Fatty acid oxidation in health and disease
van Weeghel, M.

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Fasting-induced myocardial lipid accumulation in long-chain acyl-CoA dehydrogenase knockout mice is accompanied by impaired left ventricular function

Adrianus J. Bakermans¹, Tom R. Geraedts¹, Michel van Weeghel², Simone Denis², Maria João Ferraz⁴, Johannes M.F.G. Aerts⁴, Jan Aten⁵, Klaas Nicolay¹, Sander M. Houten²,³,⁴, Jeanine J. Prompers¹,*

¹Biomedical NMR, Department of Biomedical Engineering, Eindhoven University of Technology, PO Box 513, 5600 MB Eindhoven, The Netherlands; ²Department of Clinical Chemistry, Laboratory Genetic Metabolic Diseases, ³Department of Pediatrics, Emma Children’s Hospital; ⁴Department of Medical Biochemistry; and the ⁵Department of Pathology, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands

*Authors contributed equally to this work

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Abstract

*Background*—Lipotoxicity may be a key contributor to the pathogenesis of cardiac abnormalities in mitochondrial long-chain fatty acid oxidation (FAO) disorders. Few data are available on myocardial lipid levels and cardiac performance in FAO deficiencies. The purpose of this animal study is to assess fasting-induced changes in cardiac morphology, function, and triglyceride (TG) storage as a consequence of FAO deficiency in a noninvasive fashion.

*Methods and Results*—MRI and proton magnetic resonance spectroscopy ($^1$H-MRS) were applied in vivo in long-chain acyl-CoA dehydrogenase (LCAD) knockout (KO) mice and wild-type (WT) mice (n=8 per genotype). Fasting was used to increase the heart’s dependency on FAO for maintenance of energy homeostasis. In vivo data were complemented with ex vivo measurements of myocardial lipids. Left ventricular (LV) mass was higher in LCAD KO mice compared with WT mice (P<0.05), indicating LV myocardial hypertrophy. Myocardial TG content was higher in LCAD KO mice at baseline (P<0.001) and further increased in fasted LCAD KO mice (P<0.05). Concomitantly, LV ejection fraction (P<0.01) and diastolic filling rate (P<0.01) decreased after fasting, whereas these functional parameters did not change in fasted WT mice. Myocardial ceramide content was higher in fasted LCAD KO mice compared with fasted WT mice (P<0.05).

*Conclusions*—Using a noninvasive approach, this study reveals accumulation of myocardial TG in LCAD KO mice. Toxicity of accumulating lipid metabolites such as ceramides may be responsible for the fasting-induced impairment of cardiac function observed in the LCAD KO mouse.
**Introduction**

The heart has a high energy demand, and ATP must be continuously available for the myocardium in order to maintain its contractile function. The main fuel for cardiac ATP production are long-chain fatty acids, which are degraded through mitochondrial fatty acid β-oxidation (FAO) (Neubauer, 2007). Recessively inherited defects are known for most of the enzymes involved in FAO. Neonates and infants affected by long-chain FAO disorders may present with hypoketotic hypoglycemia, hypertrophic cardiomyopathy, arrhythmia, and sudden death (Houten and Wanders, 2010; Rinaldo et al., 2002). Neonatal screening for FAO disorders can be effective in reducing death and serious adverse events in patients, as management plans to avoid periods of fasting generally prevent hypoketotic hypoglycemia (Wilcken et al., 2007). Current interventions used to prevent cardiac disease in FAO disorders are based on expert opinion and are not evidence-based (Spiekerkoetter and Mayatepek, 2010). In order to critically assess these interventions and to design novel therapeutic strategies, better understanding of the etiology of cardiac disease in FAO disorders is instrumental.

In obese and diabetic patients, an increased supply of fatty acids is responsible for an imbalance between fatty acid import and utilization. The resulting elevated myocardial lipid levels are associated with decreased cardiac function (Rijzewijk et al., 2008; Szczepaniak et al., 2003), suggesting that myocardial lipid accumulation may be detrimental for the heart. Due to a genetic defect in FAO, lipids may accumulate as well, and their toxicity may be a key contributor to the pathogenesis of cardiac abnormalities found in FAO disorders. Previously, histological and biochemical analyses revealed elevation of myocardial lipid levels and cardiac hypertrophy in mouse models of FAO disorders (Cox et al., 2001; Cox et al., 2009; Exil et al., 2003; Kurtz et al., 1998). Until now, few data are available on myocardial lipid levels in combination with measurements of cardiac performance in models for FAO defects.

