Mutation in the γ2-Subunit of AMP-Activated Protein Kinase Stimulates Cardiomyocyte Proliferation and Hypertrophy Independent of Glycogen Storage

Maengjo Kim, Roger W. Hunter, Lorena Garcia-Menendez, Guohua Gong, Yu-Ying Yang, Stephen C. Kolwicz Jr, Jason Xu, Kei Sakamoto, Wang Wang, Rong Tian

Rationale: AMP-activated protein kinase is a master regulator of cell metabolism and an attractive drug target for cancer and metabolic and cardiovascular diseases. Point mutations in the regulatory γ2-subunit of AMP-activated protein kinase (encoded by Prkag2 gene) caused a unique form of human cardiomyopathy characterized by cardiac hypertrophy, ventricular preexcitation, and glycogen storage. Understanding the disease mechanisms of Prkag2 cardiomyopathy is not only beneficial for the patients but also critical to the use of AMP-activated protein kinase as a drug target.

Objective: We sought to identify the pro–growth-signaling pathway(s) triggered by Prkag2 mutation and to distinguish it from the secondary response to glycogen storage.

Methods and Results: In a mouse model of N488I mutation of the Prkag2 gene (R2M), we rescued the glycogen storage phenotype by genetic inhibition of glucose-6-phosphate–stimulated glycogen synthase activity. Ablation of glycogen storage eliminated the ventricular preexcitation but did not affect the excessive cardiac growth in R2M mice. The progrowth effect in R2M hearts was mediated via increased insulin sensitivity and hyperactivity of Akt, resulting in activation of mammalian target of rapamycin and inactivation of forkhead box O transcription factor–signaling pathways. Consequently, cardiac myocyte proliferation during the postnatal period was enhanced in R2M hearts followed by hypertrophic growth in adult hearts. Inhibition of mammalian target of rapamycin activity by rapamycin or restoration of forkhead box O transcription factor activity by overexpressing forkhead box O transcription factor 1 rescued the abnormal cardiac growth.

Conclusions: Our study reveals a novel mechanism for Prkag2 cardiomyopathy, independent of glycogen storage. The role of γ2-AMP-activated protein kinase in cell growth also has broad implications in cardiac development, growth, and regeneration. (Circ Res. 2014;114:966-975.)

Key Words: cardiac hypertrophy ◼ glycogen storage ◼ mutation in AMPK γ2 subunit ◼ proliferation

AMP-activated protein kinase (AMPK) is known as a fuel gauge and a master regulator of cell metabolism. Activation of AMPK during cellular stress promotes ATP generation via increases of glucose uptake and fatty acid oxidation, whereas it inhibits energy consuming processes such as protein and lipid synthesis. These unique functions of AMPK make it an attractive drug target for metabolic and cardiovascular diseases, as well as cancer. AMPK is a heterotrimeric complex composed of a catalytic α-subunit and regulatory β- and γ-subunits with multiple isoforms for each subunit. The γ-subunit is the energy sensor of the complex; competitive binding of ATP against ADP or AMP to the γ-subunit regulates the kinase activity. Two isoforms of the γ-subunit are expressed in the heart, γ1 and γ2. Point mutations in the nucleotide-binding region of the γ2-subunit, encoded by the Prkag2 gene, cause a distinct form of human cardiomyopathy characterized by glycogen storage, preexcitation arrhythmia, and cardiac hypertrophy. Previous studies using mouse models expressing mutant Prkag2 genes in the heart recapitulated the characteristics of human cardiomyopathy and demonstrated that the phenotype was caused by an aberrant increase of kinase activity. Metabolic analysis of the mutant mouse hearts shows that activation of AMPK in the absence of energy deficit results in global remodeling of the

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From the Department of Anesthesiology and Pain Medicine, Mitochondria and Metabolism Center, University of Washington, Seattle (M.K., L.G.-M., G.G., Y.-Y.Y., S.C.K., J.X., W.W., R.T.); and MRC Protein Phosphorylation unit, College of Life Sciences, University of Dundee, Dundee, United Kingdom (R.W.H., K.S.). R.W.H. and K.S. are currently affiliated with Nestlé Institute of Health Sciences SA, Campus EPFL, Innovation Park, bâtiment G, Lausanne, Switzerland.

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Correspondence to Rong Tian, MD, PhD, Mitochondria and Metabolism Center, University of Washington School of Medicine, 850 Republican St. Seattle, WA 98109. E-mail rongtian@u.washington.edu

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metabolic network in favor of glycogen storage. Because the cardiac phenotype of Prkag2 mutation is similar to glycogen storage cardiomyopathy, we sought to determine whether excessive glycogen accumulation is the unifying mechanism responsible for the Prkag2 cardiomyopathy. In a mouse model with cardiac-specific overexpression of the N488I mutant of Prkag2 (R2M), we rescued the glycogen storage phenotype by targeting glucose-6-phosphate (G6P)–stimulated glycogen synthesis via genetic manipulations. Here, we show that excessive glycogen accumulation is primarily responsible for ventricular preexcitation but not cardiac hypertrophy. Rather, the mutation of γ2-AMPK stimulates proliferation and hypertrophy pathways via forkhead box O transcription factor (FoxO) and mammalian target of rapamycin (mTOR) signaling cascades, leading to abnormal cardiac growth.

**Methods**

**Animal Models**

Transgenic mice overexpressing N488I AMPK γ2 (R2M), FoxO1 (FO), and harboring a knockin (KI) mutation in glycogen synthase (GYS) 1 were generated as described. R2M-KI (DM) and R2M-FO (DM-FO) double mutant were generated on an FVB background. Wild-type littermates of transgenic mice were used as controls (NTG).

