Effects of hormone-sensitive lipase-disruption on cardiac energy metabolism in response to fasting and refeeding

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Running head: Cardiac energy metabolism in HSL-KO mice

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Abstract

Increased fatty acid (FA) flux and intracellular lipid accumulation (steatosis) give rise to cardiac lipotoxicity in both pathological and physiological conditions. Since hormone-sensitive lipase (HSL) contributes to intracellular lipolysis in adipose tissue and heart, we investigated the impact of HSL-disruption on cardiac energy metabolism in response to fasting and refeeding. HSL-knockout (KO) mice and wildtype (Wt) littermates were fasted for 24 h followed by ~6 h of refeeding. Plasma FA concentration in Wt mice was elevated 2-fold with fasting, while KO mice lacked this elevation, resulting in 2-fold lower cardiac FA uptake compared to Wt mice. Echocardiography showed that fractional shortening was 15% decreased during fasting in Wt mice and was associated with steatosis, whereas both of these changes were absent in KO mice. Compared with Langendorff-perfused hearts isolated from fasted Wt mice, the isolated KO hearts also displayed higher contractile function and a blunted response to FA. Although cardiac glucose uptake in KO mice was comparable to Wt mice under all conditions tested, cardiac VLDL uptake and lipoprotein lipase (LPL) activity were 2-fold higher in KO mice during fasting. The KO hearts showed undetectable activity of neutral cholesteryl esterase and 40% lower non-LPL triglyceride lipase activity compared to Wt hearts in refed conditions, accompanied by overt steatosis, normal cardiac function, and increased mRNA expression of adipose differentiation-related protein. Thus, the dissociation between cardiac steatosis and functional sequelae observed in HSL-KO mice suggests that excess FA influx, rather than steatosis per se, appears to play an important role in the pathogenesis of cardiac lipotoxicity.

Keywords: Lipolysis, Cardiac steatosis, Lipotoxicity.
Congestive heart failure is often associated with obese and diabetic individuals (3). Increased body fat and impaired insulin function lead to excess release of free fatty acids (FFA) from adipose tissue. The circulating fatty acids (FA) are transferred into cardiomyocytes for \( \beta \)-oxidation while excess FA are stored in triacylglycerol (TAG) droplets. Although FA are a major energy source for the heart, excess FA availability causes metabolic stress, leading to cardiac dysfunction, namely lipotoxic cardiomyopathy (26, 28, 50).

Lipotoxicity can be derived from both extra- and intra-cellular FA flux. It has been reported that saturated FA induce apoptotic cell death in rat ventricular myocytes \textit{in vitro} (6). FA perfusion has been shown to increase reactive oxygen species (ROS) in isolated rat heart (10), and to inhibit the cardio-protective action of insulin in perfused mouse heart (8). FA released from TAG droplets are also thought to be detrimental in obesity or diabetes, conditions where intracellular lipolysis is activated (51). In diabetic \textit{ob/ob} mice it has been shown that FA from intracellular TAG droplets increase intracellular ceramide, which induces cardiac apoptosis and dysfunction (4, 55).

Recently, myocardial TAG accumulation has been shown to be associated with decreased cardiac function even in physiological conditions. It has been reported that fasting leads to a decrease in cardiac diastolic function associated with myocardial accumulation of TAG in humans (14, 53). Caloric restriction has also been shown to cause phospholipid depletion, membrane remodeling, and TAG accumulation in murine myocardium (15). Thus, myocardial TAG accumulation (steatosis) appears to be a hallmark of lipotoxicity; however, the pathophysiological function and regulation of steatosis remains to be elucidated.

Cardiac energy metabolism dynamically changes in response to alterations in nutritional conditions (20, 47). Cardiomyocytes utilize FA and glucose as energy sources depending on
availability. FA derived from adipose tissue or lipoproteins are preferentially utilized in fasted conditions, while glucose provides a substantial energy source in fed conditions. Switching the preference between these energy sources, cardiac muscle efficiently utilizes them to maintain constant pump function to survive food-deprived conditions. Despite intensive investigations, however, it is not fully understood how the heart adapts to altering availability of energy sources in physiological settings.

Plasma FA are derived from adipose lipolysis, which is stimulated by hormones, such as catecholamines or glucagon, in food-deprived conditions. Adipose lipolysis is regulated by 2 major lipases, hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) (18, 56), and potentially TAG hydrolases-1 and -2 (33). A classical enzyme, HSL is expressed abundantly in adipose tissue and to a less extent in many other tissues including cardiac and skeletal muscles, pancreatic β cells, and steroid-producing glands (22). ATGL is highly expressed in adipose tissues, although ATGL knockout (KO) mice have been shown to develop cardiomyopathy with severe steatosis, suggesting that cardiac ATGL plays an important role in controlling cardiac lipid metabolism (11).

