

Partial Liver Kinase B1 (LKB1) Deficiency Promotes Diastolic Dysfunction, De Novo Systolic Dysfunction, Apoptosis, and Mitochondrial Dysfunction With Dietary Metabolic Challenge

Edward J. Miller, MD, PhD; Timothy Calamaras, BS, PhD; Aly Elezaby, BS; Aaron Sverdlov, MD, PhD; Fuzhong Qin, MD, PhD; Ivan Luptak, MD, PhD; Ke Wang, MD; Xinxin Sun, MD; Andrea Vijay, BS; Dominique Croteau, BS; Markus Bachschmid, PhD; Richard A. Cohen, MD; Kenneth Walsh, PhD; Wilson S. Colucci, MD

Background—Myocardial hypertrophy and dysfunction are key features of metabolic heart disease due to dietary excess. Metabolic heart disease manifests primarily as diastolic dysfunction but may progress to systolic dysfunction, although the mechanism is poorly understood. Liver kinase B1 (LKB1) is a key activator of AMP-activated protein kinase and possibly other signaling pathways that oppose myocardial hypertrophy and failure. We hypothesized that LKB1 is essential to the heart's ability to withstand the metabolic stress of dietary excess.

Methods and Results—Mice heterozygous for cardiac LKB1 were fed a control diet or a high-fat, high-sucrose diet for 4 months. On the control diet, cardiac LKB1 hearts had normal structure and function. After 4 months of the high-fat, high-sucrose diet, there was left ventricular hypertrophy and diastolic dysfunction in wild-type mice. In cardiac LKB1 (versus wild-type) mice, high-fat, high-sucrose feeding caused more hypertrophy (619 versus 553 μm^2 , $P<0.05$), the de novo appearance of systolic dysfunction (left ventricular ejection fraction; 41% versus 59%, $P<0.01$) with left ventricular dilation (3.6 versus 3.2 mm, $P<0.05$), and more severe diastolic dysfunction with progression to a restrictive filling pattern (E/A ratio; 5.5 versus 1.3, $P=0.05$). Myocardial dysfunction in hearts of cardiac LKB1 mice fed the high-fat, high-sucrose diet was associated with evidence of increased apoptosis and apoptotic signaling via caspase 3 and p53/PUMA (p53 upregulated modulator of apoptosis) and more severe mitochondrial dysfunction.

Conclusions—Partial deficiency of cardiac LKB1 promotes the adverse effects of a high-fat, high-sucrose diet on the myocardium, leading to worsening of diastolic function and the de novo appearance of systolic dysfunction. LKB1 plays a key role in protecting the heart from the consequences of metabolic stress. (*J Am Heart Assoc.* 2016;5:e002277 doi: 10.1161/JAHA.115.002277)

Key Words: diabetes mellitus • heart failure • metabolism • obesity

The increasing prevalence of obesity from dietary caloric excess and physical inactivity exposes patients to the risk of developing obesity-related metabolic heart disease (MHD). The earliest manifestations of MHD are left ventricular

hypertrophy (LVH) and diastolic dysfunction, which is often asymptomatic. With time, however, there may be progression to clinical heart failure related to worsening diastolic dysfunction and/or the appearance of systolic dysfunction. The cellular mechanisms underlying the development and progression of MHD are incompletely understood.

Liver kinase B 1 (LKB1; also known as serine/threonine kinase 11 [STK11]) is a tumor-suppressor kinase that is mutated in patients with Peutz-Jeghers syndrome and cancers¹ that affect cell growth and polarity.² LKB1 is also a potent activator of the cellular energy sensor AMP-activated protein kinase (AMPK).³ LKB1-mediated phosphorylation of the Thr172 site on the AMPK- α catalytic subunit is a key regulatory step in the cardioprotective activation of heart AMPK during myocardial ischemia.^{4,5} AMPK exerts several potentially beneficial actions in the myocardium including insulin-independent GLUT4 translocation,⁶ inhibition of hypertrophy,⁷ and possibly a decrease in oxidative stress.⁸ LKB1 can also activate downstream

From the Myocardial Biology Unit (E.J.M., T.C., A.E., A.S., F.Q., I.L., K.W., X.S., A.V., D.C., W.S.C.), Vascular Biology Unit (M.B., R.A.C.), and Whitaker Cardiovascular Institute (E.J.M., F.Q., M.B., R.A.C., K.W., W.S.C.), Boston Medical Center and Boston University School of Medicine, Boston, MA.

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Correspondence to: Wilson S. Colucci, MD, Section of Cardiovascular Medicine, Boston University School of Medicine, 88 E. Newton Street, Boston, MA 02118. E-mail: wilson.colucci@bmc.org

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signaling pathways in addition to AMPK,^{2,3} such as the sucrose nonfermenting 1-related kinase (SNRK)⁹ and the sucrose nonfermenting AMPK-related kinase (SNARK/NUAK2),¹⁰ for which the functions in the heart are poorly understood.

We previously found that LKB1 is posttranslationally modified by the reactive aldehyde 4-hydroxy-2-nonenal, or HNE, in mice fed a high-fat, high-sucrose (HFHS) diet,¹¹ and in vitro studies show that HNE adduction of LKB1 at lysine 97 decreases its activity.¹² This suggests that LKB1 activity may be decreased in MHD, raising the possibility that impaired LKB1 signaling contributes to the pathogenesis of MHD through a decrease in cardioprotective signaling by AMPK and/or other LKB1 targets.

Our aim was to investigate the role of LKB1 in mitigating adverse effects on the heart from metabolic stress caused by an HFHS diet, which we have shown leads to myocardial hypertrophy, diastolic dysfunction, and impaired mitochondrial function.^{11,13} Mice with homozygous deficiency of cardiac LKB1 develop severe systolic failure with 50% mortality by the age of 4 months.¹⁴ To test the consequences of a partial deficiency of cardiac LKB1, our first goal was to generate mice with cardiac-specific haploinsufficiency for LKB1 (cardiac LKB1 f/+, Cre/+ [cLKB1+/-]). Because the LKB1 axis plays an important role in noncardiac muscle, it was important that LKB1 deficiency be cardiac specific. Our second goal was to test the hypothesis that cLKB1+/- mice would be more susceptible to the adverse effects of an HFHS diet and, if so, to explore the mechanisms responsible.

Methods

Experimental Animals

Male mice heterozygous for cardiomyocyte-specific expression of the *LKB1* gene were created by crossing LKB1 flox/flox¹⁴ mice with mice expressing α MHCCre (alpha myosin heavy chain-driven Cre recombinase) (Jackson Laboratory, Bar Harbor, ME). Cardiomyocyte-specific LKB1+/- mice (LKB1 f/+, Cre/+, which has one floxed copy of the LKB1 gene and one copy of MHCCre, C-LKB1+/-) were compared with wild-type (WT) littermate controls (LKB1 f/+, no Cre). The institutional animal care and use committee of Boston University School of Medicine approved all animal experiments and were in accordance with institutional guidelines.