MRI is the modality of choice to accurately assess cardiac morphology and function due to its excellent soft tissue contrast combined with the relatively high spatial and temporal resolution needed for small animal studies. Proton magnetic resonance spectroscopy (\(^{1}\)H-MRS) is a powerful tool to non-invasively assess mobile intracellular lipid pools, predominantly present as triglyceride (TG) droplets in tissues such as skeletal muscle and liver (Machann et al., 2008). \(^{1}\)H-MRS of the mouse heart was introduced as a tool to study myocardial creatine levels in mice (Schneider et al., 2004). Recently, this approach was used to examine myocardial lipid levels in mice predisposed to cardiac steatosis. Remarkably, the lipid signal as detected by \(^{1}\)H-MRS in this study was not exclusively attributable to TG (Hankiewicz et al., 2010; Ruberg, 2010).

The purpose of this animal study is to examine, for the first time, changes in cardiac morphology, function, and TG storage as a consequence of FAO deficiency. MRI and \(^{1}\)H-MRS were applied in vivo in long-chain acyl-CoA dehydrogenase (LCAD) knock-out (KO) mice and wild type (WT) C57BL/6 mice. In the mouse, LCAD plays an essential role in long-chain FAO, and catalyzes the first of four enzymatic reactions that form the mitochondrial FAO spiral (Chegary et al., 2009). The LCAD KO mouse model displays a phenotype similar to human very long-chain acyl-CoA dehydrogenase deficiency (VLCADD) (Chegary et al., 2009; Cox et al., 2001; Kurtz et al., 1998). Fasting was used to further increase the heart’s dependency on FAO for ATP production. We hypothesized that due to the deficient FAO in LCAD KO mice, fasting would increase myocardial TG content and decrease cardiac performance.
Experimental procedures

Animals
Heterozygous LCAD KO mice (B6.129S6-Acadl^tm1UAB/Mmmh) (Kurtz et al., 1998) on a pure C57BL/6 background were obtained from Mutant Mouse Regional Resource Centers. Male LCAD KO and WT mice were generated via heterozygote as well as homozygote breeding pairs and were backcrossed 15 times with C57BL/6. Mice (n=8 per genotype) were housed at 21±1 ºC, 40-50% humidity, on a 12 h light-dark cycle, with ad libitum access to water and a standard rodent diet (ssniff Spezialdiäten, Soest, Germany). At 13 weeks of age, baseline data were acquired in the fed state as described below. Within two weeks, mice were weighed, placed solitary in a clean cage without food, but with access to water, and fasted for 24 h, followed by measurements in the fasted state. Immediately thereafter, anesthetized mice were euthanized by exsanguination from the vena cava inferior. The heart was rapidly excised, weighed, and processed for biochemical and histological analysis. All procedures were approved by the Animal Ethics Committee of Maastricht University (Maastricht, The Netherlands).

