Distinct Early Signaling Events Resulting From the Expression of the PRKAG2 R302Q Mutant of AMPK Contribute to Increased Myocardial Glycogen

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Background—Humans with an R302Q mutation in AMPKγ_2 (the *PRKAG2* gene) develop a glycogen storage cardiomyopathy characterized by a familial form of Wolff-Parkinson-White syndrome and cardiac hypertrophy. This phenotype is recapitulated in transgenic mice with cardiomyocyte-restricted expression of AMPKγ_2R302Q. Although considerable information is known regarding the consequences of harboring the γ_2R302Q mutation, little is known about the early signaling events that contribute to the development of this cardiomyopathy.

Methods and Results—To distinguish the direct effects of γ_2R302Q expression from later compensatory alterations in signaling, we used transgenic mice expressing either the wild-type AMPKγ_2 subunit (TGγ_2WT) or the mutated form (TGγ_2R302Q), in combination with acute expression of these proteins in neonatal rat cardiomyocytes. Although acute expression of γ_2R302Q induces AMPK activation and upregulation of glycogen synthase and AS160, with an associated increase in glycogen content, AMPK activity, glycogen synthase activity, and AS160 expression are reduced in hearts from TGγ_2R302Q mice, likely in response to the existing 37-fold increase in glycogen. Interestingly, γ_2WT expression has similar, yet less marked effects than γ_2R302Q expression in both cardiomyocytes and hearts.

Conclusions—Using acute and chronic models of γ_2R302Q expression, we have differentiated the direct effects of the γ_2R302Q mutation from eventual compensatory modifications. Our data suggest that expression of γ_2R302Q induces AMPK activation and the eventual increase in glycogen content, a finding that is masked in hearts from transgenic adult mice. These findings are the first to highlight temporal differences in the effects of the PRKAG2 R302Q mutation on cardiac metabolic signaling events. **(*Circ Cardiovasc Genet*. 2009;2:457-466.)**

Key Words: glycogen ■ molecular biology ■ signal transduction ■ AMPK

AMP-activated protein kinase (AMPK) is a highly conserved kinase that is responsible for controlling cellular energy homeostasis (for review, see reference 1). In the cardiomyocyte, AMPK has been implicated in stimulating myocardial fatty acid uptake[2] and oxidation[3] as well as glucose uptake,[4] glycolysis,[5,6] and possibly glycogen storage or mobilization[6–8]; all of which likely contribute to maintaining adequate ATP supply that is necessary for normal cardiac function. In noncardiac cells, AMPK plays important roles in the regulation of many pathways including gluconeogenesis, fatty acid synthesis, lipolysis, whole-body metabolism (reviewed in reference 9), and appetite.[10] As a result of these studies, and given the fact that the antidiabetic drug metformin has been shown to increase AMPK activity,[12] there has been considerable interest in developing therapies that modulate AMPK activity for the treatment of obesity and diabetes.[11] Moreover,

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pharmacological activation of AMPK has also been proposed as a treatment for limiting myocardial ischemia and reperfusion injury.[13,14] However, given the existence of naturally occurring gain- and loss-of-function mutations in AMPK and

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their association with certain cardiac abnormalities,[15–17] it is imperative that we first gain fundamental insight into the roles that AMPK plays in the heart before pursuing these therapeutic strategies. Studying and understanding these naturally occurring mutations will greatly assist in this endeavor.

AMPK consists of a catalytic subunit (α) and 2 regulatory subunits (β, γ).[18,19] The γ subunit of AMPK has been shown to bind AMP,[20] which ultimately enhances phosphorylation and activation of the α subunit to increase AMPK activity. A number of mutations within the *AMPK*γ_2 gene (*PRKAG2*) have been shown to produce a glycogen storage cardiomyopathy distinguished by ventricular preexcitation, progressive conduction system disease and in certain cases, cardiac hypertrophy.[15–17] It is believed that conduction system abnormalities present in these patients are a result of glycogen-filled myocytes causing bypass tracts and/or disruption of the annulus fibrosis between the atria and ventricle,[16,17] faster depolarization of the ventricular myocardium and resulting tachycardia.[21] This cardiac phenotype in humans can be caused by a missense mutation in the human *PRKAG2* gene that results in an arginine substitution with a glutamine at amino acid 302 (R302Q) in the γ_2 subunit.[15]