HFHS Diet

Mice were fed an HFHS diet (35.5% fat [lard] and 26.3% carbohydrates [sucrose]; Research Diets) or a control diet (CD; 4.5% fat/76.3% corn starch) matched for micronutrients for 12 weeks beginning at 12 to 20 weeks of age, as previously described.^{11,15}

Echocardiography

Two-dimensional and M-mode echocardiography was performed on nonanesthetized mice (heart rate \approx 650 beats per minute) using an Acuson Sequoia C-256 machine with a 15-MHz transducer, as described previously.^{11,16-18} Transmitral and tissue Doppler measurements of left ventricular (LV) diastolic function was performed using a VisualSonics Vevo 770 high-resolution machine and a 30-MHz transducer.^{11,16-18} All measurements of diastolic function were performed on mice anesthetized with isoflurane, titrated to a heart rate of 400 to 500 beats per minute.

Immunoblotting

Whole-heart protein lysates made from freeze-clamped hearts using lysis buffer (HEPES pH 7.4 [20 mmol/L], β -glycerol phosphate [50 mmol/L], EGTA [2 mmol/L], DTT [1 mmol/L], NaF [10 mmol/L], NaVO₄ [1 mmol/L], Triton X-100 [1%], glycerol [10%], and 1 protease inhibitor complete mini tablet-EDTA free/20 mL [Roche]) were subjected to SDS-PAGE with protein content quantified using the Bradford method (Bio-Rad). Following transfer, nitrocellulose membranes were probed with primary antibodies (LKB1 [D60C5; Cell Signaling]), LKB1 (M-18; Santa Cruz Biotechnology), phospho-LKB1 (Ser428/431; Cell Signaling), phospho-AMPK (Thr172; Cell Signaling), total AMPK α 2 (Santa Cruz Biotechnology), GAPDH (Abcam), STRAD (S-17; Santa Cruz Biotechnology), MO25 (Cell Signaling), VDAC (Abcam), PUMA (Cell Signaling), SNRK (Sigma-Aldrich), cleaved and total caspase 3 (Cell Signaling), and visualized using LI-COR secondary antibodies and the LI-COR Odyssey IR imager. Densitometry was quantified using Odyssey software. Primary antibody directed against p53 (Cell Signaling) was detected using enhanced chemiluminescence and quantified using MultiGauge software (Fujifilm).

LKB1 Immunoprecipitation and Activity Assay

LKB1 was immunoprecipitated from whole-heart lysates using LKB1 M-18 antibody conjugated to protein G beads (GE Healthcare). Samples were either subjected to immunoblotting or used for kinase activity assays by washing twice with homogenization buffer (Tris [125 mmol/L], EDTA [10 mmol/L], EGTA [10 mmol/L]), twice with kinase wash buffer (HEPES [40 mmol/L], NaCl [80 mmol/L], EDTA [0.8 mmol/L], MgCl₂ [5 mmol/L], DTT [0.8 mmol/L], glycerol [8%], pH 7.0), and then incubated with kinase assay buffer (HEPES [80 mmol/L], NaCl [160 mmol/L], EDTA [1.6 mmol/L], DTT [0.8 mmol/L], glycerol [16%], pH 7.5) containing the LKBtide synthetic substrate (300 μ mol/L; Millipore), ATP (200 μ mol/L), and [γ -³²P]ATP (5 μ Ci). After incubation at 30°C for 20 minutes,

the reaction mixture was spotted on P81 paper, washed repeatedly with cold phosphoric acid, and dried in acetone, and radioactivity was measured by liquid scintillation counting.

Mitochondrial Function Assays

Isolated mitochondrial oxygen consumption rates were measured using a Seahorse XF24 oxygen flux analyzer, as described previously,¹⁷ and ice-cold mitochondrial assay solution (70 mmol/L sucrose, 220 mmol/L mannitol, 5 mmol/L KH_2PO_4 , 5 mmol/L MgCl_2 , 2 mmol/L HEPES, 1 mmol/L EGTA, 0.2% BSA fatty acid-free, pH 7.4) plus 10 \times substrates (complex I: 50 mmol/L pyruvate and 50 mmol/L malate; complex II: 50 mmol/L succinate and 20 $\mu\text{mol/L}$ rotenone in mitochondrial assay solution). The 4 sequential injection ports of the Seahorse cartridge contained the following materials: Port A had 50 μL of 10 \times substrate and 2.5 mmol/L ADP, port B had 55 μL of 20 $\mu\text{mol/L}$ oligomycin, port C had 60 μL of 40 mmol/L FCCP, and port D had 65 μL of 40 $\mu\text{mol/L}$ antimycin A. State III was determined after port A injection, and state IV was determined after port B injection and uncoupled after port C. Isolated heart mitochondria ATP synthesis rates for complex I and II substrates were determined using modifications of the luciferin/luciferase-based

ATP Bioluminescence Assay Kit CLS II (Roche), as described previously.¹⁷

Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from frozen hearts using the mirVana miRNA Isolation Kit (Applied Biosystems), Life Technology 2X Master Mix was used for reverse transcription polymerase chain reaction to DNA, and quantitative polymerase chain reaction was performed with TaqMan PCR Master Mix and TaqMan primers (Applied Biosystems) specific for mouse SNRK (Mm00505254_m1), STK11 (LKB1) and Stk11 (LKB1; Mm00488470_m1), PRKAA2 (AMPK α 2; Mm01264789_m1), and NUAK2 (Snark; Mm00546961_m1), as described previously.¹⁷

TUNEL Staining

Apoptosis was assessed using an In Situ Cell Death Detection Fluorescein Kit (Roche Applied Science), according to the manufacturer's instructions, as described previously, using fluorescein-labeled deoxyuridine triphosphate to identify apoptotic nuclei using fluorescence microscopy.¹⁸ Random sections were counted until reaching 10 000 cardiomyocytes per

