Sucrose Nonfermenting-Related Kinase Enzyme–Mediated Rho-Associated Kinase Signaling is Responsible for Cardiac Function

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Background—Cardiac metabolism is critical for the functioning of the heart, and disturbance in this homeostasis is likely to influence cardiac disorders or cardiomyopathy. Our laboratory has previously shown that SNRK (sucrose nonfermenting related kinase) enzyme, which belongs to the AMPK (adenosine monophosphate–activated kinase) family, was essential for cardiac metabolism in mammals. Snrk global homozygous knockout (KO) mice die at postnatal day 0, and conditional deletion of Snrk in cardiomyocytes (Snrk cmcKO) leads to cardiac failure and death by 8 to 10 months.

Methods and Results—We performed additional cardiac functional studies using echocardiography and identified further cardiac functional deficits in Snrk cmcKO mice. Nuclear magnetic resonance-based metabolomics analysis identified key metabolic pathway deficits in SNRK knockout cardiomyocytes in vitro. Specifically, metabolites involved in lipid metabolism and oxidative phosphorylation are altered, and perturbations in these pathways can result in cardiac function deficits and heart failure. A phosphopeptide-based proteomic screen identified ROCK (Rho-associated kinase) as a substrate for SNRK, and mass spec-based fragment analysis confirmed key amino acid residues on ROCK that are phosphorylated by SNRK. Western blot analysis on heart lysates from Snrk cmcKO adult mice and Snrk knockout cardiomyocytes showed increased ROCK activity. In addition, in vivo inhibition of ROCK partially rescued the in vivo Snrk cmcKO cardiac function deficits.

Conclusions—Collectively, our data suggest that SNRK in cardiomyocytes is responsible for maintaining cardiac metabolic homeostasis, which is mediated in part by ROCK, and alteration of this homeostasis influences cardiac function in the adult heart. (Circ Cardiovasc Genet. 2016;9:474-486. DOI: 10.1161/CIRCGENETICS.116.001515.)

Key Words: AMPK ■ cardiac ■ echocardiography ■ metabolism ■ MYH6 ■ ROCK ■ SNRK

Cardiomyopathy is a complex disorder influenced by several factors. Some of these factors include impaired endothelial function and sensitivity to various ligands (β-agonists), altered intracellular calcium homeostasis, and accumulation of connective tissue, such as insoluble collagen.1 Recently, cardiomyopathy as a consequence of early alterations in cardiac metabolism has been proposed, particularly with respect to diabetes mellitus.2 A potential link between cardiac metabolism and function is the ROCK (Ras homolog family member A [RhoA]–associated kinase) signaling pathway. The RhoA/ROCK signaling pathway has been implicated in several cardiovascular and metabolic disorders, such as atherosclerosis, cardiac hypertrophy, and diabetes mellitus.3 ROCK is a serine threonine kinase involved in regulating various cellular processes, such as cell contraction, migration, proliferation, apoptosis/survival, and gene expression/differentiation.4 Interestingly, pharmacological inhibition of RhoA/ROCK activity has been shown to improve cardiac function in diabetes mellitus–induced cardiomyopathy.5 To date, signaling molecules regulating ROCK activity have primarily been restricted to the RhoA pathway. Our laboratory has identified a novel member of the AMPK (adenosine monophosphate–activated kinase) family, namely, SNRK (sucrose nonfermenting-related kinase), that is essential for angiogenesis6 and

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is a serine threonine kinase involved in regulating various cellular processes, such as cell contraction, migration, proliferation, apoptosis/survival, and gene expression/differentiation. Interestingly, pharmacological inhibition of RhoA/ROCK activity has been shown to improve cardiac function in diabetes mellitus–induced cardiomyopathy. To date, signaling molecules regulating ROCK activity have primarily been restricted to the RhoA pathway. Our laboratory has identified a novel member of the AMPK (adenosine monophosphate–activated kinase) family, namely, SNRK (sucrose nonfermenting-related kinase), that is essential for angiogenesis and
responsible for cardiac metabolism in mammals. Here, we implicate SNRK as a putative regulator of ROCK activity in cardiomyocytes.

Cardiomyocyte metabolism is developmentally dynamic, in that the fetal heart primarily uses glucose as its major energy substrate, and during postnatal maturation, the heart switches to primarily using fatty acid oxidation to meet its energy demands. In our previous work, we reported that the global Snrk KO mice die within 24 hours after birth and display enlarged hearts, and lethality is associated with metabolic defects in cardiac tissues. SNRK maintains metabolic homeostasis via regulation of the pACC (phosphorylated acetyl-CoA carboxylase)-phosphorylated AMPK (pAMPK) pathway during this transitional period in development. Furthermore, cardiac-specific conditional Snrk KO (Snrk cmcKO) adult mice display severe cardiac functional deficits and lethality within 8 to 10 months. We extended this work further in this study and identified ROCK as a putative substrate for SNRK that contributes to this metabolic defect in the heart. Nuclear magnetic resonance (NMR)-based substrate for SNRK that contributes to this metabolic defect in the heart. Nuclear magnetic resonance (NMR)-based metabonomics in human embryonic stem cell–derived cardiomyocytes and signaling studies in both adult heart lysates and cultured cardiomyocytes collectively imply that SNRK-mediated ROCK signaling pathway is an important regulator of cardiac function in mammals.

Methods

Detailed methods are described in the Data Supplement.

Fasudil Rescue

Male and female mice between 6 and 4 months of age were given fasudil (10 mg/kg) or saline daily for 28 days via intraperitoneal injections. Echocardiography analysis was performed immediately before the initial injection, and echocardiography imaging was repeated at 14 and 28 days. At the end of the experiment, the mice were euthanized using CO2 and cervical dislocation as per the approved Institutional Animal Care and Use Committee animal protocol.

Metabolomics Analysis

Cardiomyocytes treated with either nonsilencing shRNA (short hairpin RNA) control or shSNRK (short hairpin RNA targeting SNRK) lentiviruses were cultured in Roswell Park Memorial Institute medium containing B27 (no insulin) for 48 hours before sample collection. Sample collection and extraction was performed as described previously in Bhute and Palecek.10 H NMR spectra were acquired using Chenomx NMR Suite Profiler (version 7.7; Chenomx Inc), and the concentrations were referenced to 3-(trimethylsilyl)-2,2,3,3-tetradeteropropionic acid or TMSP-d4. Peak annotation was performed based on the existing Chenomx library and the human metabolome database. The metabolite concentrations were exported to an Excel file for further analysis.