In transgenic mice overexpressing the γ_2R302Q mutation, the γ_2N488I mutation, or the γ_2R531G mutation, the resulting phenotypes include significant glycogen accumulation within the cardiomyocyte and marked cardiac hypertrophy.[22–24] This phenotype has been attributed to alterations in AMPK activity resulting from the mutations. However, the γ_2R302Q and γ_2R531G mutations have been reported to inhibit AMPK activity, whereas the γ_2N448I mutation results in AMPK activation.[17,23,24] To date, it is not known how mutations causing either a decrease or an increase in AMPK activity might result in the same phenotype. Interestingly, evidence demonstrating changes in AMPK activity resulting from the mutations largely come from transgenic mice.[17,23,24] However, when studying adult mice from these transgenic lines, it is difficult to separate direct effects of the mutation alone from compensatory changes induced as a result of altered metabolism and glycogen accumulation. In fact, it has been shown that AMPK activity in hearts of transgenic mice expressing the γ_2N488I mutation fluctuates in response to the levels of glycogen accumulation.[25] Therefore, based on these complex interactions between AMPK and glycogen accumulation in the young and the adult heart, the objective of this investigation was to distinguish the acute, direct effects of the γ_2R302Q mutation from the chronic, potentially compensatory effects on AMPK activity and on pathways involved in glycogen accumulation and hypertrophic growth. Ultimately, the information gained from this study may provide clues as to the importance of AMPK activity, glycogen deposition, and cardiac hypertrophy in the development of the PRKAG2 syndrome. In addition, this mutation may also provide further information about the fundamental roles of AMPK in the heart and the effects of direct alteration of AMPK activity. This information is especially relevant given the interest in AMPK as a drug target for the treatment of obesity and/or diabetes and ischemia and reperfusion injury.

Methods

Animal Care

The University of Alberta adheres to the principles for biomedical research involving animals developed by the Council for International Organizations of Medical Sciences and complies with National Institutes of Health animal care guidelines.

Materials

Primary antibodies against phospho-Akt (Ser 473), Akt, phospho-AMPKα (Thr 172), AMPKα, AMPKγ_2, AMPKβ_2, phospho-acetyl CoA carboxylase (Ser 79), phospho-Ser/Thr Akt substrate, AS160, glycogen synthase (GS), and phospho-p70S6K (Thr 389) were purchased from Cell Signaling Technology (Danvers, Mass). The primary antibody against phospho-GS (Ser641/5) was purchased from Novus Biologicals (Littleton, Colo). The primary antibodies against actin- and peroxidase-labeled streptavidin as well as secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif). Radiolabeled substrates were purchased from Perkin-Elmer Life Sciences, Inc (Waltham, Mass).

Transgenic Mice

Transgenic mice with cardiomyocyte-restricted expression of the wild-type (TGγ_2WT) and the mutant human *PRKAG2* gene (TGγ_2R302Q; substitution of glutamine for arginine at residue 302) were generated with the cardiac specific promoter α-myosin heavy chain as we have previously described.[24] Male and female mice were weighed before euthanasia (whole body weight). Hearts were extracted from 2- to 5-month-old mice, rinsed in ice-cold PBS, and total heart weight (HW) was determined. Hearts from 7-day-old and 2- to 5-month-old mice that were used for biochemical analysis were frozen in liquid nitrogen immediately after extraction. In some instances, mouse ventricular cardiomyocytes were isolated using a modified version of an isolation protocol described previously.[26]

Cardiomyocyte Infection

Newborn (1- to 3-day-old, male and female) rat hearts were isolated, and neonatal rat cardiomyocytes were isolated and cultured, as we have described previously.[27] Neonatal rat cardiomyocytes were infected with green fluorescent protein- (GFP), γ_2WT-, or γ_2R302Q-expressing adenoviruses (AdGFP, Adγ_2WT, or Adγ_2R302Q, respectively) at a multiplicity of infection of 20. Forty-eight hours postinfection, cells were harvested as described.[28]

Cell Surface Area Measurements

The cardiomyocyte cell surface area measurements were obtained using ImagePro Plus software (MediaCybernetics, Bethesda, Md).