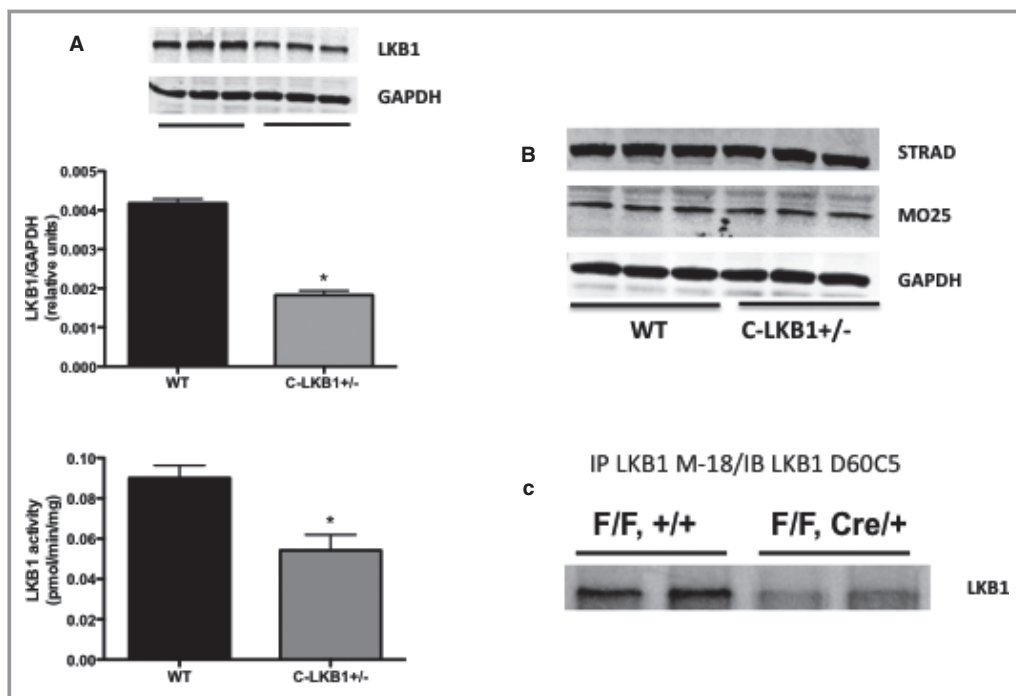


Figure 1. Characterization of the cLKB1^{+/-} mouse. A, Total cardiac LKB1 expression (n=4 to 6, -56%; * $P<0.0001$) and activity (-40%, * $P=0.01$) are decreased in cLKB1^{+/-} hearts. B, Expression of the LKB1 adapter proteins STRAD and MO25 is unchanged in cLKB1^{+/-} hearts. C, Pull-down of endogenous LKB1 protein is specific in the heart, using hearts without cardiac LKB1 (LKB1 F/F, Cre/+, or LKB1^{-/-}) as a negative control. Faint band in LKB1^{-/-} hearts represents noncardiomyocyte or atrial LKB1 protein (see Figure S1). cLKB1^{+/-} indicates cardiac-specific haplodeficiency for liver kinase B1; LKB1, liver kinase B1.

animal. The number of apoptotic nuclei was expressed per 10 000 cardiomyocytes, using colocalization of dUTP, anti- α -sarcomeric actin (Sigma-Aldrich), and Hoechst 33258 to identify apoptotic nuclei, cardiomyocytes, and nuclei, respectively.

Microscopy for Myocyte Cross-Sectional Area and Immunohistochemistry

Cardiomyocyte cross-sectional area and fibrosis were measured, as described previously.¹⁸ Briefly, sections were stained with hematoxylin and eosin and examined under a light microscope (BX40; Olympus). Five random fields from each of 4 sections per animal were analyzed, and 60 cardiomyocytes per animal were measured. The quantification of cardiomyocyte cross-sectional area was determined with ImageJ software (National Institutes of Health). To assess fibrosis, sections were stained with a Masson trichrome kit (Sigma-Aldrich) and examined under a light microscope (BX40; Olympus).

Statistical Analysis

Data are presented as mean \pm SEM for continuous variables. Differences between 2 means were tested using an unpaired, nonparametric t test (Mann–Whitney), whereas 1-way ANOVA with Bonferroni post test was used for multiple comparisons using Prism 5 (version 5.0f; GraphPad Software Inc). For all analyses, a *P* value of <0.05 was considered to be statistically significant.

Results

Phenotypic Characterization of cLKB1+/- Hearts

We generated mice with a single copy of the *LKB1* gene expressed in cardiomyocytes (cLKB1+/-). Total cardiac LKB1 expression is decreased by 56% (*P*<0.0001) in cLKB1+/- hearts, whereas the expression of the LKB1 adapter proteins STRAD and MO25 is normal (Figure 1). Decreased expression of LKB1 in the cLKB1+/- hearts is associated with a 40%

