Enhanced acyl-CoA dehydrogenase activity is associated with improved mitochondrial and contractile function in heart failure

Julie H. Rennison1, Tracy A. McElfresh1, Isidore C. Okere1, Hiral V. Patel2, Amy B. Foster2, Kalpana K. Patel2, Maria S. Stoll2, Paul E. Minkler2, Hisashi Fujioka2, Brian D. Hoit3,4, Martin E. Young5, Charles L. Hoppel2,3, and Margaret P. Chandler1*

1Department of Physiology and Biophysics, School of Medicine E553, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106-4970, USA; 2Department of Pharmacology, Case Western Reserve University, Cleveland, OH, USA; 3Department of Medicine, Case Western Reserve University, Cleveland, OH, USA; 4University Hospitals Case Medical Center, Cleveland, OH, USA; and 5Baylor College of Medicine, Houston, TX, USA

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Aims Heart failure is associated with decreased myocardial fatty acid oxidation capacity and has been likened to energy starvation. Increased fatty acid availability results in an induction of genes promoting fatty acid oxidation. The aim of the present study was to investigate possible mechanisms by which high fat feeding improved mitochondrial and contractile function in heart failure.

Methods and results Male Wistar rats underwent coronary artery ligation (HF) or sham surgery and were immediately fed either a normal (14% kcal fat) (SHAM, HF) or high-fat diet (60% kcal saturated fat) (SHAM+FAT, HF+FAT) for 8 weeks. Mitochondrial respiration and gene expression and enzyme activities of fatty acid-regulated mitochondrial genes and proteins were assessed. Subsarcolemmal (SSM) and interfibrillar mitochondria were isolated from the left ventricle. State 3 respiration using lipid substrates octanoylcarnitine and palmitoylcarnitine increased in the SSM of HF+FAT compared with SHAM+FAT and HF, respectively (242 ± 21, 246 ± 21 vs. 183 ± 8, 181 ± 6 and 193 ± 17, 185 ± 16 nAO min^-1 mg^-1). Despite decreased medium-chain acyl-CoA dehydrogenase (MCAD) mRNA in HF and HF+FAT, MCAD protein was not altered, and MCAD activity increased in HF+FAT (HF, 65.1 ± 2.7 vs. HF+FAT, 81.5 ± 5.4 nmoles min^-1 mg^-1). Activities of short- and long-chain acyl-CoA dehydrogenase also were elevated and correlated to increased state 3 respiration. This was associated with an improvement in myocardial contractility as assessed by left ventricular +dP/dt max.

Conclusion Administration of a high-fat diet increased state 3 respiration and acyl-CoA dehydrogenase activities, but did not normalize mRNA or protein levels of acyl-CoA dehydrogenases in coronary artery ligation-induced heart failure rats.

KEYWORDS Heart failure; Mitochondria; β-Oxidation; Fatty acids

1. Introduction

A variety of pathophysiological conditions including obesity, insulin resistance, diabetes, and heart failure (HF) have been shown to be associated with excess lipid accumulation in non-adipose tissue because of elevated circulating fatty acids. In particular, enhanced myocardial lipid accumulation appears to be associated with impaired myocardial contractile function and could exacerbate the progression of HF. However, the effect of enhanced lipid accumulation on mitochondrial and contractile function under pathophysiological conditions such as HF had not been systematically evaluated until recently. We reported that high saturated fat feeding in a Wistar rat model of coronary artery ligation-induced HF resulted in no impairments in mitochondrial oxidative phosphorylation, electron transport chain complex activities (ETC), or myocardial contractile function. Instead, state 3 respiration and ETC activities were elevated. These results are in agreement with another recently published study from our laboratory in a Dahl Salt-Sensitive rat model of hypertension-induced cardiomyopathy, where administration of a high saturated fat diet reduced left ventricular (LV) hypertrophy, improved contractile function, and prevented LV dilation despite elevated plasma fatty acids and myocardial triglycerides.

One possible explanation for these observations may be a fatty acid-induced activation of peroxisome proliferator...
activated receptors (PPARs), which are known to regulate the expression of genes involved in key energy producing metabolic pathways. Although the three primary PPAR isoforms, PPARα, β/δ, and γ are all known to be activated by fatty acids, PPARα is thought to be a major transcriptional regulator of fatty acid metabolism in the heart. PPARα activates the expression of genes encoding enzymes involved in fatty acid uptake and metabolism [including carnitine palmitoyltransferase-I (cpt-I), short-chain acyl-CoA dehydrogenase (scad), medium-chain acyl-CoA dehydrogenase (mcad), long-chain acyl-CoA dehydrogenase (lcad), uncoupling protein-3 (ucp3), pyruvate dehydrogenase kinase-4 (pdk4), and cytosolic thioesterase 1 (cte1)]. PPARα, its co-activator PPAR gamma co-activator-1 (PGC-1α) (a regulator of mitochondrial biogenesis7,8,9), and enzymes involved in fatty acid oxidation have been reported to be down-regulated in hypertrophied and failing hearts. Administration of a high-fat diet, however, may provide the ligand for the activation of PPARα and PGC-1α, and subsequently increase the expression of the fatty acid metabolic enzymes, thereby enabling increased uptake, utilization, and storage of excess lipids that might, under other circumstances, be cytotoxic to the myocardium.

