The lower migrating band (band B) seen on the procollagen type I immunoblot was preferentially increased and may represent a processed form of procollagen which accompanies increased collagen biosynthesis.

As shown in Figure 3D, expression of matrix metalloproteinase (MMP2) and tissue inhibitor of matrix metalloproteinase (TIMP2) increased significantly in the hearts of 32-week-old MHC-DGAT1 Tg versus NTg mice. These results support the hypothesis that these transgenic hearts are actively remodeling as a result of DGAT1 overexpression.

MMP9 expression was not different in MHC-DGAT1 Tg versus NTg hearts.

Mitochondrial Content Is Reduced in MHC-DGAT1 Tg Hearts

Mitochondrial biogenesis and function are thought to be negatively impacted by obesity and type 2 diabetes mellitus and mitochondrial dysfunction has been linked to cardiac dysfunc-
cytochrome c was reduced in the transgenic versus NTg hearts (Figure 4D).

Mitochondrial biogenesis and function are regulated by several key transcription factors, including nuclear respiration factor 1 and 2 ( NRF1 and NRF2) and mitochondrial transcription factor A (Tfam).33 We examined the levels of both NRF1 and Tfam by Q-PCR and found a significant decrease in each gene product in the 12-week-old MHC-DGAT1 Tg (versus NTg) hearts (Figure 4E). In addition, expression of both peroxisome proliferator-activated receptor (PPAR)α34 and PPARδ,31 which have been shown to be important in supporting mitochondrial function and biogenesis in the heart, were reduced in the transgenic hearts. The transcriptional cofactor PPARγ coactivator 1 (PGC1α) has been shown to be important in cardiac function.35,36 It interacts with members of the PPAR family, as well as NRF1 and NRF2, which, as noted above, serve to control mitochondrial biogenesis and function. Interestingly, we saw a reduction of PGC1α mRNA (Figure 4E) and protein (Figure 4F) levels in the MHC-DGAT1 Tg hearts compared to controls. Thus, overexpression of DGAT1 and subsequent lipid accumulation is associated with a reduction in mitochondrial biogenesis that appears to be linked to a reduction in expression of transcription factors that control this process.

**Discussion**

We have shown that transgenic expression of DGAT1 results in cardiac dysfunction in the absence of obesity, elevations in plasma lipid levels, or insulin resistance. These results support the hypothesis that cardiac steatosis has detrimental effects on myocyte function independent of those effects that accrue from excessive generalized adiposity or elevated plasma lipid levels.

Our results are seemingly in contrast with previous studies of DGAT1 and lipid accumulation. In studies of isolated fibroblasts from DGAT1-null mice, treatment with the fatty acid olate resulted in increased cell death, suggesting that the inability to esterify fatty acids into neutral triglycerides efficiently potentiates the ability of the former to promote cellular dysfunction.37 In a recent study, Liu et al.38 independently demonstrated that cardiac transgenic expression of DGAT1 results in triglyceride accumulation. In contrast to our findings, cardiac function in their transgenic model was unaffected. They go on to show that in a double transgenic model, in which both DGAT1 and ACS are coexpressed, DGAT1 expression appears to be cardioprotective. In this model, DGAT1 expression improves cardiac function and results in reduced diacylglycerol and ceramide content, as compared to the single ACS transgenic mouse.

These seemingly disparate results may be explained, in part, by the time course of the observed responses. The effect of DGAT1 overexpression on cardiac function in the Liu et al study was assessed in mice aged 3 to 4 months. The MHC-DGAT1 Tg mice in our study demonstrate modest but significant cardiac phenotypic changes at 12 weeks of age but required a year to develop severe cardiomyopathy. One might interpret these data, in aggregate, as suggesting that DGAT1-dependent triglyceride synthesis can be cardio-protective in the face of acute or subacute fatty acid overload. In this model, enhanced expression

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### Table. Echocardiographic Measurements at 12, 32, and 52 Weeks