Two-week-old mice were treated with rapamycin (2 mg/kg body weight, IP) daily for 4 weeks. Rapamycin (LC Laboratories) was dissolved in DMSO and resuspended in vehicle (0.2% carboxymethyl cellulose and 0.25% polysorbate-80) before injection. For insulin injection, mice were fasted overnight and anaesthetized with pentobarbital (80 mg/kg body weight, IP). Heart samples were freeze-clamped 20 minutes after insulin (0.5 U/kg body weight, IP). For 5-bromo-2-deoxyuridine (BrdU)–labeling experiments, 1-week-old mice were injected with BrdU (50 mg/kg body weight, IP) daily for 7 days, and hearts were subsequently harvested and fixed in 10% neutral buffered formalin for immunohistochemistry. All animal procedures were approved by the Institutional Animal Care and Use committee at the University of Washington.

**Echocardiography and ECG**

Murine echocardiography was performed using a Vevo770 high resolution imaging system (VisualSonics Inc). ECG was recorded using implantable wireless monitoring device with DSI mouse ECG transmitter ETA-F10.

**Cardiac GYS Activity and Glycogen Content**

GYS activity was measured using the method of Thomas et al. Glycogen content was determined by a glucose assay kit (Sigma-Aldrich) as described.

**Glucose Uptake and Myocardial Energetics**

11P nuclear magnetic resonance spectroscopy was used to measure glucose uptake rate, ATP, and phosphocreatine with nontracer 2-deoxyglucose as described.

**Western Blot Analysis**

Protein samples were prepared from frozen heart samples using a lysis buffer containing protease inhibitors (Sigma). Nuclear and cytosolic fractions were prepared according to instructions of NE-PER extraction kit (Pierce). Tissue lysates were matched for protein concentration and then separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories). Membranes were blocked in 5% nonfat milk and incubated with primary antibodies overnight at 4°C. Membranes were incubated with appropriate secondary antibodies conjugated to horseradish peroxidase (Pierce), and signal intensities were visualized by Chemiluminescence (Cell Signaling Technology). Films from ≥4 independent experiments were scanned, and densities of the immunoreactive bands were evaluated using National Institutes of Health Image J software.

**Immunohistochemistry**

Mouse hearts were arrested in diastole with Krebs-Henseleit buffer containing 30 mmol/L KCl and fixed in 10% neutral buffered formalin. After stained with appropriate antibodies, the positive signal was detected using confocal microscopy (Zeiss LSM510Meta).

**Real-Time Polymerase Chain Reaction**

Real-time polymerase chain reaction was performed using SYBR green (Bio-Rad) as described.

**Statistics**

Data are expressed as a mean±SD. Comparisons among the groups were performed by 1-way ANOVA and followed by a post hoc Tukey–Kramer test. The comparison between 2 groups was performed using 2-tailed Student t tests. A P<0.05 was considered statistically significant.

**Results**

**Rescue of Pathological Glycogen Storage in the R2M Heart Eliminated Ventricular Preexcitation But Did Not Affect Cardiac Hypertrophy**

AMPK is an important regulator of glucose metabolism, and R2M hearts demonstrate significant increases in glucose uptake and intracellular [G6P], the major allosteric stimulator for glycogen synthesis. To determine whether glycogen storage caused by excessive G6P accounts for the fundamental disease mechanisms of Prkag2 cardiomyopathy, we sought to block G6P stimulated glycogen synthesis in R2M hearts by introducing a KI mutation (R582A) in the muscle form of GYS1. The GYS activity was significantly reduced, and the response to G6P was eliminated in the heart of KI or DM mice (Figure 1A). Glycogen content was lower in KI heart, and glycogen accumulation was markedly reduced (by 86-fold; P<0.0001) in DM heart (Figure 1B). Reduced glycogen storage was also demonstrated by haematoxylin and eosin staining of heart sections in which intracellular vacuoles representing glycogen storage sites in live cells were prominent in R2M hearts but were absent in DM hearts (Figure 1C). The KI did not affect the expression of mutant γ2-AMPK, the AMPK activity, basal glucose uptake rate, or the myocardial energetic status of the R2M hearts (Online Figure I).

Using ambulatory ECG recording, we found that the shortened PR interval present in the ECG of R2M mice, characteristic of ventricular preexcitation, was restored in DM mice (Figure 1D). The abnormal annulus fibrosis structure was subsequently improved (Online Figure II), which is in agreement with the previous findings that glycogen causes ventricular preexcitation by disruption of annulus fibrosis in R2M mice.
However, in spite of decreased glycogen content, heart weight (HW) to body weight ratios of DM (9.3±1.24 mg/g) remained similar to that of R2M (10.3±1.44 mg/g), and both were significantly greater than that of nontransgenic littermates (NTG, 4.3±0.17 mg/g) at 2 months (Figure 1E). In addition, cross-sectional area of cardiac myocytes was 2-fold higher in either R2M or DM hearts compared with that in NTG hearts (P<0.01), suggesting marked cellular hypertrophy (Figure 1F). Abnormal cardiac growth in DM mice was further evidenced by increased left ventricular wall thickness assessed by echocardiography (Online Figure III). Noticeably, the reduction of glycogen storage partially improved cardiac function of R2M but not in DM mice. Right, Average PR intervals in the 4 groups of mice. E, Heart weight to body weight ratios (HW/BW; n=7–10). F, Left, Representative images of cardiac sections stained with wheat germ agglutinin (WGA; scale bar, 40 μm). Right, Average myocyte cross-sectional area determined from 2 cardiac sections stained with WGA in each heart showing that cross-sectional area of cardiomyocytes is increased in both R2M and DM mice (n=3 each group). All mice used for all experiments were 2-month-old (#P<0.01 vs NTG; ‡P<0.01 vs R2M).