The aim of the present study was to investigate possible mechanisms by which high fat feeding improved mitochondrial and contractile function in HF. We hypothesized that a high-fat diet during HF would result in improved mitochondrial fatty acid oxidation and increased state 3 respiration by activating genes involved in fatty acid uptake and utilization. We tested our hypothesis in rats fed with a high-saturated fat diet following coronary artery ligation surgery to induce HF.

2. Methods

2.1 Study design and induction of myocardial infarction

The investigation conforms with Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the Institutional Animal Care and Use Committee at Case Western Reserve University. Animals were maintained on a reverse 12 h:12 h light:dark cycle, and all procedures/tissue harvests were performed 2–4 h into the light cycle.

For the induction of HF, male Wistar rats were anesthetized with isoflurane (1.5–2.0%), intubated, and ventilated. A microtip pressure transducer catheter (3.5Fr, Millar Instruments) was inserted via the right carotid artery. Measurements of HR, peak LV pressure, end diastolic pressure, and +/– dp/dt were recorded over a 30 s period using a Digi-Med® Heart Performance Analyzer.12

2.2 Echocardiography

Myocardial function was evaluated by echocardiography 8 weeks post-ligation using a Sequoia C256 System (Siemens Medical) with a 15 MHz linear array transducer as previously described.10 Briefly, rats were anesthetized with 1.5–2.0% isoflurane, the chest shaved, the animal situated in the supine position and ECG limb electrodes were placed. Two-dimensional (2D), 2D-guided M-mode, and Doppler echocardiographic studies of aortic and mitral flows were performed via parasternal and foreshortened apical windows. End-diastolic and end-systolic dimensions were measured using software resident on the ultrasonograph. Myocardial performance index (MPI), cardiac index, and percent fractional shortening were calculated as previously described.10 All data were analysed in an investigator-blinded fashion.

2.3 Haemodynamic measurements

LV pressure and contractile properties were assessed 8 weeks following ligation surgery. Rats were anesthetized (1.5–2.0% isoflurane), intubated, and ventilated. A microtip pressure transducer catheter (3.5Fr, Millar Instruments) was introduced via the right carotid artery. Measurements of HR, peak LV pressure, end diastolic pressure, and +/– dp/dt were recorded over a 30 s period using a Digi-Med® Heart Performance Analyzer.12

2.4 Preparation of mitochondria

Following LV cannulation, blood samples were drawn from the inferior vena cava. Right ventricle (RV), LV, and scar mass were obtained by gravimetric measurements. A myocardial tissue sample was harvested and quick-frozen. The scar and balance of the LV were placed in KME (100 mM KCl, 50 mM 3-[N-Morpholino]propanesulfonic acid (MOPS), internal salt, and 0.5 mM EGTA, pH 7.4). Cardiac sarcolemmal (SSM) and intermitochondial mitochondria (IFM) were isolated according to Palmer et al.11 except that a modified Chappell–Perry buffer (containing 100 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM MgSO4•7H2O, and 1 mM ATP, pH 7.4, 4°C) was used for isolation of mitochondria. IFM were harvested following treatment of skinned fibres with 5 mg/gww trypsin for 10 min at 4°C.12 Mitochondrial protein concentration was determined by the Lowry method using bovine serum albumin as a standard.

2.5 Mitochondrial oxidative phosphorylation

Oxygen consumption in SSM and IFM was measured using a Clark-type oxygen electrode (Strathkelvin) at 30°C. Mitochondria were incubated in a solution consisting of 80 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM KH2PO4, and 1 mg/ml defatted bovine serum albumin at pH 7.4. The rate of oxidative phosphorylation and uncoupled respiration was measured using several substrates. Glutamate assesses complexes I, III, and IV. Succinate assesses complexes II, III, and IV. Durohydroquinone (DHQ), an analog of coenzyme Q, assesses complexes III and IV. N, N',N''-tetramethyl-p-phenylenediamine (TMPD), an electron carrier that reduces cytochrome c, used in conjunction with ascorbate, assesses complex IV. Mitochondrial respiration was measured using lipid substrates octanoylcarnitine plus malate and palmitoylcarnitine plus malate.14 State 3 (ADP-stimulated) respiration, state 4 (ADP-limited) respiration, respiratory control ratio (RCR) (state 3/state 4), and ADP/O ratio (ADP added per oxygen consumed) were determined as previously described.15

2.6 Mitochondrial electron transport chain complex activity

Samples of SSM and IFM (10 mg cholate/1 mg mitochondrial protein) were mixed in 1 ml buffer (75 mM mannitol, 220 mM sucrose, 2 mM EDTA, and 5 mM MOPS, pH 7.4) with mammalian protease inhibitor cocktail (1μl/1 ml buffer) and kept on ice. Assays were completed on the day of preparation.