<table>
<thead>
<tr>
<th>Measurement</th>
<th>NTg</th>
<th>DGAT1 Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 weeks</td>
<td>N=7</td>
<td>N=5</td>
</tr>
<tr>
<td>ESV (μL)</td>
<td>34.7±2.8</td>
<td>35.0±3.3</td>
</tr>
<tr>
<td>EDV (μL)</td>
<td>70.89±3.64</td>
<td>69.78±5.36</td>
</tr>
<tr>
<td>IVSW (mm)</td>
<td>0.85±0.06</td>
<td>0.99±0.49</td>
</tr>
<tr>
<td>LVFW (mm)</td>
<td>0.80±0.05</td>
<td>0.87±0.05*</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>51.1±1.8</td>
<td>49.9±1.7</td>
</tr>
<tr>
<td>E (m/sec)</td>
<td>0.549±0.108</td>
<td>0.738±0.160</td>
</tr>
<tr>
<td>A (m/sec)</td>
<td>0.418±0.121</td>
<td>0.384±0.130</td>
</tr>
<tr>
<td>E/A</td>
<td>1.35±0.19</td>
<td>2.11±0.87*</td>
</tr>
<tr>
<td>DT (ms)</td>
<td>30.2±3.03</td>
<td>23.4±2.61†</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>446±47</td>
<td>449±66</td>
</tr>
</tbody>
</table>

| 32 weeks    | N=5 | N=7      |
| ESV (μL)    | 34.4±7.1§ | 37.5±10.1 |
| EDV (μL)    | 71.09±9.20§ | 74.13±12.50§ |
| IVSW (mm)   | 0.87±0.08 | 0.99±0.07† |
| LVFW (mm)   | 0.83±0.03 | 0.89±0.06* |
| LVEF (%)    | 51.9±3.5 | 48.1±5.0 |
| E (m/sec)   | 0.631±0.067 | 0.695±0.172* |
| A (m/sec)   | 0.442±0.041 | 0.338±0.123 |
| E/A         | 1.43±0.14 | 2.24±0.88* |
| DT (ms)     | 35.26±5.83 | 25.10±2.3† |
| Heart rate (bpm) | 452±40 | 457±45 |

| 52 weeks    | N=6 | N=4      |
| ESV (μL)    | 40.96±4.55| 47.1±4.77* |
| EDV (μL)    | 81.77±5.59§ | 87.99±4.19*§ |
| IVSW (mm)   | 0.94±0.02 | 1.02±0.03† |
| LVFW (mm)   | 0.89±0.05 | 0.99±0.02† |
| LVEF (%)    | 50.2±2.9 | 46.5±2.8§ |
| E (m/sec)   | 0.621±0.073 | 0.637±0.078 |
| A (m/sec)   | 0.493±0.076 | 0.376±0.157 |
| E/A         | 1.34±0.13 | 1.84±0.49* |
| DT (ms)     | 30.1±6.8 | 20.65±3.23* |
| Heart rate (bpm) | 405±38 | 427±60 |

Values represent means±SD. MHC-DGAT1 Tg mice were compared, and significance was assessed to age-matched littermate controls at each time point (*P<0.05, †P<0.01, ‡P<0.001) and across time points 12 vs 52 weeks and 32 vs 52 weeks (§P<0.05, ¶P<0.01, ‡P<0.001). A indicates late mitral filling velocity; DT, deceleration time; E, early mitral inflow filling velocity; EDV, end diastolic volume; ESV, end systolic volume; IVSW, interventricular septal wall; LVEF, left ventricular ejection fraction; LVFW, left ventricular posterior wall.
of DGAT1 would provide a mechanism to sequester fatty acids and blunt their toxic potential. Indeed, it has been suggested that such a mechanism occurs in the exercise-induced accumulation of triglyceride in skeletal muscle, a phenomenon termed the athlete’s paradox. 

However, with time and even in situations where there is little or no threat of toxicity from plasma fatty acids, the DGAT1-dependent accrual of triglycerides within myocyte vesicles would provide a chronic source of endogenous triglyceride metabolites and fatty acids that could result in cellular toxicity.

