Absence of cardiac lipid accumulation in transgenic mice with heart-specific HSL overexpression

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Abnormal lipid accumulation in mice with heart-specific HSL overexpression. Am J Physiol Endocrinol Metab 281: E857–E866, 2001.—Hormone-sensitive lipase (HSL) hydrolyzes triglyceride (TG) in adipose tissue. HSL is also expressed in heart. To explore the actions of cardiac HSL, heart-specific, tetracycline (Tc)-controlled HSL-overexpressing mice were generated. Tc-responsive element-HSL transgenic (Tg) mice were generated and crossed with myosin heavy chain (MHC)-tTA TG mice, which express the Tc-responsive transactivator (tTA) in the heart. The double-Tg mice (MHC–HSL) were maintained with doxycycline (Dox) to suppress Tg HSL. Upon removal of Dox, cardiac HSL activity and protein increased 12- and 8-fold, respectively, and the expression was heart specific. Although cardiac TG content increased twofold in control mice after an overnight fast, it did not increase in HSL-induced mice. Electron microscopy showed numerous lipid droplets in the myocardium of fasted control mice, whereas fasted HSL-induced mice showed virtually no droplets. Microarray analysis showed altered expression of cardiac genes for fatty acid oxidation, transcription factors, signaling molecules, cytoskeletal proteins, and histocompatibility antigens in HSL-induced mice. Thus cardiac HSL plays a role in controlling accumulation of triglyceride droplets and can affect the expression of a number of cardiac genes.

Hormone-sensitive lipase; gene expression; microarray analysis

FREE FATTY ACIDS (FFA) are a major energy source in the heart. FFA provide up to 75% of the heart’s energy requirement in the resting state (13). Fatty acids utilized by cardiomyocytes are derived from circulating FFA and from the hydrolysis of intracellular stores of triglycerides (19). Circulating FFA are derived from either the hydrolysis of triglyceride stored in peripheral adipocytes or triglyceride in very low density lipoproteins or chylomicrons. Hormone-sensitive lipase (HSL) is responsible for the hydrolysis of triglyceride in adipocytes, whereas lipoprotein lipase (LPL) plays a role in hydrolyzing triglyceride in lipoproteins. The released FFA are taken up by cardiomyocytes and immediately utilized for β-oxidation in mitochondria or reesterified and stored as cytosolic triglyceride droplets. On the basis of the fact that HSL is expressed in the heart (11) and that the characteristics of the enzyme involved in cardiac lipolysis are similar to those of adipose HSL (27), intracellular triglyceride lipolysis is thought to be catalyzed by myocardial HSL. Thus adipose HSL, cardiac LPL, and cardiac HSL seem to control cardiac lipid supply in a coordinate manner, although the precise mechanism remains to be elucidated.

In addition to triglyceride hydrolase activity, HSL has neutral cholesterol esterase activity and also catalyzes the hydrolysis of diacylglycerol into monoacylglycerol (3). Diacylglycerol can function as a second messenger in signal transduction, mediating intracellular Ca2+ mobilization and protein kinase C activation in a variety of tissues and cells (14). Because diacylglycerol can be a substrate of HSL in the heart, HSL might potentially play a role in cardiac signal transduction by controlling diacylglycerol concentration.

Cardiomyocytes accumulate lipid droplets with fasting, whereas no lipid droplets can be observed in the heart from fed animals (8). Ischemia stimulates intracellular lipolysis, resulting in decreased triglycerides and increased FFA in the myocardium (24). However, the intracellular mechanism of accumulation and hydrolysis of lipid droplets within cardiomyocytes has not yet been clarified.

Although FFA are an essential energy source for the heart, excess FFA have detrimental effects on cardiac function. Patients with elevated serum FFA are more likely to develop arrhythmias at the onset of myocardial infarction (16), and high plasma FFA concentra-
tions have been shown to be related to the size of a myocardial infarction (18). In addition, in vitro and ex vivo studies have shown that FFA diminish contractility (17) and that FFA overload causes breakdown of membrane phospholipids and mitochondrial dysfunction (9). To investigate the effect of aberrant expression of HSL on cardiac lipid metabolism, we generated heart-specific HSL-overexpressing mice by utilization of the tetracycline-controlled gene expression (Tet) system. In this system, transgene expression is controlled by a tetracycline-responsive element (TRE), which is activated by the binding of a hybrid protein “tetracycline-responsive transcriptional activator” (tTA). The tTA binds to TRE in the absence of tetracycline, resulting in the initiation of HSL transcription. Our data show the tight regulation of foreign HSL in transgenic mice and the effects of HSL overexpression in the heart.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals were from Sigma Chemical (St. Louis, MO) unless otherwise indicated. Restriction enzymes were from Gibco-BRL (Grand Island, NY). DNA purification reagents were from Qiagen (Santa Clarita, CA). pTRE plasmid was from Clontech (Palo Alto, CA). Cholesteryl [1-14C]oleate and [α-32P]dCTP were from Amersham Life Sciences Products (Arlington Heights, IL). Organic solvents were from J. T. Baker (Phillipsburg, NJ).