MR protocol
Mice were anesthetized with 2% isoflurane in 0.4 L/min medical air and positioned supine in a purpose-built support cradle. Anesthesia was maintained by administering 1.6% isoflurane in a continuous flow of 0.4 L/min medical air through a nose cone. Body temperature was maintained at 37±0.5 ºC using a warm water flow integrated in the setup, and monitored with a rectal fiber optic probe. ECG electrodes were placed on the front paws and a respiratory sensor balloon was taped onto the abdomen. Vital signs were monitored and used for MR gating/triggering by the SA Monitoring & Gating System (Model 1025, Small Animal Instruments, NY, USA). The cradle was positioned into a horizontal-bore 9.4 T magnet (Magnex Scientific, Oxon, UK) interfaced to a Bruker Avance III console (Bruker Biospin MRI, Ettingen, Germany) and controlled by the ParaVision 5.0 software package (Bruker Biospin). A quadrature driven birdcage coil (Ø 35 mm, Bruker Biospin) was used for radiofrequency (RF) transmission and signal reception.
A segmented, prospective cardiac-triggered respiratory-gated FLASH sequence was used to acquire cinematographic MR image series of 14-16 frames per cardiac cycle. Six 1-mm contiguous left-ventricular (LV) short-axis slices were complemented with 4- and 2-chamber long-axis views. Imaging parameters: field of view=30x30 mm2; matrix=192x192; echo time (TE)=1.8 ms; repetition time (TR)=7 ms; flip angle=15º, number of averages (NA)=6. To assess diastolic function, 22-26 frames of the equatorial short-axis view were acquired with a higher temporal resolution (TR=4.8 ms; matrix=128x128) and an increased flip angle (30º) for enhanced contrast between LV cavity and myocardial wall (Wiesmann et al., 2001). Immediately after MR imaging, localized 1H-MRS was performed using a cardiac-triggered respiratory-gated point resolved spectroscopy (PRESS) sequence.(Schneider et al., 2004) A 4 μL voxel was positioned in the diastolic interventricular septum, avoiding contamination of the spectra with signal originating from pericardial lipid deposits. PRESS parameters: TE=9.1 ms; TR=2 s; 0.41 ms 90º Hermite-shaped excitation pulse; 0.9 ms 180º Mao-type refocusing pulses. Triggering was timed at ~75% of the cardiac period after ECG R-wave upslope detection. Volume selective shimming of the voxel of interest was done manually using the same PRESS sequence, until a water line width less than 40 Hz was achieved. Water suppression was performed by preceding the PRESS sequence with a cardiac-triggered
respiratory-gated chemical shift selective (CHESS) module consisting of three frequency-selective RF pulses (bandwidth=250 Hz) each followed by spoiler gradients in orthogonal directions. Total CHESS duration was 90.8 ms. Steady state of magnetization, required for accurate quantification of metabolite levels (Schneider et al., 2004), was maintained by applying dummy excitations during respiratory gates, allowing a window of <30 ms for trigger detection. Water-suppressed spectra were acquired on-resonance on creatine (1.71 parts per million (ppm) upfield of water; NA=256; acquisition time ~13 min). As a quantification reference, water spectra (NA=32) were acquired on-resonance on water with the RF pulses for water suppression turned off.

**Image analysis**

LV cavity and wall volumes were determined by semi-automatic segmentation (PIE Medical Imaging, Maastricht, The Netherlands) (Heijman et al., 2008). LV mass was calculated by multiplying the end-diastolic myocardial wall volume with a myocardial density of 1.05 g/mL (Manning et al., 1994). End-diastolic volume (EDV), end-systolic volume (ESV), stroke volume (SV), and ejection fraction (EF) were calculated as described previously (Heijman et al., 2008). Cardiac output (CO) was calculated by multiplying the SV with the average heart rate. Peak ejection rate and peak filling rate were determined from the high temporal resolution cine series as respectively the minimum and maximum in the time-derivative of the LV cavity volume (Wiesmann et al., 2001).

**Spectral analysis**

Spectral fitting was performed using AMARES in jMRUI (Vanhamme et al., 1997). The unsuppressed water spectrum was corrected for eddy-current distortions, after which DC offset was removed. The water peak was fitted to a Lorentzian line shape. The water-suppressed spectrum was phased using the corresponding unsuppressed water spectrum as a reference. The TG methylene resonance at 1.28 ppm was used as internal chemical shift reference. Peaks of metabolites were assigned according to reports in literature (Griffin et al., 2001) and fitted to Gaussian line shapes. The metabolite concentrations were expressed as a percentage of the unsuppressed water signal measured in the same voxel.

**Ex vivo myocardial lipid measurements**

Total TG content was determined biochemically from myocardial tissue collected immediately after the MR measurements in the fasted state. Tissue (50-100 mg wet weight) was homogenized on ice in 2.5 mL Dole’s extraction mixture (isopropanol:heptane:1 mol/L H$_2$SO$_4$; 40:10:1) using a dispersion tool (Ika T10 basic). (Dole, 1956) Next, 1.5 mL heptane and 0.6 mL 0.4 mol/L MOPS buffer (pH 6.4) was added, followed by thorough mixing and centrifugation (4 ºC, 3000 g). The upper phase was collected and the lower phase was extracted again with 1.5 mL heptane. Upper phases were pooled and evaporated to dryness. The residue was dissolved in 1 mL chloroform containing 1% Triton-X100, followed by evaporation to dryness and resuspension in 0.25 mL water. This solution was used for colorimetric quantification of total TG (Triglycerides LiquiColor, Instruchemie, Delfzijl, The Netherlands).

As the in vivo 1H-MRS measurements do not allow for a detailed analysis of lipid composition, we measured the total fatty acid profile. Myocardial tissue was homogenized in PBS using a dispersion tool. After sonication (twice at 8 W output, 40 J, on ice), the protein concentration was measured using the bicinchoninic acid assay. All samples were diluted to a protein concentration of 10 mg/mL. Fatty acids from a 500 μg protein sample were directly
transesterified and analyzed by gas chromatography with flame ionization detection (GC-FID) (Chegary et al., 2009; Dacremont and Vincent, 1995). Myocardial ceramide levels were determined as described previously (Groener et al., 2007).