The MHC-DGAT1 Tg hearts displayed considerable interstitial fibrosis, a hallmark of advanced cardiomyopathy and heart failure in animal models and as human patients. It has also been observed in the hearts of Zucker fatty rats and ob/ob mice. The current study suggests that this interstitial fibrosis and increased remodeling activity can result solely from cardiac steatosis in the absence of elevations in plasma lipid levels. It also implies that the changes observed in the interstitial compartment as the cardiomyopathy develops are dependent on abnormalities originating in the myocyte itself since the genetic lesion is confined to this compartment. This could represent an indirect lipotoxic effect on the myocyte since the genetic lesion is confined to this compartment. This could represent an indirect lipotoxic effect on the myocyte since the genetic lesion is confined to this compartment.

Lipotoxicity has been associated with mitochondrial dysfunction in other systems, and mitochondrial biogenesis is thought to be adversely affected in obesity and type 2 diabetes mellitus. Skeletal muscle biopsies in type 2 diabetes mellitus patients show reduced oxidative function and decreased mitochondrial area, findings that correlate with reduced insulin sensitivity. 

Both the number and function of mitochondria in the heart are linked inversely to changes in cardiac output that occur in response to physiological or pathological stress. We found a significant reduction in measures of mitochondrial number, expression of mitochondrial encoded genes, and key transcription factors known to be important in mitochondrial biogenesis in the MHC-DGAT1 Tg hearts. We also noted reduced expression of the transcriptional coactivator PGC1α, a protein which has been shown to play an important role in mitochondrial biogenesis and fatty acid metabolism. PGC1α is reduced in both heart and skeletal muscle in a rat model of congestive heart failure, and deletion of the PGC1α gene in 2 independently generated mouse lines results in a cardiomyopathic phenotype. Expression of PGC1α target genes are also reduced in myocardium of human patients with heart failure. Our findings raise the intriguing possibility that lipid accumulation and the lipotoxicity that it engenders might impair mitochondrial biogenesis and contribute to the development of cardiomyopathy, at least in part, through suppression of PGC1α expression.

Perspective

We have used cardiac selective expression of DGAT1, a rate-limiting terminal enzyme in triglyceride synthesis, to create
Mitochondrial biogenesis is reduced in MHC-DGAT1 Tg hearts at 12 weeks of age. A, Representative electron micrographs of myocardium from NTg and MHC-DGAT1 Tg mice, original magnification ×5800; bar represents 0.5 mm. Lipid droplets (LD) and mitochondria (M) are shown. B, Mitochondrial volume quantified and represented as the percentage of total ventricular area from 5 micrographs each group. C, Quantification of mitochondrial DNA copy number, assessed as the ratio of mitochondrial (mt-Co1 and mt-Cytb) to nuclear (H19) copy number. N=5 for each group. D, Expression of the mitochondrial proteins COX1 and cytochrome C (Cyt. C) assessed by Western blot analysis. Relative levels were normalized to the mitochondrial proteins COX1 and cytochrome C. N=5 for each group. E, Q-PCR analysis of PGC1α, PPARα, PPARγ, and PPARγ, in NTg and MHC-DGAT1 Tg hearts. N=4 for each group. F, Western blot analysis of PGC1α (blot in panel D was stripped and incubated with anti-PGC1α antibody) in DGAT1 Tg and NTg hearts. Two distinct bands are seen for PGC1α (A and B). Relative levels of the A and B bands were normalized to GAPDH and quantified. N=4 for each group. *P<0.05, **P<0.01.

Figure 4.


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DATA SUPPLEMENT

A MURINE MODEL OF ISOLATED CARDIAC STEATOSIS LEADS TO CARDIOMYOPATHY

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Expanded and Supplemental Methods

Materials
The following antibodies were used: anti-diacylglycerol acyl transferase 1 (DGAT1), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnologies, Santa Cruz, CA), anti-FLAG (Sigma Aldrich, St. Louis, MO), anti-pro-collagen 1A, anti-tissue inhibitor of matrix metalloproteinase 2 (TIMP2), anti-matrix metalloproteinase 2 (MMP2), anti-MMP9, anti-peroxisome proliferator-activated receptor gamma coactivator-1α (PGC1α), anti-cytochrome C (Cyt-C), anti-cytochrome C oxidase (COX1) (Abcam, Cambridge, MA), anti-phospho-Akt and anti-Akt (Cell Signaling Technologies, Danvers, MA).