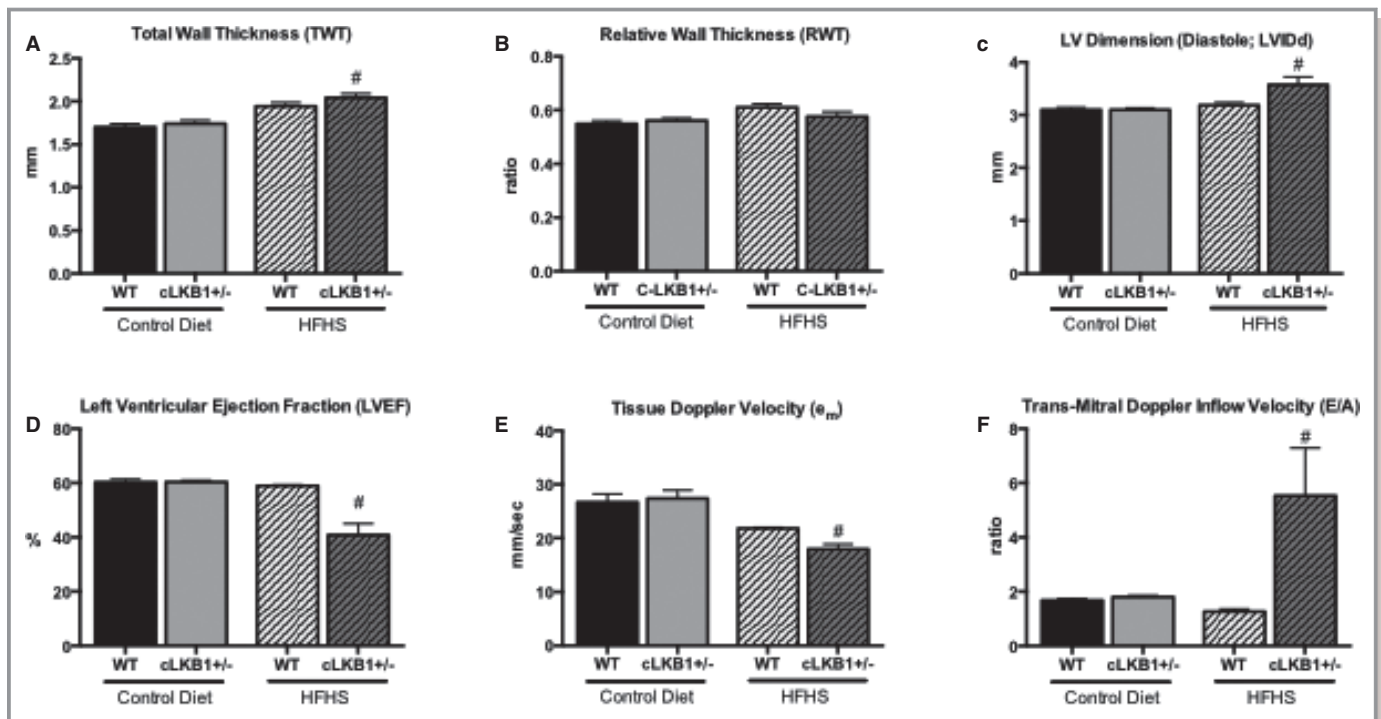


Figure 2. Detrimental effect of HFHS diet on cLKB1+/- heart function. A, An HFHS diet increases total wall thickness in WT hearts and in cLKB1+/- hearts. [#]*P*=0.14 vs WT fed HFHS. B, Relative wall thickness (LV septum plus LV posterior wall divided by LVIDd) is unchanged in cLKB1+/- hearts with and without HFHS feeding, demonstrating no evidence of eccentric LV remodeling. C, LV diastolic internal dimension is significantly increased in hearts from HFHS-fed cLKB1+/- mice. [#]*P*=0.04 vs WT fed HFHS. D, LV ejection fraction is significantly reduced in hearts from cLKB1+/- mice on an HFHS diet. [#]*P*=0.005 vs WT fed HFHS. E, Mitral annular tissue Doppler velocity (e_m) is reduced in hearts from WT fed HFHS and to a greater extent in hearts from cLKB1+/- mice fed HFHS. [#]*P*=0.008 vs WT fed HFHS. F, There is a marked increase in the transmitral Doppler inflow velocity in hearts from cLKB1+/- mice fed HFHS. [#]*P*=0.06 vs WT fed HFHS). This is consistent with marked diastolic dysfunction and restrictive LV filling. Hearts from WT fed HFHS demonstrated a mild reduction in E/A ratio consistent with impaired relaxation. n=5 to 7 per group. cLKB1+/- indicates cardiac-specific haplodeficiency for liver kinase B1; HFHS, high-fat, high-sucrose; LKB1, liver kinase B1; LV, left ventricular; LVIDd, LV diastolic internal dimension; WT, wild type.

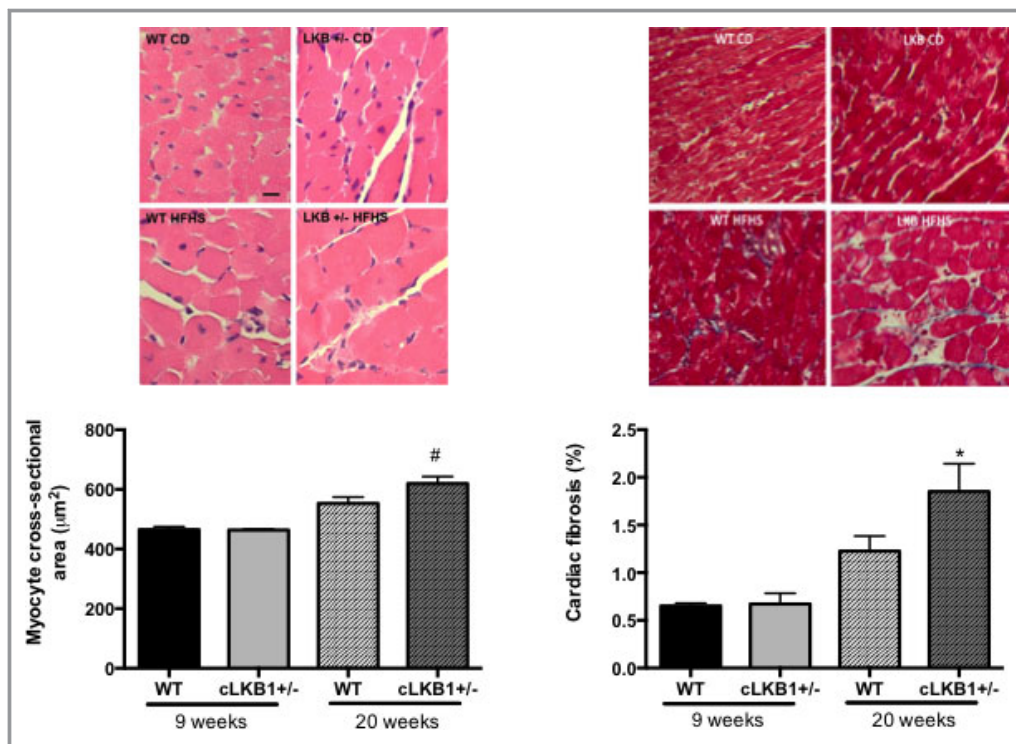


Figure 3. Increased cardiomyocyte cross-sectional area in cLKB1^{+/-} hearts after HFHS diet. Dietary challenge with HFHS feeding more severely increased (left panel) cardiomyocyte area in cLKB1^{+/-} hearts compared with hearts from WT fed HFHS and controls. [#] $P < 0.05$ vs WT fed HFHS. Cardiac fibrosis was increased in both WT and cLKB1^{+/-} hearts following HFHS feeding (right panel). CD indicates control diet; cLKB1^{+/-}, cardiac-specific haplodeficiency for liver kinase B1; HFHS, high-fat, high-sucrose; LKB1, liver kinase B1; WT, wild type.

decrease in LKB1 activity, as assessed using an LKB1 immunoprecipitation kinase activity assay ($P=0.01$) (Figure 1). The specificity for LKB1 pull-down was confirmed using lysates from cLKB1^{-/-} hearts as a negative control (Figure 1). Interestingly, there was persistence of an LKB1 phospho-Ser428/431 signal in hearts with homozygous deletion of LKB1 (LKB1^{-/-}) (Data S1 and Figure S1), suggesting that this commonly used measure of LKB1 activity is not specific in the heart.

In contrast to the severe phenotype of LKB1^{-/-} hearts, there were no changes in LV wall thickness, chamber size, or systolic or diastolic function in cLKB1^{+/-} hearts (versus WT) at ages up to 6 months (Figure S2). Consequently, partial cardiac-specific LKB1 deficiency per se does not lead to adverse cardiac remodeling or dysfunction under nonstress conditions.

HFHS Diet Causes Severe Adverse Remodeling in cLKB1^{+/-} Hearts

We previously showed that WT mice fed an HFHS diet developed cardiac hypertrophy and diastolic dysfunction with preserved systolic function.¹¹ To evaluate the role of the LKB1

axis in mediating the heart's response to metabolic stress, cLKB1^{+/-} mice ($n=24$) were fed an HFHS diet for 4 months and compared with WT littermates ($n=23$). As expected, WT mice fed an HFHS diet developed LV hypertrophy and diastolic dysfunction with decreases in Em and E/A (Figure 2). Hearts from HFHS-fed cLKB1^{+/-} mice developed more hypertrophy than CD-fed cLKB1^{+/-} mice, as assessed by echocardiography (Figure 2) and by histology with measurement of cardiomyocyte cross-sectional area (Figure 3). HFHS feeding led to an increase in oxidative stress in cLKB1^{+/-} hearts that was similar to WT (Figure S3) and induction of antioxidant gene expression (data not shown). In WT mice, HFHS feeding had no effect on LV dimensions or ejection fraction. In contrast, in cLKB1^{+/-} mice, HFHS feeding caused LV dilation (LV diastolic internal dimension; $P=0.03$ versus WT fed HFHS) and systolic dysfunction (LV ejection fraction; $P=0.001$ versus WT fed HFHS) (Figure 2). Diastolic function was also more severely impaired in hearts from cLKB1^{+/-} versus WT mice on an HFHS diet, with a larger decrease in Em by tissue Doppler (0.012 versus WT fed HFHS). Likewise, HFHS feeding caused a slowed filling pattern with a decrease in E/A by transmitral Doppler in WT mice, whereas in cLKB1^{+/-} mice, HFHS feeding caused a marked restrictive pattern with