Construction of Transgenes

A full-length cDNA of rat HSL was subcloned into pTRE plasmid at the EcoRI/XbaI site. A 3.5-kb fragment containing TRE, HSL, and the SV40 poly A signal was excised by HindIII/XhoI digestion, separated in agarose gel, and purified using QIAEX II.

Generation of Double-Transgenic Mice

The TRE-HSL gene was injected into fertilized oocytes derived from C57B/6J × CBA F1 background in the Transgenic Core Facility at Stanford University. Four identified TRE-HSL founders were crossed with C57BL/6J mice to expand the strain. Heterozygous TRE-HSL mice were then crossed with heterozygous MHCa-tTA mice, which express tTA driven by the heart-specific promoter myosin heavy chain (MHCa); kindly provided by Dr. Fishmann, Albert Einstein College of Medicine, New York, NY) (30) (Fig. 1A). Three double-transgenic strains, MHC-HSL2, -3, and -4, were established and maintained with doxycycline (Dox)-containing water until 3–5 mo of age and used for experiments. Littermates that were genotyped MHCo+/-/HSL-- were used as control animals. For some studies, animals were fasted for 16 h to induce lipid accumulation in cardiomyocytes before being killed. All animals were maintained on a chow diet (PMI 5015) with a 12:12-h dark-light cycle.

Control of Transgene Expression

Mating MHCo-tTA and TRE-HSL mice and their offspring, which were genotyped MHCo+/-/HSL-- and MHCo+/-/HSL-- were treated with drinking water supplemented with 0.2 mg/ml Dox to suppress the expression of transgenic HSL. To induce the expression of transgenic HSL, Dox-containing water was switched to normal water.

Genotyping

Genotypes were identified by Southern blot analyses and PCR with the use of genomic DNA from tail biopsies. DNA (10 μg) from each animal was digested with BamHI and fractionated on 1% agarose. DNA was transferred to a nylon membrane (Hybond N, Amersham) and probed with 32P-labeled rat HSL cDNA and tTA cDNA. Probed membranes were washed and visualized by a Phosphorimager 445 SI (Molecular Dynamics, Sunnyvale, CA). Primers used for PCR

A.

+Dox

-Dox

TRE P min CMV

HSL

B.

- mouse HSL

- Tg HSL

- tTA

E858 HEART-SPECIFIC HSL-TRANSGENIC MICE

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Fig. 1. Production of heart-specific, tetracycline-controlled hormone-sensitive lipase (HSL)-transgenic (Tg) mice. A: MHCo-tTA Tg mice that express the tetracycline-responsive transcriptional activator (tTA) under control of the heart-specific promoter myosin heavy chain-α (MHCo) were crossed with TRE-HSL Tg mice that express HSL under control of the tetracycline-responsive element (TRE). This cross yields double-Tg MHCo-HSL mice. Doxycycline (Dox) complexes with tTA, inhibiting its interaction with TRE and preventing transcription of HSL. In the absence of Dox, tTA binds to TRE and mediates HSL transcription. P min CMV, cytomegalovirus minimal promoter. B: genotyping by Southern blot analysis. Tail tip DNA (10 μg) from offspring of MHCo-tTA × TRE-HSL was digested with BamHI, size fractionated, transferred to a membrane, and probed with 32P-labeled rat HSL (top) or tTA (bottom) cDNA. Lanes 1–9: offspring; lane 10: dam (TRE-HSLA); lane 11: sire (MHCo-tTA). A representative result for the cross is shown.
are (forward/reverse) TRE-HSL: GCC GTG TAC GTT GGG AGG/GCA AAG ACG TTG GAC AGC C; MHCα-tTA: GCT GCT TAA TGA GGT CCG/CTC TGC ACC TTG GTG ATC.

**Immunoblot Analysis**

Immunoblot analyses were performed as previously described (11, 20). Two to twenty micrograms of protein from tissue homogenates were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with rabbit anti-HSL antibody, anti-p27 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or heart-type fatty acid binding protein (FABP) antibody and then with horseradish peroxidase-linked anti-rabbit IgG (Amersham). The membranes were visualized with chemiluminescence reagent ECL (Amersham), exposed to Kodak XAR film, and then analyzed by a Fluor-S multi-image analyzer (Bio-Rad, Hercules, CA).

**HSL Activity**

HSL activity was measured as neutral cholesteryl esterase activity according to a method previously described (11). The results are expressed in nanomoles of cholesteryl oleate hydrolyzed per milligram of protein per hour.

