Fatty Acid Synthase Modulates Homeostatic Responses to Myocardial Stress

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Fatty acid synthase (FAS) promotes energy storage through de novo lipogenesis and participates in signaling by the nuclear receptor PPARα in noncardiac tissues. To determine if de novo lipogenesis is relevant to cardiac physiology, we generated and characterized FAS knockout in the myocardium (FASKard) mice. FASKard mice develop normally, manifest normal resting heart function, and have normal cardiac PPARα signaling as well as fatty acid oxidation. However, they decompensate with stress. Most die within 1 h of transverse aortic constriction, probably due to arrhythmia. Voltage clamp measurements of FASKard cardiomyocytes show hyperactivation of L-type calcium channel current that could not be reversed with palmitate supplementation. Of the classic regulators of this current, Ca2+/calmodulin-dependent protein kinase II (CaMKII) but not protein kinase A signaling is activated in FASKard hearts, and knockdown of FAS in cultured cells activates CaMKII. In addition to being intolerant of the stress of acute pressure, FASKard hearts were also intolerant of the stress of aging, reflected as persistent CaMKII hyperactivation, progression to dilatation, and premature death by ~1 year of age. CaMKII signaling appears to be pathogenic in FASKard hearts because inhibition of its signaling in vivo rescues mice from early mortality after transverse aortic constriction. FAS was also increased in two mechanistically distinct mouse models of heart failure and in the hearts of humans with end stage cardiomyopathy. These data implicate a novel relationship between FAS and calcium signaling in the heart and suggest that FAS induction in stressed myocardium represents a compensatory response to protect cardiomyocytes from pathological calcium flux.

Storing energy preserves the function of many mammalian tissues during physiological and pathological stress. One of the ways that tissues store energy is through de novo lipogenesis, the synthesis of saturated fatty acids from carbohydrate precursors (1). This process, accomplished by iterative two-carbon additions to a fatty acid chain, is mediated by the multifunctional enzyme FAS2 (2, 3).

Initially relegated to cellular housekeeping, FAS was recently also shown to participate in intracellular signaling. Whole body FAS-deficient embryos die before uterine implantation (4), but liver-specific FAS knock-out mice (5) have decreased expression of fatty acid oxidation genes (classic targets of PPARα signaling) and a phenotype resembling PPARα-deficient mice (6).

Pharmacological activation of PPARα reverses these features, suggesting that FAS generates an endogenous ligand for PPARα, and this FAS-dependent molecule was recently identified as a discrete phosphatidylcholine species (7). FAS also triggers PPARα signaling in the hypothalamus (8) and in macrophages (9), raising the possibility that molecules generated by this enzyme are necessary for activating fatty acid metabolism, even in fatty acid-dependent tissues, such as the heart.

Myocardial reliance on fatty acids as an energy source is dogma in cardiac physiology (10). Under normoxic conditions, fatty acids provide ~60–70% of the energy required by the heart, with glucose, lactate, and ketones providing the remainder (10, 11). In addition to generating high energy phosphates, fatty acids also serve complex structural and signaling roles in the heart, but how intracellular lipid diversity is coordinated is unknown.

Elegant studies dating from a half century ago of myocardial fatty acid oxidation (12–14), lipid pools (14, 15), and lipid synthesis (14) demonstrated that the myocardium derives most of its fat from the circulation. In view of these findings and the notion that the heart has little need to store energy, de novo lipogenesis in myocardial physiology and pathophysiology has been largely forgotten.

To address the potential role of endogenously produced lipids in the heart, we inactivated cardiac FAS in mice. Surprisingly, we found that myocardial FAS regulates calcium signals and adaptation to stress.

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Materials and Methods

Animals and Human Tissues—The Washington University Animal Studies Committee approved protocols. Mice with the floxed FAS locus (exons 4 – 8) (5) were crossed with Cre recombinase transgenic mice under the control of the α-myosin heavy chain promoter (16) to obtain FASKard mice in a mixed genetic background. Given the potential for detrimental effects of Cre recombinase, Cre transgenic mice with a wild type FAS locus from these breeding cohorts were used as controls in all experiments, and some experiments included additional genotype controls. Littermates were utilized whenever possible; no genetically unrelated controls were used in any experiment. Unless otherwise indicated, experiments utilized male FASKard mice at 3–4 months of age eating Purina 4043 chow.