**Histology**
To detect neutral lipids, 4-μm thick cryostat sections of frozen tissue were stained with 0.3% Oil Red O (BDH, Poole, UK). After washing, sections were counterstained with hematoxylin. The sections were analyzed by light microscopy. Multispectral imaging was performed using a Nuance N-MSI-420-20 camera with Nuance 3.0 software (Cambridge Research & Instrumentation, Woburn, MA, USA). Data sets were acquired at 420-720 nm at 10 nm intervals. Spectral libraries for hematoxylin and Oil Red O, each obtained from single-stained cells, were used to unmix the staining patterns into the individual components and to separate these from background. The fraction of Oil Red O-stained surface of tissue section surface was quantified using Image-Pro 7.0 software (Media Cybernetics, Bethesda, MD, USA).

**Statistical analysis**
Statistical analyses were performed using SPSS 17.0 (SPSS, Chicago, IL, USA). Statistical significance of fasting and genotype effects on in vivo MR data were assessed using analysis of variance (ANOVA) for repeated measures, with one between-subjects factor (genotype) and one within-subjects factor (fasting). If the interaction term between the factors was significant, the effects of genotype and fasting were analyzed separately using two-sided unpaired and paired t-tests, respectively. The effect of genotype on ex vivo determined lipid content was determined using a two-sided t-test. Relations between variables were assessed by Pearson r correlation coefficients. All data are presented as mean±standard deviation (SD), with the level of significance set at P<0.05.

**Results**

**Left ventricular morphology and function**
We assessed cardiac LV morphology and function from cine MR images acquired in fed and fasted LCAD KO mice and controls (figure 1, Table 1). At baseline, body weight was similar for both genotypes. During 24 h fasting, body weight decreased by 17% compared to body weight at the start of fasting. LV mass was 10% higher in fed LCAD KO mice compared to fed WT mice (P<0.05), indicating mild LV myocardial hypertrophy. Minor loss of LV mass (-7%, P<0.01) was observed in fasted WT mice, whereas LV mass of LCAD KO mice was unaffected by fasting. EDV was 14% lower in fed LCAD KO mice than in fed WT mice (P<0.05). After fasting, EDV decreased (-10%, P<0.01) in WT mice, while ESV remained the same in fasted WT mice. In contrast, ESV increased in fasted LCAD KO mice (+54%, P<0.05), whereas EDV did not change in fasted LCAD KO mice. Peak ejection rate, SV, and CO were similar in both genotypes, and were not affected by fasting. In the fed state, EF was not different between genotypes. After fasting, EF was preserved in WT mice, but decreased in LCAD KO mice (-13%, P<0.01). Additionally, in LCAD KO mice, peak filling rate was significantly reduced by fasting (-23%, P<0.01), indicating decreased LV diastolic function. Combined, these data reveal impaired LV performance in the fasted LCAD KO mouse.
Chapter 5

Table 1: Animal characteristics, LV morphology, and LV function of wild type and LCAD KO mice in fed conditions and after 24 h fasting (N=8/group)