The concentration data matrix was normalized by the total concentration of metabolites, which was equivalent to normalization by total spectral area in each sample to evaluate the metabolite fractions and also to account for the differences in cell number and efficiencies of extraction. Statistical and pathway analysis was performed using MetaboAnalyst 3.0.13 The concentration data were autoscaled (mean centering followed by dividing each variable by the standard deviation) before principal component analysis, and student’s t test was performed assuming unequal group variance, and p was chosen to be 0.05. Pathway topology analysis was performed in MetaboAnalyst’s pathway analysis module using global test algorithm for pathway enrichment (adjusted for multiple testings) and out-degree centrality to assess importance. The Homo Sapiens library was used for analysis, and the metabolic pathways with an impact score >0 and false discovery rate <0.05 were considered to be significantly enriched.

Protein Kinase Array

Purified SNRK protein was provided to Invitrogen (Life Technologies) for kinase substrate identification service on ProtoArray human protein microarray. In short, SNRK was assayed at concentrations of 5 and 50 nmol/L on ProtoArray human protein microarrays v5.0. All possible kinase substrates at each concentration of SNRK were evaluated by their Z-factor rank within the array and were compared with the negative control assay. A protein was defined as being a candidate substrate if it met the following conditions: the Z-factor, or signal-to-noise ratio, was >0.35 on at least one array, indicating a signal >1.5-fold above the noise. The signal-used value was >2000 relative units on at least one array probed with SNRK and was >2-fold higher than the signal-used value for the corresponding protein in the negative control assay. The replicate spot coefficient of variance was <5% on the corresponding array. The interassay coefficient of variance was <5%. Additional experimental details are available on request.

In Vitro ROCK Phosphorylation Protocol

Human umbilical vein endothelial cells (Lonza CC2519) were lysed with radioimmunoprecipitation assay buffer (Sigma) containing complete mini EDTA-free protease inhibitor cocktail (Roche) and PhosSTOP phosphatase inhibitor (Roche). Samples were incubated on ice for 20 minutes and cleared at 13300 rpm at 4°C for 30 minutes. ROCK1 was immunoprecipitated using an anti-ROCK1 antibody (Cell Signaling Technology) with protein G agarose beads (Thermo Scientific). Immunoprecipitates were washed 3x with dilution buffer (10 mmol/L Tris, 130 mmol/L NaCl, 0.05% Triton-X-100, 0.1% BSA, protease inhibitors). Additional washes with 50 mmol/L Tris (pH 8.0) and Tris-Saline (10 mmol/L Tris HCl, 140 mmol/L NaCl; pH 8.0) were also performed. The Lowry quantification method was used to determine the protein concentration. Immunoprecipitated ROCK1 protein was incubated with or without 5 to 30 nmol/L purified SNRK protein in the presence of 3 μmol/L γ-ATP-32 (Perkin Elmer) and 1 μmol/L ATP (Cell Biolabs) in kinase buffer (20 mmol/L HEPES [pH 7.7], 20 mmol/L MgCl2, 2 mmol/L DTT, 1x protease inhibitor [Roche] and 1x phosphatase inhibitor [Roche]). The samples were allowed to incubate for 15 minutes at 30°C. After incubation, the samples were washed 2 to 3 times with kinase buffer and resuspended in Lamelli sample buffer. Five microliter of the reaction was reserved for radioactive analysis, and the remaining samples were resolved on a 10% MiniPROTEAN TGX precast gel (BioRad) and subjected to SDS-PAGE. The gel was then dried and exposed to autoradiography film.

Western Blot Analysis

Adult heart tissues were collected immediately after euthanization, and the proteins were isolated using homogenization in radioimmunoprecipitation assay buffer (Sigma) with complete mini EDTA-free protease inhibitor cocktail (Roche) and PhosSTOP phosphatase inhibitor (Roche) using a Quagen TissueRuptor. Methodologies related to protein estimation and quantification were described previously. The following antibodies were used: anti-myosin-binding subunit (MBS; BioLegend), anti-phospho MBS (pMBS; Cell Signaling...
Cardiac Fibrosis Mice Show Myocardial Dysfunction and Increased Cardiac (cmcKO) Conditional Knockout Adult P. Peters. For all of the in vivo and in vitro ROCK signaling and inhibitor value of <0.05 was considered significant when comparing 2 parameters. For the trichrome staining analysis and the Western blot experiments are provided in Table I in the Data Supplement. Sample data values for the metabolomics data are provided in Table II in the Data Supplement.

Results

Snrk Cardiac (cmcKO) Conditional Knockout Adult Mice Show Myocardial Dysfunction and Increased Cardiac Fibrosis

Previously, our laboratory assessed the role of Snrk in mammalian development and concluded that SNRK is essential for cardiac metabolism.7 In that study, we generated and validated the global and cardiac-specific (MYH6CRE)14 conditional Snrk cmcKO mouse reagents.7 Homozygous loss of Snrk (Loxp/Loxp) in cardiomyocytes results in lethality between 8 and 10 months of age, and heterozygous loss of Snrk (Loxp/WT) showed lethality shortly after 1 year of age. Echocardiography analysis on 6-month-old adult male mice showed significant enlargement of the left ventricle internal diameter in systole and diastole, significant increase in end-diastolic volume and end-systolic volume, and significant decrease in the ejection fraction (EF) and the fractional shortening (FS) in the Snrk cmcKO (MYH6CRE Snrk Loxp/Loxp) mice compared with the CRE-negative littermates.7 We now performed strain and strain rate imaging analysis (Figure 1A), which showed that Snrk cmcKO mice display significantly worsening strain patterns, including decreased radial strain (P=0.0286), increased circumferential strain (P=0.0286), and decreased strain rates S (P=0.0286). The strain rate changes correlate with a decrease in systolic function. The Snrk cmcKO mice also display an increased strain rate E (P=0.0286) that is associated with worsening diastolic function. Furthermore, the pulmonary acceleration time (P=0.0571), ejection time (P=0.0571; Figure 1B), and right ventricular outflow tract time–velocity integrals (TVI) were also decreased in Snrk cmcKO mice (P=0.0571; Figure 1C). All together, these findings are consistent with those of a dilated cardiomyopathy, with a resultant increase in pulmonary artery pressure.