HSL-KO mice have been created and shown to have impaired adipose lipolysis and male infertility (5, 34, 35). In addition, HSL-KO mice have increased diacylglycerol (DAG) content and lipoprotein lipase (LPL) activity in adipose tissue and muscles (12, 13), impaired corticosterone (23, 24) and glucose-stimulated insulin secretion (40, 42), resistance to obesity (16, 43), and muscle insulin resistance (30). Despite these investigations, however, the cardiac phenotype of HSL-KO mice has not been fully elucidated. In the current study, HSL-KO mice were fasted and refed, and the hearts analyzed to explore the impact of impaired lipolysis on cardiac energy metabolism. The results demonstrate how the heart responds to an imbalance in
energy supply and provide insights into the pathogenesis of cardiac lipotoxicity.
EXPERIMENTAL PROCEDURES

Animal study. Heterozygous HSL-KO mice in a C57BL/6 background were bred and homozygous KO mice and wildtype (Wt) littermates aged 6 to 12 months were used for all experiments (35). The mice were maintained on a chow diet (MF, Oriental Yeast Co., Japan) with a 12:12-h dark-light cycle and housed in individual cages 4 weeks prior to the experiments. The mice were fasted for 24 h (9 AM to 9 AM) followed by refeeding ad libitum, and killed at the indicated times. All procedures were performed in accordance with National Institutes of Health guidelines for the care and use of animals, and approved by the guidelines for animal experiments, Fukui University.

Blood Chemistry. Blood was collected when animals were killed (9 AM for fed/fasted, 12 AM for 3h-refed, and 3 PM for 6h-refed). Plasma concentrations of FFA, TAG, total cholesterol (T-Ch) and insulin were measured by commercially-available kits (WAKO or Shibayagi, Japan). Plasma glucose concentration was measured using Freestyle™ (NIPRO, Japan).

Tissue fatty acids uptake. Tissue FA uptake was analyzed by injecting $[^{125}\text{I}]$beta-methyl iodophenyl pentadecanoic acid (BMIPP, Nihon Medi-Physics, Japan) (48). Since plasma FA concentration varies, it was measured just prior to the injection, and $0.1 \mu Ci\ [^{125}\text{I}]$BMIPP/mM FFA/g body weight (BW) was bolus-injected via a tail vein and the specific activity was calculated from the blood samples drawn 2 min after the injection. The mice were killed 20 min after the injection and cardiac ventricle and liver were excised. Tissue radioactivity was measured and tissue FA uptake was calculated using the plasma specific activity. The animal experiments were repeated with a modified method in which animals were injected with a fixed dose of $[^{125}\text{I}]$BMIPP (0.1 $\mu$Ci /g BW), and tissue FA uptake was calculated with variable specific activities. The results were consistent using both methods and representative data are presented.
**Tissue glucose uptake.** Tissue glucose uptake was analyzed as previously described with minor modifications (9). Briefly, 0.1 $\mu$Ci/g BW of $[^{14}C]$D-deoxyglucose (GE Healthcare, UK) was bolus-injected via a tail vein. The mice were killed 40 min after the injection, plasma glucose measured, and cardiac ventricle and liver were excised. The tissues were then dissolved in Solvable™ (Perkin-Elmer, MA) and radioactivity was measured using a liquid scintillation counter. Tissue glucose uptake was calculated from tissue radioactivity and plasma specific activity.

**Tissue VLDL uptake.** VLDL was obtained from rabbits fed a high-cholesterol diet (0.5% w/w) by ultracentrifugation (44). The VLDL was radio-labeled with $[^{125}I]$(GE Healthcare) using IODOBEASE™ (Pierce, IL), purified by 3 passages through desalting columns (Pierce) and dialyzed against EDTA-saline. Approximately 92% of the radioactivity was precipitated by trichloroacetic acid and 50% of the radioactivity was recovered in the water-phase after lipid extraction. Mice were injected with 120,000 cpm/g BW of $[^{125}I]$VLDL and killed 20 min afterwards. Cardiac ventricle and liver were collected to measure their radioactivity. Ninety-five% of the tissue radioactivity was recovered in the water phase after lipid extraction.

**Lipase assays.** Hearts were homogenized in 20 mM Tris, 1 mM EDTA, pH 7.4 containing 255 mM sucrose, 1 $\mu$M leupeptin and 0.1 $\mu$M okadaic acid, and the supernatant was used for the assays. LPL and non-LPL TAG lipase activities were measured as previously described with minor modifications (34, 54). For the LPL assay 60 $\mu$l of the supernatant was incubated at 37°C for 30 min in 200 $\mu$l of a reaction mixture containing 105 $\mu$M tri[3$^3$H]oleoylglycerol (99.4 $\mu$Ci/$\mu$mol), 23.7 $\mu$M lecithin and 4.2% of heated rat serum in 100 mM potassium phosphate buffer (pH 8.0) in the absence or presence of 1 M NaCl. LPL activity was determined by subtracting the activity in 1 M NaCl from the one in the absence of 1 M NaCl. Non-LPL TAG
lipase activity was measured using a substrate used in the LPL assay including 5 mM sodium taurocholate. Sixty μl of the heart samples were incubated in a reaction mixture without rat serum in 1M NaCl and 100 mM potassium phosphate (pH 7.4). Neutral cholesteryl ester hydrolase (NCEH) activity was determined using 100 μl of the heart samples and cholesteryl-[14C]oleate as described previously (21).