**Tissue Lipid Content**

Mice were anesthetized with 0.11 mg/kg ketamine and 0.013 mg/kg xylazine, and hearts were perfused with 3 ml of PBS via the left ventricle to wash out red blood cells. Lids were extracted from 30–40 mg of the heart with 42 volumes of chloroform-methanol-PBS (10:5:6) and analyzed by TLC as previously described by Ruiz and Ochoa (23). Briefly, an internal standard of cholesteryl formate (0.2 μg) was added to each sample, and aliquots of extracted lipids that had been dried and redissolved in toluene were spotted onto EDTA-treated TLC plates. Standards for each of the lipid classes were spotted at various concentrations to construct a calibration curve. The plates were developed in a stepwise fashion with chloroform-methanol-water 60:40:5 (vol/vol/vol) to 2 cm, ethyl acetate-2-propanol-ether-methanol-water 60:40:5 (vol/vol/vol/vol) to 2 cm, toluenediethyl ether-ether-methanol-water 60:40:3 (vol/vol/vol) to 5 cm, toulene-diethyl ether-ether-methanol-water 60:40:3 (vol/vol/vol) to 7.5 cm, heptane-diethyl ether 94:8 (vol/vol) to 10.5 cm, and pure heptane to 12.5 cm. The plates were charred by dipping in 10% cupric sulfate in 8% phosphoric acid and were heated to 200°C for 2 min. The charred plates were then analyzed on a Fluor-S.

**Serum Lipid Analysis**

Blood was drawn by a puncture of the heart. Animals of both genders were used. Serum was separated immediately, and total cholesterol, triglyceride, and free fatty acid concentrations were determined with commercially available kits (Sigma, Wako Chemicals).

**Morphological Studies**

Tissues were fixed with 1.5% glutaraldehyde, 4% polyvinylpyrrolidone, 0.05% calcium chloride, and 0.1 M sodium cacodylate, pH 7.4, for 16 h. Light microscopy was performed as previously described (21). For electron microscopy, the fixed tissue was washed with 0.15 M imidazole, treated with 4% osmium tetroxide (30 min) and 2% uranyl acetate (1 h at 4°C), and then dehydrated and embedded. Thin sections were stained with lead citrate and uranyl acetate and examined.

**Microarray Expression Analysis**

Experiments were basically performed following the Affymetrix GeneChip expression analysis protocol by use of Affymetrix Hybridization Oven 640 and Fluidics Station. Cardiac ventricular RNA was prepared from fed or fasted MHCα-tTA and HSL-induced MHC-HSL mice. Four animal samples per group were pooled and used for subsequent preparation. Four hundred micrograms of pooled total RNA (100 μg/animal) were used for mRNA preparation, double-strand (ds) cDNA synthesis, and biotin-labeled cRNA synthesis. Labeled cRNA (10 μg) was applied to an Affymetrix oligonucleotide array GeneChip Murine 11K set. Comparison analyses were performed for fed HSL-induced MHC-HSL vs. fed MHCα-tTA and fasted HSL-induced MHC-HSL vs. fasted MHCα-tTA mice.

**Semiquantitative PCR Analysis**

Expression of alcohol dehydrogenase-1 (ADH-1), CAAT box enhancer binding protein (C/EBP)-β, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase, and sulfotransferase (M-STP1), maltohexiin (MTI) and -II, cyclin-dependent kinase (CDK) inhibitor p27, tubulin α-4 and -5, and β-actin were analyzed by semiquantitative PCR. For each target gene, PCR conditions for the linear phase of amplification were established by a series of experiments with various numbers of cycles of PCR. Once quantitative conditions were established, 0.4 μl of equally diluted ds-cDNA samples were amplified in duplicate, visualized on agarose, scanned, and quantified using a Fluor-S. Each quantitative experiment was performed two to four times to confirm reproducibility. The sequences of primers are (forward/reverse) ADH-1: TGG CTC TGC CGT CAA AGT/CAG GGC AGA AGT CAT GGT; C/EBP-β: GCG CGA GCG CAA CAA CAT CT/TGC TTG AAC AGG TTC CGC AG; HMG-CoA synthase: CTC ACC ACT TTC CCG AAG AA/ACC CCT GTG ATG ATC CTG ATC CA; M-STP1: AGA TCC CTA CCA CTG AGC AC/GGC ACA CTT ATC TAG CTT GCC; MT-1: ATG GAC CCC AAC TGC TGC TAC TGG AAC TAC AGC TGC ACG TGC TCG ACT T; MT-2: CCA ACT GCT CCT GTG CCT/CAC AGC AGC TAC TTG CT C; α-27: AGC GAT TAT GCT GAC CCT/CCA ACC TCT CAC GGC AAC TAC; tubulin α-4: TCA ACT ACC AAC CAC CAG CTG TCT CCC TCA CCC TG TCC TC; tubulin α-5: AGG TGT CCA GAT GGG CAA TC/GCA CAT GTT CCC TCC AG; β-actin: ATG GTG GGA AGT GTG CAG AAC/ACG GCA GCT CAT TGT AGA AGG.

**Statistical Analysis**

Values were compared among three groups: MHCα-tTA, MHC-HSL with Dox, and MHC-HSL without Dox. Three to six animals per group were normally used for experiments. ANOVA was used to determine a significant difference using STATVIEW (Abacus Concepts) on a Macintosh computer. A value of P < 0.05 was considered significant. Results are presented as means ± SE.