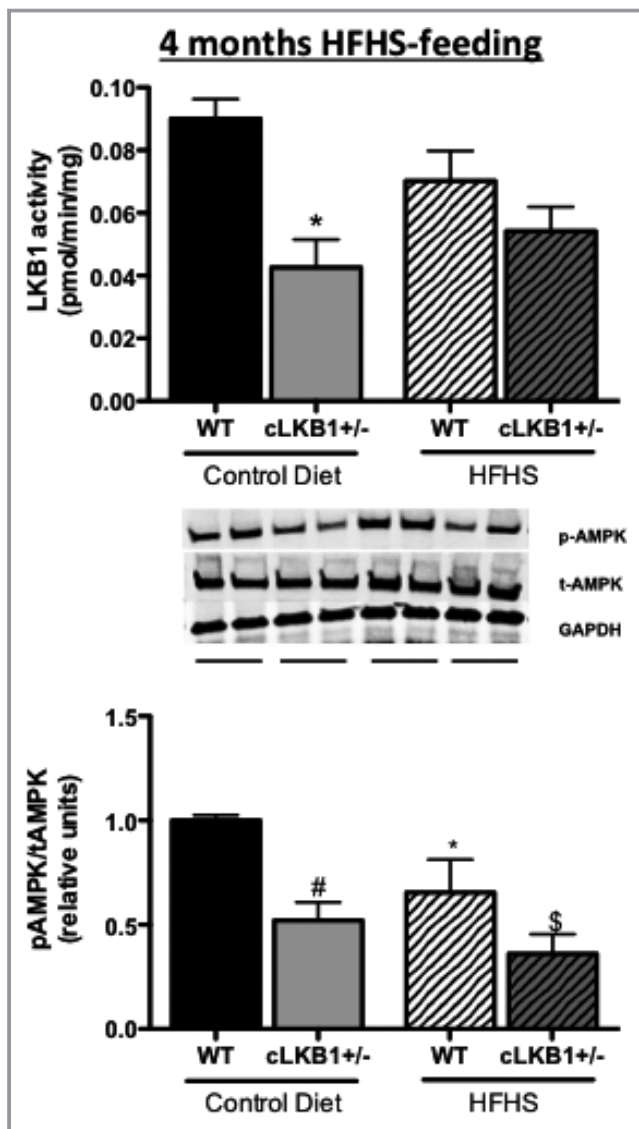


Figure 4. LKB1 activity and AMPK phosphorylation are reduced after an HFHS diet. Cardiac LKB1 activity was reduced in cLKB1+/- mice vs WT on CD (* $P=0.003$) and nonsignificantly reduced at 4 months of an HFHS diet in WT mice ($P=0.19$) (top panel). The HFHS diet did not further reduce LKB1 activity in cLKB1+/- hearts. AMPK Thr172 phosphorylation was reduced in cLKB1+/- hearts (# $P=0.001$ vs WT) (bottom panel) on a CD and in WT hearts on an HFHS diet ($P=0.07$ vs CD). On an HFHS diet, AMPK Thr172 phosphorylation in cLKB1+/- was decreased by 31%, which tended to be lower than in HFHS-fed WT hearts ($P=0.15$). $n=5$ to 7 per group. CD indicates control diet; cLKB1+/-, cardiac-specific haplodeficiency for liver kinase B1; HFHS, high-fat, high-sucrose; LKB1, liver kinase B1; p-AMPK, phospho-Thr172 AMPK; t-AMPK, total AMPK; WT, wild type.

a ≈ 5 -fold increase in E/A ($P=0.012$ versus WT CD). The increased myocyte hypertrophy and diastolic dysfunction occurred in concert with increased myocardial fibrosis in both WT and cLKB1+/- hearts (Figure 3).

Of note, 17% (4 of 24) of cLKB1+/- mice on an HFHS diet died compared with none of the WT mice on an HFHS diet (0

of 23). No WT or cLKB1+/- mice on the CD died. In contrast to WT mice on the HFHS diet, cLKB1+/- mice developed LV systolic dysfunction with chamber dilation, more severe diastolic dysfunction with progression to restrictive filling, and increased mortality with premonitory hunched posture and poor grooming, which can be a sign of congestive heart failure.

Effect of an HFHS Diet on LKB1 Activity and AMPK Activation

In WT mice, cardiac LKB1 activity was reduced 28% after 4 months on the HFHS diet, but the decrease did not reach statistical significance ($P=0.19$) (Figure 4). Of note, we found that in WT mice, HFHS feeding for 8 months decreased LKB1 activity by $\approx 32\%$ ($P=0.02$) with no change in LKB1 expression (Figure S4). In cLKB1+/- hearts, 4 months of an HFHS diet did not further reduce LKB1 activity.

Downstream AMPK Thr172 phosphorylation was reduced by 48% in cLKB1+/- hearts ($P=0.001$ versus WT) on CD and by 35% ($P=0.07$) in WT hearts on the HFHS (versus CD) diet (Figure 4). On the HFHS diet, AMPK Thr172 phosphorylation in cLKB1+/- hearts was decreased by 31%, which tended to be lower than in hearts of HFHS-fed WT mice ($P=0.15$).

Increased Expression of SNRK, a Downstream LKB1 Target, in Hearts From HFHS-Fed Mice

Although AMPK is the best recognized LKB1 downstream target, other targets undergo direct activation by LKB1, such as the >12 members of the AMPK-related kinase (ARK) family.^{2,19} A member of the ARK family, SNRK, may be important for limiting cellular proliferation in colon cancer⁹ and inhibiting neuronal apoptosis.²⁰ Of note, it was shown recently that cardiomyocyte-specific loss of SNRK causes mitochondrial dysfunction, LV dilation, and reduced systolic function.²¹ The similarity of this phenotype to the one we observed in HFHS-fed cLKB1+/- mice raised the possibility that SNRK is an important target for LKB1 in MHD.