**Insulin Sensitivity Was Increased in R2M Hearts**

It has been shown that AMPK inhibits mTOR signaling via phosphorylation of tuberin/TSC (Ser1387) and raptor (Ser792).16,17 Consistent with increased AMPK activity in R2M, we observed increased phosphorylation of TSC and raptor at these sites (Online Figure IV). However, we also found that the phosphorylation level of Akt (S473) was significantly increased in R2M hearts (Figure 2A). Furthermore, a greater phosphorylation of TSC (Ser939, Thr1462) and raptor (Ser863) at the insulin-stimulated phosphorylation sites was observed (Figure 2A). The increased insulin-signaling pathway was also observed in DM hearts (Online Figure V). To determine whether insulin signaling is enhanced in R2M hearts, we subjected R2M mice to overnight fasting and compared Akt activation to that in fed mice. Fasting completely inhibited the phosphorylation of Akt and its downstream targets, mTOR and ribosomal protein S6, compared with ad lib feeding in both control and R2M mutant hearts (Figure 2B). To determine whether the greater phosphorylation of Akt in fed R2M heart was because of increased sensitivity to insulin, we injected a low dose of insulin (0.5 U/kg, IP) in fasted mice. R2M hearts responded to insulin injection with a greater increase in the phosphorylation level of Akt (S473; Figure 2C). It is unlikely that this is due to hyperinsulinemia because the cardiac-specific R2M mice did not develop systemic insulin resistance and showed no
Activation of the mTOR Pathway Partially Contributes to Cardiac Hypertrophy in R2M

Consistent with increased insulin signaling, the phosphorylation level of mTOR was significantly increased in R2M hearts (Figures 2B and 3A). Increased mTOR activity was further evidenced by increased phosphorylation of its downstream targets, p70S6 kinase (by 3.5-fold), ribosomal protein S6 (by 3.7-fold), and eukaryotic translation initiation factor 4E-binding protein 1 (by 3.2-fold). We next sought to rescue the abnormal cardiac growth phenotype in R2M mice by decreasing mTOR activity through treatment with rapamycin. Two-week-old mice were treated with either vehicle or rapamycin (2 mg/kg IP) daily for 4 weeks. This time point was chosen because activation of Akt and mTOR was first detected in R2M mice at 2 weeks of age (Figure 3B). Rapamycin treatment effectively inhibited mTOR activity, manifested as the loss of S6 phosphorylation (Figure 3C). Rapamycin treatment as expected did not affect phosphorylation of Akt (S473) in either genotype. After 4-week treatment, heart size of rapamycin-treated R2M was reduced by 45% compared with that of vehicle-treated R2M, as measured by HW to tibia length ratios. The reduced HW was attributed to a decrease in the cardiomyocyte size because the cross-sectional area of myocytes in rapamycin-treated R2M hearts was significantly decreased (Online Figure VII). Rapamycin-treated control mice also showed a 20% reduction of HW tibia length ratios compared with vehicle-treated control. As a result, HW tibia length ratios of R2M-rapamycin remained ≈87% greater than that of NTG-rapamycin (Figure 3D). Thus, inhibition of mTORC1

Figure 2. Insulin sensitivity was increased in the hearts of R2M mice. A. Left, Representative immunoblot of whole heart lysates of NTG and R2M mice probed for phosphorylated Akt at Ser473 (P-Akt [S473]), total Akt (T-Akt), P-TSC2 (S939), P-TSC2 (T1462), T-TSC2, P-raptor (S863), and T-raptor with β-actin as a loading control. Right, Quantification of band intensity on the immunoblot. Protein levels were normalized to β-actin, and data were expressed as fold change relative to NTG (n=4). B. Representative immunoblot of whole heart lysates of NTG and R2M mice after ad libitum feeding (Fed) or overnight fasting (Fast) for P-Akt (S473) and T-Akt, P-mammalian target of rapamycin (mTOR; S2448), T-mTOR, P-S6 (S235/236), and T-S6 (n=4). C. Top, Representative immunoblot of whole heart lysates of NTG and R2M mice 20 minutes after insulin (0.5 U/kg IP) or PBS injection and probed for P-Akt (S473) and T-Akt. Bottom, Quantification of band intensity on the immunoblot. Protein levels were normalized to β-actin as a loading control.

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pathway by rapamycin only partially reduced the cardiac hypertrophy of R2M hearts.

**FoxO Pathway Was Inactivated in R2M Hearts**

In searching for additional mechanisms responsible for the abnormal cardiac growth, we found that another downstream target of Akt, FoxO, was highly phosphorylated and inactivated in R2M hearts (Figure 4A). In R2M hearts, increased phosphorylation of FoxO3a at multiple Akt target sites was observed, and this was associated with decreased nuclear localization of FoxO3a protein and a lower level of total FoxO3a protein (Figure 4B), suggesting that increased phosphorylation of FoxO3a promoted its export from the nucleus and subsequent degradation in the cytosol. Increased phosphorylation of FoxO1a at Akt target sites was also observed, but there was no change in total FoxO1a protein. Inactivation of FoxO was further indicated by changes in the expression of its downstream targets, MURF1 and cyclin D1 (Figure 4A). Upregulation of cyclin D1 resulted in a higher protein level in both the nuclear and cytosolic fractions (Figure 4C).