All ETC complex activities were measured as specific donor-acceptor oxidoreductase activities using a diode array spectrophotometer.16 Donors and acceptors were chosen to span specific regions of the complete ETC.

2.7 Electron microscopy

Freshly isolated SSM and IFM were prepared for transmission electron microscopy as previously described3,21 with the exception...
that samples were fixed using one-quarter-strength Karnovsky’s fixative. A myocardial tissue sample from the LV was obtained during the terminal surgery, immediately fixed, and prepared as previously described. Electron micrographs were assessed in an investigator-blinded fashion.

2.8 RNA extraction and quantitative RT–PCR
RNA extraction (and subsequent quantitative RT–PCR) was performed on frozen powdered LV tissue as previously described. Specific quantitative assays were designed from rat sequences available in GenBank for expression of ppar α, atrial natriuretic factor (anf), and genes regulated by PPARα: scad, mcad, ldad, cpt-1, pdk4, ucpx, and cte-1. Primer and probe sequences for these Taqman assays have previously been published with the exception of scad. Sequences for scad are 5’-CATAACGCGCT GTTTTGGC-3’ (forward primer), 5’-AAGCCTTTGGTGCGGGGTTGAG-3’ (reverse primer), and 5’-FAM-CAGTGAGCCAGGCAATGGCAGTG-CA TAMRA-3’ (probe). Standard RNA was made for all assays by the T7 polymerase method (Ambion), using total RNA isolated from rat hearts. The correlation between Ct (the number of PCR cycles required for the fluorescent signal to reach a detection threshold) and the amount of standard was linear over a 5-log range of RNA for all assays. The PCR data are reported as the number of transcripts/nanogram RNA.

2.9 Western immunoblot analysis
For the analysis of MCAD protein expression, SSM and IFM protein samples (100 μg) were resuspended in a standard Laemmli buffer (XT sample buffer, BioRad) and boiled for 5 min. To assess the potential contribution of proteins involved in substrate flux across the plasma membrane, fatty acid translocase (FAT/CD36) and fatty acid transport protein (FATP-1) protein expression was measured in myocardial tissue. Protein was extracted from frozen powdered LV tissue as previously described. In order to allow for gel-to-gel comparisons, a standard sample was loaded onto each gel and protein bands detected in samples were then normalized to their respective gel control band. Sample proteins were separated using SDS–PAGE on a 10% Tris–glycine polyacrylamide gel and transferred onto a nitrocellulose membrane. Protein transfer was verified with Ponceau staining. Membranes were washed in TBST and blocked with 5% non-fat dry milk in TBST. Membranes were incubated with a mouse polyclonal antibody for MCAD (1:500; Abnova), a goat polyclonal antibody for FATP-1 (1:100; Santa Cruz), a mouse polyclonal antibody for CD36 (1:5000; a gift from Maria Febbraio, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland OH), or a mouse monoclonal antibody for calsequestrin (1:1000; Affinity Bioreagents) overnight at room temperature. Membranes were incubated for 1 h with an HRP-linked secondary antibodies for MCAD and calsequestrin (goat anti-mouse, Pierce), for FATP-1 (donkey anti-goat, Jackson Laboratories), or for CD36 (goat anti-rabbit, Pierce) for 1 h. Protein bands were visualized via chemiluminescent detection (Supersignal, Pierce) on film and bands were quantified using Image J (http://rsb.info.nih.gov/ij/). For MCAD, each protein band was normalized to citrate synthase activity in the individual mitochondrial fractions. For CD36 and FATP-1, each protein band was normalized to calsequestrin.