Experimental Animals and Cell Culture
All experiments were approved by the Institutional Animal Care and Use Committee at University of California at San Francisco (UCSF) and comply with the guidelines for the care and use of laboratory animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Ventricular myocytes were prepared from 1-2 day-old neonatal Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) as previously described 1. Mice were fed a standard chow diet (PicoLab Mouse Diet 20 5058, which contains 21.8% protein and 9.0% total fat).

Generation of a Cardiac Specific DGAT1 Transgenic Mouse
The murine-DGAT1 sequence containing a N terminal FLAG coding sequence was subcloned into a vector containing the α-MHC promoter, kindly provided by Dr. Jeffery Robbins 2. The construct was introduced into fertilized mouse eggs in the UCSF Diabetes Center Mouse Core Facility and independent founder lines were established. The initial MHC-DGAT1 Tg line was propagated in the C57/B6 strain; however, expression of the transgene was not detectable in subsequent generations. Recovery of transgene expression was achieved by backcrossing into the DBA/2J mouse strain. The transgene was propagated in this strain in all subsequent generations (> 5 crosses). The presence of the transgene was confirmed by Southern blot and by PCR based analysis using tail DNA (Mo Bio Laboratories, Carlsbad, CA). For Southern blot analysis genomic DNA was digested with EcoR1. Hybridization was performed with a 32P end-labeled -PCR product generated using transgene specific primer pairs (5’ ATCACTCCAGTGTTTCCG and 3’ TGGAGTGGCAACTCCAGC). Unlabeled primers were used for PCR screening of genotype. All experiments were performed on NTg and MHC-DGAT1 Tg littermates after more than 5 generations of propagation in the DBA/2J background.

Lipid Extraction
Heart tissues were harvested, snap frozen in liquid nitrogen and stored at -80 ºC. The tissue was weighed (wet weight) and homogenized in 150 mmol/l NaCl. Triglycerides were extracted according to the method of Bligh and Dyer 3. Prior to extraction, 3H triolein, (0.25 μCi/ 200μl, Perkin Elmer, Waltham, MA) was added to each sample to control for extraction efficiency. Briefly, the homogenate was extracted in 750 μl 1:2
chloroform: methanol overnight. An additional 250 μl chloroform was added to the suspension followed by 250 μl of H2O with vigorous mixing. The organic phase was collected dried and resuspended in chloroform. The triglyceride content (Cayman Chemical, Ann Arbor, MI) and free fatty acid (Wako Diagnostics, Richmond, VA) were quantified using colorimetric assays. Results were normalized for the wet weight of the myocardium. Tissue ceramide and DAG measurements were carried out according to previously described methods 4. DAG was resolved by thin layer chromatography using 30:20:2 ratio of heptane: isopropyl ether: acetic acid.

**Lipid Peroxide**
Heart tissue was quickly harvested and snap frozen in liquid nitrogen. Tissue was weighed and the malondialdehyde + 4-hydroxyalkenal levels were quantified using a colorimetric assay (OxisResearch, Biotech MDA 586, Portland, OR) as previously described 5.