To restore FoxO activity, we crossed the R2M mice with transgenic mice overexpressing FoxO1a in the heart (FO) because FoxO3a overexpression mice are not viable and FoxO1a has been shown to inhibit cardiac hypertrophy by similar mechanisms as FoxO3a. Overexpression of FoxO1a in R2M hearts (DM-FO) significantly reduced the HW (Figure 4D) and the cardiomyocyte cross-sectional area (236±28 μm² versus 328±55 μm²; P<0.01; Online Figure VIII). At the molecular level, the overexpression of FoxO1a decreased phosphorylation of FoxO3a and normalized total Fox3a protein in R2M hearts.

**Cardiomyocyte Proliferation Was Increased in R2M Hearts During Postnatal Cardiac Growth**

Because the upregulation of cyclin D1 suggests a potential increase of cell proliferation in R2M, we determined whether...
cellular hyperplasia contributes to the abnormal cardiac growth in R2M mice. Measurements of HW at multiple ages showed that increases in HW to body weight ratios started between 1 and 2 weeks, progressed through the postnatal period, and leveled off after weaning (Figure 5A). At 2 weeks when HW to body weight ratios increased by 50% in R2M, cardiomyocyte size did not change in terms of the length and width (Figure 5B). Rather, protein level of proliferating cell nuclear antigen, a cell proliferation marker, was elevated during the first 3 weeks after birth in R2M hearts, whereas its level declined sharply in NTG hearts after 1 week (Figure 5C). In 2-week-old R2M, mRNA levels were increased for cyclin D1 (1.7-fold), D2 (1.7-fold), and so were protein levels of cyclin D1 (4.1-fold) and D2 (1.7-fold; Figure 5D and 5E).

We subsequently performed immunohistological analysis of 2-week-old hearts using antibodies against proliferation markers, Ki67 and phospho-histone 3 (PH3). R2M hearts contained more Ki67+ or PH3+ cells, suggesting an increased cell proliferation (Figure 6A). The total number of cells (based on 4',6-diamidino-2-phenylindole staining) or cardiomyocytes (based on GATA binding protein 4 staining) per cross section of the heart was also increased in R2M (Figure 6B). There was no difference in the distribution of mono- versus multi-nucleated cardiomyocytes between the 2 groups (Online Figure X). BrdU labeling between 1 and 2 weeks after birth demonstrated that there was a modest increase in the percentage of all cell types that incorporated BrdU in R2M hearts compared with age-matched NTG (25% versus 30%). However, the fraction of cardiomyocytes in R2M hearts that incorporated BrdU was increased by 2-fold, indicating that a higher rate of cardiomyocyte proliferation during this period (Figure 6C). Comparing hearts at 2 weeks with that at 2 months, we found that the number of

Figure 4. The forkhead box O transcription factor (FoxO) pathway was inactivated in the hearts of R2M mice. A, Left, Representative immunoblot of P-FoxO3a (S318), P-FoxO3a (S253), P-FoxO3a (T32), T-FoxO3a, P-FoxO1a(S256), T-FoxO1, MURF1, and cyclin D1 in whole heart lysates of NTG and R2M mice with β-actin as a loading control. Right, Quantification of band intensity normalized to β-actin, and data are expressed as fold change relative to NTG (n=4). B, Representative immunoblot of P-FoxO3a (S318), T-FoxO3a in the nuclear and cytosolic fractions of hearts of NTG and R2M mice. Histone H3 was used as a nuclear protein loading control, and GAPDH was used as a cytosolic protein loading control (n=3). C, Representative immunoblot of cyclin D1 in the nuclear and cytosolic fractions of NTG and R2M hearts (n=4). D, Heart weight to body weight (HW/BW) in NTG, R2M, transgenic mice overexpressing wild type of FoxO1 in the hearts (FO), and double mutant overexpressing both N488I mutant form of γ2-AMP-activated protein kinase and wild type of FoxO1 in the hearts (DM-FO; n=8). E, Immunoblot and expression levels of cyclin D1, P-Akt (S473), T-Akt, P-S6 (S235/236), T-S6, P-FoxO3a (S318), T-FoxO3a, and T-FoxO1a in whole heart lysates of NTG, R2M, FO, and DM-FO mice (n=4). All mice used for all experiments were 2-month-old (#P<0.01 vs NTG; ‡P<0.01 vs R2M).
PH3+ cells decreased substantially in the adult hearts in both genotypes (Figure 7A–7C). However, at either age, the number of proliferating cells was 3- to 5-fold higher in R2M hearts compared with age-matched NTG. Importantly, the PH3+ cardiomyocyte was absent in NTG hearts but present albeit at a much lower frequency in adult R2M hearts. Overexpression of FoxO1a in R2M eliminated the proliferating cardiac myocytes in the adult heart (Figure 7C). Taken together, these findings show that increased heart size in R2M resulted from increases of both cell number and cell size, demonstrating that the mutation of γ2-AMPK stimulates cardiac growth by promoting cardiomyocyte proliferation and enhancing hypertrophic growth.

Discussion

This study demonstrates that point mutation of Prkag2 causes glycogen storage and cardiac hypertrophy via distinct mechanisms. By abrogating G6P-stimulated activity of GYS1, we have rescued pathological glycogen storage and consequently the preexcitation phenotype in the mouse model of Prkag2 cardiomyopathy. However, the effects of Prkag2 mutation on cardiac growth are independent of glycogen storage or preexcitation and cannot be rescued by reducing glycogen content of the heart. The mutation, via enhanced insulin sensitivity and activation of Akt, stimulates cardiomyocyte proliferation during postnatal growth by downregulating FoxO signaling and promoting cardiac hypertrophy via mTOR activation in the developed hearts. These findings elucidate important mechanisms underlying Prkag2 cardiomyopathy, and furthermore, reveal a novel function of γ2-AMPK that is critical for the use of AMPK as a therapeutic target.