**RESULTS**

**Generation of Transgenic Strains**

From the first set of injections, four animals of 37 live births were identified as TRE-HSL positive by Southern blot analysis. These four founders (TRE-HSL1, -2, -3, and -4) contained three, one, three, and four copies of the integrated TRE-HSL gene, respectively. All founders were crossed with C57BL/6J...
mice to expand the strain. TRE-HSL2, -3, and -4 strains were successfully bred, although only two of the integrated three copies of TRE-HSL gene were transmitted in the TRE-HSL3 strain. None of the three copies of TRE-HSL was transmitted in the TRE-HSL1 strain. Heterozygous F1 offspring of the TRE-HSL2, -3, and -4 mice (HSL+/−) were then crossed with heterozygous MHCα-tTA mice (MHC+/−) to produce double-transgenic strains MHC-HSL2, -3, and -4. Figure 1B shows a representative result of a Southern blot analysis for F1 offspring from the cross. Offspring 1, 2, 3, 5, 6, and 7 have the two copies of TRE-HSL gene transmitted from the dam, whereas offspring 2, 3, 7, and 8 have the MHCα-tTA gene transmitted from the sire. Thus offspring 2, 3, and 7 were identified as MHC-HSL double-transgenic mice. The identified double-transgenic mice (MHC+/−/HSL+/−) were used for subsequent experiments. The general appearance, growth, activity, and fertility of MHC-HSL mice were normal.

HSL expression was induced by removal of Dox from the drinking water for 3 wk. Animals were then killed for tissues to be harvested and for investigation of HSL expression. As shown in Fig. 2A, cardiac HSL protein expression was induced eightfold in Dox(−) MHC-HSL4 (MHC+/−/HSL+/−) mice compared with control MHCα-tTA (MHC+/−/HSL+/−) mice. In contrast, transgenic HSL was completely suppressed in Dox(+) MHC-HSL4 mice. Cardiac HSL activity in Dox(−) mice was 12-fold higher than in Dox(+) mice or control mice. A similar level of cardiac HSL induction was seen in MHC-HSL2 mice, whereas none of the MHC-HSL3 mice displayed inducible cardiac HSL expression. There was no significant difference in HSL induction between genders. The MHC-HSL4 strain was mainly used in subsequent experiments unless otherwise stated.

To test the tissue specificity of the induction of HSL expression, HSL activity was investigated in various tissues. As shown in Fig. 2B, removal of Dox from the drinking water induced HSL activity only in the heart. In Dox(−) MHC-HSL mice, HSL activity in the heart was 4–18 times higher than in other tissues except fat. Fat showed the highest HSL expression (60–70 nmol·mg protein−1·h−1) among any of the tissues in all three groups. Dox(+) MHC-HSL mice and control MHCα-tTA mice showed no HSL induction in any tissues examined. These data indicate that the inducible expression of HSL is cardiac specific in MHC-HSL transgenic mice.

Fig. 2. Induction of Tg HSL expression. A, top: immunoblot of HSL. Tg HSL was induced for 3 wk, and animals were killed. Mouse hearts were homogenized, and 20 µg of protein were fractionated on SDS-PAGE, transferred to nitrocellulose, and probed with anti-rat HSL-fusion protein antibodies. Bottom: HSL activity was assayed as neutral cholesterol esterase activity, as described in MATERIALS AND METHODS. C, control MHCα-tTA mice; +Dox, HSL-suppressed MHC-HSL mice; −Dox, HSL-induced MHC-HSL mice. Six animals in each group were studied, and representative results are shown. B: HSL activity in various tissues. After 3 wk of induction, tissues were excised and homogenized, and HSL activity was measured as neutral cholesterol esterase activity. C, control MHCα-tTA mice; +, HSL-suppressed MHC-HSL mice; −, HSL-induced MHC-HSL mice. Six animals (2 males, 1 female) in each group were studied. Values represent means ± SE.

Fig. 3. Low magnification electron micrograph of left ventricle muscle from control and HSL-induced mice. Tg HSL was induced for 4 wk, and the animals were killed after 16 h of fasting. Whereas muscle fibers from the control MHCα-tTA mice (A) show numerous lipid droplets (L) associated with clusters of mitochondria, no lipid droplets were found in similar regions of muscle from HSL-induced MHC-HSL mice (B).
Effect of Cardiac HSL Overexpression

Microscopy. A histological examination of tissues from left ventricle muscle from fed control and 4-wk HSL-induced mice revealed no special changes. The wet weights of the hearts were also not affected by HSL overexpression for 4 wk (male: 162 ± 6 vs. 153 ± 5 mg for control and HSL-induced, respectively). To bring out potential differences, the mice were fasted for 16 h. Heart muscle was removed and processed for both light and electron microscopy. Light microscopy staining with toluidine blue showed numerous small blue droplets (presumed to be lipid) dispersed in the muscle of the fasted control MHC-o-tTA mice, but there were no such droplets in the muscle of the HSL-induced MHC-HSL mice (data not shown). When sections of the fasted animals were viewed with the electron microscope, a similar story was found; i.e., many lipid droplets were located within clusters of mitochondria in the muscle fibers of control MHC-o-tTA mice (Fig. 3A), but not within the mitochondrial clusters, or elsewhere, in cardiac muscle fibers of the HSL-induced MHC-HSL mice (Fig. 3B). No other morphological differences were observed in the samples taken from the mice.