**Plasma Triglycerides, Glucose, Insulin Tolerance and Sensitivity**
Plasma was collected at the time of animal sacrifice and stored at -80 ºC prior to sample assay. Triglycerides, cholesterol, high density lipoprotein and low density lipoprotein were measured by a commercial laboratory (IDEXX Laboratories, West Sacramento, CA). Mice were subjected to an overnight fast prior to the glucose tolerance and insulin tolerance testing. Mice were weighed and fasting blood glucose level was measured using a commercially available glucometer. Mice were injected intraperitoneally with either 20% glucose (2g/kg body weight) or human insulin (0.75 units/kg body weight). Blood glucose levels were measured every 15 min for 1-2 h after injection. Tissue insulin sensitivity was assessed by Western blot analysis of phospho-Akt versus total Akt in cardiac extracts. Mice received an intraperitoneal injection of either saline (sham) or 5 units/kg insulin. After 10 minutes, mice were euthanized and the hearts extracted and snap frozen prior to Western blot analysis for phospho-Akt and total Akt. The ratio of phospho-Akt:total Akt represents an indirect measure of insulin sensitivity 6.

**Luciferase Activity Assay**
Neonatal rat ventricular myocytes were transiently transfected with an expression vector containing the murine DGAT1 coding sequence downstream of an amino terminal FLAG epitope (kindly provided by Dr. Robert Farese) and/or the –1595 bp human BNP luciferase reporter plasmid 7 by electroporation (Gene Pulser, Bio Rad Laboratories, Hercules, CA) at 280 mV and 250 μF. Cells were plated and cultured in DMEM H-21 containing 10% ECS for 24 hours, then changed to media containing 50 μM palmitic acid or oleic acid using fatty acid-free bovine serum albumin as a carrier, for 24 hours to promote triglyceride synthesis. Neonatal rat ventricular myocytes were shifted into fatty acid-free media. After 48 hours, luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Where indicated, firefly luciferase levels were normalized for Renilla luciferase. Lipid accumulation was verified in fixed cells stained for intracellular lipid using oil red-O (ORO).

**Western Blot**
Extracts of total protein were prepared from tissues that were collected and snap frozen in liquid nitrogen prior to homogenization in lysis buffer, containing 20 mmol/L Tris pH 8.0, 150 mmol/L NaCl, 1% Triton X100 and Complete Protease Inhibitor (Roche Applied Science, Indianapolis, IN). Total protein was subjected to SDS-polyacrylamide gel electrophoresis and transferred to membranes. Membranes were probed with antibodies as indicated. Blots were stripped and re-probed with anti-GAPDH to normalize for protein loading. Blots were washed and incubated with horseradish peroxidase-conjugated secondary antibody and immune complexes were visualized by chemiluminescence (SuperSignal West Femto, Pierce Protein Research Products, Rockford, IL). Signal intensities were quantified using Kodak Scientific Imaging systems.

**RNA Isolation and Quantitative PCR**

Heart or liver tissue, preserved in RNAlater (Applied Biosystems, Foster City, CA.), was used to isolate total RNA with the RNeasy kit (Qiagen, Valencia, CA). Total RNA was reverse transcribed into cDNA. Quantitative PCR was carried out using Taqman primer sets (Applied Biosystems) for the following gene transcripts: DGAT1 (Mm00515643_m1), ANP (Mm01255748_g1), BNP (Mm00435304_g1), αSA (Mm00808218_g1), PGC1α (Mm00447183_m1), NRF (Mm01163627_m1), and Tfam (Mm00447485_m1) PPARα (Mm00627559_m1) PPARδ (Mm01305434_m1) and PPARγ (Mm00440945_m1).

**Mitochondrial volume and number**

Mitochondrial volume, as described by Wang et al. ⁸, was quantified from five independent ventricular section electron micrographs per group (original magnification 5800×) using ImageJ software. Mitochondrial volume is expressed as a percent of total micrograph area.

The relative quantity of mitochondrial and nuclear DNA was assessed by Q-PCR according to methods previously described ⁹. Total DNA was isolated by extraction in phenol: chloroform from left ventricular homogenates digested with proteinase K. Primer sequences for the mitochondria genes mt-Co1, mt-Cytb and the nuclear gene H19 were used as described ⁹. SYBR Green was used as a detection agent (Applied Biosystems).