2.10 Acyl-CoA dehydrogenase activity
Short-, medium-, and long-chain acyl-CoA dehydrogenase activities were measured in isolated SSM and IFM as the phenazine ethosulfate stimulated reduction of cytochrome c using 2μM butyryl-CoA (a 4-carbon substrate), 0.2 mM octanoyl-CoA (an 8-carbon substrate), or 50μM palmitoyl-CoA (a 16-carbon substrate), respectively, as described previously. Glucose, free fatty acids, and triglyceride concentrations in plasma were measured using an enzymatic spectrophotometric kit. Tissue triglyceride content was measured in homogenate extracts using an enzymatic spectrophotometer method (Wako Chemicals). Insulin and leptin concentrations in plasma were assayed by ELISA (ALPCO Diagnostics). Adiponectin concentrations in serum were measured by ELISA (B-Bridge, San Diego, CA). Carnitine and acylcarnitine content in LV myocardial tissue were assessed as previously described. Total carnitine was the sum of carnitine and acylcarnitine. Citrate synthase was measured as previously described.

2.11 Plasma and tissue metabolic products
Glucose, free fatty acids, and triglyceride concentrations in plasma were measured using an enzymatic spectrophotometric kit. Tissue triglyceride content was measured in homogenate extracts using an enzymatic spectrophotometer method (Wako Chemicals). Insulin and leptin concentrations in plasma were assayed by ELISA (ALPCO Diagnostics). Adiponectin concentrations in serum were measured by ELISA (B-Bridge, San Diego, CA). Carnitine and acylcarnitine content in LV myocardial tissue were assessed as previously described. Total carnitine was the sum of carnitine and acylcarnitine. Carnitine synthase was measured as previously described.

2.12 Statistical analysis
Differences among SHAM, SHAM+FAT, HF, and HF+FAT were determined using a ZW-ANOVA followed by Bonferroni t-tests for multiple comparisons. Data are expressed as group means ± SEM. Significance was established at P < 0.05.

3. Results
3.1 Body and heart mass
HF increased LV and RV mass when normalized to body mass (see Supplementary material online, Table S1). High fat in SHAM animals did not alter LV or RV mass, but in HF+FAT high fat attenuated the increases in RV/body mass and biventricular mass/body mass seen in HF. Mean scar tissue mass did not differ between the two ligated groups (see Supplementary material online, Table S1).

3.2 Cardiac function and echocardiographic measures
High fat did not alter LV contractile function or remodelling in SHAM animals. Coronary ligation resulted in impaired LV contractile function and remodelling in HF and HF+FAT compared with SHAM and SHAM+FAT as assessed by increased MPI, end-diastolic, and end-systolic area, as well as decreased peak LV +dP/dt, cardiac index, and area fractional shortening (Table 1). Interestingly, peak LV +dP/dt, an index of myocardial contractility, was increased in HF+FAT compared with HF. Thus, LV contractile function and the progression of LV remodelling were not exacerbated by high fat feeding in HF; instead, peak LV +dP/dt was improved.

3.3 Metabolic substrates and humoral factors
Plasma glucose, plasma insulin, and tissue triglycerides were not altered by HF or by high fat feeding. As expected, plasma free fatty acids, leptin, and adiponectin were elevated in both SHAM+FAT and HF+FAT. Plasma triglycerides were elevated in HF compared with SHAM (see Supplementary material online, Table S2).

3.4 Mitochondrial morphology
Neither HF nor high fat feeding altered the morphology of isolated SSM and IFM (Figure 1). In keeping with previous studies of cardiac mitochondria, these organelles have assumed a spherical shape. A small number of mitochondria exhibit a degree of extraction of matrix material. A few mitochondria show blebbing of their outer membrane. This description applies equally to SSM and IFM, however, IFM (Figure 1E–H) appear to be, on average slightly larger.
bars = 1 μm.

than the SSM (Figure 1A–D). Myocardial tissue samples of the LV were fixed in order to assess the integrity of both SSM and IFM within the myocardial tissue as well as the overall structure of the cardiomyocytes per se. Mitochondrial shape, size, and density in situ also were not altered by HF or high fat feeding (Figure 2). Although the myofibrils show a degree of contraction, they are identical in appearance under all conditions of this study. In a similar fashion, all cardiomyocyte nuclei have the same appearance, i.e. they are euchromatic, often exhibiting a single nucleolus.

3.5 Oxidative phosphorylation

Protein yield in SSM was not altered by HF or high fat (SHAM 11.8 ± 0.8, SHAM+FAT 10.2 ± 0.4, HF 10.8 ± 1.1, HF+FAT 11.9 ± 1.1 mg gww⁻¹), but was decreased in IFM in both HF groups compared with SHAM (SHAM 8.35 ± 0.39, SHAM+FAT 8.37 ± 0.49, HF 6.46 ± 0.55, HF+FAT 6.20 ± 0.69 mg gww⁻¹). Citrate synthase activity in myocardial tissue was also decreased in both HF groups (SHAM 115 ± 3, SHAM+FAT 120 ± 3, HF 99 ± 4, HF+FAT 102 ± 5 μmol min⁻¹·gww⁻¹), consistent with the decreased IFM protein yield.