Lipid analysis. To biochemically analyze the changes observed by microscopy, cardiac lipid content was then assessed. As shown in Table 1, cardiac triglyceride content increased approximately twofold in control MHC-o-tTA mice after an overnight fast. In contrast, cardiac triglyceride content was not changed in HSL-induced heart. Cardiac free cholesterol and FFA content were increased with fasting in both MHC-o-tTA and HSL-induced MHC-HSL mice but were not affected by overexpression of HSL. Total phospholipids were not changed by fasting in hearts from either control or HSL-induced mice (data not shown). No cholesteryl esters were detected in the hearts of control or HSL-induced mice. Serum lipid concentrations were analyzed after 3 wk of HSL induction. In both fed and fasted conditions, serum triglyceride, total cholesterol, and FFA were not changed by the overexpression of HSL in the heart (Table 2).

Gene expression. To study the effects of HSL-overexpression on cardiac gene expression, microarray analysis was performed. From an analysis comparing control and HSL-induced mice, cardiac expression of ~100 genes was changed at least twofold in either the fed or fasted condition. The known genes whose expression was altered greater than twofold are listed in Tables 3 and 4. Among these genes, the expression of ADH-1, C/EBP-β, HMG-CoA synthase, M-STP1, MT-1 and -II, p27, and tubulin α-4 and -5 were altered by HSL overexpression under both fed and fasted conditions. These nine genes were investigated by semiquantitative PCR analysis to confirm the consistency of the results of microarray analysis. As shown in Fig. 4, all of the changes seen in microarray analysis were consistent with the results from semiquantitative PCR analysis.

In the category of lipid/glucose/energy metabolism, expression of ADH-1, C/EBP-β, and HMG-CoA synthase was increased in HSL-induced heart in both fed and fasted conditions (Tables 3 and 4). In addition to these genes, genes involved in fatty acid oxidation, i.e., cytochrome P450, carnitine palmitoyltransferase I, and uncoupling protein-2 were upregulated with fasting. M-STP1 and MT-1 and -II were also upregulated in both fed and fasted conditions in HSL-induced heart, suggesting significant effects of HSL overexpression on other aspects of cardiac metabolism. Interestingly, expression of many genes involved in the cell cycle, growth, and signaling was affected by overexpression of HSL. In this category, p27 was downregulated in both fed and fasted conditions. A Western blot of p27 (Fig. 5) confirmed that the expression of p27 protein

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### Table 2. Serum lipid profile

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mice</th>
<th>TG, mmol/l</th>
<th>TC, mmol/l</th>
<th>FFA, mg/l</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed</td>
<td>MHC-o-tTA</td>
<td>0.77 ± 0.07</td>
<td>2.90 ± 0.10</td>
<td>136 ± 28</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>MHC-HSL (+Dox)</td>
<td>0.95 ± 0.12</td>
<td>2.87 ± 0.31</td>
<td>119 ± 14</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>MHC-HSL (−Dox)</td>
<td>1.02 ± 0.14</td>
<td>3.54 ± 0.23</td>
<td>136 ± 17</td>
<td>8</td>
</tr>
<tr>
<td>Fasted</td>
<td>MHC-o-tTA</td>
<td>1.14 ± 0.17</td>
<td>2.35 ± 0.18</td>
<td>223 ± 34</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>MHC-HSL (+Dox)</td>
<td>0.90 ± 0.10</td>
<td>2.43 ± 0.21</td>
<td>178 ± 25</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>MHC-HSL (−Dox)</td>
<td>0.81 ± 0.07</td>
<td>2.17 ± 0.25</td>
<td>195 ± 17</td>
<td>12</td>
</tr>
</tbody>
</table>

Values represent means ± SE; n = no. of mice per group.
was reduced in the hearts of HSL-induced mice compared with control. HSL overexpression also affected cardiac cytoskeletal genes; genes encoding myofibrils, i.e., 64-kDa autoantigen D1, MHC-β homolog, and epidermal keratin type 1 were altered in the fed state. Tubulin α-4 and -5 were downregulated in both fed and fasted conditions. A remarkable difference in gene expression pattern between fed and fasted conditions was shown in the category of immune-related genes. A number of genes for MHC antigens and genes for antigen processing were downregulated by HSL overexpression only with fasting.

LPL and HSL are thought to regulate cardiac FFA supply in a coordinate manner (19). Because microarray analysis showed no change in LPL expression, Northern blot analysis was performed to confirm the stable expression of LPL with the use of another set of transgenic mice. Consistent with the array analysis, LPL mRNA expression was not changed in Dox(-) MHC-HSL mice compared with that of Dox(+) MHC-HSL mice or control MHC-α-tTA mice in either fed or fasted conditions (data not shown). Although microarray analysis did not show any changes in cardiac FABP mRNA expression, it was of interest whether posttranscriptional regulation of FABP was affected by HSL overexpression, because we have shown (25) that FABP interacts with HSL in adipocytes. However, cardiac FABP protein expression in Dox(-) MHC-HSL

### Table 3. Microarray comparison analysis of cardiac gene expression between HSL-induced MHC-HSL and MHC-α-tTA mice in fed condition

