Sucre Nonfermenter-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 (sucre 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 putative 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. Recently, cardiomyopathy as a consequence of early alterations in cardiac metabolism has been proposed, particularly with respect to diabetes mellitus. 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. ROCK 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 (sucre 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 deficit in the heart. Nuclear magnetic resonance (NMR)-based metabolomics 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.

**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 mmol/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, BioLegend), phosSTOP phosphatase inhibitor (Roche), anti-ROCK1 (Cell Signalin...
the global and cardiac-specific (MYH6CRE) 14 conditional CRE-negative littermates. 7 We now performed strain and cardiac metabolism. 7 In that study, we generated and validated malian development and concluded that SNRK is essential for SNrk

Snrk

Mice Show Myocardial Dysfunction and Increased Snrk

Statistics

Student’s unpaired t test was used for comparison analysis for the cardiomyocyte western blot, metabolomics, and trichrome staining analysis. Mann–Whitney test was used for comparison analysis for the adult heart Western blot analysis and all of the echocardiographic data analysis. The results are described as means (± standard error of the mean). A P value of < 0.1 was considered to be approaching significance. A P value of < 0.05 was considered significant when comparing 2 parameters. For all of the in vivo and in vitro ROCK signaling and inhibitor experiments, a P value of < 0.05 was considered to be slightly significant, and a P value of < 0.0125 was considered significant because of P value correction for multiple comparisons. Sample data values 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 nonsilencing short hairpin RNA control or efficacyle-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 1A in the Data Supplement) and decreased (Figure 1B 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
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. 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

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**Figure 3.** SNRK phosphorylation of ROCK and influences on its activity in Snrk cmcKO adult hearts. A, Direct phosphorylation of immunoprecipitated ROCK1 from human umbilical vein endothelial cells. Quantification of the radioactive band in A is shown, and the successful immunoprecipitation of ROCK1 is demonstrated with the ROCK antibody on immunoprecipitated lysates. Arrow denotes ROCK1 band. Data represent 3 to 4 independent experiments. B, Western blot quantification on lysates from cardiomyocyte-deleted SNRK adult hearts. C, Representative Western blot images from 4 independent heart lysates. The number of mice per group equals 3. AKT indicates protein kinase B; ERK, extracellular signal-regulated kinase; IKK, inhibitor of kappa B kinase; KO, knockout; 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.
Figure 4. Loss of Snrk in cardiomyocytes 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.
Western 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.4,21,22 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.23 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).

![Image of Figure 5](https://example.com/image.png)

**Figure 5.** Inhibition of ROCK signaling improved cardiac function in cardiomyocyte-conditional SNRK null mice (Snrk cmcKO). Echocardiography (ECHO) was performed on 4- to 6-month-old adult male and female Snrk WT (CRE negative) and Snrk cmcKO (MYH6CRE SNRK L/L) mice immediately before treatment and after 4 weeks. The mice received daily injections of saline or fasudil as described in the Materials and Methods. A and B, Left ventricular (LV) function parameters ejection fraction (EF) % and fractional shortening (FS) %. C and D, The end-diastolic volume (EDV) and end-systolic volume (ESV) that were normalized to body weight (BW). E, The isovolumic relaxation time (IVRT) in milliseconds. F, Representative M-mode images from the mouse hearts at baseline or after 4 weeks of daily fasudil or saline injections. There were 2 males and 2 females in the saline-treated Snrk cmcKO and Snrk WT groups and 2 males and 3 females in the fasudil-treated Snrk cmcKO and Snrk WT groups. See Table V in the Data Supplement for complete data set. cKO indicates Snrk cmcKO;
Diastolic volume did not show a significant change in the cardiac functional improvements. cmcKO mice and that inhibition of ROCK results in Snrk involved in generating the cardiac functional deficits observed in the fasudil-treated cmcKO mice (14.45 at baseline to 12.41 at 4 weeks; $P=0.0286$). 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 Ampkα1 and Ampkα2. 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 knockdown cardiomyocytes, indicating the activation of compensatory mechanisms to increased osmotic stress, and further supporting the notion that SNRK is an important regulator of cardiac cellular homeostasis.

To assess the contribution of SNRK to cardiac function, we conditionally deleted Snrk in cardiomyocytes in vivo using MYH6CRE14 mouse line. The Snrk cmcKO mice develop cardiomyopathy and die at 8 to 10 months of age. The 6-month echocardiographic studies showed changes in left ventricular dimensions and decreased ventricular function consistent with a dilated cardiomyopathy. Left heart failure often result in increased pulmonary artery pressures, and our echocardiography–Doppler interrogation of right heart hemodynamics corroborate this relationship. These mice over time progressively show increased fibrosis and cardiac stiffening, phenotypes associated with dysregulated ROCK signaling. Indeed, we identified 2 key molecules, namely the transforming growth factor-β signaling pathway and ROCK signaling pathways, in our SNRK substrate screen, both of which have been implicated in cardiac stiffening and fibrosis. We posit that SNRK’s involvement in the 2 signaling pathways may not be mutually exclusive because both have been implicated in cardiomyocyte function previously. For example, transforming growth factor-β and ALK2 regulate ROCK1 expression during valvuloseptal endocardial cushion formation and upregulation of bone morphogenetic protein-2 and antagonized transforming growth factor-β1/ROCK-enhanced cardiac fibrotic signal through activation of Smurf1/Smad6 complex. In this study, we only focused on ROCK because of the overwhelming evidence, suggesting ROCK as a cardiovascular risk factor and its role in cardiovascular physiology and pathophysiology.
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 Ca\(^{2+}\) 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 phosphatase light chain (inactivating). The exchange factor RhoA is the chief upstream activator of ROCK1 to date and has been the established regulator of this pathway\(^4\); 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.\(^41\) Indeed, 2 abstracts report that SNRK overexpression in the heart improves cardiac metabolic efficiency and response to myocardial ischemia,\(^42,43\) 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.\(^23\) 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.
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**Disclosures**

None.

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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.