Although the most common clinical complaint of Prkag2 cardiomyopathy is preexcitation arrhythmia, the unique characteristics of the disease, ventricular preexcitation associated with severe cardiac hypertrophy and glycogen storage, distinguish it from Wolff–Parkinson–White syndrome with structurally normal hearts.5,6,20,21 Rather, the cardiomyopathy phenotype seems to be similar to glycogen storage diseases caused by mutation in lysosomal associated membrane protein 2 and α-L-arabinofuranosidase A.20,22–24 Different from most forms of glycogen storage disease, mouse hearts expressing mutant Prkag2 are able to use glycogen while faithfully recapitulating the human cardiomyopathy phenotypes, suggesting that the disease mechanism is attributable to an imbalance of glycogen synthesis and use.8,11 Elimination of excessive glycogen accumulation by targeting the G6P-stimulated activity of GYS1, shown here, corroborates the prior metabolic flux analysis and provides definitive evidence that enhanced glycogen synthesis because of high intracellular glucose, and G6P is responsible for the glycogen storage in Prkag2 cardiomyopathy.10 Furthermore, it provides an opportunity to distinguish the direct consequence of Prkag2 mutation from changes secondary to glycogen storage. The rescue of preexcitation in the double mutant heart shows that the arrhythmia is a consequence of glycogen storage rather than a direct effect of AMPK on ion channels as previously speculated.25–27 It is also noteworthy that the reduction of glycogen storage is associated with improvement of cardiac function, suggesting that strategies of preventing/reducing glycogen storage are of therapeutic value. The mechanisms by which glycogen causes cardiac dysfunction remain unclear. We speculate the possible mechanisms for that. First of all, preexcitation can induce cardiac dysfunction. The accessory pathway causing preexcitation has been implicated as the cause of cardiac dysfunction. Many reports have shown that normalization of preexcitation with depletion of the accessory pathway can restore cardiac function.28,29 Second, extraordinary glycogen accumulation displaces contractile elements, distorts the overall cell morphology, and causes cardiac dysfunction.30,31 Third, when accumulated glycogen is used and metabolized, glycogen metabolism increases lactate production, lowers pH, and hampers contractile ability because of its action on sarcomeric protein. The improved cardiac function by lowering glycogen content may result from any one of the above-mentioned mechanisms.

Surprisingly, cardiac hypertrophy remains even after glycogen storage is prevented and ventricular preexcitation rescued in the double mutant heart, indicating that the abnormal cardiac
growth was regulated by separate mechanisms. Previous studies have shown that the Prkag2 cardiomyopathy is dependent on the kinase activity of AMPK, but the cardiac hypertrophy phenotype is contrary to the classic paradigm of AMPK function in cell growth, thus presenting a puzzle for the field. Several studies have shown that AMPK inhibits protein synthesis and cell growth under stress conditions by inhibiting mTOR signaling via phosphorylation of TSC2 and raptor. Consistent with this notion, activation of AMPK by 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside in cultured neonatal cardiac myocytes prevents agonist-induced hypertrophy. In the Prkag2 mutant heart, however, increased phosphorylation of TSC2 and raptor is observed both at the AMPK and the Akt sites reflecting increases both in AMPK activity and insulin sensitivity of these hearts. This is unique to the Prkag2 mutant heart in which the activation of AMPK occurs under nutrient rich and energy sufficient conditions in contrast to stress-induced activation of AMPK, which is invariably associated with energy deficit. The resultant activation of the mTOR pathway suggests that the insulin-stimulated phosphorylation cascade overrides the inhibitory regulation of AMPK on mTOR activity, leading to a progrowth phenotype in the mutant heart. Similarly, increased Akt activity and p70S6K phosphorylation have been reported in another mouse model harboring T400N point mutation of Prkag2 with increases of AMPK activity in the heart.

It has recently been shown that γ2-subunit is abundantly expressed in the fetal heart; its expression falls sharply after birth but reappears in cardiac hypertrophy and failure. Our results show that activation of γ2-containing AMPK in the postnatal period results in failure to arrest cardiomyocyte proliferation.

Figure 6. Cell proliferation was increased in the hearts of R2M mice during postnatal growth. A, Left, Representative confocal image of 2-week-old NTG and R2M hearts stained for phosphorylated-histone 3 (PH3, red, top), Ki67 (green, bottom), 4',6-diamidino-2-phenylindole (DAPI; blue), and merged image (merge). Right, Quantification of cells stained positive for PH3 or Ki67 in 1 cross section of the hearts from 2-week-old mice (n=6 for each genotype; scale bar, 50 μm). B, Top, Representative confocal image of a 2-week-old R2M heart stained for α-sarcomeric actin (α-SA, red), GATA binding protein 4 (GATA4) (green), DAPI (blue), and merged image (merge). Bottom, Quantification of all nuclei (DAPI+) and cardiomyocyte nuclei (GATA4+α-SA+) in each cross section of the hearts (n=4 for each genotype; scale bar, 10 μm). C, Top, 5-bromo-2-deoxyuridine (BrdU) was injected into 1-week-old pups for 7 days. Hearts were harvested at 2 weeks. Heart sections were stained for GATA4 (green), BrdU (red), and DAPI (blue). Arrow indicate cardiomyocyte with BrdU incorporation. Bottom, Percentage of proliferative cells (BrdU+/DAPI+) and percentage of proliferative cardiomyocyte (GATA4+BrdU+/GATA4+) in the 2-week-old heart. Thirty random fields were counted in each section (n=3; scale bar, 10 μm; *P<0.05 vs NTG).
In summary, we show that mutation of the γ2-subunit of AMPK accentuates insulin signaling, increases cell proliferation during the postnatal growth period, and stimulates myocyte hypertrophy in adulthood, leading to abnormal cardiac growth in Prkag2 cardiomyopathy. These changes are attributable to mechanisms independent of glycogen accumulation in the heart, thus providing new insights to the pathogenesis of Prkag2 cardiomyopathy. The findings also underscore the importance of isoform-specific function of AMPK that is critical for the pharmacological targeting of the AMPK.