<table>
<thead>
<tr>
<th>Category</th>
<th>Gene Name</th>
<th>FoldΔ</th>
<th>Accession No.</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid/glucose/energy</td>
<td>Alcohol dehydrogenase</td>
<td>2.4+</td>
<td>M18480</td>
<td>Fatty acid oxidation</td>
</tr>
<tr>
<td>metabolism</td>
<td>Apolipoprotein D</td>
<td>4.1+</td>
<td></td>
<td>Component of high-density lipoprotein</td>
</tr>
<tr>
<td>C/EBP-β</td>
<td>HMG-CoA synthase</td>
<td>2.0+</td>
<td>X62600</td>
<td>Transcriptional factor, cell differentiation</td>
</tr>
<tr>
<td>6-Phosphofructo-2-kinase</td>
<td>Prostaglandin D synthetase</td>
<td>6.9+</td>
<td>U12791</td>
<td>Cholesterol synthesis, ketogenesis</td>
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<td></td>
<td>ATP synthase protein</td>
<td>2.9+</td>
<td></td>
<td>Glycolysis regulation</td>
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<td></td>
<td>Vacuolar H(+)-ATPase (mvp)</td>
<td>2.5+</td>
<td>AB006361</td>
<td>Prostaglandin D synthesis</td>
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<tr>
<td></td>
<td>Amino levulinate synthase</td>
<td>2.4+</td>
<td></td>
<td>ATP synthesis in mitochondria</td>
</tr>
<tr>
<td>Other</td>
<td>Aryl sulfotransferase</td>
<td>3.0+</td>
<td></td>
<td>Steroid and thyroid hormone receptor family</td>
</tr>
<tr>
<td>metabolism</td>
<td>Fumarylacetosetate hydrolase</td>
<td>2.5+</td>
<td>M841145</td>
<td>CAT, CAAT box enhancer protein; HMG, 3-hydroxy-3-methylglutaryl; MHC, myosin heavy chain.</td>
</tr>
<tr>
<td></td>
<td>Metallothionein-II</td>
<td>2.8+</td>
<td>V00835</td>
<td>Essential metal metabolism, detoxification</td>
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<tr>
<td></td>
<td>N10 nuclear hormonal binding receptor</td>
<td>3.2+</td>
<td>X16995</td>
<td>Essential metal metabolism, detoxification</td>
</tr>
<tr>
<td></td>
<td>24pβ</td>
<td>2.7+</td>
<td>X81627</td>
<td>Steroids and thyroid hormone receptor family</td>
</tr>
<tr>
<td></td>
<td>Amino levulinate synthase</td>
<td>2.3+</td>
<td></td>
<td>Transports of hydrophobic ligands</td>
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<tr>
<td></td>
<td>N-type calcium channel α1-subunit</td>
<td>5.2+</td>
<td>U094999</td>
<td>Heme and porphyrin synthesis</td>
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<tr>
<td>Cell cycle/</td>
<td>c-fos</td>
<td>7.2+</td>
<td>V00727</td>
<td>Calcium signaling</td>
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<tr>
<td>growth/signaling</td>
<td>Choline kinase homolog</td>
<td>3.1+</td>
<td></td>
<td>Cell growth, signal transduction</td>
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<td></td>
<td>Cold-inducible RNA-binding protein</td>
<td>2.7+</td>
<td>D78135</td>
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<td></td>
<td>Cyrt61</td>
<td>3.4+</td>
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<td>Growth factor-inducible immediate-early gene</td>
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<td>Leucine zipper-containing transcription factor</td>
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<td>X07411</td>
<td>Cell cycle regulation</td>
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<td>M13444</td>
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<td>Tubulin α-5</td>
<td>2.4+</td>
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<td>Component of bipolar spindle</td>
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<td>Others</td>
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<td>Intracysternal A particle IAP-IL3</td>
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<td>X04120</td>
<td>Related to type B and type D retroviruses</td>
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<td>Neutrophil elastase</td>
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<td>L04961</td>
<td>X inactivation</td>
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<td>Pentyltetetetrazol-related mRNA</td>
<td>2.1+</td>
<td></td>
<td>Neuronal pentyltetetrazol-induced gene</td>
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</table>

Known genes whose expression was changed more than twofold are listed. Boldface characters show the genes whose expression was changed in both fed and fasted conditions. C/EBP-β, CAAT box enhancer protein; HMG, 3-hydroxy-3-methylglutaryl; MHC, myosin heavy chain.
Table 4. Microarray comparison analysis of cardiac gene expression between HSL-induced MHC-HSL and MHCα-4TA mice in fasted condition