**Histology and Immunofluorescence**

Animals were anesthetized with isoflurane and the hearts perfused with PBS followed by Z-Fix (Anatech Ltd, Battle Creek, MI), excised and then embedded in paraffin and subjected to transverse sectioning. Standard hematoxylin and eosin (H&E), Masson’s trichrome, and Oil-Red-O staining were performed with the assistance of the UCSF Pathology Core. Sections were also incubated with antibodies to FLAG (Sigma Aldrich) or DGAT1 (Santa Cruz Biotechnologies) and then anti-mouse Alexa Fluor 488 (Invitrogen) or anti-rabbit Cy3 (Invitrogen) secondary antibodies. The slides were then mounted with Vectashield mounting medium containing 4’,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Immunofluorescent images were acquired using an Olympus IX-70 inverted fluorescent microscope. FITC-WGA stained sections were used to evaluate myocyte size with published methodology ¹⁰. Apoptosis was assessed by terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL)
staining of cardiac ventricular sections counterstained with hematoxylin. TUNEL staining was quantified using ImageJ software.

Collagen Volume
Analysis of collagen volume fraction was performed with 5-µm-thick transverse cardiac cross sections stained with Masson's trichrome. Four to five photomicrographs were obtained and the volume of muscle and collagen was measured using Metamorph analysis software (Molecular Devices, Sunnyvale, CA). Collagen volume fraction was then calculated for the heart as the sum of connective tissue areas divided by the sum of connective tissue plus muscle areas in all fields

Blood Pressure Measurement
BP and HR were measured by the tail-cuff plethysmography method in unanesthetized mice using a Hatteras Instruments SC1000 blood pressure analysis system.

Echocardiography
Mice were anesthetized with 1.5% isoflurane and echocardiography was carried out using a Vevo 660 system (VisualSonics, Toronto, Canada) equipped with a 30-MHz real-time microvisualization scan head according to the method of Zhang et al

Statistical Analysis
Data were analyzed using 1 way ANOVA with Newman-Keuls posthoc test or Students t test. Results are presented as mean ±standard deviation (SD) or standard error of the mean (SEM) as indicated.

SUPPLEMENTAL RESULTS

DGAT1 expression in primary cardiomyocyte cultures.
The effect of DGAT1 expression in neonatal rat cardiomyocytes was examined following transient transfection of a CMV-FLAG-tagged DGAT1 expression vector. Expression of DGAT1 resulted in increased lipid droplet formation, as assessed by ORO staining in the setting of palmitate supplementation (Fig. S1A). The ORO stain appeared to co-localize with expressed FLAG-DGAT1, assessed by immunoreactivity with the anti-FLAG antibody. This result suggests that increased expression of DGAT1 results in increased lipid stores in these myocytes.

To assess the phenotypic response to DGAT1 overexpression and resulting triglyceride accumulation, we measured the activity of a transfected BNP luciferase reporter, a surrogate marker for activation of the hypertrophic gene transcription program in these neonatal myocytes. Co-transfection of the DGAT1 expression vector resulted in enhanced BNP promoter-dependent luciferase reporter activity when the cultures were supplemented with either saturated (palmitate) or unsaturated (oleate) fatty acids (Fig. S1B) implying that increased triglyceride synthesis and storage is associated with activation of the hypertrophic gene transcription program in these cells.

DGAT1 expression in the heart.
A cardiac myocyte selective DGAT1 transgenic mouse was achieved using a transgene containing the αMHC promoter linked to FLAG-tagged, murine DGAT1 coding sequence (Fig. S2A). Independent founder lines were established, and the presence of the transgene was confirmed by Southern blot and PCR analysis. A single line (line D) which showed moderate expression of the transgene by Western blot analysis was chosen for analysis. Selectivity of transgene expression was confirmed by Western blot using an antibody to the FLAG epitope. While robust FLAG expression was seen in the heart, no significant expression was detected in lung, liver, kidney or skeletal muscle (Fig. S2B). Expression of DGAT1 was visualized by immunofluorescence in fixed heart sections using antibody to the FLAG epitope, as well as an antibody to DGAT1. Again, this selectively demonstrated increased expression of DGAT1 in the transgenic vs. NTg hearts (Fig. S2C).