_Echocardiography._ Cardiac function was studied by echocardiography on awake mice using ultrasonography equipped with a 13-MHz linear transducer (Prosound α-10, ALOKA, Japan) as previously described (31).

_Langendorff-perfused heart._ Experiments were basically performed as previously reported (29). Briefly, after anesthesia, all mice were treated with sodium heparin (500 USP units/kg intravenously) and hearts were quickly excised and connected to a modified Langendorff apparatus. Each preparation was perfused under constant flow conditions with oxygenated (95% oxygen, 5% CO2) Tyrode solution containing in mM: NaCl, 141.0; KCl, 5.0; CaCl2, 1.8; NaHCO3, 25.0; MgSO4, 1.0; NaH2PO4, 1.2; HEPES, 5; dextrose, 7.0; and 3% FA-free BSA, pH 7.4 at 36°C, and placed in a semi-closed circulating water-warmed (36°C) chamber. Perfusion pressure was measured with a pressure transducer (Nihon Kohden Co, Tokyo, Japan) and maintained within a pressure range (60 to 65 mm Hg) by adjusting flow. To prepare FA-containing Tyrode, 5% vol of 120 mM palmitic acid (PA) in ethanol was slowly added to the Tyrode containing 6% FA-free BSA at 37°C, dialyzed against BSA-free Tyrode for 3 times, 2x diluted and filtered. Final concentration of PA was 2.08 ± 0.03 mM. The PA-free Tyrode was also prepared as 2x concentrated, dialyzed and filtered in parallel. All the hearts were subjected to a 15-min stabilization period, followed by 15 min of PA infusion and then 15 min of washout. Some hearts were manually flushed with 1 ml of BSA-free Tyrode immediately after the PA
infusion ended and snap-frozen to measure cardiac TAG content. The hearts were electrically stimulated at twice the diastolic threshold current with a duration of 1 ms using a polytetrafluoroethylene-coated silver bipolar electrode. Pacing was performed from the epicardial surface of the left ventricular (LV) wall at a basic cycle length of 150 ms. A polyethylene balloon was inserted into the cavity of the LV through the left atrium to measure the LV pressure. The balloon was filled with water to adjust the LV diastolic pressure (LVdP) to approximately 8 mmHg. The LV developed pressure (LVDP) was calculated using the following formula: LVDP = LV systolic pressure minus LVdP.

**Electron microscopy.** Experiments were basically performed as previously described using a transmission electron microscope (Hitachi H-7500) (49).

**Tissue lipid and glycogen content.** The hearts were perfused with 3 ml of PBS from the left ventricle and lipids were extracted from 30-40 mg of the left ventricles. The lipids were separated by thin-layer chromatography (TLC) and analyzed as previously described (46). Cardiac glycogen content was measured by commercially-available kits (BioVision Research Products, CA).

**Gene expression analysis.** Total RNA was extracted from cardiac ventricle using Trizol® reagent (Invitrogen, CA), and reverse-transcribed using Quantitect® reverse transcription kit (Qiagen, Germany). The target genes were amplified and analyzed in triplicate using TaqMan® probes as described previously (49).

**Statistical analysis.** All values are expressed as mean ± SE. Significance was determined by ANOVA followed by Fisher’s protected least significant difference. \( P<0.05 \) was considered significant and the differences between genotypes are indicated in figures unless otherwise noted.
RESULTS

Physiological parameters in fed ad libitum condition. When mice were maintained on a chow diet for longer than 6 months, plasma FA concentration in Wt mice was relatively elevated (0.99 ± 0.08 mmol/L), while it was 40% lower in KO mice (0.61 ± 0.11 mmol/L) compared to Wt mice (Table 1). Plasma concentration of TAG, T-Ch and glucose were comparable in both genotypes, however, plasma insulin concentration was markedly elevated in KO mice, indicating their insulin resistance. Blood pressure (BP) and heart rate (HR) were comparable in both genotypes.

Plasma energy sources in response to fasting and refeeding. To study the impact of fasting and refeeding on circulating energy sources, the mice were fasted for 24 h and then refed for 3 or 6 h. As shown in Fig. 1A, plasma FA concentration was elevated 2-fold in Wt mice with fasting, while KO mice showed a marginal elevation. Plasma TAG concentration in KO mice declined markedly with fasting (96 to 26 mg/dl), while it remained over 100 mg/dl in Wt mice (Fig. 1B). High performance liquid chromatography analysis showed that the decreased TAG was in the VLDL fraction (data not shown). KO mice showed 30% higher plasma glucose concentration in fasted and refed conditions, and marked hyperinsulinemia in fed and refed conditions compared to Wt mice (Fig. 1, D and E). Food and water intake were comparable in both genotypes during the experiments (data not shown).