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<thead>
<tr>
<th>Category</th>
<th>Gene Name</th>
<th>FoldΔ</th>
<th>Accession No.</th>
<th>Function</th>
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<td>Lipid/glucose/energy</td>
<td>Alcohol dehydrogenase</td>
<td>5.5+</td>
<td>M18480</td>
<td>Fatty acid oxidation</td>
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<td>metabolism</td>
<td>C3H cytochrome P450 (Cyp1b1)</td>
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<td>U03283</td>
<td>Fatty acid oxidation etc.</td>
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<td>C/EBP-β</td>
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<td>AF017175</td>
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<tr>
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<td>C/EBP-β</td>
<td>2.1+</td>
<td>X62680</td>
<td>Transcriptional factor, cell differentiation</td>
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<tr>
<td></td>
<td>HMG-CoA synthase</td>
<td>3.1+</td>
<td>X61800</td>
<td>Transcriptional factor, cell differentiation</td>
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<tr>
<td></td>
<td>Mitochondrial RNA splicing protein (MRS4)</td>
<td>3.4+</td>
<td></td>
<td>Mitochondrial functions (?)</td>
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<td>Steroid dehydrogenase (Ke 6)</td>
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<td>U34072</td>
<td>Short-chain alcohol dehydrogenase family</td>
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<td>Uncoupling protein-2 (UCP 2)</td>
<td>2.7+</td>
<td>U69135</td>
<td>Fatty acid oxidation</td>
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<td>Protein targeting to glycogen (PTG)</td>
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<td>U89924</td>
<td>Glycogen synthesis</td>
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<td>Catecholamine, thyroid hormone metabolism</td>
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<td>U87456</td>
<td>Detoxification: oxidation of xenobiotics</td>
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<td>V00835</td>
<td>Essential metal metabolism, detoxification</td>
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<td>Metallothionein-II</td>
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<td>Essential metal metabolism, detoxification</td>
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<td>Thioether S-methyltransferase</td>
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<td>Metabolism of sulfur-containing compounds</td>
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<td>Tissue inhibitor of metalloproteinases-3</td>
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<td>Z30970</td>
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<td>Xanthine dehydrogenase</td>
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<td>X75129</td>
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<td>GFR α-3</td>
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<td>Proenin-converting enzyme</td>
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<td>c-fos</td>
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<td>Prolinecogene</td>
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<td>Cytoskeleton</td>
<td>Tubulin α-4</td>
<td>2.4−</td>
<td>M13444</td>
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<td>Tubulin α-5</td>
<td>2.5−</td>
<td></td>
<td>Component of bipolar spindle</td>
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<td>Topoisomerase-inhibitor suppressed gene</td>
<td>2.7−</td>
<td>D86344</td>
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</table>

Known genes whose expression was changed more than twofold are listed. Boldface characters show the genes whose expression was changed in both fed and fasted conditions.
mice was not changed compared with that of Dox(+) MHC-HSL mice or MHCα-tTA mice by Western blot analysis (data not shown).

DISCUSSION

In this study, we have established heart-specific, tetracycline-controlled, HSL-overexpressing mice. Transgenic HSL was expressed specifically in the heart, and its expression was tightly regulated by the administration of Dox. Cardiac HSL activity was induced 12-fold and protein mass 8-fold in heterozygous MHC-HSL mice. Dox-dependent suppression of transgenic HSL was complete in our transgenic mice, as documented by the fact that cardiac HSL activity and protein levels in Dox-treated MHC-HSL mice were as low as those of control MHCα-tTA mice. Although some leakage of the target gene has been reported in transgenic mice with the Tet system (30), our data showed no significant leakage of transgene expression.

Upon fasting, serum FFA increase, and cardiomyocytes accumulate triglyceride droplets. It is thought that an imbalance between influx and utilization of FFA causes cardiac lipid droplet formation. In this study, we have demonstrated that cardiac HSL is capable of hydroylizing accumulated lipid droplets in vivo, preventing lipid accumulation. Cardiac lipid accumulation can be harmful; excess FFA can lead to cytotoxicity, and it has been reported that diabetic Zucker rats accumulate lipid droplets in cardiomyocytes, leading to lipoapoptosis (31). Lipid droplet accumulation is also observed in certain diseases such as dilated and hypertrophic cardiomyopathies (12, 29). Controlling cardiac lipid accumulation might possibly be beneficial in these conditions.

Overexpression of heart HSL affected the expression of several cardiac genes associated with metabolism, cell growth, immune response, and the cytoskeleton. We propose that the overexpression of HSL increased intracellular fatty acid flux by hydrolyzing any triglycerides produced by esterification and that the change in fatty acid metabolism is a common mechanism responsible for the alteration in these genes. Thus, under both fed and fasted conditions, ADH, C/EBP-α and -β, MSTP, and MT-I were downregulated in fasted MHC-HSL mice compared with MHCα-tTA control mice under both fed and fasted conditions. The degree of changes detected by PCR and microarray analysis in HSL-induced MHC-HSL experiments. The degree of changes detected by PCR and microarray analysis in HSL-induced MHC-HSL experiments.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fed C</th>
<th>Fasted C</th>
<th>Tg C</th>
<th>Fold Change Tg vs C PCR</th>
<th>Fasted Tg</th>
<th>Fold Change Tg vs C micro array</th>
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<td>1.2</td>
<td>3.0</td>
<td>2.4</td>
<td>5.5+</td>
<td>2.0</td>
<td>2.1+</td>
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<td>CEBP-β</td>
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<td>7.0+</td>
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<td>2.0-</td>
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<td>4.4-</td>
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<td>2.7-</td>
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<td>β-actin</td>
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</table>