Streptozotocin-induced diabetes involved injecting mice with streptozotocin (150 mg/kg single-dose i.p.) or vehicle and harvesting tissue after 10 days. β-Cell-specific KATP mutant (Kir6.2[K185Q,AN30]) transgenic mice have been described (17). Leptin receptor-deficient (db/db) mice were from Jackson Laboratories. Heart-specific acyl-CoA synthase transgenic mice have been described (18). Heart-specific tumor necrosis factor receptor-associated factor 2 (TRAF2)-overexpressing mice are a tetracycline-responsive (“tet-off”) transgenic model that develop progressive LV systolic dysfunction by 8 weeks of age in the absence of tetracycline (19). Cardiac tissue was obtained from controls and patients with severe ischemic cardiac failure. Animals and human tissues have been described (5). Assays were performed in duplicate and normalized to amplified mRNA for ribosomal protein L32.

Immunoblot Analysis and Immunoprecipitation—Techniques were performed as described (24, 25) using the following antibodies: fatty acid synthase (ab22759) from Abcam Inc. (Cambridge, MA); β-actin (A2066) from Sigma-Aldrich; phospho-Ca2+/calmodulin-dependent protein kinase IIα (CaMKIIα) (A-17) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); sarcoplasmic reticulum Ca2+/ATPase (SERCA2a) (A010-20), phospho-Thr-17-PLB (A010–13), phospho-Ser-16-PLB (A010-12), and total-PLB A1 (A010-14) from Badrilla Ltd. (Leeds, UK); and phospho-Thr-286-CaMKII (catalog no. 3361) from Cell Signaling Technology Inc. (Danvers, MA).

Histology—Hearts were cut at the mid-ventricle in a short axis orientation, fixed in 10% neutral buffered formalin, embedded in paraffin, cut in 5-mm sections with a microtome, and stained with hematoxylin and eosin (H&E) or Masson’s trichrome.

Substrate Metabolism Using Isolated Working Heart Perfusion—Whole mouse heart fatty acid and glucose oxidation were assessed ex vivo using the isolated working heart perfusion technique essentially as described (26). Mice were heparinized (100 units i.p.) and anesthetized with sodium pentobarbital, and excised hearts were placed in ice-cold Krebs-Henseleit bicarbonate solution. The ascending aorta was cannulated and retrograde perfused with Krebs-Henseleit bicarbonate solution (containing 95% O2, 5% CO2), and the left atrium was also cannulated. Aortic and left atrial lines were perfused with Krebs-Henseleit bicarbonate solution containing 10 mM palmitate, 0.4 mM palmitate, and 10 microunits/ml insulin. Left atrial (preload) and aortic (afterload) pressures were set at 15 and 50 mm Hg, respectively. Palmitate and glucose oxidation rates were assessed by using either [3H]palmitate (0.1 μCi/ml) or [14C]glucose (0.1 μCi/ml) and quantifying released 3H2O or 14CO2 in 10-min intervals for a total of 60 min of aerobic perfusion. Cardiac output and aortic flows were measured using inline flow probes (Transonic Systems, Inc.) placed in the left atrial and aortic lines, respectively. Heart rate and pressures were measured using a pressure transducer (TSD 104A, BIOPAC Systems, Inc.). Cardiac work was calculated as the product of peak systolic pressure and cardiac output.

Echocardiography—Procedures were performed using the VisualSonics Vevo 770 Imaging System (VisualSonics Inc., Toronto, Canada) and analyzed with VisualSonics software. Anesthesia was induced with isoflurane (4–5%) and maintained by continuous inhalation (1.5–2%). M-mode images of the parasternal short axis, two-dimensional images of parasternal long and short axis, and velocity Doppler images of mitral valve inflow, mitral valve annulus, and the LV septum were obtained. Parameters were used to calculate LV mass, fractional shortening, mitral inflow deceleration time, E/A ratio, E/E′ ratio, and systolic strain rate (27).