<table>
<thead>
<tr>
<th></th>
<th>Fed</th>
<th>LCAD KO</th>
<th>Fed</th>
<th>LCAD KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (weeks)</td>
<td>13.8 ± 0.5</td>
<td>13.0 ± 0.4</td>
<td>15.4 ± 0.2</td>
<td>14.9 ± 0.2</td>
</tr>
<tr>
<td>Body weight (g)****</td>
<td>29.9 ± 1.7</td>
<td>29.7 ± 0.8</td>
<td>25.3 ± 0.8</td>
<td>24.8 ± 1.1</td>
</tr>
<tr>
<td>Heart mass at autopsy (mg)</td>
<td>120.5 ± 10.2</td>
<td>132.3 ± 12.2</td>
<td>122.9 ± 5.3</td>
<td>151.6 ± 7.3‡‡‡</td>
</tr>
<tr>
<td>LV mass (mg)</td>
<td>77.5 ± 5.9</td>
<td>66.4 ± 9.4‡</td>
<td>112.3 ± 11.7§§</td>
<td>138.5 ± 9.2‡‡‡</td>
</tr>
<tr>
<td>End-diastolic volume (μL)</td>
<td>24.9 ± 4.5</td>
<td>19.4 ± 3.31</td>
<td>21.8 ± 4.4</td>
<td>29.9 ± 7.51, §</td>
</tr>
<tr>
<td>Stroke volume (μL)</td>
<td>52.6 ± 3.1</td>
<td>47.0 ± 7.4</td>
<td>48.3 ± 5.0</td>
<td>47.1 ± 6.6</td>
</tr>
<tr>
<td>Ejection fraction (%)</td>
<td>68.0 ± 3.9</td>
<td>70.6 ± 3.7</td>
<td>69.1 ± 3.7</td>
<td>61.7 ± 4.7‡‡, §§</td>
</tr>
<tr>
<td>Heart rate (1/min)</td>
<td>526 ± 49</td>
<td>512 ± 57</td>
<td>511 ± 57</td>
<td>509 ± 43</td>
</tr>
<tr>
<td>Cardiac output (mL/min)</td>
<td>27.7 ± 3.1</td>
<td>24.0 ± 4.0</td>
<td>24.5 ± 2.4</td>
<td>23.9 ± 3.3</td>
</tr>
<tr>
<td>Peak ejection rate (mL/s)</td>
<td>0.30 ± 0.04</td>
<td>0.31 ± 0.03</td>
<td>0.31 ± 0.04</td>
<td>0.27 ± 0.03</td>
</tr>
<tr>
<td>Peak filling rate (mL/s)</td>
<td>0.25 ± 0.01</td>
<td>0.26 ± 0.02</td>
<td>0.22 ± 0.02</td>
<td>0.20 ± 0.01§§</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD. General fasting effect: ***, P<0.001. If interaction was significant, the results of two-sided t-tests are given: ‡, P<0.05; ‡‡, P<0.01; ‡‡‡, P<0.001 vs. WT, §, P<0.05; §§, P<0.01 vs. fed.

FIGURE 1. Equatorial short-axis MR images at end-diastole from a WT mouse (A) and a LCAD KO mouse (B) in fed condition. Note the increased left ventricular (LV) wall thickness in the LCAD KO mouse, indicating LV hypertrophy. (C) LV cavity volume in the equatorial short-axis slice plotted against time after ECG R-wave upslope detection for a WT mouse, before and after fasting. (D) LV cavity volume-time curves for a LCAD KO mouse. (E) LV ejection fraction. **, P<0.01 vs. WT, §§, P<0.01 vs. fed.
Chapter 5

In vivo myocardial metabolite levels

In order to assess myocardial metabolite levels, we applied in vivo localized ¹H-MRS to the mouse heart. Several resonances can be distinguished in the water-suppressed ¹H-MR spectra (figure 2). Concentrations of nine metabolites were quantified relative to the unsuppressed water signal obtained from the same voxel (Table 2). At baseline, the prominent TG methylene signal at 1.28 ppm was higher in LCAD KO myocardium than in controls, indicating elevated myocardial TG levels in fed LCAD KO mice. Importantly, the TG methylene signal strongly increased in fasted LCAD KO mice (+63±56%, P<0.05), whereas this signal decreased after fasting in WT mice (-40±33%, P<0.05). Other TG associated peaks showed a similar pattern, such as those from the α-methylene protons (CH₃COO, 2.21 ppm) and the allylic methylene protons (CH₂=CH-CH-CH₂, 1.99 ppm). The residual water peak at 4.7 ppm hampered accurate quantification of nearby peaks. Consequently, the resonances originating from the glycerol backbone at 4.1 ppm and the olefinic protons (CH=CH) at 5.3 ppm could not be quantified. Nevertheless, the peak originating from olefinic protons was more prominent in the spectra obtained from LCAD KO myocardium, illustrating that the increased TG methylene signal was paralleled by increased signal associated with unsaturated bonds in LCAD KO myocardial TG. This observation is corroborated by a higher signal of allylic methylene in LCAD KO mice, which increased further upon fasting. Taurine, choline/carnitine, and total creatine levels were similar in both genotypes, and were not affected by fasting. These data show that in WT mice, myocardial TG pools are depleted during fasting, while in LCAD KO mice, myocardial TG levels are elevated in fed conditions and further increase upon fasting.

Table 2: Myocardial metabolite concentrations as a percentage of the total water signal.