We next investigated whether myocardial stiffening and impaired cardiac function could be the result of increased extracellular matrix deposition, a phenomenon described as myocardial fibrosis.15 We performed trichrome staining on Snrk cmcKO heart tissue sections (Figure 1D). Snrk cmcKO showed a significant increase in fibrosis staining (CRE-negative 10.38%, cmcKO 19.32%; P=0.0212; Figure 1E; Table I in the Data Supplement). These data indicate deformation in the heart segments, resulting in altered myocardial function and suggest that Snrk in cardiomyocytes is critical for maintaining heart contractility.

SNRK Knockdown Cardiomyocytes In Vitro Show Defective Metabolism

To investigate what might be amiss in cardiomyocytes in the absence of SNRK, we investigated changes in metabolism in SNRK knockdown cardiomyocytes in vitro because previous data on Snrk cmcKO mice showed cardiac metabolic defects.7 Cardiomyocytes treated with either nonsi-lencing short hairpin RNA control or efficacy-confirmed shSNRK lentivirus were subjected to NMR-based metabolomics measurement as described in the Methods section. The heat maps (Figure 2A) and subsequent graphical representation (Figure 2B) clearly depict extensive metabolic changes in SNRK knockdown cardiomyocytes. Metabolites that significantly increased (Figure IA in the Data Supplement) and decreased (Figure IB in the Data Supplement) in SNRK knockdown cardiomyocytes have been indicated. Among the increased metabolites, we observed significant increase in multiple osmoieties, including glycerophosphocholine (fold change, 1.44; P=0.0046) and taurine (fold change, 1.27; P=0.038). We also observed an increase in a few essential amino acids, including valine and threonine, while majority of the nonessential amino acids, including alanine (P<0.1), asparagine, aspartate, glutamate, and glutamine, showed a significant decrease because of SNRK knockdown (P<0.05). This indicates significant changes in amino acid–related metabolic pathways, which is also observed in pathways analysis (Figure 2C). Interestingly, glycerol was significantly increased because of SNRK knockdown relative to control (fold change, 1.38; P=0.018). Glycerol can serve as a substrate for energy in cardiomyocyte,16 and we observe several other metabolites associated with energy metabolism, including citrate, fumarate, malate, succinate (P<0.05), pyruvate, and nicotinamide adenine dinucleotide (P<0.1), to be significantly reduced because of SNRK knockdown in cardiomyocytes. This indicates that SNRK can play a crucial role in energy homeostasis in cardiomyocytes.

Further pathway topology analysis (Figure 2C) and metabolite set enrichment analysis (Figure 2D) suggested significant changes in alanine, aspartate, and glutamate metabolism (P<0.002; Figure 2C; Figure II in the Data Supplement), citrate cycle (P=3.47E-06; Figure 2C; Figure III in the Data Supplement), taurine and hypotaurine metabolism (P<0.002), aminoacyl t-RNA biosynthesis (P=0.009), and glycine, serine, and threonine metabolism (P=0.01; Figure 2C). Specific metabolites and amounts that changed in SNRK knockdown cardiomyocytes are indicated in Tables II and III in the Data Supplement. Taking the in vivo and in vitro data together, SNRK in cardiomyocytes plays an instrumental role in maintaining metabolic homeostasis, which is critical for the normal functioning of the heart.
ROCK Is a Putative Substrate of SNRK

To identify signaling proteins responsible for the cardiac function deficits observed in Snrk cmcKO mice in vivo, we performed a phosphopeptide proteomic screening using pure SNRK protein generated in the laboratory. Human histidine-tagged SNRK protein was generated in bacteria (Figure IV in the Data Supplement), purified (Figure IVA in the Data Supplement), and confirmed by mass spectral analysis (23% peptide coverage) and Western blotted for SNRK antibody (Figure IVD in the Data Supplement). Using this purified SNRK protein (Figure IVE in the Data Supplement), we outsourced the kinase profiling to Life Technologies. An in vitro protein kinase experiment was performed on 9000 N-terminal Glutathione S-transferase fusion proteins on an array at 2 SNRK protein concentrations (5 and 50 nmol/L). This screen identified ROCK1 as one of the top hits along with several members of the transforming growth factor-β signaling pathway (Activin A receptor type 1 also known as ALK2, Activin A receptor like type 1 also known as ALK1, Mothers against decapentaplegic homolog 3, Bone morphogenetic protein receptor type 2) that showed 2-fold or more phosphorylation compared with the negative control (Table IV in the

Figure 1. Cardiomyocyte-conditional SNRK null mice (Snrk cmcKO) echocardiography (ECHO) results. ECHO was performed on 6-month-old adult Snrk WT (CRE negative) and Snrk cmcKO (MYH6CRE SNRK L/L) as described in the Materials and Methods. A, Strain and strain rate imaging analyses by ECHO. B, Isovolumic relaxation time (IVRT) in milliseconds, a parameter for diastolic function, ratio of mitral peak velocity of early filling (E) in m/s to early diastolic mitral annular velocity (e'; E/e' ratio), pulmonary acceleration time (PAT) in milliseconds, ejection time (ET) in milliseconds, and ratio of PAT to ET. C, Time–velocity integrals (TVI) in centimeter along with mitral peak velocity (E) in m/s and diastolic mitral annular velocity (e') in m/s. D, Representative images of Gomori’s Trichrome staining in CRE negative or cardiomyocyte-specific conditional null (MYH6CRE SNRK L/L) adult mouse hearts. E, Average percent collagen staining in CRE negative and MYH6CRE SNRK L/L adult mouse hearts. The number of mice per group equals 3 to 5. *P<0.05, #P<0.1. Scale bar =1 mm. KO indicates knockout; and SNRK, sucrose nonfermenting related kinase.
We confirmed the ROCK1 phosphorylation in vitro using a ROCK kinase assay (Figure 3A). ROCK1 protein was immunoprecipitated from human umbilical vein endothelial cells (Figure 3A, immunoprecipitation blot) and was incubated with purified SNRK protein in the presence of radiolabeled phosphate (P32). As shown (Figure 3A, lane 2), a P32-labeled band is observed in the lane with immunoprecipitated ROCK plus SNRK protein at the correct size for the ROCK1 protein, which was quantified in 3 independent experiments (Figure 3A, quantification panel). In addition, we performed multistage fragmentation tandem mass spectrometry on ROCK1 immunoprecipitated from human umbilical vein endothelial cells in the presence or absence of SNRK. The ROCK1 band was excised and subjected to in-gel tryptic digestion. The resulting peptides were analyzed by multistage fragmentation mass spectral analysis.17 The data for ROCK1 identified residues S27, T237, and S479 (Figure V in the Data Supplement, blue circles) that resides in the N-terminal region. These sites are distinct from those reported in the literature (Figure V in the Data Supplement, magenta circles) or previously identified using proteomic approaches (Figure V in the Data Supplement, yellow highlighted residues). Collectively, these data suggest that ROCK1 is a direct target of SNRK, and phosphorylation of ROCK by SNRK at specific residues is likely to influence its downstream activity.