Transverse Aortic Constriction (TAC)—TAC was performed as described with minor modifications (28, 29). Mice were appropriately anesthetized, the chest was opened, and the aorta was identified after blunt dissection through the intercostal...
muscles. A 7-0 silk suture was tied around the aorta and a blunt needle with subsequent removal of the needle. The chest and incision were appropriately closed, and sham operations were performed using the same procedures without TAC. Postprocedural echocardiography was performed 30 min after TAC. TAC experiments with simultaneous telemetry and invasive hemodynamic monitoring were performed as described (30). TAC experiments performed in the setting of CaMKII inhibition used vehicle versus water-soluble KN93 (Calbiochem) i.p. injected daily at 10 \( \mu \)g/kg for 2 days and 1.5 h preceding the surgery (adapted from previously reported in vivo dosing protocols (31–33)).

Assessment of Contractility with Dobutamine—Cardiac catheterization was performed using a 1.4F Millar\textsuperscript{®} catheter advanced into the left ventricle via percutaneous cannulation of the right carotid artery. Escalating doses of dobutamine were administered via a catheter inserted in the left internal jugular vein. Hemodynamic data were analyzed using appropriate software.

Myocyte Electrophysiology—Mouse ventricular myocytes were isolated as described (21). Whole-cell voltage clamp recordings were performed (34, 35) using an Axopatch 200A amplifier (Molecular Devices, Sunnyvale, CA) connected to a Digidata 1322A digitizer board (Molecular Devices) and a MP-225 micromanipulator (Sutter Instrument Co., Novato, CA).

Cardiomyocytes were continuously perfused with an extracellular solution containing 137 \( \text{mM} \) NaCl, 5.4 \( \text{mM} \) KCl, 0.5 \( \text{mM} \) MgCl\(_2\), 3 \( \text{mM} \) NaHCO\(_3\), 0.2 \( \text{mM} \) Na\(_2\)HPO\(_4\), 5 \( \text{mM} \) HEPES (pH 7.4), and 10 \( \text{mM} \) glucose, with additions as described. For L-type Ca\(^{2+}\) current recordings, 1.8 \( \text{mM} \) CaCl\(_2\) and 10 \( \mu \)M tetrodotoxin were added to the bath solution. For the assessment of palmi
tate effects on L-type Ca\(^{2+}\) current, the bath solution was first perfused with fatty acid-free bovine serum albumin (BSA) (60 \( \mu \)M) and then a complexed palmitate-BSA (30 \( \mu \)M, 60 \( \mu \)M) solution, providing an unbound palmitate concentration of 42 \( \text{nM} \), as described previously (36). For \( K_{ATP} \) current recordings, the pipette solution contained 130 \( \text{mM} \) potassium aspartate, 20 \( \text{mM} \)

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FIGURE 1. Cardiac FAS and generation of FASKard mice. A, FAS protein in heart tissue from saline-injected (Ctrl) mice and animals with streptozotocin-induced diabetes (STZ). B, FAS protein in heart tissue from WT and \( \beta \)-cell-specific \( K_{ATP} \) mutant transgenic mice with insulin-deficient diabetes. C, FAS protein in heart tissue from WT and leptin receptor-deficient (db/db) mice with insulin-resistant diabetes. D, simplified representation of the strategy yielding mice with FAS deficiency in the heart. E, FAS mRNA by quantitative RT-PCR in cardiac tissue of 6-week-old control and FASKard mice expressed relative to control message (L32). F, FAS protein by immunoblotting of mouse hearts using an anti-FAS polyclonal antibody. G, FAS enzyme activity in heart lysates using an assay measuring malonyl-CoA-dependent consumption of NADPH. No difference was detected using livers from the same mice (not shown). H, thin layer chromatographic assessment of de novo synthesized palmitate by \(^{14} \text{C} \)acetate radiolabeling of cultured cardiac myocytes. The number of mice and significant results are indicated. Error bars, S.E.
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KCl, 4 mM K2HPO4, 1 mM MgCl2, 10 mM EGTA, 1 mM K2ATP, and 10 mM HEPES (pH 7.2–7.3). For L-type Ca2+ current recordings, the pipette solution contained 130 mM CsCl, 20 mM tetraethylammonium chloride, 10 mM EGTA, 5 mM K2ATP, and 10 mM HEPES (pH 7.2–7.3). KATP Currents were assessed using a voltage ramp from −120 to +40 mV at a rate of 40 mV/s from a holding potential of −70 mV. L-type Ca2+ currents were assessed at −55 to +35 mV for 100 ms at a 10-mV step after a 400-ms voltage ramp from −70 to −55 mV to inactivate Na+ channels. Series resistance compensation was set at 70–90%, and the 4-pole low pass Bessel filter on the amplifier was set at 2 kHz. Whole-cell currents were digitized at 10 kHz using pCLAMP 9 (Molecular Devices). Current traces were analyzed using pCLAMP 9 software (Molecular Devices). Assessment of other ion channels (shown in Fig. 5, C–H) was as described (35).