**Body weight, lipid profile, glucose homeostasis and blood pressure are not altered in the MHC-DGAT1 Tg mice.**

Body weight, serum triglycerides, and cholesterol did not differ significantly in the NTg and MHC-DGAT1 Tg mice. (Fig. S3A). Low density lipoprotein was also assessed but was near or below the level of detection in the assay (data not shown). The results are comparable to previously reported values for the DBA/2J strain and suggest that systemic lipid metabolism was not altered in the DGAT1 Tg mouse. Fasting blood glucose was normal in the DGAT1 Tg mice (Fig. S3B). In addition, the response to a glucose or insulin challenge was not altered in the transgenic mice, suggesting that the presence of the transgene did not alter systemic glucose metabolism (Fig. S3C-D). Insulin sensitivity was assessed in the hearts of insulin-injected mice by determining the ratio of phospho-Akt (ie, activated Akt) levels to total Akt levels in cardiac extracts using Western blot analysis. No significant differences in these ratios were seen in the NTG vs. MHC-DGAT1 Tg mice (Fig. S3E). These results are similar to those in which transgenic expression of DGAT1 or DGAT2 in hepatocytes also failed to induce insulin resistance.

**Cardiac Hypertrophy and Function**

Blood pressure was assessed in NTg and MHC-DGAT1 Tg mice previously acclimated to the tail cuff procedure. No significant differences were noted (Fig. S4A). Biventricular heart weight (HW) was assessed at 12, 32 and 52 weeks of age in NTg and MHC-DGAT1 Tg. No significant differences between littermates were seen when normalized for body (BW) weight or tibial length (TL) (Fig. S4). Interestingly both the HW/BW and HW/TL ratio were increased in both NTg and MHC-DGAT1 Tg mice at 52 weeks (compared 12 and 32 weeks) suggesting an age-dependent effect, independent of the presence of the transgene. Representative echocardiographs are shown in Fig. S5 obtained from 52 week old NTg and MHC-DGAT1 Tg mice.

**Apoptosis**

The apoptotic index was assessed by quantification of TUNEL positive nuclei in ventricular sections from 12 week old NTg and MHC-DGAT1 Tg mice and quantified (Fig. S6A and B). Apoptosis was significantly increased in the transgenic hearts, suggesting that DGAT1-mediated lipid accumulation and subsequent increase in
programmed cell death may contribute to the observed cardiomyopathy in this model. Ceramide has been associated with lipotoxicity in some models, however, ceramide levels were not significantly different in the transgenic vs. non-transgenic hearts (Fig. S6C).

Lipid Peroxides
Lipotoxicity has been associated with the generation of reactive oxygen species (ROS) in several models. Malondialdehyde, a product of lipid peroxide and marker of ROS, was measured in the NTg and MHC-DGAT1 Tg cardiac tissue. However, no significant difference was found (Fig. S6D). While this might suggest that ROS does not significantly contribute to the phenotype in this model of lipid accumulation, it is also possible that the low levels of ROS that are likely to accrue in this chronic model of lipotoxicity are not readily detected in this assay.