Mitochondrial respiration was measured using lipid substrates octanoylcarnitine and palmitoylcarnitine to assess the ability of the mitochondria to oxidize fatty acids of different chain lengths. State 3 respiration in SSM was not altered in SHAM+FAT or HF using either lipid substrate (Figure 3A). Using both octanoylcarnitine and palmitoylcarnitine, however, state 3 respiration was elevated in HF compared with SHAM+FAT and HF. Similar to our data using lipid substrates, state 3 respiration was not altered in SSM of SHAM+FAT or HF using glutamate, succinate, DHQ, or TMPD-ascorbate as respiratory substrates (data not shown), but was elevated using glutamate in HF+FAT (Figure 3A). State 3 respiration did not differ in IFM with any of the respiratory substrates used (see Supplementary material online, Figure S1). The differential effects

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Left ventricular functional measurements obtained by left ventricular cannulation and echocardiography in SHAM, SHAM+FAT, HF, and HF+FAT 8 weeks following coronary artery ligation surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV cannulation</td>
<td>SHAM</td>
</tr>
<tr>
<td>Heart rate (b.p.m.)</td>
<td>331 ± 16</td>
</tr>
<tr>
<td>Peak systolic LV pressure (mmHg)</td>
<td>119 ± 7</td>
</tr>
<tr>
<td>Peak LV end diastolic pressure (mmHg)</td>
<td>7.6 ± 1.0</td>
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<tr>
<td>Peak LV +dP/dt (mmHg/s)</td>
<td>7014 ± 592</td>
</tr>
<tr>
<td>Peak LV −dP/dt (mmHg/s)</td>
<td>5950 ± 484</td>
</tr>
<tr>
<td>Echocardiography</td>
<td></td>
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<tr>
<td>End-diastolic area (cm²)</td>
<td>0.91 ± 0.06</td>
</tr>
<tr>
<td>End-systolic area (cm²)</td>
<td>0.382 ± 0.058</td>
</tr>
<tr>
<td>Cardiac index (ml·min⁻¹·mg⁻¹)</td>
<td>171 ± 26</td>
</tr>
<tr>
<td>Area fractional shortening (cm²)</td>
<td>0.593 ± 0.046</td>
</tr>
<tr>
<td>Myocardial performance index</td>
<td>0.408 ± 0.025</td>
</tr>
</tbody>
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LV, left ventricular.
Cardiac index was calculated by dividing cardiac output by body weight. Area fractional shortening was calculated as the sum of left ventricular end-systolic and end-diastolic areas divided by end-diastolic area. Myocardial performance index was defined as the sum of the isovolumic contraction and relaxation times divided by the ejection time. Values are expressed as mean ± SEM.

*P < 0.05 vs. SHAM.
**P < 0.05 vs. SHAM+FAT.
***P < 0.05 vs. HF.
observed in SSM and IFM are not surprising as they have previously been shown to be distinct populations that respond differently under pathological conditions.32

In SSM, state 4 respiration was not altered in SHAM + FAT or HF, but was elevated using octanoylcarnitine only in HF + FAT compared with SHAM + FAT (see Supplementary material online, Figure S2A). In IFM, state 4 respiration was not altered in SHAM + FAT or HF + FAT, but was increased in HF compared with SHAM using octanoylcarnitine and glutamate (see Supplementary material online, Figure S2B).

RCR was not altered in either high fat group, but was decreased in SSM of HF compared with SHAM and HF + FAT using octanoylcarnitine and palmitoylcarnitine (see Supplementary material online, Figure S3A). In IFM, RCR was decreased in HF with palmitoylcarnitine, octanoylcarnitine, and glutamate compared with SHAM, but was increased in HF + FAT compared with HF with glutamate only (see Supplementary material online, Figure S3B). ADP/O did not differ in the SSM or IFM with any lipid substrate (see Supplementary material online, Figure S4).

3.6 Mitochondrial electron transport chain complex activities

Activity of complex II + coenzyme Q in SSM (SHAM 78.0 ± 8.8, SHAM + FAT 61.7 ± 5.8, HF 60.6 ± 5.7, HF + FAT 85.4 ± 10.0 nmoles min⁻¹ mg⁻¹) and IFM (SHAM 88.9 ± 7.7, SHAM + FAT 71.5 ± 7.0, HF 72.4 ± 8.3, HF + FAT 93.9 ± 8.8 nmoles min⁻¹ mg⁻¹) was elevated in HF + FAT compared with SHAM + FAT. In the SSM, the activity of complex II + coenzyme Q was also elevated in HF + FAT compared with HF. Complex III activity in IFM was elevated in HF + FAT compared with SHAM + FAT (SHAM 5623 ± 620, SHAM + FAT 5066 ± 594, HF 6766 ± 498, HF + FAT 7437 ± 1373 nmoles min⁻¹ mg⁻¹). Complex I, complex I + III, NADH-dehydrogenase, complex II, complex II + III, complex IV, and succinate dehydrogenase activities Figure 2

Electron micrographs of mitochondria in left ventricular myocardial tissue in (A) SHAM, (B) SHAM + FAT, (C) HF, (D) HF + FAT. SSM are situated beneath the sarcolemma (↑). IFM (▲) are located between the myofibrils. The cluster of SSM (D) is not the result of treatment; such aggregates occasionally are present in untreated cardiomyocytes. Bars = 1 μm.