Statistics—Data represent means ± S.E. Significance of differences between two groups was calculated using a two-tailed unpaired t test. Survival curves were compared using the log-rank test.

RESULTS

Cardiac FAS and Generation of FASKard Mice—FAS is known to be expressed in the heart (2). Because FAS is regulated by insulin in lipogenic tissues (37), we showed that FAS protein was decreased in the hearts of two insulin-deficient models, streptozotocin-treated mice and animals with diabetes (17, 38), and in the hearts of FAS knockout mice (2). Because FAS is regulated in response to insulin in lipogenic tissues (37), we showed that FAS expression and activity in FASKard mice probably reflects regulation by insulin in lipogenic tissues (37).

Mice carrying the floxed (flanked by loxP, the target of Cre recombinase) FAS gene were crossed with transgenic mice expressing Cre driven by the α-myosin heavy chain promoter to yield FASKard progeny (Fig. 1D) that were born at the expected Mendelian frequency and developed normally. FAS mRNA, protein mass, and enzyme activity (Fig. 1, E–G) were decreased relative to Cre−/− controls (animals expressing Cre recombinase with wild type FAS alleles). Additionally, adult cardiac myocytes derived from FASKard hearts had a significantly lower capacity to synthesize palmitate, the principal fatty acid synthesized by FAS, than control myocytes (Fig. 1H). Residual cardiac FAS expression and activity in FASKard mice probably reflects expression in nonmyocardial cells and perhaps non-myosin heavy chain-driven Cre mosaicism (40). Fasting levels of glucose, cholesterol, triglycerides, and free fatty acids were unaffected in FASKard mice (not shown).

Base-line Cardiovascular Parameters in FASKard Mice—There were no differences in multiple variables determined by echocardiography and no difference in blood pressure between FASKard and “Control” mice (Table 1). Myocardial morphology and architecture were normal in FASKard mice (not shown).

We assessed myocardial substrate metabolism in the isolated working heart model and, given PPARα-deficient phenotypes in mice with FAS deficiency in liver, hypothalamus, or macrophages, predicted that isolated working heart studies would show decreased fatty acid oxidation (41) in FASKard myocardium. This prediction was incorrect. Substrate metabolism, cardiac output, and work (Fig. 2) as well as expression of PPARα-dependent genes (supplemental Fig. 1) were unaffected in FASKard hearts. In addition, lipid mass spectrometry of cardiac tissue from FASKard mice revealed no reduction in the specific phosphatidylcholine species (i.e. 16:0/18:1 phosphatidylcholine) that has previously been identified as an endogenous ligand for PPARα in liver tissue (7) (supplemental Fig. 2).

Susceptibility to Hemodynamic Stress in FASKard Mice—Pressure overload induced by TAC (28, 42) unexpectedly caused death within 1 h in most of the FASKard but not in the control mice (Fig. 3A). Periprocedural echocardiography showed overt systolic dysfunction by 30 min (Fig. 3B). This phenotype did not appear to be due to intrinsic contractile defects because myocardial contractility was not overtly dysfunctional in FASKard mice in response to dobutamine, a potent β1-adrenergic agonist (data not shown).

Periprocedural LV, aortic, and telemetry tracings during TAC for representative control and FASKard mice are shown in