We next evaluated ROCK activity by determining the amount of phospho-Threonine 853 in the pMBS of myosin light chain phosphatase, a key enzyme in the RhoA-ROCK muscle contraction signaling cycle. We performed Western blots for ROCK1, ROCK2, and pMBS protein levels in Snrk cmcKO adult (Figure 3B) heart lysates. Of the proteins investigated, pMBS levels were higher in adult Snrk cmcKO hearts (MYH6CRE SNRK L/L; Figure 3B; P=0.1) compared with CRE-negative controls. We also investigated SNRK knockdown...
cardiomyocytes in vitro for pMBS and observed an increase that was not significant (Figure 4A; \(P=0.36, n=3\)). Interestingly, when control and shSNRK knockdown cardiomyocytes were treated with the ROCK inhibitor Y27632 (Figure 4A), there was a slightly significant decrease in pMBS levels compared with those in the control-treated shSNRK knockdown cardiomyocytes (40.75%; \(P=0.041, n=3\)). Specific sample data values are indicated in Table I in the Data Supplement. Collectively, the phosphoproteomic analysis and signaling data suggest that ROCK is a substrate of SNRK, and ROCK activity is attenuated by SNRK in vivo and in vitro in cardiomyocytes.

SNRK-ROCK Signaling in Cardiomyocytes

We next investigated signaling pathways that can be regulated by ROCK signaling, such as AKT and mitogen-activated protein kinase (ERK) in both in vivo Snrk cmcKO mice (Figure 3B) and in vitro SNRK knockdown cardiomyocytes (Figure 4B–4D). ROCK is known to regulate the PI3K (phosphoinositide 3-kinase)-AKT pathway by activating the phosphatidylinositol (3,4,5)-trisphosphate phosphatase called phosphatase and tensin homolog, which results in a reduction in pAKT levels. \(^{18,19}\) In addition, ROCK has also been shown to influence ERK signaling in smooth muscle cells. \(^{20}\) Western
Figure 4. Loss of Snrk in cardiomyoctyes results in increased ROCK activity. Western blots were performed on cardiomyocytes as described in Materials and Methods. A–D, Western blot quantification of cardiomyocytes treated with water (control) or 5 μM ROCK inhibitor Y27632. Data represent 3 to 4 independent experiments. *P<0.05, **P<0.0125. AKT indicates protein kinase B; ERK, extracellular signal-regulated kinase; IKK, inhibitor of kappa B kinase; MBS, myosin-binding subunit; pAKT, phospho protein kinase B; pERK, phospho extracellular signal-regulated kinase; pIKK, phospho inhibitor of kappa B kinase; pMBS, phospho myosin-binding subunit; ROCK, Rho-associated kinase; and SNRK, sucrose nonfermenting related kinase.
blot analysis was conducted on Snrk WT (CRE Negative) and Snrk cmcKO (MYH6CRE SNRK L/L) heart lysates, as well as on control and shSNRK knockdown cells, with and without the ROCK inhibitor Y27632. Loss of SNRK in the heart resulted in decreased pAKT (Figure 3B; 46.15%, P=0.1, n=3) and the AKT downstream effector pIKK (Figure 3B; 44.62%, P=0.1, n=3), which suggests decreased AKT signaling in the Snrk cmcKO hearts. We also observed a trend toward increased pERK (Figure 3B; 183.47%, P=0.2, n=3) in the heart lysates. Specific sample data values are indicated in Table I in the Data Supplement.

Surprisingly, knockdown of SNRK in cardiomyocytes resulted in a nonsignificant change in pAKT (Figure 4B; 171.07%, P=0.136, n=4) and pERK (Figure 4D; 311.64%, P=0.212) and a significant increase in pIKK (Figure 4C; 254.38%, P=0.012, n=3). Inhibition of ROCK signaling using the ROCK inhibitor Y27632 was able to significantly decrease the pMBS levels in the shSNRK cardiomyocytes compared with the control-treated shSNRK cardiomyocytes (Figure 4A; control 123.35%, shSNRK 40.75%; P=0.041, n=3). pAKT, pERK, and pIKK levels did not significantly change in the presence of the ROCK inhibitor (Figure 4C), suggesting that ROCK signaling is not the only molecule involved in regulating pAKT, pERK, and pIKK signaling in cultured cardiomyocytes. These results collectively suggest that SNRK directly influences ROCK signaling in cardiomyocytes in vivo and in vitro.

**SNRK-ROCK Signaling Is Required for Normal Cardiac Function**

Altered ROCK signaling has been previously implicated in cardiac hypertrophy and ventricular remodeling. To determine whether the cardiac defects observed in the Snrk WT (CRE Negative) and Snrk cmcKO (MYH6CRE SNRK L/L) mice are attributed to increased ROCK activation, Snrk WT (CRE Negative) and Snrk cmcKO (MYH6CRE SNRK L/L) mice were treated with the ROCK inhibitor fasudil. To assess changes in cardiac function, echocardiography was conducted before drug treatment and after 4 weeks of daily fasudil or saline injection (Figure 5; Table V in the Data Supplement). Fasudil treatment in Snrk cmcKO mice resulted in stabilization of EF and FS phenotypes, from 55.55% (cKO-fasudil) to 50.66% (cKO-saline)
EF and from 24.77% to 22.7% FS before treatment to 53% EF and 23.23% FS after fasudil treatment compared with the saline-treated Snrk cmcKO mice, which displayed continued declines in EF (44.55%; P=0.0286) and FS (18.9%; P=0.0286). End-diastolic volume did not show a significant change in the Snrk cmcKO mice (0.0064) treated with fasudil compared with baseline (0.0070; P=0.6905), whereas end-diastolic volume continued to increase in the saline-treated Snrk cmcKO mice from 0.0066 at baseline to 0.0082 at 4 week (P=0.0286). End-systolic volume did not increase in the fasudil-treated Snrk cmcKO mice but did increase in the saline-treated mice (baseline 0.0029–4 weeks, 0.0046; P=0.0286), indicating a preserved EF in the fasudil-treated mice. IVRT showed a similar trend, with a slight decrease in the fasudil-treated Snrk cmcKO mice (14.45 at baseline to 12.41 at 4 weeks; P=0.3413) and an increase in the Snrk cmcKO saline-treated mice (12.4 at baseline to 13.22 at 4 weeks; P=0.0286). These data indicate that increased ROCK activity is involved in generating the cardiac functional deficits observed in the Snrk cmcKO mice and that inhibition of ROCK results in cardiac functional improvements.