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Disclosures
None.

References

What Is Known?
- AMP-activated protein kinase (AMPK) is an energy sensor and master regulator of cell metabolism, growth, and survival.
- Point mutations in the regulatory γ2 subunit of AMPK, encoded by PRKAG2, cause human cardiomyopathy characterized by myocyte hypertrophy, glycogen storage, and ventricular preexcitation.
- AMPK stimulates myocyte hypertrophy in adulthood, leading to these changes was independent of increases cell proliferation during the postnatal growth period, and stimulates myocyte hypertrophy in adults. Mol Cell. 2012;30:214–226.

Novelty and Significance

What Is New?
- Pathological glycogen storage could be rescued by genetically targeted glycogen synthesis in a mouse model for PRKAG2 cardiomyopathy (R2M).
- Reduced glycogen content in the R2M heart normalizes ventricular preexcitation but does not affect cardiac hypertrophy.
- Increased insulin sensitivity in R2M leads to activation of mammalian target of rapamycin and inactivation of forkhead box 0 transcription factor pathways, thus causing abnormal cardiac growth by increasing both the number and size of cardiomyocytes.

Activation of AMPK during stress conditions maintains energy homeostasis by stimulating substrate metabolism for ATP production and by suppression of growth and promotion of autophagy. These functions make AMPK an attractive drug target. Interestingly, point mutations in γ2 regulatory subunit increased AMPK activity in the absence of energy deficit but caused cardiomyopathy with glycogen storage, arrhythmia, and excessive cardiac growth in humans. These observations raise concern about using AMPK activation as a therapeutic approach and also the question of isoform-specific function of γ2-AMPK. We show that mutation of γ2-AMPK accentuates insulin signaling, increases cell proliferation during the postnatal growth period, and stimulates myocyte hypertrophy in adulthood, leading to abnormal cardiac growth. These changes were independent of glycogen accumulation in the heart. The findings provide new insights into the pathogenesis of PRKAG2 cardiomyopathy and underscore the importance of isoform-specific function of AMPK, critical for the pharmacological targeting of AMPK.
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Supplemental Material

Detailed Methods

Animal models

Transgenic mice with cardiac-specific overexpression of N488I AMPK γ2 (R2M) were generated on an FVB genetic background as previously described\(^1\). The mouse line harboring a knock-in mutation in the muscle form of glycogen synthase (GYS\(^1\)\(^{RS82A/R82A}\) KI) was generated on a CJ57B/L6 genetic background\(^2\) and was backcrossed to FVB for 8 generations. R2M-KI double mutant (DM) was generated on an FVB background. Transgenic mice with cardiac-specific overexpression of FoxO (Forkhead Box O transcription factor)\(^1\) (FO1) were generated on an FVB genetic background\(^3\). Wild type littermates of each transgenic mouse line were used as controls (NTG).

Two weeks old R2M or NTG were treated with rapamycin (2 mg/kg body weight, i.p.) or vehicle daily for 4 weeks. Rapamycin (LC Laboratories) was dissolved in DMSO and re-suspended in vehicle (0.2% carboxymethyl cellulose and 0.25% polysorbate-80) before injection. For insulin injection, mice were fasted overnight and anaesthetized with pentobarbital (80 mg/kg body weight, i.p.). Heart samples were freeze-clamped 20 minutes after insulin (0.5 U/kg body weight, i.p.) or PBS injection. For glucose tolerance test, mice were fasted overnight and injected with glucose (2 mg/kg body weight, i.p.). Blood glucose level was measured by contour blood glucose monitoring system (Bayer) at 0, 15, 30, 60, 90, and 120 minutes after glucose injection. For BrdU labeling experiments, one week old mice were injected with BrdU (50 mg/kg body weight, i.p.) daily for 7 days, and hearts were subsequently harvested and fixed in 10% neutral buffered formalin for immunohistochemistry.
All animal procedures were performed in accordance with the National Institute of Health’s Guide for the Care and Use of Laboratory Animals. The protocols were approved by the institutional IACUC committee at the University of Washington.

**Echocardiography and ECG**

Transthoracic echocardiography was performed in mice under light anesthesia using a Vevo770 high resolution imaging system with 30-MHz RMV-707B scanning head (VisualSonics Inc.). Left ventricular wall thickness (anteroseptal and posterior) as well as chamber dimensions were measured in end-systole and end-diastole with M-mode echocardiography. Electrocardiogram (ECG) was recorded for 2 hours using implantable wireless monitoring device with DSI mouse ECG transmitter ETA-F10.