| Table 1 Comparison of physiological parameters between control and FASKard mice |
|--------------------------------------|--------------------------------------|
| Echo parameter                      | Control                              | FASKard                             |
| LVId                                  | 3.99                                 | 4.07                                |
| LVIDs                                 | 2.72                                 | 2.82                                |
| LVPWd                                 | 0.72                                 | 0.77                                |
| LVPW's                                | 1.12                                 | 1.11                                |
| IVSd                                  | 0.76                                 | 0.74                                |
| IVSs                                  | 1.18                                 | 1.17                                |
| LVM                                  | 104.3                                | 103.1                               |
| LVMi                                 | 4.3                                  | 4.2                                 |
| RWT                                  | 0.37                                 | 0.36                                |
| FS                                    | 31.9                                 | 30.6                                |
| RIMP                                  | 0.68                                 | 0.69                                |
| E                                    | 634.3                                | 598.3                               |
| A                                    | 482                                  | 444                                 |
| E/A                                  | 1.33                                 | 1.37                                |
| DT                                   | 27.0                                 | 30.8                                |
| S'                                   | 18.57                                | 19.68                               |
| E'                                   | 19.42                                | 20.73                               |
| A'                                   | 17.1                                 | 19.3                                |
| E/E'                                  | 32.7                                 | 28.9                                |
| IVCT                                  | 16.5                                 | 17                                  |
| IVRT                                  | 19.13                                | 18.75                               |
| SR S                                 | 6.63                                 | 5.65                                |
| SR D                                 | −6.25                                | −5.8                                |
| Non-invasive BP                       | Control                              | FASKard                             |
| SBP                                  | 97.1                                 | 98.2                                |
| DBP                                  | 81.0                                 | 82.7                                |

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LV pressure decreases acutely as expected in both genotypes soon after TAC (segments 2 and 3 in Fig. 4A and segments 1 and 2 in Fig. 4B). Control mice recover (Fig. 4A, segments 2–5), but FASKard mice develop an arrhythmia (QRS complex widening; Fig. 4B, segment 2) that precedes subsequent LV or aortic pressure compromise. The QRS complex continues to widen with progressive contractile failure (Fig. 4B, segments 3 and 4), probably contributing to the rapid demise. Arrhythmia following TAC was documented in three FASKard mice and in none of five control animals.

Altered Calcium Homeostasis In FASKard Hearts—Because mice deficient in Kir6.2 (the major subunit of ATP-sensitive inward rectifying potassium (KATP) channels) are also acutely susceptible to TAC (due mostly to arrhythmias) (43, 44), we pursued the possibility that ion channel alterations explain the cardiac FASKard phenotype. Whole-cell voltage clamp studies showed that KATP-specific currents were unchanged in FASKard cardiomyocytes (Fig. 5A, left). Time to maximal channel opening was also unchanged (Fig. 5A, right). We next focused on the L-type calcium channel (LTCC). In the basal state, FASKard cardiomyocytes demonstrated hyperactivation of LTCC (Fig. 5B, left), which may be proarrhythmic (45). LTCC currents were maximally activated with isoproterenol, a β-adrenergic agonist and LTCC activator (46) (Fig. 5B, right). Under these conditions, LTCC currents in both control and FASKard cells increased to the same level, suggesting that the calcium influx apparatus has similar capacity in both genotypes but FAS deficiency induces accelerated L-type current in the basal state. LTCC hyperactivation appeared to be relatively selective because the activity of several other ion channels was unaffected in FASKard cardiomyocytes (Fig. 5, C–H).

Protein abundance of the LTCC, ryanodine receptor, and cardiac isoform of the SERCA2a were unaffected in FASKard mice (Fig. 6A), consistent with functional changes in channel activity. Both protein kinase A and CaMKII positively regulate LTCC activity via direct phosphorylation (47, 48). Using site-specific phosphorylation of phospholamban (PLB) as a surrogate of protein kinase A and CaMKII activity, we found a selective increase in CaMKII activity in FASKard hearts (Fig. 6B). Increased CaMKII activity was supported by the presence of increased autophosphorylation of CaMKII, an independent marker of activation (Fig. 6C). The FAS-CaMKII relationship was recapitulated in vitro because shRNA-mediated knockdown of FAS in H9c2 cells (known to express key components of the calcium signaling apparatus (49)) increased CaMKII signaling (Fig. 6D). Increased CaMKII signaling represents a potential mechanism underlying arrhythmia with pressure overload (Fig. 4B).