We attempted, without success, to measure endogenous SNRK activity via an immunoprecipitation kinase activity approach (analogous to LKB1 activity) using 6 different anti-SNRK antibodies. Furthermore, there is no known posttranslational marker, analogous to AMPK phospho-Thr172, for measuring SNRK activity; however, we found that HFHS feeding increased SNRK expression, as reflected by a 40% ($P=0.01$) increase in SNRK mRNA and a 100% increase in SNRK protein ($P=0.03$) in WT hearts. Likewise, in hearts from HFHS-fed cLKB1+/- mice, there were 52% ($P=0.002$) and 64% ($P=0.03$) increases in SNRK mRNA and protein, respectively (Figure 5). There were no changes in the expression of

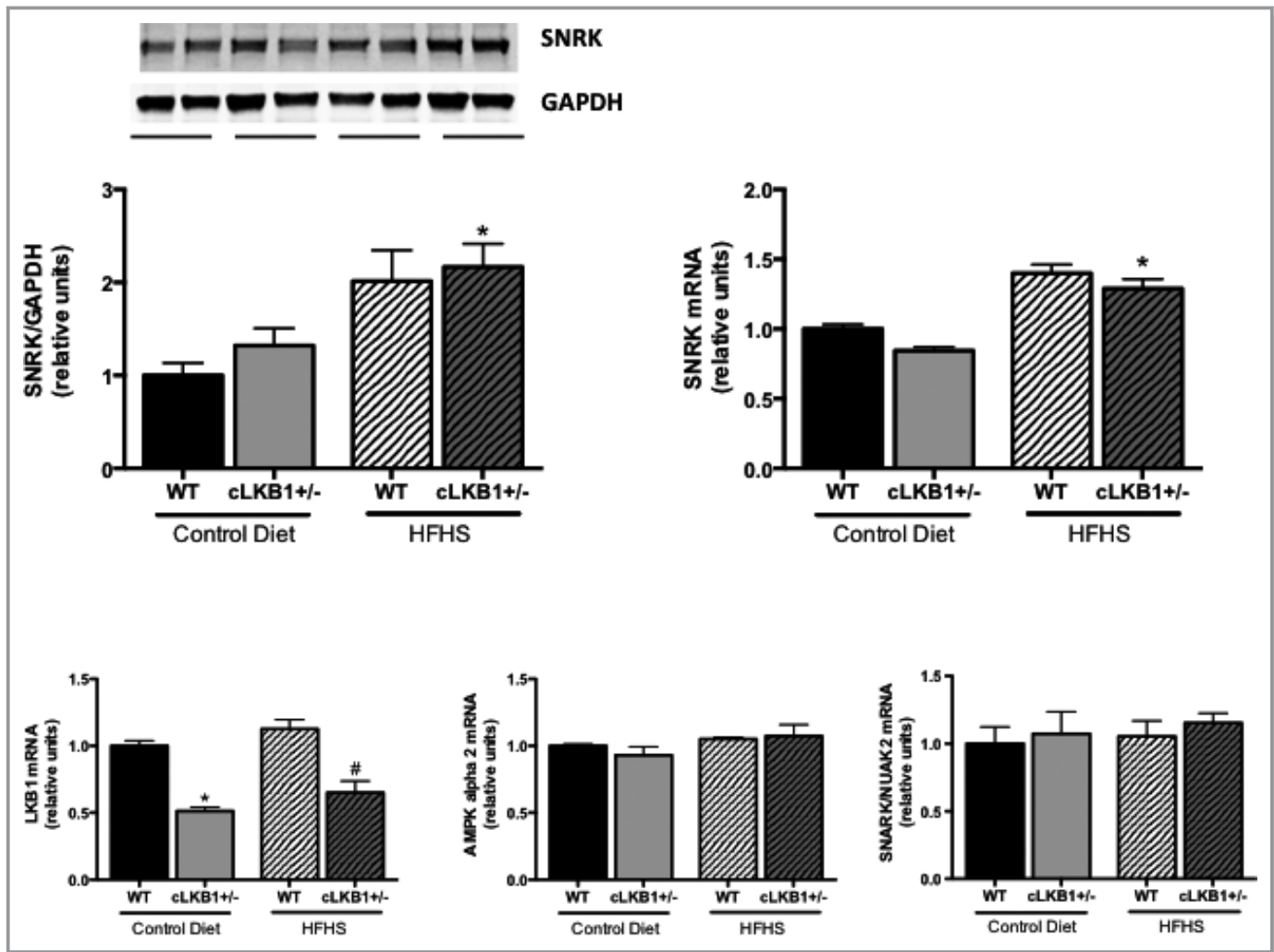


Figure 5. SNRK expression is upregulated by HFHS diet. SNRK protein level (upper left panel) is upregulated by HFHS diet in both WT and cLKB1+/- (* $P=0.04$ vs CD) and is measured at the transcript level by quantitative reverse transcription polymerase chain reaction (upper right) (* $P=0.0004$ vs WT fed HFHS). LKB1 expression is reduced $\approx 50\%$ in cLKB1 hearts and is not induced by an HFHS diet (lower left panel) (# $P=0.005$ vs WT fed HFHS). AMPK $\alpha 2$ and SNARK/NUAK2 transcript expression is not changed in cLKB1+/- hearts with a control or HFHS diet. $n=5$ to 8 per group. CD indicates control diet; cLKB1+/-, cardiac-specific haplodeficiency for liver kinase B1; HFHS, high-fat, high-sucrose; LKB1, liver kinase B1; SNARK, sucrose nonfermenting AMPK-related kinase; SNRK, sucrose nonfermenting 1-related kinase; WT, wild type.

either AMPK $\alpha 2$ or SNARK/NUAK2 (another ARK) with the HFHS diet, showing that induction of SNRK was not ubiquitous among members of the ARK family.

Increased Apoptosis in Hearts From HFHS-Fed cLKB1+/- Mice

The HFHS diet increased the percentage of TUNEL-positive cardiomyocyte nuclei in both WT ($P=0.04$ versus WT CD) and cLKB1+/- hearts ($P=0.02$ versus cLKB1+/- CD) (Figure 6). The HFHS diet-induced increase in TUNEL-positive nuclei tended to be higher in cLKB1+/- versus WT hearts ($P=0.19$). This trend was associated with increases in cleaved caspase 3 and the expression of proapoptotic p53 and PUMA (Figure 6).

There were no changes in the expression of the antiapoptotic proteins BCL2, BCL_{xl}, and MCL1 (data not shown).

Mitochondrial Dysfunction in Hearts From HFHS-Fed cLKB1+/- Mice

Mitochondrial respiration and maximal ATP production were not different in mitochondria isolated from cLKB1+/- versus WT hearts of CD-fed mice (data not shown); however, after 4 months of HFHS feeding, maximal ATP production with both complex I and II substrates was decreased in mitochondria from cLKB1+/- (versus WT) hearts (Figure 7). Likewise, complex II-driven state III, state IV, and uncoupled respiration were decreased in mitochondria from cLKB1+/- (versus WT) hearts