Cardiac uptake of energy sources. We then asked how hearts of KO mice adapt to insufficient FA supply from the circulation. To answer this, cardiac uptake of the major energy sources, FA, glucose and VLDL, were studied by injecting each radioactive tracer. As shown in Fig. 2A, cardiac FA uptake was increased 2-fold with fasting in Wt mice in parallel to elevated plasma FA concentrations, whereas it was not altered with fasting in KO mice. Cardiac FA uptake during refeeding was lower than the level of the fed condition in both Wt and KO mice. Hepatic FA
uptake was increased 3-fold with fasting in both Wt and KO mice, while there was no significant
difference between the genotypes (Fig. 2D). As shown in Fig. 2B, cardiac glucose uptake was
~95% decreased with fasting in both Wt and KO mice, although there was no significant
difference between the genotypes, indicating that glucose does not compensate for the decreased
FA influx in KO hearts. In contrast to heart, hepatic glucose uptake did not show any changes in
response to fasting and refeeding in either genotype, reflecting the differential expression and
regulation of glucose transporters (GLUT) in heart (GLUT4) and liver (GLUT1 and 2 etc.) (Fig.
2E). Finally, Cardiac VLDL uptake was 2-fold increased in KO mice during fasting (Fig. 2C)
without any changes in liver (Fig. 2F), suggesting that VLDL-TAG potentially compensates for
decreased FA uptake in the fasted KO hearts.

Cardiac function. Echocardiography was performed to investigate whether HSL-disruption
affects cardiac function in response to fasting and refeeding. As shown in Fig. 3, both diastolic
and systolic LV internal diameter (LVID) were increased with fasting in Wt mice (Fig. 3, A and
B), which resulted in a 15% (49% to 42%) decrease in systolic function, indicated by fractional
shortening (FS) (Fig. 3C). In contrast, KO hearts showed unaltered LVID during fasting and
refeeding (Fig. 3, A and B), and FS was even increased with fasting (45% to 48%, Fig. 3C)
despite slower HR compared to Wt mice (Fig. 3D). In addition, systolic BP was elevated with
fasting in KO mice, while it remained unaltered in Wt mice (Fig. 3E). These results indicate that
fasting-induced functional depression, which occurs in Wt mice, is masked in the hearts of
HSL-KO mice. To confirm these findings the effects of FA on LV contractility was also
elucidated in Langendorff-perfused hearts isolated from fasted mice (Fig. 4). Consistent with the
FS observed with echocardiography, KO hearts displayed 54% higher LVDP compared to Wt
hearts (86 vs 56 mmHg) after a 15 min stabilization period of perfusion in the absence of fatty
acids. Interestingly, PA infusion increased LVDP by 55% in Wt hearts while the increase was absent in KO hearts. Cardiac TAG content was barely detectable in Wt mice hearts and below the detectable range in KO mice hearts when the content was measured immediately after the PA infusion ended (data not shown).

**Cardiac steatosis.** Histological changes were investigated with electron microscopy. As shown in Fig. 5B Wt hearts accumulated appreciable lipid droplets (LDs) during fasting, whereas KO hearts had virtually no LDs in either fed or fasted conditions (Fig. 5, D and E). In contrast, KO hearts accumulated numerous LDs at 6 h of refeeding, a time when LDs had disappeared in Wt hearts (Fig. 5, C and F). No other morphological changes were observed in the hearts of either genotype in any of the conditions studied. Compatible with the microscopic findings, cardiac TAG content was increased 2-fold in Wt mice, although it was decreased 2-fold in KO mice during fasting. In contrast, cardiac TAG content in Wt mice was decreased 3-fold during refeeding compared to that during fasting, while TAG in KO mice was increased 5-fold during 3-6h of refeeding compared to that during fasting, and gradually decreased afterwards (Fig. 5G). Cardiac cholesteryl ester (CE) content remained trace amount throughout the experiments in both Wt and KO mice, and cardiac content of FA and free cholesterol (FC) were not altered in KO hearts compared to Wt hearts (data not shown). Cardiac glycogen content was equally decreased with fasting in both genotypes (Fig. 5H). Thus, KO mice displayed distinct cardiac steatosis during refeeding, accompanied by no morphological or functional changes.

**Cardiac lipases.** We then analyzed cardiac hydrolase activities against TAG and CE in various conditions to explore potential mechanisms associated with the steatosis in KO mice. As shown in Fig. 6A, the activity of non-LPL TAG lipase, which involves HSL and ATGL, was comparable during fed and fasted conditions; however, it was 40% lower in KO hearts after 6 h
of refeeding. Cardiac NCEH activity was increased with fasting and remained elevated with refeeding in Wt hearts, although the activity was undetectable during any condition in KO hearts, indicating that HSL totally accounts for cardiac NCEH activity (Fig. 6B). Cardiac LPL is located on the surface of capillary endothelia and hydrolyzes VLDL-TAG to release FA for the heart (41). As shown in Fig. 6C, in parallel with cardiac VLDL uptake, cardiac LPL activity was 2-fold elevated with fasting in KO mice, suggesting that FA from VLDL-TAG might compensate for the energy deprivation in fasted KO mice.