**Cardiac glycogen synthase activity and glycogen content**

Glycogen synthase (GYS) activity in cardiac tissue was measured using the method of Thomas et al.4. Hearts were freeze-clamped and homogenized in ice-cold buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.27 M sucrose, 1 % (w/v) Triton X-100, 50 mM NaF, 5 mM Na₃P₂O₇, 1 μM microcystin-LR, 1 mM DTT, 1 mM benzamidine and 0.5 mM PMSF. Lysates were desalted over Penefsky columns (2.5 ml Sephadex G-50 fine equilibrated with lysis buffer) to remove an inhibitory component in heart extracts prior to assay in reactions containing 1.67 mM [¹⁴C(U)]-UDP-glucose (0.12 mCi.mmol) in the presence and absence of 10 mM glucose-6-phosphate (G6P). Homogenates were added to assay buffer containing 10 mM G6P and [¹⁴C] UDP-glucose. The radioactivity was read by scintillation counting. Myocardial glycogen content was determined by measuring the amount of glucose released from glycogen by amylglucosidase and a glucose assay kit (Sigma-Aldrich) as previously described5.

**Glucose uptake and myocardial energetics**
$^{31}$P nuclear magnetic resonance (NMR) spectroscopy was used to measure the rate of glucose uptake in isolated hearts perfused with nontracer 2-deoxyglucose (2-DG) as previously described$^5$. Mice were heparinized and anesthetized with sodium pentobarbital (100 mg/kg body weight, i.p.), and the hearts were perfused in Langendorff mode at 37°C. All hearts were stabilized for 25 minutes and a $^{31}$P NMR spectrum was collected to assess the baseline high energy phosphate content. To determine the rate of glucose uptake, hearts were switched to a buffer in which glucose was replaced with 5 mM 2-DG and monitored for the time-dependent accumulation of 2-DG-phosphate (2-DG-P). The rate of 2DG uptake was estimated by the slope of the fitted line as previously described$^5$. The $^{31}$P NMR resonance areas corresponding to ATP, phosphocreatine (PCr), inorganic phosphate (Pi), and 2-DG-P were fitted to Lorentzian functions and corrected for saturation (ATP, 1.0; PCr, 1.2; Pi, 1.15; 2-DG-P, 1.8). The mean value of ATP concentration previously measured by HPLC for hearts of WT or R2M mice was used to calibrate the ATP peak area of the baseline $^{31}$P NMR spectrum$^6$.

**HEK293T cell culture**

HEK293T cells (ATCC) were cultured in DMEM medium in the presence of 10% FBS and 100U/ml penicillin/100ug/ml streptomycine. The cells were transfected with plasmid constructs harboring N488I mutant (R2M) of γ2-AMPK or GFP control for 24 hours, and were serum-starved overnight then treated with insulin for 20 minutes.

**Preparation of protein extracts and Immunoblotting**

Protein samples were prepared from frozen heart samples using a lysis buffer containing protease inhibitors (Sigma). Nuclear and cytosolic fractions were prepared according to instructions of NE-PER extraction kit (Pierce). Tissue lysates were matched for protein concentration and then separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Bio-Rad laboratories). Membranes were blocked in 5% non-fat milk and
incubated with primary antibodies overnight at 4°C. The following primary antibodies were obtained from Cell Signaling Technology unless otherwise noted. Phospho-AMPK (T172), Phospho-AMPK (S491), AMPK, P-ACC (S79), Phospho-Akt (S473), Akt, Phospho-mTOR (S2448), mTOR, Phospho-S6 (S235/236), S6, Phospho-4EBP1 (S65), 4EBP1, Phospho-FoxO3a (T32), Phospho-FoxO3a (S253), Phospho-FoxO1a (S256), Phospho-FoxO3a (S318), FoxO3a, Phospho-TSC2 (T1462), Phospho-TSC2 (S939), Phospho-TSC2 (T1387), TSC2, Phospho-Raptor (S792), Raptor, Phospho-IRS1(S636/639), Phospho-IRS1(S1101), Phospho-IRS1 (S302), IRS1, Cyclin D1, β-actin, FoxO1a (Epitomics), PCNA (Abcam) and MURF1 (Abcam), Phospho-Raptor (S863) (Thermo scientific). Membranes were incubated with appropriate secondary antibodies conjugated to horseradish peroxidase (HRP) (Pierce) and signal intensities were visualized by Chemiluminescence (Cell Signaling Technology). Films from at least four independent experiments were scanned and densities of the immunoreactive bands were evaluated using NIH Image software.

Histology and immunohistochemistry

Mouse hearts were arrested in diastole with KH buffer containing 30 mM KCl and fixed in 10% neutral buffered formalin. Longitudinal sections of hearts were stained with wheat germ agglutinin conjugated to FITC (Sigma) for assessment of myocyte cross-sectional area and with Haematoxylin and Eosin for assessment of heart morphology. The cardiac sections were used for trichrome and CD31 staining to assess annulus fibrosis and vascularity, respectively. For determination of cell proliferation, heart sections were blocked in 10% normal goat serum and incubated with primary antibodies overnight at 4°C: Ki67 (Vector labs), Phospho-Histone 3 (S10, Cell Signaling Technology), GATA4 (Santa Cruz Biotechnology), α-sarcomeric actin (Sigma), BrdU (Roche). Sections were incubated with secondary antibodies conjugated to TRITC and FITC (Jackson ImmunoResearch). After mounting with DAPI-containing medium, the positive signal was detected using confocal microscopy (Zeiss LSM510Meta).
**RNA isolation and RT-PCR**