<table>
<thead>
<tr>
<th>Chemical Shift (δ)</th>
<th>Fed (ppm)</th>
<th>WT (n=8)</th>
<th>Fed LCAD KO (n=8)</th>
<th>WT (n=8)</th>
<th>Fasted LCAD KO (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taurine (1)</td>
<td>3.39</td>
<td>0.10±0.03</td>
<td>0.13±0.07</td>
<td>0.13±0.03</td>
<td>0.12±0.03</td>
</tr>
<tr>
<td>Choline/carnitine (2)</td>
<td>3.21</td>
<td>0.13±0.06</td>
<td>0.15±0.08</td>
<td>0.20±0.05</td>
<td>0.15±0.07</td>
</tr>
<tr>
<td>Creatine -CH₃ (3)</td>
<td>2.99</td>
<td>0.08±0.04</td>
<td>0.12±0.08</td>
<td>0.12±0.05</td>
<td>0.08±0.03</td>
</tr>
<tr>
<td>TG CH=CH-CH CH (4)</td>
<td>2.72</td>
<td>0.05±0.02</td>
<td>0.08±0.04</td>
<td>0.07±0.02</td>
<td>0.08±0.03</td>
</tr>
<tr>
<td>TG CH₃COO (5)</td>
<td>2.21</td>
<td>0.08±0.03</td>
<td>0.13±0.04</td>
<td>0.07±0.02</td>
<td>0.19±0.06†††, §</td>
</tr>
<tr>
<td>TG CH₃CH=CH CH (6)</td>
<td>1.99</td>
<td>0.12±0.03</td>
<td>0.21±0.06†</td>
<td>0.13±0.05</td>
<td>0.31±0.09†††, §</td>
</tr>
<tr>
<td>TG CH₃COO (7), ††</td>
<td>1.57</td>
<td>0.06±0.02</td>
<td>0.11±0.04</td>
<td>0.07±0.03</td>
<td>0.17±0.08</td>
</tr>
<tr>
<td>TG -CH₂ (8)</td>
<td>1.28</td>
<td>0.65±0.16</td>
<td>1.10±0.20†††</td>
<td>0.39±0.16§</td>
<td>1.79±0.60†††, §</td>
</tr>
<tr>
<td>TG -CH₃ (9)†</td>
<td>0.84</td>
<td>0.14±0.05</td>
<td>0.24±0.11</td>
<td>0.14±0.04</td>
<td>0.27±0.08</td>
</tr>
</tbody>
</table>

Chemical shift (δ) given in ppm (parts per million). Numbers in parentheses refer to the peaks assigned in Figure 2. Data are expressed as mean±SD. General fasting effect: *, P<0.05. General genotype effect: †, P<0.05; ††, P<0.01. If interaction was significant, the results of two-sided t-tests are given: ‡, P<0.05; ‡‡, P<0.01; ‡‡‡, P<0.001 vs. WT, §, P<0.05 vs. fed.

Ex vivo myocardial lipid analysis

We compared the results obtained with ¹H-MRS to biochemical TG measurement. As shown in figure 3, TG methylene levels measured in vivo with ¹H-MRS correlated with the biochemical measurements of total TG (r=0.91, P<0.0001). Corresponding to in vivo observations, total myocardial TG in fasted LCAD KO mice was higher than in fasted WT mice (P<0.01). Histology confirmed the accumulation of lipid droplets in cardiomyocytes of fasted LCAD KO mice as compared to fasted WT mice, indicating development of microvesicular steatosis (figure 4).

Next, GC-FID was used to obtain total fatty acid profiles. Total myocardial fatty acid content was significantly higher in fasted LCAD KO mice compared to fasted WT mice (LCAD KO,
FIGURE 2. Examples of myocardial 1H-MR spectra obtained in vivo from a WT mouse (A) and a LCAD KO mouse (B) in fed condition and after 24 h fasting. Spectra are scaled equally. Metabolite peaks were assigned according to literature. Taurine, 3.39 ppm; choline/carnitine, 3.21 ppm; creatine -CH3, 2.99 ppm; triglyceride CH=CH-CH2-CH=CH, 2.72 ppm; TG CαH2COO, 2.21 ppm; TG CH2-CH=CH-CH2, 1.99 ppm; TG CβH2CH2COO, 1.57 ppm; TG -(CH2)n-, 1.28 ppm; TG -CH3, 0.84 ppm. Glc, composite resonances from the glycerol backbone, glucose and other carbohydrates; TG, triglyceride. (C) Myocardial TG derived from the methylene peak. ***, P<0.001 vs. WT, §, P<0.05 vs. fed.