Cardiac gene expressions. The expression of cardiac genes involved in energy metabolism was analyzed to investigate the impact of HSL-disruption. As shown in Fig. 7, the expression of mitochondrial 3-hydroxy-3-methylglutaryl coenzyme A synthase (mHMG-CS), which is known to catalyze ketogenesis, was robustly increased with fasting and refeeding in Wt hearts, whereas this increase was markedly blunted in KO hearts. Similarly, the increased expression of uncoupling protein (UCP)3 with fasting was blunted in KO mice, while the expression of UCP2 showed no significant difference between genotypes. Among the genes related to glucose and lipoprotein metabolism, the expression of GLUT4 was 20 to 40% increased in KO hearts compared with Wt hearts in fasted and refed conditions (Fig. 7), while glycolytic enzymes, including hexokinase 2 and pyruvate kinase, did not show any difference between genotypes (data not shown). The expression of LDL receptor (LDLR) was decreased with fasting and increased with refeeding in both genotypes; however, the increase was markedly blunted in KO mice. The expression of VLDL receptor (VLDLR) was increased with fasting, while there was no difference between genotypes (Fig. 7). The expression of LDLR-related protein 1 (LRP1) and LPL did not show differences between genotypes (data not shown). In a group of genes involved in LD metabolism, the expression of ATGL was 3-fold increased with fasting in both genotypes,
while it was 45% decreased in KO hearts compared to Wt hearts in the 6 h-refed condition. Diacylglycerol acyltransferase (DGAT)2, which catalyzes the final step of TAG synthesis, showed higher expression in KO hearts during fasting and refeeding, and the expression of adipose differentiation-related protein (ADRP), an LD-associated protein, was 60% decreased with fasting, but 2-fold increased with 6 h-refeeding in KO hearts compared to Wt hearts, paralleling the changes in cardiac TAG content. The mRNA expression of mHMG-CS, GLUT4, HSL, LPL, VLDLR, and UCP2 was also analyzed by Northern blotting and consistent results were documented (data not shown).
DISCUSSION

In the current study utilizing HSL-KO mice, we demonstrate how hearts respond to altering energy sources during fasting and refeeding. In Wt mice cardiac systolic function was depressed during fasting, and this was associated with increased FA uptake and steatosis (Figs. 2-5). This phenomenon is compatible with previous reports showing that fasting or caloric restriction leads to cardiac steatosis and decreased cardiac function in humans and mice (14, 15, 53). Increased FA availability would appear to play a role in the decreased systolic function in Wt mice, because, in contrast to Wt mice, KO mice have unaltered cardiac function, which was associated with decreased FA influx and scarce steatosis during fasting. Supporting the impact of FA on decreased cardiac function, it has been reported that a high-fat diet leads to increased FA uptake, TAG accumulation, mitochondrial degeneration, and contractile dysfunction in rodents (36-38). Although other factors, including the autonomic nervous system and cardiac insulin signaling, might be involved (2), given those findings, decreased FA influx is likely to be one of the mechanisms to explain the unaltered cardiac function in fasted KO mice.

Increased systolic function was also observed in isolated perfused hearts of fasted KO mice compared to Wt hearts (Fig. 4). KO hearts also displayed stable LVDP during PA perfusion. These findings are compatible with the results from echocardiography, and might suggest that KO hearts rely on glucose rather than FA as a major energy source during fasting and that cardiac HSL per se plays a distinct role in controlling cardiac energy metabolism. Unexpectedly, PA perfusion increased LVDP in fasted Wt hearts. This phenomenon might be explained by hypothesizing that Wt hearts had become adapted to FA-excess conditions during 24h of fasting, resulting in relative energy deprivation during the stabilization period of the perfusion that was then corrected by the perfused PA supplied. Although PA resulted in a positive effect during this
short period (15 min) of perfusion, longer exposure might have provoked adverse effects. Indeed, PA perfusion induced a significant number of ventricular premature contractions which compelled electrical pacing as previously reported (27). The precise mechanisms for the PA-effects will require further investigation.

It was unexpected that Wt and KO mice would develop cardiac steatosis under quite different conditions; Wt mice developed steatosis during fasting, while KO mice developed steatosis during refeeding (Fig. 5). Since cardiac TAG content is determined by FA uptake, β–oxidation, TAG synthesis and lipolysis, some of these factors were expected to be altered between Wt and KO mice. During fasting, the source of cardiac energy shifts from glucose to FA, and β–oxidation dominates energy production over glucose oxidation (Fig. 2) (20). In fasted Wt mice excess FA are provided from the circulation and unused FA appear to be packaged and stored in LDs. In contrast, in fasted KO mice cardiac FA uptake remains low, whereas glucose uptake is markedly decreased and cardiac workload is even increased (Figs. 2, 3 and 4). Thus, in this setting KO hearts would not have excess FA to be stored in LDs. Moreover, the absence of steatosis in fasted KO hearts suggests that the increased delivery of FA from VLDL-TAG hydrolysis was insufficient to fully replace the high uptake of FFA observed in Wt mice.