[^{3}H]Phenylalanine Incorporation

[^{3}H]phenylalanine (1 μCi/mL) was added to isolated neonatal rat cardiomyocytes for 24 hours, and incorporation was determined, as described previously.[29]

Measurement of Nuclear Factor of Activated T Cells Activity

To determine nuclear factor of activated T cells (NFAT) transcriptional activity, cardiomyocytes were cultured as described earlier and infected with adenoviruses harboring either the GFP (AdGFP) or the NFAT-Luc-Promoter (AdNFAT; Seven Hills Bioreagents) and assayed for luciferase activity as we have described previously.[30] Luciferase activity was standardized to protein content.

In Vitro AMPK Assay

AMPK activity was measured using the in vitro AMPK peptide substrate activity assay as described previously.[31]

Immunoblot Analysis

Boiled samples of heart or isolated cardiomyocytes homogenates were subjected to SDS-PAGE in gels containing 5% to 10%

acrylamide and transferred to nitrocellulose and immunoblotted, as described previously.[32]

Histology

One-micrometer sections of human heart endomyocardial biopsy samples fixed in 10% formalin were prepared and stained with hematoxylin-eosin stain, as described previously.[24] The left atrial appendage samples were collected as part of a post-transplant assessment for the PRKAG2 sample and as part of a clinical evaluation following a clinically indicated procedure for the "control" sample.

Glycogen Assay

Glycogen was extracted from frozen powdered mouse heart ventricular tissue or isolated cardiomyocytes, converted to glucose, and quantified using a range of glucose standards as described previously.[33] Glycogen was measured as micromole glucosyl units per gram wet weight of tissue or cells.

Measurement of Glycogen Synthase and Phosphorylase Activity

Activity was measured in cardiomyocyte homogenates essentially as described previously.[34,35]

Measurement of Glycolytic Rates in Isolated Cardiomyocytes

Rates of glycolysis were measured using 5-[^{3}H] D-glucose as described by Folmes et al.[36]

Statistical Analysis

All data are presented as mean±SEM. For comparison of 3 groups, ANOVA followed by the Bonferroni multiple comparisons test was used for the determination of statistical analysis. For comparison with AdGFP (set at an arbitrary value of 1), a 1-sample *t* test to a hypothetical mean was used. For comparison of 2 groups, a 2-tailed *t* test was used. A value of $P<0.05$ was considered significant.

Results

AMPK Activity and Subunit Expression

To confirm that hearts from TGγ_2R302Q mice demonstrated reduced AMPK activity as published previously,[24] whole heart homogenates were assayed for AMPK activity. Although hearts from both γ_2 wild-type overexpressing transgenic mice (TGγ_2WT) and γ_2 mutant overexpressing transgenic mice (TGγ_2R302Q) displayed decreases in AMPK activity compared with nontransgenic (NTG) hearts, AMPK activity in TGγ_2R302Q hearts was also significantly decreased compared with TGγ_2WT hearts (Figure 1A). Consistent with the reductions in total AMPK activity, α_1 and α_2 activities were both reduced to ≈50% of NTG levels in TGγ_2WT hearts, whereas the activities of both isoforms in TGγ_2R302Q hearts were decreased to ≈35% (data not shown). These data indicate that there are no specific effects of the mutation on either of the 2 catalytic isoforms of AMPK. In agreement with an overall decrease in AMPK activity, AMPKα phosphorylation at Thr172, a surrogate marker of the in vivo activation status of AMPK, was significantly reduced in hearts from TGγ_2R302Q mice compared with NTG and TGγ_2WT (Figure 1B). Interestingly, the γ_2 subunit was more highly expressed in the TGγ_2WT mouse hearts as compared with the TGγ_2R302Q mouse hearts, whereas γ_2 was not detected in NTG hearts at this exposure (Figure 1C). Although the expression of all other subunits was unchanged (data not shown), expression of β_2 was also increased in both TGγ_2WT and TGγ_2R302Q mouse hearts as compared with NTG hearts (Figure 1D).