**Discussion**

In this study, we have identified that a member of the AMPK family, namely SNRK, influences ROCK signaling to maintain cardiac function in the adult. Snrk was first identified in 3T3-L1-differentiated adipocyte cells and compared with Ampla1 and Ampla2; Snrk expression in white adipose tissue, brown adipose tissue, heart, and brain is significantly higher. The expression profile of SNRK and the family that it belongs to suggests a role for this enzyme during high metabolic needs like the heart contraction and relaxation cycles. Indeed, in our previous report, we identified that SNRK is a critical regulator of metabolic function during embryonic cardiovascular development and identified the metabolic pAMPK-pACC signaling pathway deregulation in the heart. These results directly support current NMR-based metabolomic analysis data in SNRK knockdown cardiomyocytes, which shows decreased metabolites, such as those involved in alanine, aspartate, and glutamate metabolism (Figure II in the Data Supplement), as well as citric acid cycle metabolism (Figure III in the Data Supplement), and increases in metabolites involved in lipid synthesis, such as glycerol (Figure I in the Data Supplement), which are processes directly influenced by pAMPK-pACC signaling.

Interestingly, perturbations in cardiac metabolic output have been observed in patients with heart failure. In normal hearts, the primary metabolic energy source is derived by lipid metabolism/fatty acid oxidation, and in failing hearts, the metabolic substrate changes to glucose metabolism. Furthermore, accumulation of lipids (free fatty acids) in the failing heart can create additional metabolic stress by increasing energy uncoupling and proton leakage and decreasing energy production.

In the SNRK knockdown cardiomyocytes, we did not observe any significant changes in glycolysis or glucose metabolism, but we did observe significant alterations in oxidative phosphorylation and lipid metabolism, indicating an important role for SNRK in cardiac metabolism, and loss of SNRK can result in phenotypes similar to those observed during cardiac failure. In addition, taurine and hypotaurine metabolism was found to be increased in the Snrk cmcKO mice, which displayed continued declines in EF (44.55%; P=0.0286), indicating a preserved EF in the fasudil-treated mice. IVRT showed a similar trend, with a slight decrease in the fasudil-treated Snrk cmcKO mice (14.45 at baseline to 12.41 at 4 weeks; P=0.3413) and an increase in the Snrk cmcKO saline-treated mice (12.4 at baseline to 13.22 at 4 weeks; P=0.0286). These data indicate that increased ROCK activity is involved in generating the cardiac functional deficits observed in the Snrk cmcKO mice and that inhibition of ROCK results in cardiac functional improvements.

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Interestingly, perturbations in cardiac metabolic output have been observed in patients with heart failure. In normal hearts, the primary metabolic energy source is derived by lipid metabolism/fatty acid oxidation, and in failing hearts, the metabolic substrate changes to glucose metabolism. Furthermore, accumulation of lipids (free fatty acids) in the failing heart can create additional metabolic stress by increasing energy uncoupling and proton leakage and decreasing energy production.

In the SNRK knockdown cardiomyocytes, we did not observe any significant changes in glycolysis or glucose metabolism, but we did observe significant alterations in oxidative phosphorylation and lipid metabolism, indicating an important role for SNRK in cardiac metabolism, and loss of SNRK can result in phenotypes similar to those observed during cardiac failure. In addition, taurine and hypotaurine metabolism was found to be increased in the Snrk cmcKO mice, which displayed continued declines in EF (44.55%; P=0.0286), indicating a preserved EF in the fasudil-treated mice. IVRT showed a similar trend, with a slight decrease in the fasudil-treated Snrk cmcKO mice (14.45 at baseline to 12.41 at 4 weeks; P=0.3413) and an increase in the Snrk cmcKO saline-treated mice (12.4 at baseline to 13.22 at 4 weeks; P=0.0286). These data indicate that increased ROCK activity is involved in generating the cardiac functional deficits observed in the Snrk cmcKO mice and that inhibition of ROCK results in cardiac functional improvements.
ROCKs are ubiquitously expressed proteins, and both isoforms ROCK1 and ROCK2 are expressed in the heart. Signaling mechanisms associated with contraction in the vascular smooth muscle cells in the heart are well studied for ROCK. Briefly, intracellular Ca2+ level increases through activated G-protein-coupled receptors, which in turn stimulate downstream phosphorylation of myosin light chain kinase (activating) and phosphorylation of the MBS (also known as MYPT1) of myosin light chain phosphatase (inactivating). The exchange factor RhoA is the chief upstream activator of ROCK1 to date and has been the established regulator of this pathway; the net result of which is contraction of smooth muscle cells. Our studies suggest that SNRK is a second player upstream of ROCK, but whether the phosphorylation directly activates or inhibits ROCK activity or whether additional proteins along with SNRK are required for altered ROCK activity is currently unknown. Our data suggest that SNRK is a negative regulator of ROCK signaling, and loss of SNRK increases ROCK activity in cardiomyocytes. Indeed, loss of SNRK in Snrk cmcKO adult (6 months old) mice showed higher levels of pMBS, reinforcing the hypothesis that phosphorylation of ROCK1 by SNRK may be inhibitory (Figure 3). The converse experiment would be the overexpression or gain of SNRK in cardiac tissue, which should decrease ROCK activity and consequences associated with it. The resulting phenotype would include improved cardiac function because ROCK1 is necessary for the transition from cardiac hypertrophy to failure. Indeed, 2 abstracts report that SNRK overexpression in the heart improves cardiac metabolic efficiency and response to myocardial ischemia, which support our predictive hypothesis.

We also examined the activation status of additional downstream effectors of ROCK, such as AKT and ERK, and identified altered in vivo expression levels of pAKT and pERK, further suggesting that ROCK activity is altered in vivo when SNRK is absent (Figures 3 and 6). Interestingly, we did not see a significant difference in the in vitro expression levels of pAKT, pERK, and pMBS in SNRK knockdown cardiomyocytes. These observations could be attributed to the inherent differences between in vitro culturing conditions and in vivo responses that contain additional non-cardiomyocytes cell types influencing the experimental readout. Additional in vitro studies are necessary to specifically address these differences.