Figure 3
State 3 respiration in SSM (A) using glutamate, octanoylcarnitine, and palmitoylcarnitine as respiratory substrates and (B) Activity of short-, medium-, and long-chain acyl-CoA dehydrogenases in SSM of SHAM, SHAM + FAT, HF, and HF + FAT. Activity of medium-chain acyl-CoA dehydrogenase correlated to state 3 respiration in the SSM using (C) palmitoylcarnitine and (D) octanoylcarnitine. Values are mean ± SEM, †P < 0.05 compared with SHAM + FAT; ‡P < 0.05 compared with HF.
were not altered in SSM or IFM (see Supplementary material online, Tables S3 and S4). The increase in complex II + coenzyme Q and complex III activities was not reflected in increased state 3 respiration using complex I (glutamate), complex II (succinate), and complex III (DHQ) substrates. Additionally, without a concomitant increase in the activity of the other complexes of the ETC, the increase in complex II + coenzyme Q is unlikely to account for the increased state 3 respiration evident in the SSM of HF+FAT.

3.7 RNA expression

Expression of *anf* was not altered with high fat feeding in sham animals (SHAM 105 ± 23, SHAM+FAT 100 ± 16). HF resulted in an 8-fold increase in *anf* expression; however, *anf* did not differ between HF and HF+FAT (HF 920 ± 268, HF+FAT 814 ± 168). mRNA expression of fatty acid-responsive genes cte-1 (Figure 4) and *mcad* (Figure 5) was significantly decreased in HF compared with SHAM; a similar trend was observed for *pdk4* (*P* = 0.065). Whereas high fat diet increased the expression of *pdk4* and cte-1 in both SHAM+FAT and HF+FAT, there was no effect of high fat on the expression of other genes investigated (Figure 4).

3.8 Acyl-CoA dehydrogenase protein expression and enzyme activity

Despite decreased mRNA expression of *mcad* in HF compared with SHAM (Figure 5A), MCAD protein expression (Figure 5B) and activities of SCAD, MCAD, and LCAD (Figure 3B) were not altered. MCAD protein expression also was not altered by high fat feeding in SSM (Figure 5B) or IFM (see Supplementary material online, Figure S5). Interestingly, the activities of acyl-CoA dehydrogenases (SCAD, MCAD, and LCAD) were elevated in SSM of HF+FAT despite no alterations in mRNA and protein expression (Figure 3B). Furthermore, there was a strong positive relationship between MCAD activity and state 3 respiration in SSM using palmitoylcarnitine (Figure 3C) and octanoylcarnitine (Figure 3D) as respiratory substrates. This positive relationship with state 3 respiration also existed for SCAD (octanoylcarnitine \( R^2 = 0.631, P < 0.0001 \); palmitoylcarnitine \( R^2 = 0.651, P < 0.0001 \) ) and LCAD activity (octanoylcarnitine \( R^2 = 0.606, P < 0.0001 \); palmitoylcarnitine \( R^2 = 0.609, P < 0.0001 \)).

CD36 and FATP-1 protein expression were not altered by HF or high fat diet (data not shown), suggesting that the elevated state 3 respiration using lipid substrates and acyl-CoA dehydrogenase activity is not a result of changes in fatty acid uptake due to alterations in fatty acid transport protein content. However, because we measured protein expression of CD36 and FATP-1 in whole tissue, this assessment did not differentiate between CD36 and FATP-1 localized to the plasma membrane from that found in intracellular compartments.

3.9 Acylcarnitines

Total carnitine (carnitine + acylcarnitine) content in myocardial tissue was decreased in HF compared with SHAM (Figure 6A). High fat also decreased total carnitine in SHAM+FAT and HF+FAT compared with SHAM and HF.
respectively. Because acylcarnitine content did not differ between groups, the decrease in total carnitine can be attributed to decreased carnitine. Additionally, the decreased carnitine content resulted in an increased acylcarnitine-to-carnitine ratio in SHAM+FAT and HF+FAT (Figure 6A).