FIGURE 3. Regression analysis shows good correlation (r=0.91, P<0.0001) between myocardial triglyceride levels as measured in vivo with 1H-MRS, and ex vivo with a standard biochemical assay in WT and LCAD KO mice after 24 h fasting. Solid line represents the linear fit.
570.9±104.8 nmol/mg protein versus WT, 407.5±47.8 nmol/mg protein; P<0.01). The relative contributions of both saturated (SFA) and polyunsaturated fatty acids (PUFA) to the total myocardial fatty acid content were slightly lower, whereas the relative content of monounsaturated fatty acid (MUFA) was higher in LCAD KO mice (figure 5A). Detailed analysis of the individual fatty acid species in the myocardium revealed a prominent, 2.5 fold higher level of oleate (C18:1[n-9]) in LCAD KO mice (P<0.001), and a mild elevation of linoleate (C18:2[n-6]) (P<0.05). Notably, of all fatty acid species, only docosahexaenoic acid (DHA, C22:6[n-3]) content was lower in the LCAD KO myocardium, both in absolute terms (P<0.05), and relative to the total myocardial fatty acid content (P<0.0001, figure 5B). Downstream of oleate and linoleate in the FAO pathway are C14:1[n-9] and C14:2[n-6], respectively. These fatty acids were strongly elevated in the LCAD KO myocardium (P<0.0001, figure 5B), although the absolute levels were low. These elevations are paralleled by prominently increased levels of the corresponding C14:1 and C14:2 acylcarnitines in blood, plasma, and tissue samples of LCAD KO mice as reported previously.(Chegary et al., 2009; Cox et al., 2001; Kurtz et al., 1998) Taken together, these data underscore the essential role of LCAD in the oxidation of oleate and linoleate.

In order to establish whether increased TG accumulation coincides with an increase in lipotoxic compounds, we measured myocardial ceramide levels. Ceramide content was elevated in fasted LCAD KO mice compared to fasted WT mice (P<0.05, figure 5C).

**Discussion**

An imbalance between fatty acid utilization and supply may lead to cardiac steatosis. As such, excessive lipid accumulation has been proposed as a contributor to the pathogenesis of cardiac abnormalities in long-chain FAO disorders. Until now, limited data were available on the effects of impaired FAO on myocardial performance and lipid accumulation. We assessed cardiac morphology, function, and myocardial TG storage of WT and LCAD KO mice using non-invasive MR methods. Previous studies have shown that the LCAD KO mouse phenotype resembles human VLCADD, and that it is currently the best preclinical model for long-chain FAO disorders (Chegary et al., 2009; Cox et al., 2001; Cox et al., 2009; Kurtz et al., 1998). We show that fasting decreased myocardial TG content in WT mice, while cardiac performance was not affected. In contrast, elevated levels of myocardial TG in fasted LCAD KO mice were accompanied by decreased LV EF and reduced peak filling rate. Cardiac steatosis was confirmed by histology, revealing prominent accumulation of lipid droplets in cardiomyocytes of fasted LCAD KO mice. The MR images obtained in fed conditions illustrate cardiac hypertrophy and maintenance of systolic cardiac performance in LCAD KO mice compared to WT mice, confirming previous M-mode echocardiography measurements (Cox et al., 2009). In addition, we show that, due to an increase of ESV, EF decreases in fasted LCAD KO mice, which suggests that fasting induces contractile dysfunction of the LCAD KO myocardium. No quantitative measures of diastolic function were previously reported. Here, we show that diastolic performance, in terms of peak filling rate, is similar for both genotypes in the fed state, but decreases after 24 h fasting in the LCAD KO mice. Myocardial fibrosis, previously reported for 10% of 14-16 week old male LCAD KO mice (Kurtz et al., 1998), may alter LV filling rates (Jellis et al., 2010). It is unlikely that fibrotic tissue will develop within a 24 h fasting period, since in mouse models of myocardial ischemia or progressive heart failure, fibrosis occurs only after several days or
FIGURE 4. Oil Red O staining of frozen LV myocardium of a WT mouse (A) and a LCAD KO mouse (B) after 24 h fasting. Intracellular lipid droplets are abundant in the cardiomyocytes of the LCAD KO mouse. (C) Percentage of Oil Red O-positive surface area, as determined with spectral imaging analysis. *, P<0.05 vs. WT.