Susceptibility to the Stress of Aging in FASKard Mice—To assess age-dependent effects, we compared histology in 3-, 6-, and 12-month-old FASKard hearts. By 6 months, FASKard hearts develop small foci of chronic inflammation/fibrosis in a pattern suggesting a metabolic (myocyte-intrinsic) origin (data not shown). FASKard mice died before controls due in part to LV dilatation and overt heart failure (Fig. 7A, including representative cardiac appearances). Compared with age-matched controls, CaMKII activity was increased in 1-year-old FASKard hearts (Fig. 7B). In order to gain insight into the etiology of such progressive heart failure, we performed a limited screen for
transcriptional changes of genes involved in various cellular processes, such as stress response, inflammation, apoptosis, adhesion, metabolism, and proliferation, using a commercially available PCR Array (supplemental Fig. 3A). Mice were studied at 3 months of age, when FASKard hearts are histopathologically normal. The modulation of several of these genes and relevant related genes were verified by quantitative RT-PCR in an independent cohort of mice (supplemental Fig. 3B). Of the genes that showed the most increase in FASKard compared with control hearts, several have been implicated in cardiac remodeling (e.g. TGFβ1, tenascin C, KLF2, αSK-actin, and β-myosin heavy chain (50–53)). Alterations in such genes appear to be selective because expression of atrial natriuretic peptide and brain natriuretic peptide, classic markers of heart failure/remodeling (53), was unchanged (supplemental Fig. 3B). Although several signaling processes can coordinately activate these genes, activation of the CaMKII signaling axis is known to play a major role (54, 55).

CaMKII Inhibition Rescues FASKard Mice—In an attempt to directly implicate CaMKII signaling in the FASKard phenotype, we subjected FASKard mice to the hemodynamic stress of TAC in the presence and absence of a well validated CaMKII inhibitor, KN-93. FASKard mice injected with KN-93 were less likely to succumb to pressure overload (Fig. 8).

FAS Induction in Murine and Human Heart Failure—We next studied FAS in hearts with overt systolic dysfunction. Heart-specific acyl-CoA synthase-overexpressing mice are normal in early life and then develop heart failure by ~8 weeks of age (18). FAS protein was marginally increased in 4-week-old acyl-CoA synthase transgenic mice (Fig. 9A, top) but clearly induced in the setting of systolic dysfunction at 8 weeks of age (Fig. 9A, bottom). FAS was also increased in the hearts of heartspecific TRAF2 transgenic mice (Fig. 9B) with inflammation-mediated heart failure (19, 56).

Tissues from structurally normal hearts and from class IV cardiomyopathic hearts subjected to LVAD placement as a bridge to heart transplantation showed that FAS mRNA levels were dramatically increased with heart failure (Fig. 9C). This induction relative to controls was confirmed at the protein level in hearts from two class IV cardiomyopathy patients (Fig. 9D, top). FAS protein decreased following LVAD placement in these patients (compare pre and post lanes in Fig. 9D, bottom), indicating FAS induction in decompensated human heart failure and the reversibility of this response with mechanical unloading of the failing myocardium. These results suggest that FAS is increased in both mouse and human heart failure.

FAS, LTCC, and Lack of Palmitate Effect on Currents—The hyperactivation of calcium signaling in FASKard hearts (i.e. significantly increased LTCC currents and CaMKII activation) raised the possibility that FAS might act to modulate the function of specific members of the calcium signaling cascade.
by direct interaction. Co-immunoprecipitation experiments revealed that although FAS has no identifiable interaction with CaMKII, SERCA2a, PLB, or ryanodine receptor (data not shown), a portion of myocardial FAS appears to interact with Cav1.2, the LTCC predominantly expressed in the heart (Fig. 10A). Because the activation of voltage-gated Ca\(^{2+}\) channels can be regulated by lipids (57–62) and the main product of FAS is palmitate, such an interaction might provide a mechanism by which \textit{de novo}-synthesized lipids regulate calcium homeostasis. The addition of palmitate to FASKard cardiac myocytes failed to reduce the hyperactivated LTCC currents (Fig. 10B), but we cannot rule out the possibility that more complex lipid species with \textit{de novo}-synthesized lipid components subserve this role.

**DISCUSSION**

About a third of advanced heart failure patients die, often from arrhythmia, within 30 months (63). Myocardial dysfunc-
tion is common in diabetes (64), an epidemic disorder contributing to the increasing prevalence of heart failure. Generating force to eject blood relentlessly requires energy, derived mostly from metabolism of exogenous lipids (12–15). Potential roles for endogenous lipids, such as those produced by FAS, have not been explored. We now show that FAS may be involved in maintaining homeostasis with hemodynamic stress and that cardiomyocyte calcium signaling appears to be involved in this process.