The content of individual acylcarnitines, palmitoylcarnitine, stearoylcarnitine, and oleoylcarnitine were not altered in HF (Figure 6B). Only stearoylcarnitine was elevated in SHAM+FAT compared with SHAM. Palmitoylcarnitine, stearoylcarnitine, and oleoylcarnitine were elevated in HF+FAT compared with HF, but interestingly, stearoylcarnitine and oleoylcarnitine were elevated as well in HF+FAT compared with SHAM+FAT.

4. Discussion

The present study shows that administration of a high fat diet in HF increased state 3 respiration and acyl-CoA dehydrogenase (SCAD, MCAD, and LCAD) activities, but did not affect the decreased mRNA expression of PPAR target genes associated with coronary artery ligation-induced HF. These findings are summarized in Table 2. The activity of acyl-CoA dehydrogenases showed a strong positive relationship with increased state 3 respiration when lipid respiratory substrates were utilized. These effects of high fat were not evident in normal animals, suggesting that the high fat effect on mitochondrial capacity is limited to HF. Additionally, 8 weeks of high fat feeding in normal animals did not adversely affect mitochondrial respiration, the expression or activity of enzymes involved in β-oxidation, or LV contractile function.

We hypothesized that feeding rodents a high fat diet during HF would result in improved mitochondrial fatty acid oxidation and increased state 3 respiration by activating genes involved in fatty acid uptake and utilization. In the present study, mitochondrial respiration was assessed using the lipid substrates, octanoylcarnitine and palmitoylcarnitine. We observed increased state 3 respiration using both lipid substrates and an elevation in the activity of each chain-length specific acyl-CoA dehydrogenase. The first and rate-limiting step of β-oxidation, the α,β-dehydrogenation of fatty acyl-CoA is catalysed by a family of flavoproteins known as the acyl-CoA dehydrogenases. This process shortens a fatty acid by two carbons resulting in acetyl-CoA units that enter the citric acid cycle and generate reducing equivalents that enter the ETC at complex I or II. A long-chain fatty acid would initially utilize LCAD, but as the fatty acid chain-length is repetitively shortened by two carbons, MCAD and SCAD would be utilized as well. Therefore, a diet high in long-chain fatty acids (like the one used in the present study) would provide substrate not just for LCAD, but for MCAD and SCAD as well. Both octanoylcarnitine and palmitoylcarnitine enter the mitochondria by the carnitine transport system; however, for the eight carbon octanoylcarnitine, the first step of β-oxidation is catalysed by MCAD while the 16 carbon palmitoylcarnitine utilizes LCAD. We have shown an increase in state 3 respiration using both octanoylcarnitine and palmitoylcarnitine as respiratory substrates. Because both substrates enter the mitochondria by the same mechanism (the carnitine transport system), but utilize a different acyl-CoA dehydrogenase for the initial step of β-oxidation, this data indicates that the increase in state 3 respiration is not specific to the chain-length of a fatty acid.

In the current study, the activity of the acyl-CoA dehydrogenases was increased in the HF group fed high fat and showed a strong positive relationship with the increased state 3 respiration using lipid substrates. Previous studies using a direct PPARα agonist, clofibrate, also demonstrated good correlations between state 3 respiration using palmitoylcarnitine and acyl-CoA dehydrogenase activity in liver mitochondria.33,34 We observed an elevation in the activity of each chain-length specific acyl-CoA dehydrogenase. One possible mechanism by which mitochondrial activity of these enzymes increases during high fat feeding may involve the absence of inhibitory effect of certain proteins...
on the activity of acyl-CoA dehydrogenases. Proteins that normally inhibit acyl-CoA dehydrogenase activity by targeting either the glutamate catalytic residue or the FAD cofactor may themselves be altered so as to allow increased enzyme activity. It is also important to note that the activity of the fatty acid oxidation enzymes SCAD, MCAD, and LCAD were increased in SSM despite no alterations in scad, mcad, lcad, or the protein expression of MCAD by high fat feeding in HF. These results suggest that the enhanced state 3 respiration observed in the present study was independent of transcriptional events, but might reflect post-transcriptional alterations of fatty acid oxidation enzymes. For example, Chu et al. reported post-translational modifications of SCAD when fatty acid oxidation was up-regulated in an effort to meet the energetic demands of enhanced sarcoplasmic reticular calcium cycling and hyperdynamic function in phospholamban knockout hearts. These potential modifications affecting enzyme activity require further investigation to examine their potential role in the increased enzyme activity in HF following high fat feeding.