A drastic shift of the source of energy from FA to glucose occurred with refeeding, since cardiac FA uptake was depressed below the level of the fed state, while glucose uptake was fully recovered in both genotypes (Fig. 2). Consequently, in Wt hearts the stored TAG in LDs appear to have been hydrolyzed by intracellular lipases and disappeared (Fig. 5). In contrast, KO mice unexpectedly developed marked steatosis with refeeding. Our lipase assays have demonstrated that HSL accounts for virtually 100% of NCEH activity in the heart and ~40% of non-LPL TAG lipase activity in the 6 h-refed condition. The decreased TAG lipase activity was associated with
cardiac steatosis in KO mice during refeeding when cardiac FA and VLDL uptake were unaltered (Fig. 2, 5 and 6). Although cardiac TAG synthesis, β-oxidation and lipoprotein secretion (32) were not directly assessed, the results suggest that the decreased TAG lipase activity might contribute to the development of steatosis in this setting. Similarly, ATGL-KO mice develop overt steatosis associated with 31% lower cardiac lipolytic activity compared to Wt mice (11). Although mechanisms for the steatosis remain to be definitively established, the results suggest a significant function of cardiac HSL in controlling intracellular TAG in concert with ATGL.

The pathophysiological function of LDs or steatosis, i.e., whether they are toxic or protective, has been controversial. It is of note that KO mice have unaltered cardiac function despite overt steatosis during refeeding (Fig. 3C), suggesting that LDs (steatosis) are not necessarily detrimental. Supporting the concept that LDs are protective, Listenberger et al. have reported that oleate (mono-unsaturated FA) inhibits cellular apoptosis by channeling toxic palmitate (saturated FA) into LDs in Chinese hamster ovary cells (25). In addition, Urahara et al. have recently shown that LD-associated proteins ADRP and Tip47 protect renal tubular cells from FA-induced apoptosis by packaging FA in LDs and reducing oxidative stress (52). Although many reports have demonstrated that lipotoxicity can be derived from LDs in certain conditions, i.e., diabetes where lipases are activated (4, 7, 50, 55), our results indicate that steatosis per se is not necessarily detrimental in the absence of aberrant lipolysis, but rather increased FA influx from the circulation might have negative impact for cardiomyoctes.

The energy tracer experiments demonstrate that VLDL-TAG, rather than glucose, compensates for the impaired FA supply from adipose tissue during fasting in KO mice (Fig. 2). Initially we expected that cardiac glucose uptake might be altered in fasted KO mice, because KO mice had elevated plasma glucose concentrations and marked hyperinsulinemia (Fig. 1).
However, cardiac glucose uptake was not altered in KO mice in any condition currently studied compared to Wt mice. The results suggest that KO mice might have severe insulin resistance in the heart, which is compatible with a previous report describing systemic insulin resistance (30). Given the fact that HSL is a potent DAG lipase and KO mice accumulate DAG in the heart (12), activated protein kinase C might be involved in the pathogenesis of cardiac insulin resistance (19). Further investigations are necessary to identify the mechanisms. During fasting KO hearts showed increased VLDL-TAG uptake associated with increased activity of cardiac LPL (Figs. 2C and 6C). Haemmerle et al. have reported that HSL-KO mice have increased LPL activity in skeletal and cardiac muscle, suggesting that they have increased hydrolysis of circulating lipoprotein-TAG in tissues expressing LPL (13). In the current study we have directly shown that cardiac VLDL uptake is increased in fasted KO mice associated with increased activity of cardiac LPL. These results support the concept that activated LPL plays a role in hydrolysis and uptake of VLDL-TAG in fasted KO hearts, and this might explain, at least in part, the marked decrease in plasma TAG concentration observed in KO mice with fasting (Fig. 1B). It is also possible that the activated LPL increased local release of FA which in turn competed with circulating FFA, resulting in lower uptake of FFA as shown in Fig. 2A.

Disruption of HSL affects many cardiac genes related to metabolism (Fig. 7). Among the genes, expression of mHMG-CS and UCP3 was remarkably blunted in KO mice. mHMG-CS catalyzes ketogenesis in response to FA-loading in hepatocytes, and its transcription is known to be regulated by peroxisome proliferator-activated receptor (PPAR)α (17). The mRNA expression of cardiac UCP3 is also known to be regulated by PPARα and its induction with fasting has been shown to be blunted in PPARα-KO mice (39). Thus, the blunted expression of mHMG-CS and UCP3 may also reflect decreased FA influx into HSL-KO hearts. Despite unaltered glucose
uptake, mRNA expression of GLUT4 was increased in fasted and refed conditions in KO hearts. The increase might be a compensatory effect in response to energy deprivation, and might be explained by the decreased FA influx, since FA have been shown to inhibit transcription of GLUT4 in H9C2 cardiomyotubes (1). Interestingly, LDLR mRNA expression was increased in Wt hearts with refeeding, while the increase was blunted in KO hearts (Fig. 7). If intracellular cholesterol content is decreased by HSL-deficiency, LDLR mRNA expression could have been increased, as reported in KO adrenal cells (23). However, the expression was decreased in KO hearts. Since cardiac CE content is trivial and free cholesterol content is unaltered, intracellular cholesterol does not seem to have an impact on cardiac LDLR mRNA expression in KO mice. Instead, a prompt insulin secretion upon refeeding might have stimulated the expression, since insulin has been shown to increase LDLR mRNA expression in cultured hepatic cells (45). If this is the case, the result would be consistent with impaired insulin signaling in the heart of HSL-KO mice.