In contrast to the findings in hearts from transgenic mice, acute adenoviral-mediated expression of the γ_2 mutant (Adγ_2R302Q) in isolated cardiomyocytes resulted in significantly higher AMPK activity (Figure 1E), with a trend to increase phosphorylation of the AMPKα subunit (Figure 1F) compared with control GFP-expressing cardiomyocytes (AdGFP). This increase in AMPK activity was also observed in hearts from 7-day-old TGγ_2R302Q mice (Figure 1G). Consistent with activation of AMPK, phosphorylation of acetyl CoA carboxylase, a downstream target of AMPK, was also significantly increased in γ_2R302Q expressing cardiomyocytes compared with control (1.63±0.15-fold increase, $P<0.05$ versus control). Together, these data suggest that the reduction in AMPK activity observed in hearts from adult transgenic mice is likely a secondary effect rather than a direct result of the mutation per se. Interestingly, as observed in adult transgenic mice, the γ_2WT subunit was present at higher levels than the γ_2R302Q subunit in adenovirally transduced cardiomyocytes (Figure 1H), whereas expression of the β_2 subunit was increased in both groups compared with GFP control (Figure 1I), suggesting that increased γ_2 levels may have a specific effect on β_2 expression and/or stability.

The Development of Cardiac Hypertrophy

TGγ_2R302Q mice exhibited significant cardiac hypertrophy as indicated by the increase in heart weight (HW) (Figure 2A) compared with NTG and TGγ_2WT mice without a significant change in body weight (34.2±2.7 g, 27.6±1.0 g, 35.2±3.0 g for NTG, TGγ_2WT, and TGγ_2R302Q, respectively, P=NS). Although many different myocardial cell types may contribute to increased HW/body weight, we show a direct increase in cardiomyocyte size in TGγ_2R302Q mice as compared with both NTG and TGγ_2WT controls (Figure 2B). Interestingly, TGγ_2WT mice also developed a significant increase in HW and an increase in cardiomyocyte cell surface area compared with NTG controls (Figure 2A and 2B, respectively), suggesting that expression of the nonmutated form of the γ_2 subunit also has a modest effect on cardiomyocyte growth. As we have previously shown that AMPK is a negative regulator of hypertrophy[29] and that inhibition of AMPK signaling may lead to a permissive environment for development of hypertrophy,[37] the inhibition of AMPK activity observed in both transgenic mouse models may contribute to the hypertrophic phenotype in these mice. However, phosphorylation of both Akt and p70S6K at their activating sites[38] was significantly decreased in the TGγ_2R302Q hypertrophic hearts compared with NTG hearts (Figure 2C and 2D, respectively), suggesting that protein synthesis is not increased in adult TGγ_2WT or TGγ_2R302Q mouse hearts. In addition, acute expression of either the γ_2WT or the γ_2R302Q subunit in isolated cardiomyocytes did not result in increased cardiomyocyte size (Figure 2E), accelerated protein synthesis (basal or phenylephrine stimulated; Figure 2F), or stimulation of prohypertrophic growth pathways (Figure 2G), suggesting that alterations in AMPK activity by these mutations do not promote