Alterations in cardiac calcium cycling are implicated in both arrhythmias and impaired contractile function (65). In normal hearts, plasma membrane depolarization prompts calcium influx through LTCCs, triggering calcium release from the sarcoplasmic reticulum through ryanodine receptors, generating a transient elevation in cytosolic calcium that induces myofilament contraction. Calcium is lowered by reuptake into the sarcoplasmic reticulum by SERCA2a or extrusion from the cell. CaMKII, thought to be activated by calcium itself, is a critical regulator of this change in cytosolic calcium.

Hyperactivation of CaMKII signaling may be responsible for arrhythmias and cardiomyopathy in FASKard mice. CaMKII regulates components of the intracellular calcium apparatus, including LTCCs (66, 67), and CaMKII hyperactivity is implicated in the generation of proarrhythmic early after-depolarizations (45), and CaMKII hyperactivation induces both early after-depolarizations and delayed after-depolarizations (32). Heart-specific CAMKIIβ transgenic mice develop a progressive cardiomyopathy (68), transgenic mice expressing a CAMKII inhibitory peptide resist ischemia-induced pathologic remodeling (31), and CAMKIIβ-deficient mice are protected from pressure overload-induced hypertrophy and heart failure (69, 70). Supporting the notion that CaMKII hyperactivation contributes to the cardiac FAS deficiency phenotype, FAS knock-
down in cultured cells induces CaMKII activation (Fig. 6D), and inhibition of CaMKII activity in vivo rescues FAS-deficient hearts from the decompensation resulting from acute pressure overload (Fig. 8).

How does FAS deficiency lead to CaMKII activation? Because FASKard hearts have no detectable perturbations in fatty acid or glucose metabolism, it is unlikely that this effect is prompted by gross alterations in cardiac substrate selection or shifts in intracellular lipid pools utilized for energy. PPARα (a major mediator of myocardial lipid metabolism) is not affected by FAS in the heart, unlike liver, hypothalamus, and macrophages (5, 8, 9), where FAS deficiency impairs PPARα-dependent gene expression that is restored by agonist treatment. Instead, FAS appears to serve a novel calcium signaling function in cardiomyocytes. Our demonstration that FAS co-immunoprecipitates with LTCC supports a role for FAS in the regulation of upstream events in calcium signaling. Although the addition of palmitate did not rescue the LTCC hyperactiva-

**FIGURE 6. CaMKII signaling is hyperactivated in FASKard hearts.** A, immunoblot analysis of LTCC, ryanodine receptor (RYR), and SERCA2a in control and FASKard hearts. B, immunoblots of CaMKII-activated (phospho-Thr-17) and protein kinase A-activated (phospho-Ser-16) PLB in hearts. The arrows indicate PLB of the correct size; higher bands could represent hyperphosphorylated PLB. C, immunoblots of total and activated (phospho-Thr-286) CaMKII in hearts. D, shRNA-mediated knockdown of FAS expression in H9c2 cells with immunoblots for FAS, activated (phospho-Thr-286) CaMKII, and total CaMKII. Increased activated CaMKII with FAS knockdown was seen in a total of four independent experiments. Error bars, S.E.

**FIGURE 7. Premature death and spontaneous ventricular dilatation in FASKard mice.** A, survival curves are shown for FASKard and control mice over time. The numbers for each genotype are indicated. Representative mid-ventricular H&E-stained cardiac sections from representative mice for each corresponding genotype are shown on the right. B, immunoblots of total and activated (phospho-Thr-286) CaMKII in 1-year-old hearts.
tion, the presence of more complex de novo-synthesized lipids (akin to the regulation of PPARα signaling by distinct phospholipid species) remains a possibility. Alternatively, because palmitoylation affects intracellular signaling mostly through membrane localization, protein palmitoylation driven by de novo lipogenesis may be involved as described in other cell types (71–73). There is precedent for gating of ion channels by palmitoylation (74), raising the possibility that defective lipidation in the absence of FAS increases base-line calcium influx to activate CaMKII. De novo-generated lipids might also modify the composition of compartments, such as caveolae, known to orchestrate signaling (75). Altering substrate and enzyme localization at caveolae regulates the magnitude of phosphorylation by CaMKII (76–78).