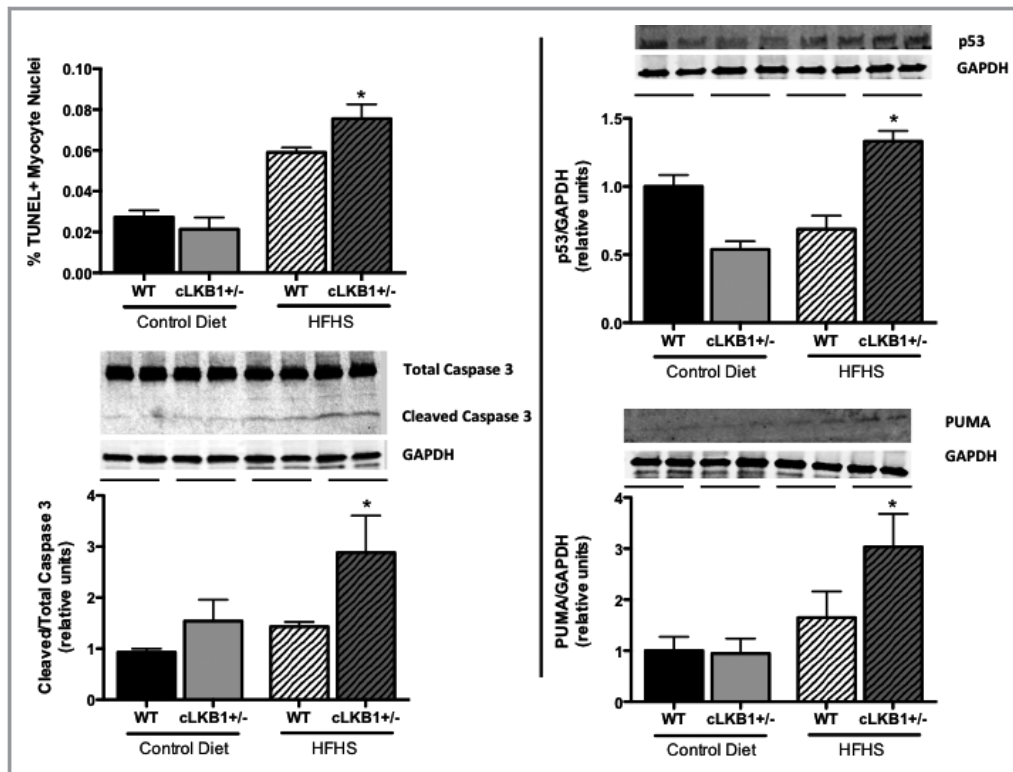


Figure 6. Increased apoptosis in cLKB1^{+/-} hearts. HFHS feeding increased the percentage of TUNEL-positive cardiomyocyte nuclei (top left panel) in both WT and cLKB1^{+/-} hearts (**P*=0.02 vs cLKB1^{+/-} fed CD). This coincided with an increase in cleaved/total caspase 3 (**P*=0.03 vs WT fed HFHS) (bottom left panel), increased p53 expression (**P*=0.0004 vs WT fed HFHS) (top right panel), and PUMA expression (**P*=0.02 vs WT fed HFHS) (bottom left panel). *n*=5 to 7 per group. CD indicates control diet; cLKB1^{+/-}, cardiac-specific haplodeficiency for liver kinase B1; HFHS, high-fat, high-sucrose; WT, wild type.

(Figure 7). Mitochondrial mass, as reflected by VDAC expression and complex II succinate dehydrogenase subunit A expression, was not different in cLKB1^{+/-} versus WT hearts (data not shown). Respiration using complex I substrates was unchanged.

Discussion

Obesity-related MHD is increasing at an alarming rate.²² Hallmarks of obesity-related MHD include LVH and diastolic dysfunction, and with time, there is often progression to clinical heart failure due to more severe diastolic dysfunction and/or the development of systolic dysfunction.²³ Our goal was to investigate the role of the key energetic signaling enzyme LKB1 in mitigating the response of the heart to the metabolic challenge of an HFHS diet. As we expected, the adverse effects of HFHS feeding were worse in cLKB1^{+/-} mice, which exhibited greater LVH, more severe diastolic dysfunction, and the development of de novo systolic dysfunction that was associated with increased mortality. Because we previously observed that HFHS feeding per se decreases LKB1 activity by means of HNE adducts,¹² we

expected that cLKB1^{+/-} mice with a 50% decrease in LKB1 protein would exhibit a further decrease in LKB1 activity when exposed to HFHS feeding. Contrary to this thesis, we found that LKB1 activity in cLKB1^{+/-} mice was not lowered further by HFHS feeding. These findings suggest that a 50% decrease in LKB1 activity per se is not detrimental to cardiac function but rather makes the heart more susceptible to the adverse effects of metabolic stress.

Specifically, this study provides several new findings. First, in contrast to mice with complete absence of cardiac LKB1, the 50% decrease in LKB1 in cLKB1^{+/-} mice had no effect on cardiac structure or function. Second, HFHS feeding, which led to LVH and moderate diastolic dysfunction, decreased LKB1 and AMPK signaling and increased the expression of SNRK, a member of the ARK family that is activated by LKB1.²⁴ Third, in cLKB1^{+/-} (versus WT) mice, HFHS feeding caused more severe diastolic dysfunction, cardiomyocyte hypertrophy, the de novo appearance of systolic dysfunction, early death, increased apoptotic signaling, more fibrosis, and worse mitochondrial function. Together, these findings suggest that LKB1 plays a key role in mitigating the cardiac response to a dietary metabolic challenge.

Phenotype of the cLKB1+/- Mouse

In prior studies, homozygous ($\approx 100\%$) deletion of cardiomyocyte LKB1 (LKB1-/-) led to a severely dysfunctional cardiac phenotype with atrial and LV dilation, profound systolic failure, and arrhythmias beginning at 4 weeks of age and progressing to death before 6 months.^{14,25} The cLKB1+/- mouse is novel and provides novel insight regarding the role of LKB1 in the response to pathophysiological stimuli. It is noteworthy that partial deficiency of cardiac LKB1 expression and activity in the cLKB1+/- mouse had no effect on cardiac structure or function in the absence of dietary stress (Figure S2). The lack of a cardiac phenotype in the cLKB1+/- mouse indicates that LKB1 activity on the order of 50% is sufficient to support normal cardiac structure and function.

Effects of HFHS Feeding on the LKB1-AMPK Axis

We previously showed that HFHS feeding for 4 to 8 months causes LVH, diastolic dysfunction, increased oxidative stress, and mitochondrial impairment.^{11,15} In the present study, HFHS feeding for 4 months likewise caused LVH and diastolic dysfunction that coincided with increased oxidative stress defined by accumulation of cardiac HNE, a reactive aldehyde generated by lipid peroxidation. HNE has been shown to inhibit LKB1 activity,⁷ at least in part, via adduction to lysine 97.¹² In the present study, we found that HFHS feeding for 4 and 8 months decreased LKB1 activity by 28% and 32%, respectively, although only the decrease at 8 months reached significance; however, the modest decrease in LKB1 activity with 4 months of HFHS feeding was associated with a significant 35% decrease in AMPK activity.