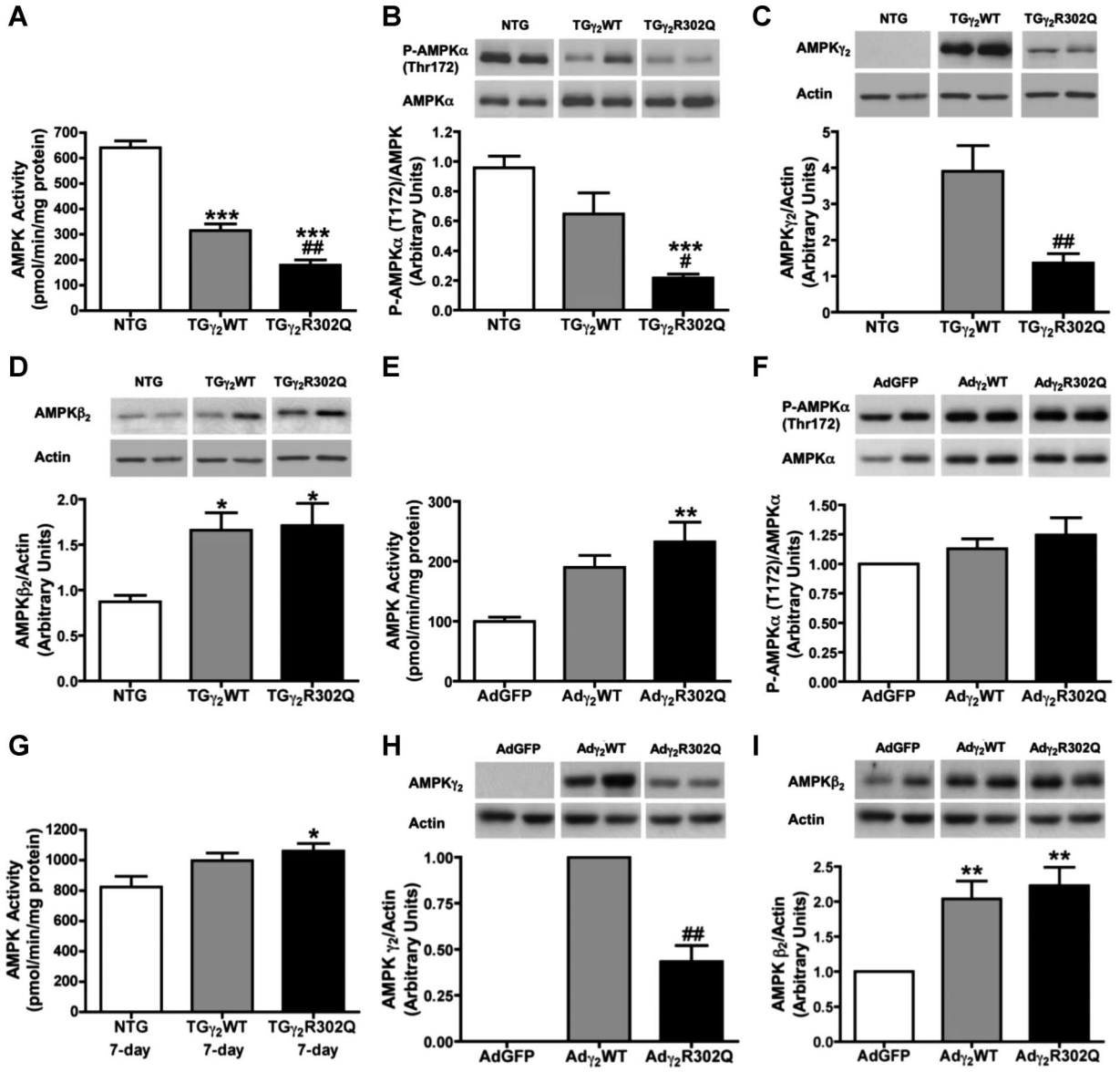

Figure 1. AMPK activity is differentially affected by acute and chronic expression of γ_2R302Q. Whole heart homogenates (n=6, 7) from adult TGγ_2WT and TGγ_2R302Q mice display decreased AMPK activity (pmol/min/mg protein) compared with NTG mice (A) whereas AMPK activity is increased in cell lysates (n=4) from adenovirally transduced cardiomyocytes expressing γ_2R302Q (Adγ_2R302Q) (E) and in heart homogenates from 7-day-old TGγ_2R302Q mice (G). Phosphorylation of AMPKα (Thr172) is reduced in TGγ_2R302Q heart homogenates (B) and unchanged in cell lysates from cardiomyocytes expressing γ_2R302Q (F), as measured by immunoblot analysis using antiphospho-AMPKα (Thr 172) and antitotal-AMPKα antibodies. Expression of γ_2 is increased in whole heart homogenates (C) and in cell lysates from cardiomyocytes expressing γ_2WT (H), whereas β_2 is increased in heart homogenates (D) and cell lysates from cardiomyocytes expressing either γ_2R302Q or γ_2WT (I), as measured using anti-AMPKγ_2, anti-AMPKβ_2, and antiactin antibodies. ***$P<0.001$ versus NTG or AdGFP; **$P<0.01$ versus NTG or AdGFP; ##$P<0.01$ versus TGγ_2WT or Adγ_2WT; *$P<0.05$ versus NTG or AdGFP; and #$P<0.05$ versus TGγ_2WT or Adγ_2WT.

cardiomyocyte cell growth by means of conventional mechanisms.