In general terms, the current transcriptional analysis is consistent with previous studies showing that genes promoting fatty acid oxidation are down-regulated in hypertrophy and failing hearts. As reported in this current investigation, however, alterations in mRNA expression are not always reflective of protein expression or enzyme activity. Morgan et al. reported a repression of fatty acid oxidation genes in HF, but a 45% high fat diet initiated 8 weeks following ligation surgery had no effect on mRNA, protein expression, or enzyme activity of MCAD. Interestingly, fenofibrate, a direct PPARα ligand, induced mRNA expression of PPARα regulated genes, as well as protein expression and activity of MCAD. In a study by Sack et al., LV hypertrophy was associated with decreased mRNA expression of mcad, with no change in protein or enzyme activity. However, when LV hypertrophy progressed to HF, decreased mcad was accompanied by decreased protein and enzyme activity. These data support the existence of a temporal pattern for changes in mRNA expression, protein expression, and enzyme activity that are contingent upon the stage or progression of contractile dysfunction/failure. In the present study, the level of LV dysfunction would be considered mild-to-moderate, having not progressed to more severe or decompensated HF; therefore, our findings may reflect an earlier stage in disease progression.

Conditions known to elevate fatty acids such as high fat feeding, obesity, and diabetes are associated with activation of PPARα and genes involved in fatty acid oxidation. We have shown that high fat feeding was associated with increased expression of cte-1 and pdk4, but the expression of additional fatty acid-responsive genes (pparα, pparδ, scad, mcad, lcad) was not altered. Variations in the response of the PPARα regulated genes examined in this study might reflect differential effects of distinct fatty acid species (specifically in this study, palmitate, stearate, and oleate) on gene expression. For example, we have previously demonstrated that the fatty acid composition of high fat diets (specifically high saturated vs. high unsaturated fat diets) differentially affected PPARα regulated gene expression. Furthermore, in isolated adult cardiomyocytes, long-chain monounsaturated fatty acids such as oleate induced fatty acid responsive genes to a greater extent than long-chain saturated fatty acids such as palmitate (Young et al., unpublished results). Future studies should examine the differential effects of distinct fatty acid species and also possible alternative pathways that could regulate the expression of genes involved in the transport and utilization of fatty acids.

It was also important to determine whether the effects of a high fat diet were independent of HF, so we assessed the effects of high saturated fat feeding on mitochondrial and contractile function in normal rats. An interesting observation in the current study is that although high fat feeding in HF increased state 3 respiration and the activities of the acyl-CoA dehydrogenases, there was no effect on mitochondrial or contractile dysfunction in normal rats. Additionally, expression of anf, a gene marker of LV hypertrophy, was not different in normal animals. These results indicate that the effects of high fat on cardiac mitochondrial function are exclusive to HF and that 8 weeks of high fat feeding in normal animals does not adversely affect myocardial contractile or mitochondrial function. However, the effects of longer duration high fat feeding on mitochondrial and myocardial contractile function have not been assessed and should be a target of future investigations.

Carnitine facilitates the translocation of acyl-CoA across the mitochondrial membrane and buffers mitochondrial acetyl-CoA content. Decreased myocardial carnitine content is associated with hypertrophic cardiomyopathy, whereas administration of propionyl-l-carnitine prevents the decrease in cardiac work typically associated with hypertrophy. Consistent with these findings, free and total carnitine were decreased by HF (Figure 6). A high fat diet might be expected to induce a decrease in carnitine and a concomitant increase in acylcarnitine, but in our study high fat caused a further decrease in myocardial carnitine while acylcarnitine levels remained unchanged. The decreased carnitine content, though significant, is not severe enough to be associated with contractile dysfunction. However, the effect of the decreased carnitine content on the acylcarnitine-to-carnitine ratio is noteworthy. An increase in the acylcarnitine-to-carnitine ratio (which reflects the acyl-CoA-to-CoA ratio) indicates that more acyl-CoA is being synthesized than is being oxidized, and therefore serves as a marker of metabolic distress. The increased acylcarnitine-to-carnitine ratio, in both SHAM-FAT and HF-FAT, may serve as an early indicator that mitochondrial dysfunction would be evident with a longer duration of high fat feeding.

In summary, our results clearly show that state 3 respiration and acyl-CoA dehydrogenase (SCAD, MCAD, and LCAD) activities are elevated in high fat fed HF animals. High fat attenuated an HF-induced repression of only a few of the PPAR target genes. Although high fat feeding did not influence expression of the acyl-CoA dehydrogenases investigated, activity of these enzymes are increased and show a strong positive relationship with state 3 respiration using lipid substrates. These effects of high fat on mitochondrial and contractile function are not evident in normal animals, indicating the high fat effect on mitochondrial capacity is specific to HF. The increased activity of the acyl-CoA dehydrogenases may be due to a decrease of inhibitory proteins or...
a post-translational modification of these enzymes, and requires further investigation to examine their potential role in the increased mitochondrial capacity associated with high-fat-fed HF animals.

Supplementary material
Supplementary Material is available at Cardiovascular Research online.

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