Fig. 4. Semiquantitative PCR analysis of the genes that were changed on microarray analysis in both fed and fasted conditions. Double-strand cDNA samples were amplified by PCR in duplicate and visualized on 1.5% agarose gel containing ethidium bromide. Lanes 1 and 2: fed MHCα-tTA; lanes 3 and 4: fasted MHCα-tTA; lanes 5 and 6: fed MHC-HSL (–Dox); lanes 7 and 8: fasted MHC-HSL (–Dox). ADH, alcohol dehydrogenase-1; HMG, 3-hydroxy-3-methylglutaryl-CoA synthase; MSTP, aryl sulfotransferase; MT-I and II, metallothionein-I and -II; Tub4, tubulin α-4; Tub5, tubulin α-5. Each analysis was repeated in 2–4 independent experiments. The degree of changes detected by PCR and microarray analysis in HSL-induced MHC-HSL mice compared with MHCα-tTA control mice under both fed and fasted conditions are displayed.

Fig. 5. Western blot of p27 in hearts of control and HSL-induced mice. Tg HSL was induced for 3 wk, and animals were killed. Mouse hearts were homogenized, and 20 µg of protein were fractionated on SDS-PAGE, transferred to nitrocellulose, and probed with anti-p27 antibodies.

HMG-CoA synthase, which is located in both the cytosol and mitochondria, catalyzes the condensation of acetoacetyl-CoA and acetyl-CoA to form HMG-CoA. The HMG-CoA produced by cytosolic HMG-CoA synthase is converted to mevalonate by HMG-CoA reductase, the rate-limiting enzyme in cholesterol bio-
synthesis, whereas the HMG-CoA produced within mitochondria is converted to acetoacetate by HMG-CoA lyase and then to hydroxybutyrate and acetone. In this manner, mitochondrial HMG-CoA synthase is an important enzyme for ketogenesis. Fatty acids increase the transcription of mitochondrial HMG-CoA synthase both in vitro and in vivo (2, 5), and this is mediated by peroxisome proliferator-activated receptors (22). The upregulation of HMG-CoA synthase by HSL overexpression suggests that chronic FFA release stimulated intracellular ketogenesis. Accordingly, other ketogenic and fatty acid-oxidative genes, i.e., carnitine palmitoyltransferase I, cytochrome P450, steroid dehydrogenase, and uncoupling protein-2 were also upregulated in fasted HSL-induced mice. On the other hand, the cholesterol biosynthetic pathway does not seem to be activated by HSL overexpression, because mRNA levels of other enzymes involved in cholesterol synthesis were not affected.

A number of genes involved in cell growth or intracellular signaling were also affected by HSL overexpression. Among this category, p27 was downregulated in both fed and fasted conditions, and this was confirmed by Western blots. Although the upregulation of genes involved in cell growth or intracellular signaling could suggest the possibility that HSL overexpression might result in cardiac hypertrophy, we did not observe any evidence of cardiac hypertrophy. However, HSL overexpression was maintained for only 3–4 wk in the current studies, and longer time periods might be required for these changes to be seen. FFA can affect cell growth, differentiation, and intracellular signaling (15). In addition, linoleic acid induces depletion of p27 and increases CDK2 activity, which is required for G1/S transition (7), and a fatty acid inhibitor, cerulenin, increases expression of the CDK inhibitors p21 and p27 (4). These reports are consistent with our data and suggest that chronically overreleased intracellular FFA possibly led to these changes in our transgenic mice.

Previous studies have also shown that unsaturated fatty acids are able to cause alterations in the normal distribution pattern of cytoskeletal proteins, including tubulins, actins, and myosins (6). In both fed and fasted conditions, expression of tubulin α-4 and -5 was decreased in HSL-induced heart. These data also support the idea that overexpression of HSL causes chronic excess exposure of FFA, affects cardiac gene expression of these antigens and cytoskeletal molecules. Thus the changes in gene expressions seen in HSL-induced mice convincingly suggest the existence of chronic exposure to high intracellular fatty acid flux. However, cardiac fatty acid content was not changed in HSL-induced mice compared with pair-fed or pair-fasted control mice. An increase in intracellular FFA release by the action of HSL might have been compensated for by an accelerated efflux or oxidation of FFA in HSL-induced mice.

HSL and LPL are thought to be coordinately regulated. Shimada et al. (26) reported that HSL activity and mRNA expression are elevated in adipose tissue in LPL-transgenic mice, which overexpress LPL in adipose tissue, skeletal muscle, and heart. In contrast, our data show stable LPL mRNA expression despite high expression of HSL in the hearts of transgenic mice. These data might indicate independent roles of LPL and HSL in cardiac lipid metabolism. It is possible that overexpressed HSL only accelerates the rate of intracellular hydrolysis and reesterification of triglyceride and does not affect the demand for extracellular FFA provided by the action of LPL.

In summary, in the current study, we created heart-specific HSL-overexpressing mice with a Tet system. The results show a role for cardiac HSL in controlling the accumulation of triglyceride droplets in the heart. HSL overexpression also affected the expression of a number of genes involved in lipid/energy metabolism, cell growth/cell cycle, and cellular antigen presentation. This animal model will be an excellent tool to study further the function of cardiac HSL.

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