FIGURE 5. (A) Relative contributions of saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids to the total myocardial fatty acid content of fasted WT and LCAD KO mice, as determined using gas chromatography with flame ionization detection. (B) Myocardial fatty acid profiles after 24 h fasting. (C) Total myocardial ceramide after 24 h fasting. *, P<0.05; **, P<0.01; ***, P<0.001 vs. WT.
even weeks from disease onset (Cleutjens et al., 1995). Furthermore, the LV hypertrophy already present in LCAD KO mice at baseline was not exacerbated after fasting. Indeed, histological analysis did not show prominent myocardial fibrosis in fed and fasted LCAD KO mice (data not shown). Moreover, expression of brain natriuretic peptide and atrial natriuretic factor, markers for hypertrophic myocardial adaptation, was not different between LCAD KO mice and controls, and was not affected by (repeated) fasting (data not shown). Apparently, the heart of LCAD KO mice is able to compensate the relatively mild metabolic defect in the fed condition, and this compensation fails upon fasting. A shift from FAO towards glucose metabolism for maintaining energy homeostasis may account for normal cardiac function as observed under fed conditions, but may become inadequate when glucose levels drop after a prolonged period of fasting (Kurtz et al., 1998). Diastolic dysfunction often precedes systolic failure, which may develop over longer time, in more severe long-chain FAO deficiencies, or after a more strenuous nutritional challenge. Altogether, our findings suggest that the LV functional alterations in the LCAD KO mouse are not caused by fasting-induced fibrotic changes of the myocardium, but more likely by a metabolic effect of fasting.

In healthy human volunteers, it was shown that short-term caloric restriction induces impaired diastolic LV function without altering systolic function. Myocardial phosphocreatine-to-ATP ratio was maintained, suggesting that energy shortage is not the main cause of impaired diastolic function after caloric restriction in healthy subjects. Increased levels of myocardial TG correlated negatively with diastolic function, suggesting that lipotoxicity plays a role (van der Meer et al., 2007). In line with these findings, Hammer et al. showed that, after a three-day period of complete starvation, myocardial TG content increases up to three-fold compared to baseline levels in healthy humans, and that diastolic function decreases concomitantly (Hammer et al., 2008). Insufficient FAO leads to accumulation of TG in non-adipose tissue such as the myocardium, as was demonstrated in a rodent model for extreme obesity (Zhou et al., 2000). As expected, LCAD KO mice display elevated myocardial TG levels, which increase even further after fasting. In accordance with findings in lean healthy men (Hammer et al., 2008; van der Meer et al., 2007) and diabetic patients, (Rijzewijk et al., 2008) increased myocardial TG levels were accompanied by decreased diastolic function in LCAD KO mice. In addition, total myocardial ceramide content was higher in fasted LCAD KO mice compared to fasted WT mice, showing that elevated myocardial TG levels may be accompanied by increased levels of lipotoxic compounds such as ceramide. Further investigations of the myocardial energy homeostasis in the LCAD KO mouse are required, before the detrimental effects of fasting on cardiac function can be fully attributed to lipotoxicity.

In sharp contrast with LCAD KO mice, WT mice in the current study showed a decrease in myocardial TG content after 24 h fasting. For these mice, a fasting period of 24 h may be sufficient to shift the equilibrium between cardiac storage of fatty acids and their utilization towards the latter, resulting in a depletion of the myocardial TG pool. The loss of LV mass found in fasted WT mice is consistent with a previous study, and may be explained by starvation-induced autophagy (Kanamori et al., 2009).

In apparent contrast to a recent 1H-MRS study of the mouse heart, (Hankiewicz et al., 2010) we find a good correlation between myocardial lipid levels measured in vivo and the TG content determined biochemically. In addition, histology supports that the TG signal in the 1H-MR spectra originates from lipid droplets inside cardiomyocytes. So far, assessment of myocardial TG levels in laboratory animals has been limited to histology, biochemical assays on tissue samples, 1H-MRS of isolated intact or dissected hearts, or a combination of those
techniques (Szczepaniak et al., 2003). The current work demonstrates that non-invasive, in vivo cardiac $^1$H-MRS at physiological heart rates can be applied to animal models of disease in a longitudinal study design, allowing monitoring changes in myocardial TG levels and other $^1$H-MRS observable metabolites.

In conclusion, this study reveals accumulation of myocardial TG in LCAD KO mice, which increases even further upon fasting. Concomitantly, LV EF and diastolic function in LCAD KO mice decrease after fasting, suggesting adverse effects of increased lipid metabolite levels. The results obtained here provide a basis for future longitudinal investigations in animal models of FAO defects to non-invasively assess the impact of therapeutic interventions on myocardial TG levels and cardiac function.

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References


Chapter 5