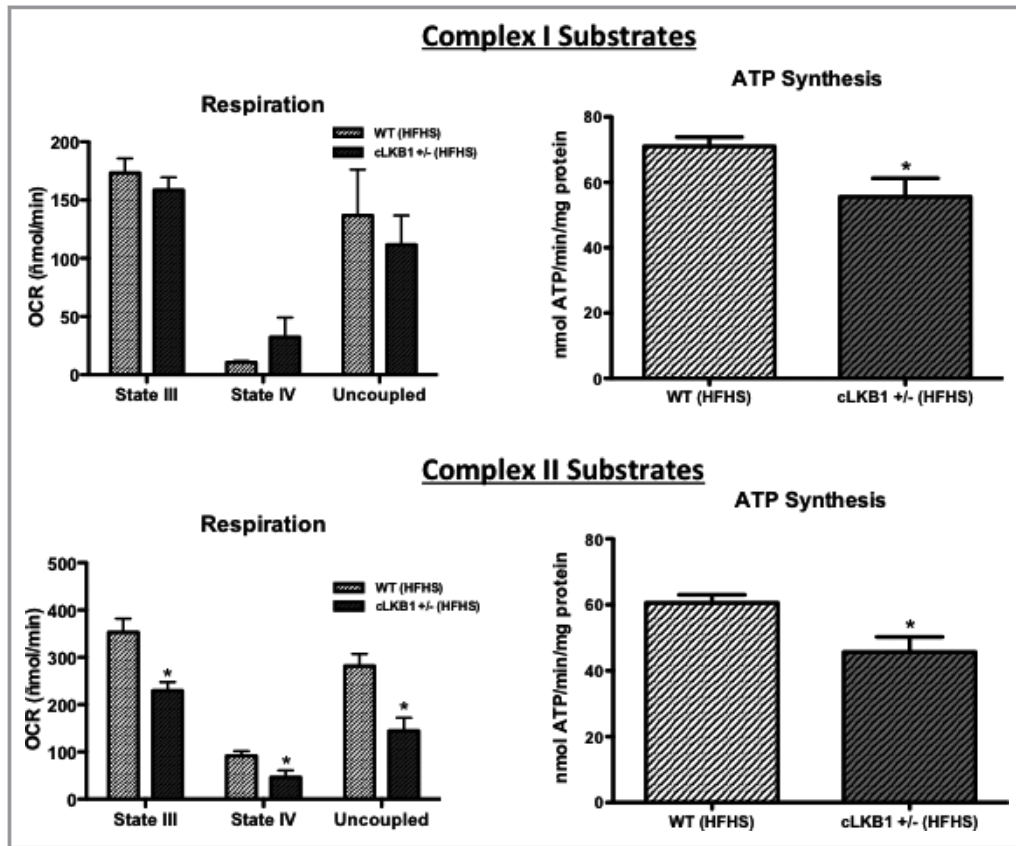


Figure 7. Mitochondrial dysfunction in cardiac mitochondria isolated from HFHS-fed hearts. Using mitochondrial complex I substrates, there was no difference in mitochondrial respiration measured in isolated heart mitochondria (top left), but there was a significant reduction in the maximal rate of ATP synthesis ($*P=0.04$ vs WT fed HFHS). Using complex II substrates, the rates of maximal ADP-stimulated respiration (state III), proton leak (state IV), and FCCP-uncoupled respiration were all significantly decreased in cLKB1+/- hearts from HFHS-fed mice ($*P<0.05$ vs WT fed HFHS) (bottom left). In addition, the maximal rate of ATP synthesis was reduced in HFHS-fed cLKB1+/- hearts ($*P=0.02$ vs WT fed HFHS). $n=7$ per group. cLKB1+/- indicates cardiac-specific haplodeficiency for liver kinase B1; HFHS, high-fat, high-sucrose; WT, wild type.

HFHS Feeding in cLKB1+/- Mice

Although cLKB1+/- mice have no cardiac phenotype at baseline, they have a markedly worse response to HFHS feeding, with more severe diastolic dysfunction and the de novo appearance of systolic dysfunction and early death. This finding suggests that the LKB1-AMPK axis may play an important role in opposing the adverse effects of HFHS feeding. In WT mice, the ≈28% decrease in LKB1 activity caused by HFHS feeding was less than the ≈50% decrease in the cLKB1+/- mouse; therefore, these observations suggest that LKB1, despite a modest decrease in activity, exerts a protective effect in the setting of metabolic stress.

We previously showed that HFHS feeding leads to mitochondrial dysfunction with increased reactive oxygen species production, decreased ATP synthesis, and oxidative post-translational modifications of mitochondrial proteins that may contribute to dysfunction.¹⁵ In this regard, it is noteworthy that in cLKB1+/- mice, HFHS feeding caused more severe mitochondrial dysfunction, reflected by larger decreases in maximal ATP production with both complex I and II substrates. LKB1 deficiency may thus contribute to worsening myocardial dysfunction by causing energetic limitation of key energy-dependent processes such as sarcoplasmic reticulum calcium reuptake. Acting through AMPK, LKB1 may oppose the effects of HFHS feeding on mitochondrial function by supporting mitochondrial biogenesis²⁶ or decreasing oxidative stress.²⁷ In cLKB1+/- mice, HFHS feeding was also associated with evidence of increased apoptotic signaling and activity that may have contributed to the appearance of systolic heart failure, reflected by dilation and a fall in ejection fraction. Apoptotic myocytes were increased with HFHS feeding and tended to be more increased in cLKB1+/- mice and were associated with significant increases in caspase 3 activation and increased expression of p53 and PUMA. LKB1 interacts with p53 in the nucleus,²⁸ and an inverse relationship between LKB1/AMPK activity and p53 abundance has been observed.²⁹ Consequently, diminished LKB1 activity in the hearts of HFHS-fed cLKB1+/- mice may be a potential factor in promoting a deleterious proapoptotic signaling cascade.

Role of Decreased AMPK Activity and Other LKB1 Targets

HFHS feeding caused a greater decrease in AMPK activity in cLKB1+/- mice than in WT mice, although the difference was small and did not reach significance. It is possible that this modest decrease in AMPK in the cLKB1+/- mice contributes to more deleterious effects of an HFHS diet in these mice. Other downstream LKB1 targets are now recognized such as SNRK and SNARK/NUAK2.^{2,19,30} Although the functions of

these LKB1 targets are not fully understood, it is noteworthy that mice with cardiomyocyte-specific deletion of SNRK develop cardiac and mitochondrial dysfunction similar to that observed in HFHS-fed cLKB1+/- mice.²¹ It is of interest that expression of SNRK is increased by HFHS feeding, suggesting that expression may be a compensatory response to dietary stress. Although it is not currently possible to measure SNRK activity, it seems likely that LKB1-SNRK signaling would be impaired in the cLKB1+/- mice, possibly removing a potentially important compensatory mechanism.

In summary, partial LKB1 deficiency promotes a deleterious response to HFHS feeding with more severe diastolic dysfunction, the de novo appearance of systolic dysfunction, mitochondrial dysfunction, and increased apoptotic signaling. An alternative view of these results is that HFHS feeding of cLKB1+/- mice unmasks a phenotype not dissimilar to that observed with severe (homozygous) LKB1 deficiency. In either case, our findings indicate that LKB1 signaling plays an important role in protecting the heart from the structural and functional effects of dietary stress. Our observation that SNRK, a newly recognized target of LKB1, is upregulated by HFHS feeding raises the possibility that the adverse effects of LKB1 deficiency involve a decrease in SNRK signaling. Future studies that elucidate the mechanisms by which LKB1 protects the heart from pathological stimuli such as dietary excess may lead to novel approaches to metabolic and other stresses.

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Disclosures

None.

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