To assess whether attenuation of ROCK signaling in Snrk cmcKO mice is consequential, we treated Snrk cmcKO mice with the ROCK inhibitor fasudil. Before treatment, the Snrk cmcKO mice already displayed cardiac functional defects. After 4 weeks of treatment with the inhibitor, the Snrk cmcKO mice began showing signs of improved cardiac function and stabilization of the phenotype compared with the untreated Snrk cmcKO mice that continued to show decreased cardiac function (Figure 5; Table V in the Data Supplement). These findings suggest that some of the cardiac functional deficits observed in the Snrk cmcKO mice are most likely a result of increased ROCK activity and that SNRK regulation of ROCK signaling is an important component of normal cardiac function.

In summary, our data suggest that an AMPK family member SNRK is a critical metabolic sensor in cardiac tissues and directly influences ROCK signaling in cardiomyocytes, effects that directly contribute to maintaining cardiac metabolic homeostasis and cardiac functional output in mammals.
Acknowledgments

We thank members of the Developmental Vascular Biology Program for their invaluable input and insight during the course of this study. We also thank Dmitry L. Sonin for assistance with the initiation of the mouse echocardiography studies and the Children’s Research Institute imaging core facilities for Hamamatsu slide scanner and viiapharm image analysis support and the Histology core for slide preparations. This publication was supported by the National Center for Advancing Translational Sciences, National Institutes of Health, through Grant Number UL1TR001436. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health (NIH).

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Disclosures

None.

References


CLINICAL PERSPECTIVE

Dilated cardiomyopathy is a disorder where the heart becomes enlarged and its function is compromised. Dilated cardiomyopathy is influenced by several factors, and recent evidences point to alteration in cardiac metabolism, especially in conjunction with diabetes mellitus as a possible factor. Previously, our laboratory has identified a serine threonine kinase in the AMPK (adenosine monophosphate–activated protein kinase) family, namely, SNRK (sucrose nonfermenting-related kinase), that plays a critical role in cardiac metabolism in mammals. Others have implicated SNRK as a suppressor of adipocyte inflammation. In this study, we investigated the hypothesis that SNRK-induced signal transduction in cardiomyocytes is critical for maintaining its cardiac function in mammals. Using echocardiography on Snrk cardiac conditional knockout mice, we show here that these mice have cardiac function deficits that resemble dilated cardiomyopathy phenotype, and these deficits are in part because of major changes in the metabolites involved in fatty acid oxidation in cardiomyocytes. We then identified using phosphopeptide-based proteomic screen ROCK1 (Rho-associated kinase 1) that serves as a putative substrate for SNRK in cardiomyocytes. ROCK1 is a kinase that is involved in regulating muscle contraction and has been previously implicated in several cardiovascular diseases, such as atherosclerosis, cardiac hypertrophy, and diabetes mellitus. Importantly, elevated ROCK activity and endothelial dysfunction was associated as chief risk factors that lead to the progression of cardiovascular disease and outcome in the Framingham heart study. This study, therefore, provides the molecular link associated with the elevated ROCK activity in cardiomyocytes of the mammalian heart and delineates SNRK as a target for dilated cardiomyopathy. Biochemical analysis in vivo confirmed that Snrk cardiac conditional mice showed elevated ROCK activity, which was rescued partially by ROCK inhibitor fasudil. Importantly, the fasudil rescue restored some of the cardiac functional parameters in Snrk cardiac conditional knockout mice, thus, implying SNRK-ROCK signaling pathway as a critical pathway in cardiomyocyte that mediates cardiac function and elevates this interface for drug discovery in cardiovascular disease.
METHODS

Mouse Experiments. The mice were housed in the Medical College of Wisconsin Biological Resource Center, and all experiments were performed in accordance with an IACUC approved animal procedure protocol 1022. The Snrk cmcKO adult mice were generated from MYH6CRE positive Snrk LoxP/wild type (WT) males mated to Snrk LoxP/LoxP females. When possible age and litter matched male and female mice were used for each animal experiment. Mixed backcrossed animals were used for all of the mouse experiments. Primer information for mouse genotyping were provided previously1.

Human Cardiomyocyte System. Directed differentiation of CMs from human embryonic stem cells (hESCs) is described in previous studies2,3. Briefly, hESCs maintained on matrigel in mTeSR1 were dissociated into single cells using Accutase (Life Technologies) at 37°C for 5 min and then were seeded onto a matrigel-coated 12-well plate at 100,000-200,000 cell/cm² in mTeSR1 supplemented with 5 μM Y-27632 (Tocris) for 24 h. After 24 h, fresh mTeSR1 medium was used every day to expand stem cells. When cells achieved a density of 500,000 cell/cm², cells were treated with 12 μM CHIR99021 (Selleckchem) in RPMI/B27 without insulin for 24 h (day 0 to day 1). The medium was changed to RPMI/B27 without insulin on day 1, followed by 5 μM IPW2 (Tocris) treatment on day 3. During the day 5 medium change IWP2 was removed. Cells were maintained in the RPMI/B27 starting from day 7, with the medium changed every 3 days.

Lentiviral Transduction of CMs and Drug Treatments. Day six CMs differentiated from hESCs were dissociated into single cells with Accutase at 37°C for 5-8 mins, and then were spun down into aliquots of 1.5×10⁶ cells. Cells were re-suspended in 30 μL concentrated lentivirus medium in the presence of 6 μg/mL polybrene (Sigma), and then incubated at room temperature for 10 mins. At the end of
incubation, 2 mL of DMEM/10% FBS + 5 μM Y-27632 (Tocris) was added and the suspension was transferred into 2 wells of a 12-well plate pre-coated with Matrigel, and incubated at 37°C, 5% CO₂ overnight. The medium was replaced with fresh DMEM/10% FBS on the second day of lentiviral infection, and afterwards cells were treated with water (control), and 5 μM Y27632 for 9 days. Two days later, infected cells were selected and enriched for 3 days based on resistance to 1 μg/mL puromycin (Sigma). 48 h before sample collection, cells were switched to RPMI containing B27 (no insulin) supplement. On day 15, the cells were rinsed with PBS and the proteins were collected for western blot analysis as previously described1,4,5.