REFERENCES


Fig. S1. Expression of DGAT1 in neonatal cardiac myocytes results in lipid accumulation and enhanced BNP reporter activity. (A) Cardiac myocytes were transfected with an either an empty expression vector (CMV) or a vector expressing FLAG-DGAT1 and treated with BSA or BSA: palmitate (4:1 molar ratio). Lipid accumulation was visualized with ORO staining where red identifies neutral lipid. Expression of FLAG-DGAT1 was confirmed by immunocytochemistry (brown stain). (B) The -1595 bp BNP luciferase reporter was cotransfected with the FLAG-DGAT1 expression vector. Cultures were treated with BSA, BSA: acid palmitate (saturated fatty acid) or oleate (unsaturated fatty acid) at a 4:1 molar ratio. After 48 hours, cells were collected and lysates were prepared for measurement of firefly or Renilla luciferase activity. Pooled data from 3 independent experiments are presented as mean ±SEMs.
**Fig. S2.** Construction of a cardiac myocyte selective MHC-DGAT1 Tg mouse. (A) Representation of the MHC-Flag-DGAT1 Tg construct (left panel). The presence of the transgene was confirmed by Southern Blot analysis in 4 independent founders, and Line D was used for subsequent studies (middle panel). A PCR based screen was used to identify the transgene in subsequent generations (right panel). (B) Western blot analysis of expression of the FLAG-DGAT1 and GAPDH from protein extracts derived from heart, skeletal muscle, lung, kidney and liver in the NTg and MHC-DGAT1 Tg tissue. (C) Expression of DGAT1 in NTg and MHC-DGAT1 Tg heart sections using anti-FLAG (green, top panel) or anti-DGAT1 (red, bottom panel). As a control, anti-mouse Alexa fluor 488 and anti-rabbit cy3 fluorescent secondary antibodies were incubated with the MHC-DGAT1 Tg heart sections in the absence of primary antibody. Original magnification 200×. *p<0.05, ***p<0.001. All data represent mice aged 12 weeks.
**Figure S3**

**A**

Body weight (g) of female (F) and male (M) NTg (N=9 males and N=6 females) and MHC-DGAT1 Tg (N=4 males and N=5 females) littermates. No statistical differences were noted.

**B**

Serum lipid profile in NTg and MHC-DGAT1 Tg mice. No statistical differences were noted.

**C**

Glucose (mmol/L) over time (min.). No statistical difference between groups was noted.

**D**

Plasma triglycerides (TG), total cholesterol (chol.), high density lipoprotein (HDL) and free fatty acid (FFA) in NTg and MHC-DGAT1 Tg mice. N=4 for each group. No statistical differences were noted.

**E**

Fasting blood glucose, glucose tolerance and insulin tolerance. (NTg, N=6, MHC-DGAT1, N=5). No statistical difference between groups was noted. Western blot analysis of phospho-Akt, total Akt and GAPDH from heart extracts derived from NTg and MHC-DGAT1 Tg mice in control mice (E) and following insulin treatment (F). Phospho-Akt was quantified and normalized against total Akt. No significant differences were noted. N=4 each group. All data represent NTg and MHC-DGAT1 Tg mice aged 12 weeks.
Fig. S4. Assessment of blood pressure and cardiac hypertrophy. (A) Blood pressure, N=6 each group, mice aged 12 weeks. Systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial pressure (MAP). No statistical differences between groups were noted. Biventricular weight was measured and normalized to (B) body weight (HW/BW, mg/g) and (C) tibial length (HW/TL, mg/mm) in mice aged 12 weeks (N=4 each group), 32 weeks (NTg, N= 5 and MHC-DGAT1 Tg N= 7) and 52 weeks (NTg, N =14 and MHC-DGAT1 Tg, N=6). No significant differences were noted.
Fig. S5. Evaluation of cardiac function in 52 week old NTg and MHC-DGAT1 Tg mice by M-mode and Doppler echocardiography. M-mode echocardiographic images (A and B) and transmitral blood flow (C and D) were obtained in NTg (A, C) and MHC-DGAT1 Tg (B, D) mice aged 52 weeks. Mitral inflow early filling velocity (E), mitral inflow late filling (atrial contraction) velocity (A), and deceleration time (DT). Representative images are shown.
Fig. S6. Apoptosis, ceramide and lipid peroxidation. (A) Representative TUNEL stained fixed ventricular sections. Nuclei were counterstained with hematoxylin. Arrows indicate positive stained nuclei. (B) The number of TUNEL positive nuclei was quantified using ImageJ software. *p < 0.05. (C) Quantification of extracted cardiac ceramide (N=5 each group). No significant difference was noted. (D) Lipid peroxidation as assessed by total malondialdehyde (MDA) + 4-hydroxyalkenals (4-HAE) concentration in NTg and MHC-DGAT1 Tg heart tissue extracts. N= 5 each group. No significant difference was noted. All data represent NTg and MHC-DGAT1 Tg mice aged 12 weeks.