Glycogen Deposition

Humans with the γ_2R302Q mutation develop PRKAG2 syndrome, a characteristic of which is excessive glycogen accumulation within the cardiomyocytes. Previous studies have shown that large vacuoles detected with hematoxylin-eosin staining of cardiomyocytes are representative of areas of glycogen deposition.[22] Consistent with this study and positive PAS staining in TGγ_2R302Q mouse hearts,[24] large vacuoles and distended cardiomyocytes, indicative of glycogen accumulation, were evident in the atria of a patient with the γ_2R302Q mutation (Figure 3A) but were absent in the atria of a patient without this mutation (Figure 3B). In agreement with these data and previous results,[24] TGγ_2R302Q mouse hearts displayed a significant 37-fold increase in glycogen compared with NTG hearts (Figure 3C). In addition, TGγ_2WT hearts had a 10-fold increase in glycogen compared with NTG hearts but still had significantly lower glycogen levels than TGγ_2R302Q hearts (Figure 3C). In agreement with these findings, acute expression of either the γ_2WT or the γ_2R302Q

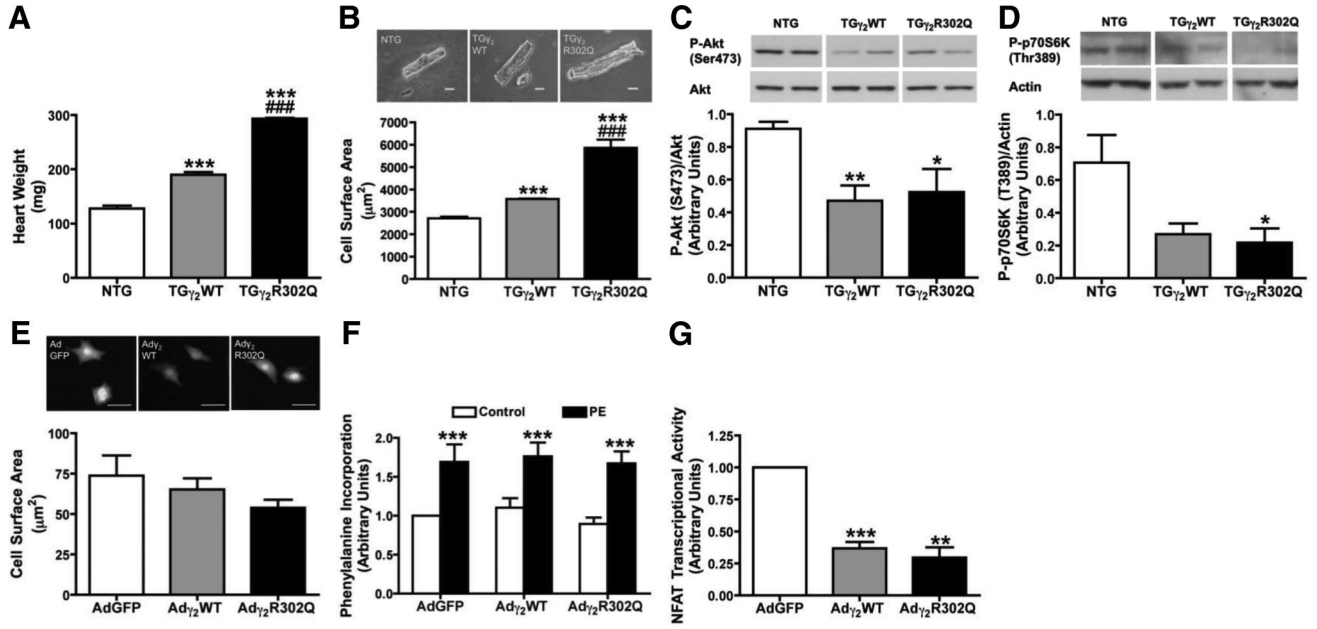

Figure 2. Cardiac hypertrophy is induced in transgenic mice with chronic expression of γ_2WT and γ_2R302Q but is absent in isolated cardiomyocytes acutely expressing γ_2WT and γ_2R302Q. HW (A) and ventricular myocyte cell surface area (99 to 125 cells/group isolated from 3 hearts in each group; B) were increased in adult TGγ_2WT and TGγ_2R302Q mice compared with NTG mice. Isolated neonatal rat cardiomyocytes transduced with adenoviruses expressing GFP, γ_2WT, or γ_2R302Q (AdGFP, Adγ_2WT, or Adγ_2R302Q, respectively) (238 to 255 cells/group; n=20) displayed no changes in cell surface area (E). Representative photographs of cardiomyocytes in each group are shown, where the white bar is 20 μm (B and E-inset). Phosphorylation of Akt and p70S6K were decreased in whole heart homogenates from TGγ_2R302Q mice, as measured by antiphospho-Akt (Ser473), antiphospho-p70S6K (Thr389) and antiactin antibodies (C and D). Basal or phenylephrine-induced protein synthesis was not affected by increased γ_2WT or γ_2R302Q expression in neonatal rat cardiomyocytes, as measured by [^{3}H]Phenylalanine incorporation (F). Coinfection of neonatal rat cardiomyocytes with the NFAT-luciferase reporter gene (AdNFAT) and AdGFP, Adγ_2WT, or Adγ_2R302Q resulted in impaired NFAT transcriptional activity in cardiomyocytes expressing γ_2WT or γ_2R302Q (G). ***$P<0.001$ versus NTG or AdGFP; ###$P<0.001$ versus TGγ_2WT or Adγ_2WT; **$P<0.01$ versus NTG or AdGFP; and *$P<0.05$ versus NTG.