**Echocardiography and Image Analysis.** Transthoracic echocardiography was performed in anesthetized (2% isoflurane) six months old litter matched males. An investigator (L.M.H) who was blinded to the study groups performed the measurements and data analyses. A comprehensive 2D transthoracic echocardiogram with Doppler was performed on animals using Animals were studied with a commercially available echocardiographic system (Vivid 7, General Electric, Wauwatosa, WI), with an 11-MHz M12-L linear array transducer. A comprehensive 2D transthoracic echocardiogram with Doppler was performed from the cardiac short axis of the left ventricle at the papillary muscle level. Using the anatomical M-mode feature of the Vivid 7 echo, an M-mode display was generated from raw data 2D images of the short-axis of the left ventricle at the level of the papillary muscle with the line selected passing through the anterior and inferior segments. Ejection fraction % (EF) was measured using left ventricle end diastolic volume (LVEDV) and left ventricle end systolic volume (LVESV) using the formula EF= LVEDV - LVESV / LVEDV x 100. Fractional shortening % (FS) was calculated with the formula: left ventricle end diastolic dimension (LVEDD)-left ventricle end systolic dimension (LVESD)/LVEDD x 100. To assess diastolic function, pulse wave Doppler was used to assess transmitral inflow velocities in the apical 4-chamber view to obtain E velocities and isovolumic relaxation times (IVRT). Tissue Doppler was
used to assess tissue motion velocity from the mitral annulus to obtain $e'$ velocities. $E/e'$ is a calculated ratio. Pulse wave Dopplers was also used to sample velocities at the right ventricular outflow tract to assess for pulmonary acceleration time (PAT) and ejection time (ET). For two-dimensional strain analysis, the images were processed with EchoPac Q analysis software, (General Electric, Wauwatosa, WI). A cardiac cycle was defined from the peak of one R wave to the peak of the following R wave. Unless stated otherwise, for every measurement, three consecutive cardiac cycles were measured, and the average used for reporting. The method has been previously described. Briefly, for radial and circumferential strain, the endocardial border was traced in an end-systolic frame in the short-axis view at the mid ventricle as identified by the prominent papillary muscles. The software automatically selected 6 equidistant tissue-tracking regions of interest in the myocardium. The outer border was adjusted to approximate the epicardial border. The computer then provided a profile of radial (myocardial deformation toward the center) and circumferential (myocardial deformation along the curvature) strain (%) with time.

**Purification of SNRK.** Cloned full length human SNRK sequence was transformed into BL21(DE3) competent cells (New England Biolabs). Bacterial cells were cultured overnight at 37°C in LB and induced with 1mM IPTG and incubated at 25°C for 6 h. The bacteria were then pelleted and the supernatant was removed. The pelleted bacteria were lysed using a bacterial lysis buffer containing 13 mL BPER (Pierce Technologies), 50 μL DNAse (2 U/μL), 41 μL lysozyme (50 mg/mL) and 40 μL 1M MgCl2, protease inhibitor (Roche). The pellet was incubated with the lysis buffer at room temperature for 15 mins. One tablet of complete Mini EDTA-free protease inhibitor cocktail (Sigma) was add to the mixture and centrifuged at 7,600 x g for 15 mins. The supernatant was removed and dialyzed in a Slide-A-Lyzer with 10K molecular cutoff (ThermoFisher) overnight at room temperature in 1X binding buffer (57mM NaH2PO4 pH8.0, 300mM NaCl). The dialyzed supernatant was subjected to Nickel-NTA column
purification, and pure protein eluted in 10 mM, 25 mM and 250 mM Imidazole elution buffer. Samples of the fractions were resolved on a 10% Mini-PROTEAN TGX precast gel (BioRad), subjected to SDS-PAGE and subsequently stained with coomassie blue. Fractions eluted with 250 mM Imidazole were concentrated using Amicon Ultra 15 mL 50 MWL, kDa centrifugal filter units (EMD Millipore). Purified protein was subjected to Mass Spectometry analysis for identity confirmation. SNRK kinase activity was confirmed using the ADP-Glo Kinase Assay (Promega). In short, purified SNRK protein was incubated with 10mM ATP, different amounts Histone H3 (0, 500ng, 1000ng, 2000ng, 4000ng) and kinase reaction buffer. ADP-Glo reagent was then added to stop the kinase reaction and deplete the unconsumed ATP. After 40 mins, the kinase detection reagent was added to convert the ADP to ATP for luciferase detection of the ATP. The luciferase was measured using a plate-reading luminometer (SpectraMaxGeminiEM, Molecular Devices) with SoftMax Pro software.

**Identification of SNRK Candidate Substrates.** All possible kinase substrates at each concentration of SNRK were evaluated by the Z-Factor rank within the array and were compared to the negative control assay. A protein was defined as being a candidate substrate for SNRK if it met the following conditions: 1) The Z-Factor, or signal-to-noise ratio, was greater than 0.35 on at least one assay, indicating a signal greater than 1.5-fold above the noise. 2) The Signal Used value was greater than 2,000 relative unites on at least one array probed with SNRK and was greater than 2-fold higher than the Signal Used value for the corresponding protein in the negative control assay. 3) The replicate spot coefficient of variation (CV) was less than 50% on the corresponding array. 4) The inter-assay CV was less than 50%.

**Mass Spectral Analysis.** Immunoprecipitated ROCK1 protein was incubated with or without 50 nM purified SNRK protein in the presence of 4 $\mu$ M ATP (Cell Biolabs) in kinase buffer [20 mM HEPES (pH 7.7), 20 mM MgCl$_2$, 2 mM DTT, 1X protease inhibitor (Roche) and 1X phosphatase inhibitor (Roche)]. The
samples were allowed to incubate for 15 mins at 30°C. After incubation the samples were washed 2-3 times with kinase buffer and re-suspended in Lamelli sample buffer. The samples were resolved using gel electrophoresis and then subjected to in-gel tryptic digestion followed by Mass Spectral analysis.

Mass spectral analysis was performed on an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific, Waltham, MA, USA) coupled with a nanoAQUITY ultraperformance liquid chromatographer (UPLC) (Waters Corp.) interfaced with a nanoelectrospray ion source. Peptide separation was carried out on a capillary column built in-house with 5µm C18 nanoparticles to 10 cm long column with 75 µm inner diameter tubing. The fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) for the columns were pulled by a micropipette puller P-2000 (Sutter Instrument Company, CA, USA) and packed with C18 resin using a bomb-loader. The solvents A and B used for chromatographic separation of peptides were 2% acetonitrile in 0.1% formic acid and 98% acetonitrile in 0.1% formic acid, respectively. The peptides injected onto the microcapillary column were resolved at the rate of 200 nl/min, using the following gradient conditions: 0 to 10 min, 2 to 20% B; 120 min, 40% B; 135 min, 60% B; 150 min, 70% B; 158 min, 98%B, which was held for 4 min and then switched to 98% A and held for another 18 min. The ions eluted from the column were electrosprayed at a voltage of 1.70 kV. The ion transfer temperature was kept at 250°C. No auxiliary or sheath gas was used.