In summary, the current study demonstrates that inhibiting adipose and cardiac lipolysis potentially ameliorates the negative effects of FA on the heart even in physiological conditions, and steatosis per se is not necessarily detrimental to the heart in the absence of increased lipase activity.
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FIGURE LEGENDS

Fig. 1. Plasma concentration of FFA (A), TAG (B), total cholesterol (C), glucose (D), and insulin (E) during fasting and refeeding experiments. r3h and r6h represent refed for 3 h and 6 h, respectively. *P < 0.05 versus Wt mice. Values are the mean ± SE for 8-9 mice in each group except FFA (n=5).

Fig. 2. Tissue uptake of FA (A and D), glucose (B and E), and VLDL (C and F) in heart and liver. A and D: 0.1 μCi [125I]BMIPP/mM FFA/g BW of BMIPP was bolus-injected intravenously and tissues were collected 20 min after the injection. Tissue FA uptake was calculated from tissue radioactivity, tissue weight and plasma specific activity of [125I]BMIPP. The graph represents 3 independent animal experiments. *P < 0.05 versus Wt mice. Values are the mean ± SE for 5-6 mice in each group. B and E: 0.1 μCi /g BW of [14C]D-deoxyglucose was bolus-injected intravenously and tissues were collected 40 min after the injection. Tissue glucose uptake was calculated from tissue radioactivity, tissue weight and plasma specific activity of [14C]D-deoxyglucose. The graph represents 2 independent animal experiments. *P < 0.05 versus Wt mice. Values are the mean ± SE for 4-5 mice in each group. C and F: 120,000 cpm/g BW of [125I]VLDL was bolus-injected intravenously and tissues were collected 20 min after the injection. Tissue radioactivity was measured by a gamma counter and VLDL uptake is expressed as μg VLDL/tissue weight/h. The graph represents 3 independent animal experiments. *P < 0.05 versus Wt mice. Values are the mean ± SE for 4-6 mice in each group.

Fig. 3. Cardiac function assessed with echocardiogram in fasted and refed (5 h) conditions. A:
Left ventricular internal diameter at end diastole (LVIDd). B: Left ventricular internal diameter at end systole (LVIDs). C: Fractional shortening (FS). D: Heart rate (HR). E: Systolic blood pressure (SBP). F: Diastolic blood pressure (DBP). G: Body weight (BW). *P < 0.05 versus Wt mice. Values are the mean ± SE for 10 male mice except BP and BW (n=7).

Fig. 4. Effects of palmitic acid (PA) on the left ventricular developed pressure (LVDP) in Langendorff-perfused Wt and HSL-KO hearts. A: Representative changes in LVDP during 15 min of 2 mM PA infusion followed by 15 min of washout. B: The time courses of LVDP in the Wt and KO hearts during PA infusion (n = 6 of each) and at 15 min of washout (n = 3 of each). The numbers in parentheses indicate the number of observations at each point. See text for details. ***p < 0.001 vs. value before PA infusion. ††p < 0.01, †††p < 0.001 vs. values at the corresponding duration of PA infusion in Wt. Note that the LVDP before PA infusion was significantly greater in KO hearts compared with WT hearts (p < 0.01).

Fig. 5. Electron microscopy of Wt (A, B, C) and HSL-KO (D, E, F) hearts. Left ventricles were excised in the indicated conditions and fixed with glutaraldehyde and osmium tetroxide. Thin sections (80 nm) were stained with lead citrate and uranyl acetate and investigated with a transmission electron microscope (Hitachi H-7500). Three mice/group, 10 positions/sample were randomly investigated and the representative photographs are presented. Arrows indicate lipid droplets. Magnification ×9,200. G: Cardiac TAG content. The mice were killed in the indicated conditions and hearts were perfused with PBS via the left ventricle. Cardiac lipids were extracted with chloroform:methanol (2:1), separated by TLC, visualized and quantified. r3h, r6h, r9h and r12h represent refed for 3, 6, 9 and 12 h, respectively. The graph represents 2 independent animal
experiments. *$P < 0.05$ versus Wt mice. Values are the mean ± SE for 3 mice in each group. H: Cardiac glycogen content. The hearts were perfused with PBS, homogenized with water, and glycogen content was measured as described in *EXPERIMENTAL PROCEDURES*. r6h represent refed for 6 h. Values are the mean ± SE for 3-4 mice in each group.

Fig. 6. Cardiac activities of non-LPL TAG lipase (*A*), NCEH (*B*) and LPL (*C*) in response to fasting and refeeding. Wt and HSL-KO mice were killed in the indicated conditions and heart homogenate was used for the assays as described in *EXPERIMENTAL PROCEDURES*. r3h and r6h represent refed for 3 h and 6 h, respectively. The graph represents 3 (non-LPL TAGL and LPL) or 2 (NCEH) independent animal experiments. *$P < 0.05$ versus Wt mice. Values are the mean ± SE for 4-5 mice in each group.