mutation in isolated cardiomyocytes also significantly increased glycogen content (70% and 270% versus control, respectively; Figure 3D), suggesting that cellular alterations induced by the expression of either the WT or the mutant form of the γ_2 subunit can both promote glycogen accumulation.

Glycogen Turnover

To determine the mechanism by which glycogen accumulation occurs, we examined the enzymes involved in glycogen synthesis and degradation. In TGγ_2R302Q hearts, activity of glycogen synthase, the rate-limiting enzyme in glycogen synthesis, was significantly decreased compared with NTG

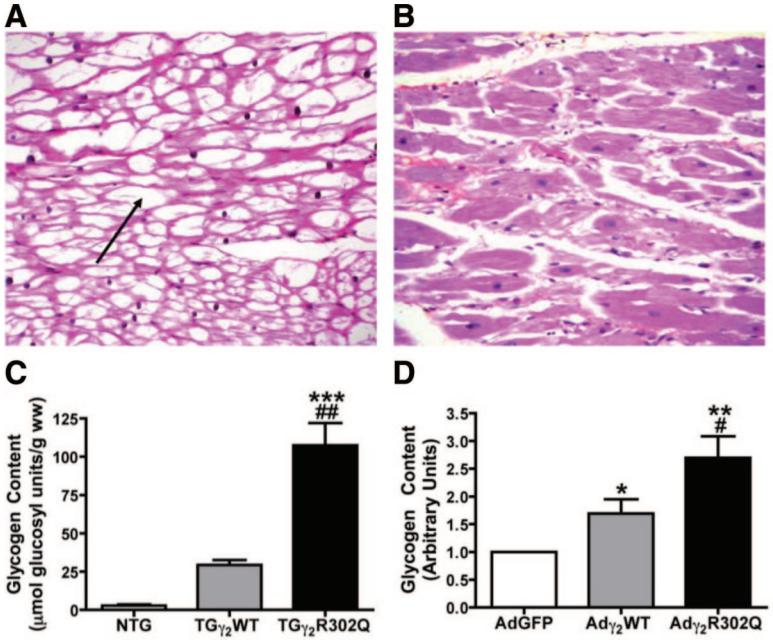

Figure 3. Glycogen levels are increased with acute and chronic expression of γ_2WT and γ_2R302Q. Hematoxylin-eosin staining of a human heart endomyocardial biopsy sample from a patient with the γ_2R302Q mutation (A) shows evidence of glycogen accumulation (arrow points to a vacuolated myocyte indicative of glycogen deposition) compared with a control patient with no γ_2R302Q mutation (B). Glycogen was measured from frozen powdered mouse heart ventricles from adult NTG, TGγ_2WT, and TGγ_2R302Q mice and from AdGFP, Adγ_2WT, and Adγ_2R302Q infected cardiomyocytes. Myocardial glycogen was increased in TGγ_2WT and TGγ_2R302Q hearts (μmol glucosyl units/gram wet weight of tissue, n=3, 4) and in Adγ_2WT and Adγ_2R302Q infected cardiomyocytes (standardized to AdGFP, n=7,8) (C and D). ***$P<0.001$ versus NTG or AdGFP; **$P<0.01$ versus NTG or AdGFP; ##$P<0.01$ versus TGγ_2WT or Adγ_2WT; *$P<0.05$ versus NTG or AdGFP; and #$P<0.05$ versus TGγ_2WT or Adγ_2WT.