Survey full-scan mass spectrometry (MS) spectra (m/z, 300 to 2,000) were recorded in the Orbitrap analyzer at a resolution of 30,000 followed by tandem mass spectrometry (MS/MS) of the 10 most intense peptide ions in the linear ion trap analyzer. Neutral loss-triggered multistage activation for simultaneous fragmentation of neutral loss product and precursor was enabled at m/z of -98, -49 and -32.6 relative to the precursor ion, corresponding to a neutral loss of phosphate moiety from +1, +2, and +3 charged ions. The chromatographic and mass spectral functions were controlled using the Xcalibur data system (ThermoFinnigan, Palo Alto, CA, USA). Peptides were identified using the Mascot search
algorithm for human protein database at a global false-discovery rate of 5%. The resulting files were evaluated using Visualize tool\textsuperscript{7}.

**Tissue Sectioning, Staining and Image Analysis.** Adult mouse hearts were fixed in 4% paraformaldehyde/PBS for 1 to 2 days at 4°C. After fixation tissues were washed extensively in PBS and embedded in paraffin or sucrose/OCT as previously described\textsuperscript{1}. Seven micron thick sections were stained with Gomori’s Trichrome staining and Hematoxylin counter staining. The microscopic images of the entire tissue section were scanned at 40x magnification using the Nanozoomer 2.0-HT (Hamamatsu, Japan) digital slide scanning system (Children’s Research Institute’s Imaging Core). The amount of fibrosis was quantified using software assisted, unbiased microimage quantification method. The scanned images were imported into Visiopharm software (Denmark) and using the imager module, three 20x ROI images were extracted from left, center and right regions of the heart. Collagen positive regions (navy blue stains) were identified by thresholding the specific image channel filter and the rest of the tissue (muscle - stained red and nucleus - stained deep blue) classified as tissue region with total tissue region defined as sum of muscle, nuclear and collagen region without any interstitial space. All original images were processed with this preset thresholds and linear Bayesian classification to generate a processed image. Total collagen positive area per ROI was measured in microns and represented as a percentage of the total tissue area.

**References**


Supplemental Figures and Tables

Figure S1. Loss of SNRK in cardiomyocytes results in altered metabolic profiles. Panel A shows the upregulated metabolites and B shows the downregulated metabolites. *p<0.05, # p<0.1.
Figure S2. Loss of SNRK in cardiomyocytes results in altered alanine, aspartate, and glutamate metabolism. Panel A is the Pathway topology analysis impact score, p value and FDR. Panel B is the pathway view showing the corresponding Alanine, aspartate and glutamate metabolic pathways. The matched metabolites are highlighted according to their P values. Panel C is the normalized concentration from the individual metabolites. *p<0.05, # p<0.1.
Figure S3. Loss of SNRK in cardiomyocytes results in altered Citric acid cycle metabolites. Panel A is the Pathway topology analysis impact score, p value and FDR. Panel B is the pathway view showing the altered metabolites in the citric acid cycle. The matched metabolites are highlighted according to their P values. Panel C is the normalized concentration from the individual metabolites. *p<0.05, # p<0.1.
Figure S4. Human histidine tagged SNRK protein generation and purification. Panel A indicates a SDS-PAGE gel resolving the different fractions. Lys: lysate, FT: flow through. Numbers indicate the fraction number post 10 mM (1-5), 25 mM (6-8) and 250 mM (9-11) imidazole elution. The numbers on top of each lane corresponds to fraction elution profile in panel B. Panel B indicates elution profile and arrows indicate imidazole elution points with each number corresponding to concentration of imidazole. Only IPTG induced bacterial profile is shown. Panel C shows fraction #10 (black asterisks), re-purified a second round on Nickel NTA column and eluted with 250 mM imidazole. Panel D shows a western blot with SNRK antibody in uninduced (UI) and induced (I) lysate. Red arrow indicates the full-length protein and black arrow indicates degraded products. Numbers on left of each gel indicate protein size markers in kDa. Panel E indicates the three fractions from 250 mM imidazole purification that were subsequently concentrated and run on Coomassie-stained SDS-PAGE gel before the final product was sent for array experiment.
Figure S5. Mass spectral (MS) analysis of the SNRK mediated phosphorylation sites on ROCK1. MS analysis was used to identify the residues on ROCK which are phosphorylated by SNRK in vitro. Full length ROCK was incubated with or without SNRK. The SNRK dependent phosphorylated residues are depicted with a blue circle and the previously published phosphorylated residues are depicted with a magenta circle. MS identified amino acids are shown in bold red (current study), and the yellow highlighted residues were previously identified but not yet confirmed by proteomic analysis on PhosphoSitePlus.
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Adult Heart Lysate Western Blot Quantification

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**Table S2.** Individual data values for the Metabolomics data analysis.
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**Table S3.** Full metabolomic data set. HMDB: human metabolic database ID number.
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Table S4. Putative SNRK targets identified in the human ProtoArray microarray.

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<th>Ki (nM)</th>
<th>Fold change</th>
<th>p-value</th>
<th>Inhibition (%)</th>
<th>Concentration (nM)</th>
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Table S4. Putative SNRK targets identified in the human ProtoArray microarray.
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<th>LVIDs/BW cm/g</th>
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<th>% FS</th>
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</table>

Table S5. Comparison of the Fasudil treatment between Snrk WT and Cardiomyocyte-conditional

**SNRK null mice (Snrk cmcKO) – ECHO Analysis.** ECHO was performed on 6-4 month adult male and female Snrk WT and cmcKO mice. The mice received daily injections of saline or fasudil as described in the materials and methods. Left ventricular dimensions were normalized to body weight (BW) g, interventricular septal end diastole (IVSd) cm, left ventricular internal diameter end diastole and end systole (LVIDd, LVIDs) cm, left ventricular posterior wall end diastole (LVPWd) cm, end diastolic volume (EDV), end systolic volume (ESV) parameters, ejection fraction (EF) %, fractional shortening (FS) %, left ventricular mass in diastole (LVD Mass ASE) g, stroke volume index (SVI) ml/g, isovolumic relaxation time (IVRT) msec, early mitral inflow velocity (E) m/s / early diastolic mitral annular velocity (e') m/s, pulmonary acceleration time (PAT) msec, ejection time (ET) msec and time-velocity integral (TVI) cm.

There were 2 males and 2-3 females per experimental group.