Real Time PCR was performed as previously described\(^7\). Total RNA was isolated from frozen hearts using the RNeasy kit (Qiagen), and cDNA synthesized using Omniscript reverse synthase and random hexamers according to manufacturer’s guidelines. Real time PCR was performed using SYBR green (Bio-Rad) using the following primers: cyclin D1, 5’-CTGGCCATGAACCTGGA-3’ and 5’-ATCCGCTCTGGCATTTTG; cyclin D2, 5’-CTGGCCATGAACCTGGA-3’ and 5’-GTTCACTTCATCATCCTGCTG-3’; cyclin E1, 5’-GAGCTTGAATACCCTAGGACTG-3’ and 5’-CGTCTCTCTGAGCTTTATAGAC-3’; MURF1, 5’-ACGAGAAGAGAGAGCTTGAGG-3’ and 5’-CTTGGCAGGAGAGGAAAGG-3’; p21, 5’-CACAGCTCAGTGAGTGGAA-3’ and 5’-CCACCACCACACACCAGATA-3’. The Real Time PCR results for the mRNA levels of each gene were normalized to 18S rRNA levels.

**Statistics**

Data are expressed as a mean ± s.d. Comparisons among the groups were performed by 1-way ANOVA and followed by a post hoc Tukey-Kramer test. The comparison between 2 groups was performed using 2 tailed Student’s \(t\) tests. A \(p<0.05\) was considered statistically significant.

**References**


Online Figure I. AMPK activity, glucose uptake rate and myocardial energetics in the hearts of R2M, KI and double mutant (DM) mice. (A) Representative immunoblot of P-AMPK (T172), T-AMPK, P-ACC (S79) and T-ACC in whole heart lysates of NTG, R2M, and DM mice. n=3. (B) Basal glucose uptake rate measured by time-dependent 2-deoxyglucose-phosphate accumulation and (C) myocardial energetic status assessed by phosphocreatine to ATP ratio (PCr/ATP) in isolated perfused hearts from 2-month old mice using 31P NMR spectroscopy. *p< 0.05 versus NTG; #p< 0.01 versus NTG.
Online Figure II. Trichrome-stained cardiac sections from NTG (A), R2M (B), and DM (C). (A) A normal annulus fibrosis in NTG separating the interatrial septum (IAS) from the interventricular septum (IVS). (B) A bundle of glycogen containing vacuolated myocytes (arrowheads) in the R2M heart interrupted the annulus fibrosis (arrows) at the atrioventricular junction. (C) Normal annulus fibrosis in the DM heart. Scale bar, 100 μm.
Online Figure III. Echocardiographic measurements of 2 month old mice. LVPWD, left ventricular posterior wall thickness at the end-diastole; LVDD: left ventricular end-diastolic internal dimension; LVIDS: left ventricular end-systolic internal dimension; FS, Fractional shortening. n=10; #p< 0.01 versus NTG; ‡p< 0.01 versus R2M.
Online Figure IV. TSC2 and raptor protein expression in the hearts of 2-month old NTG and R2M mice. Representative immunoblot of P-TSC2 (T1387), T-TSC2, P-raptor (S792), and T-raptor in whole heart lysates of NTG and R2M mice. N=4
Online Figure V. Insulin signaling pathway in R2M-KI (DM) double mutant hearts. n=3
Online Figure VI. Characterization of R2M mice. (A) Body weight. n=8. (B) Blood glucose level of fed and fasted mice. n=6, (C) Glucose tolerance test. n=6.
Online Figure VII. Rapamycin reduced cardiomyocyte size. (A) Representative images of cardiac sections stained with Wheat Germ Agglutinin (WGA). (B) Quantification of cardiomyocyte cross-sectional area. Scale bar, 40 μm. n=3. #p< 0.01 versus NTG; ‡p< 0.01 versus vehicle.
Online Figure VIII. Effects of mTOR and FoxO pathways in enhanced cardiac growth of R2M. Top panels, Heart size of NTG, R2M, and DM-FO mice with and without rapamycin (Rap) treatment. Bottom Panels, Representative images of cardiac sections stained with WGA to determine the difference in cardiomyocyte cross-sectional area. Average value of 10 images per heart is shown for each group. Scale bar, 40 μm.
Online Figure IX. Relative change of the mRNA level of FoxO target genes. n=4. *p< 0.05 versus NTG; ‡p< 0.05 versus R2M
Online Figure X. Distribution of cardiomyocytes containing different number of multiple nuclei in 2-week old NTG and R2M mice. Top, representative confocal image of cardiomyocytes from a 2-week old R2M heart stained with DAPI. Bottom, percentage of mono- or multi-nucleated cardiomyocytes out of the total number of cardiomyocytes counted. A total of 200-250 cardiomyocytes were counted in each heart. n=3. Scale bar, 20 μm.
Online Figure XI. Vascularity in the DM mutant mice. Representative CD31 stained image of NTG (A), R2M (B), and DM (C). (D) Vascularity index expressed as a ratio of CD31 positive and total section areas. n=5-7.
Online Figure XII. Effects of rapamycin treatment on cell proliferation. (A), Representative confocal image of heart sections from 6-week old treated with rapamycin (Rap) or vehicle showing a proliferating cardiomyocyte stained positive for PH3 (green), alpha-sarcomeric actin (α-SA, red), and DAPI (blue) in R2M and a proliferating non-cardiomyocytes stained positive for PH3 and DAPI but not α-SA in NTG. (B), Quantification of proliferating cardiomyocytes in hearts. All proliferating cells were counted as PH3+ cells per section and proliferating cardiomyocytes (PH3+ CMs) were stained positive for both PH3 and alpha-sarcomeric actin in stained hearts. n=3 for each genotype. Scale bar, 10 μm; *p< 0.01 versus NTG.