Cardiomyopathy in experimental diabetes has been known for decades to be associated with abnormal calcium flux (64, 79), and our demonstration of decreased cardiac FAS in experimental diabetes (Fig. 1) suggests that this may be mediated in part by activation of CaMKII. We also demonstrate that cardiac FAS is induced in two established mouse models of heart failure generated through distinct mechanisms and elevated in patients with severe cardiomyopathy (Fig. 9). Furthermore, this up-regulation appears to be dynamic (as gauged by comparing tissues in the same individuals before and after LVAD-mediated relief of decompensated failure) in patients with end stage cardiomyopathy. To our knowledge, FAS has not been reported to be regulated in either human or murine heart failure (80, 81). A search of microarray data for human cardiomyopathies available through the Harvard CardioGenomics data base did not yield evidence for FAS regulation (not shown), but our results indicate that FAS is up-regulated in the setting of heart failure. This response is unexpected because FAS consumes energy to synthesize fat, hinting that this process is worth its energy cost even in the setting of increased myocardial energy demand.

**FIGURE 8.** CaMKII inhibition ameliorates effects of TAC in FASKard mice. Survival curves for FASKard mice subjected to TAC after daily i.p. injection of the CaMKII inhibitor KN-93 at 10 μmol/kg for 2 days prior to the procedure and 1.5 h before surgery or injection of vehicle (Control) at the same times. The p value represents a comparison between the KN-93 and control groups.

**FIGURE 9.** FAS induction in mouse models of heart failure and in human heart failure. A, immunoblots of FAS protein in heart tissue from 4-week-old (top) and 8-week-old (bottom) control and acyl-CoA synthase transgenic (ACS Tg) mice. B, immunoblots of FAS protein in heart tissue from control and TRAF2 transgenic mice at 8 weeks of age. C, FAS mRNA in cardiac tissue from individuals with structurally normal hearts who died of non-heart-related causes (Normal) and patients with class IV cardiomyopathy requiring LVAD support (CM). D, FAS protein in heart tissue from two control subjects (Normal) and two cardiomyopathy patients (CM) (top panels), and FAS protein in heart tissue from the two CM patients of the top panels before and after LVAD support (bottom panels). Error bars, S.E.
The susceptibility of FASKard mice to arrhythmias and cardiomyopathy suggests that this induction of FAS in heart failure is compensatory, perhaps limiting CaMKII activity, which is increased in the failing heart (82). This putative compensatory up-regulation of FAS obviously does not prevent pathological remodeling in advanced heart failure, but the inability to induce FAS, as occurs with insulin deficiency and resistance in diabetes, could exacerbate heart failure. Conversely, stimulating heart-specific FAS activity might provide a mechanism to replenish distinct lipid species that are critical to myocardial function, thus aiding the recovery of the failing heart.

Such stimulation would probably involve nuclear events because FAS is mostly transcriptionally regulated (2, 37). At least three factors are important. Carbohydrate response element binding protein responds to glucose and in other tissues coordinates up to half of the lipogenic process (83). Endoplasmic reticulum stress, characteristic of heart failure and other pathologies, is mediated in part by XBP-1, a factor that promotes fatty acid biosynthesis independent of known lipogenic nuclear factors (84). The most studied lipogenic transcription factor is sterol regulatory element binding protein-1 (SREBP-1), an insulin-regulated factor with a broad range of target genes. Given the wide net cast by its induction, SREBP-1 activation might be counterproductive. For example, various SREBP-1 transgenic mice develop fatty liver, β-cell failure, and other lipotoxic phenotypes (85). However, the myocardium has marginal capacity for de novo lipogenesis, suggesting that increasing cardiac SREBP-1 might elicit responses distinct from other tissues. In support of this notion, cardiac SREBP-1 activates the G protein-coupled inwardly rectifying K⁺ channel (GIRK1/4), leading to enhanced acetylcholine-sensitive K⁺ currents and reduced arrhythmias postmyocardial infarction (86).

Our findings indicate that deletion of FAS in cardiomyocytes abrogates the ability of the heart to respond to stress through a process that appears to involve calcium flux and activation of CaMKII and that FAS is increased in heart failure. Identifying specific molecule(s) involved in this process could help clarify some of the complex relationships between cardiac metabolism and heart failure.

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