Fig. 7. Cardiac expression of genes related to FA, glucose, lipoprotein, and lipid droplet metabolism. The mRNA expressions were analyzed by RT-qPCR and normalized to GAPDH. The relative expressions are presented against fed Wt mice. 3h and 6h represent refed for 3 h and 6 h, respectively. *$P < 0.05$ between genotypes. Values are the mean ± SE for 4 mice in each group.
### TABLE 1.

Physiological parameters in Wt and HSL-KO mice in fed *ad libitum* condition

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wt (n)</th>
<th>KO (n)</th>
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</thead>
<tbody>
<tr>
<td><strong>Body weight (g)</strong></td>
<td></td>
<td></td>
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<tr>
<td>(male)</td>
<td>33.5 ± 1.6 (11)</td>
<td>33.4 ± 0.9 (11)</td>
</tr>
<tr>
<td>(female)</td>
<td>27.7 ± 0.9 (11)</td>
<td>26.4 ± 0.7 (11)</td>
</tr>
<tr>
<td><strong>Heart rate (BPM)</strong></td>
<td>669 ± 11 (10)</td>
<td>674 ± 9 (10)</td>
</tr>
<tr>
<td><strong>Systolic blood pressure (mmHg)</strong></td>
<td>114 ± 11 (8)</td>
<td>89 ± 10 (7)</td>
</tr>
<tr>
<td><strong>Diastolic blood pressure (mmHg)</strong></td>
<td>74 ± 11 (8)</td>
<td>75 ± 11 (7)</td>
</tr>
<tr>
<td><strong>Plasma concentration</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free fatty acids (mmol/L)</td>
<td>0.99 ± 0.08 (5)</td>
<td>0.61 ± 0.11* (5)</td>
</tr>
<tr>
<td>Triacylglycerol (mg/dl)</td>
<td>124 ± 12 (8)</td>
<td>96 ± 9 (8)</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>89 ± 3 (8)</td>
<td>104 ± 6 (8)</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>148 ± 9 (8)</td>
<td>134 ± 9 (8)</td>
</tr>
<tr>
<td>Insulin (ng/mL)</td>
<td>0.6 ± 0.2 (8)</td>
<td>5.7 ± 2.9* (8)</td>
</tr>
</tbody>
</table>

Values are the mean ± SE. Each parameter consists of the data from both genders.

*P < 0.05 versus Wt mice.
<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Wt (n=7) KO (n=7)</td>
<td>Wt (n=7) KO (n=7)</td>
</tr>
<tr>
<td><strong>Food intake (g)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad libitum 24h</td>
<td>1.99 ± 0.69</td>
<td>3.22 ± 0.44</td>
</tr>
<tr>
<td>Fasting 24h</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Refeeding 0-3h</td>
<td>0.42 ± 0.18</td>
<td>0.65 ± 0.12</td>
</tr>
<tr>
<td>Refeeding 3-6h</td>
<td>0.29 ± 0.13</td>
<td>0.88 ± 0.43</td>
</tr>
<tr>
<td>Refeeding 6-24h</td>
<td>3.35 ± 0.74</td>
<td>3.21 ± 0.23</td>
</tr>
<tr>
<td>Refeeding 0-24h</td>
<td>4.06 ± 0.71</td>
<td>4.74 ± 0.57</td>
</tr>
<tr>
<td><strong>Water intake (ml)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad libitum 24h</td>
<td>2.72 ± 0.65</td>
<td>3.20 ± 0.87</td>
</tr>
<tr>
<td>Fasting 24h</td>
<td>1.20 ± 0.34</td>
<td>1.13 ± 0.20</td>
</tr>
<tr>
<td>Refeeding 0-3h</td>
<td>1.11 ± 0.32</td>
<td>1.60 ± 0.16</td>
</tr>
<tr>
<td>Refeeding 3-6h</td>
<td>0.63 ± 0.20</td>
<td>0.86 ± 0.16</td>
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<tr>
<td>Refeeding 6-24h</td>
<td>4.17 ± 0.16</td>
<td>4.12 ± 0.27</td>
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<tr>
<td>Refeeding 0-24h</td>
<td>5.92 ± 0.46</td>
<td>6.58 ± 0.27</td>
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Values are the mean ± SE. There is no significant difference between genotypes.
Fig. 1
Fig. 2
Fig. 3
Fig. 4

A

LVDP

PA  Washout

Wt

KO

5 min

100 mmHg

B

LVDP before PA
Wt = 56 ± 5 mmHg
KO = 86 ± 5 mmHg

% changes in LVDP

0 10 20 30 40 50 60 70

0 5 10 15 15 min

Duration of PA infusion (min) After washout

Wt  KO

***  ***  ††  †††  †††
Fig. 5
Fig. 6
Fig. 7

Lipid droplet metabolism

Fatty acid metabolism

Glucose/ lipoprotein metabolism

Relative Expression

mHMG-CS

UCP2

UCP3

Relative Expression

GLUT4

LDLR

VLDLR

Relative Expression

HSL

ATGL

DGAT2

ADRP

Relative Expression

* * *