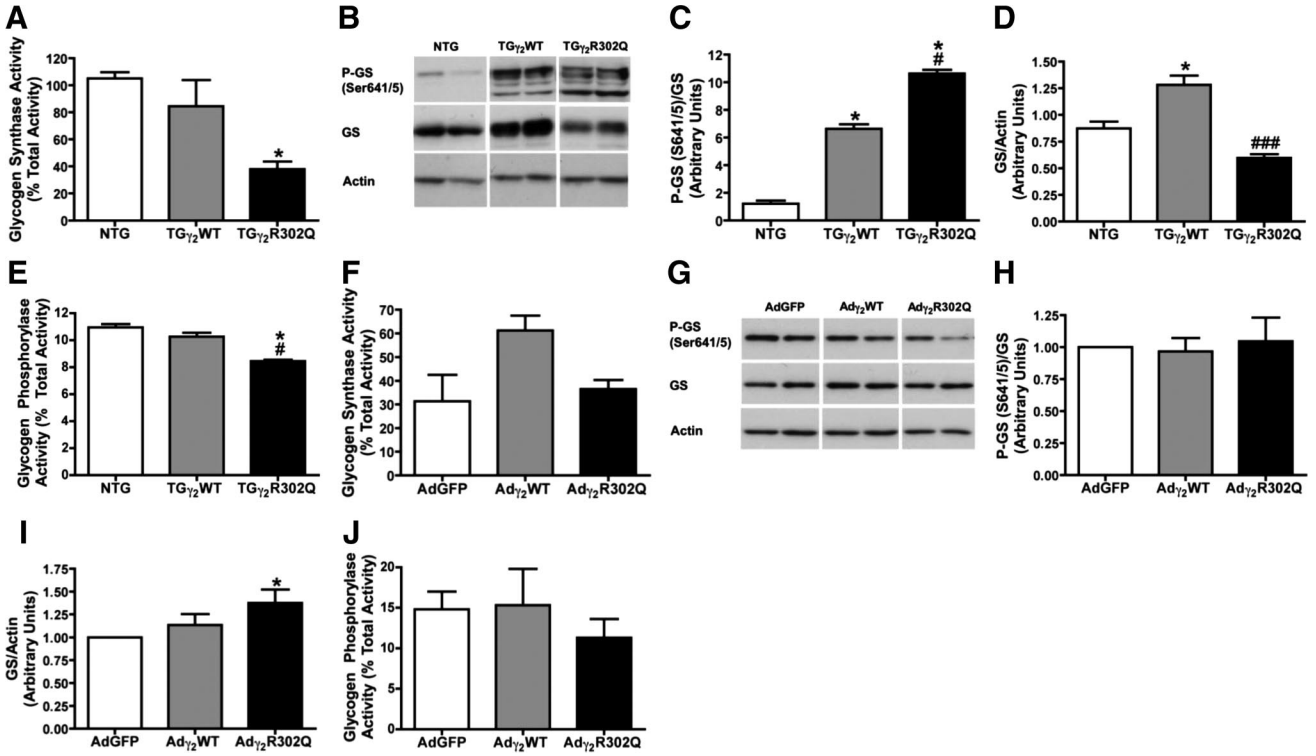

Figure 4. GS and GP are differentially regulated by acute and chronic expression of γ_2R302Q. GS and GP activities, measured as percent of total activity using 15 mmol/L glucose-6-phosphate or 200 mmol/L glycogen, respectively, were decreased in TGγ_2R302Q hearts (A and E). GS protein expression was decreased (B, middle panel), whereas inhibitory phosphorylation was increased (B, upper panel) in TGγ_2R302Q hearts, as measured using antiphospho GS (Ser641/645), anti-GS and antiactin antibodies. Densitometry of phospho-GS/GS and GS/actin ratios are shown (C and D, respectively). GS (F) and GP (J) activities were unchanged in cell lysates from neonatal rat cardiomyocytes expressing GFP, γ_2WT, or γ_2R302Q. GS protein expression was increased in cell lysates from neonatal rat cardiomyocyte expressing γ_2R302Q (G, middle panel), whereas GS phosphorylation was unchanged, (G, upper panel) as measured using antiphospho GS (Ser641/645), anti-GS, and antiactin antibodies. Densitometry of phospho-GS/GS and GS/actin ratios are shown (H and I, respectively). ###$P<0.001$ versus TGγ_2WT or Adγ_2WT; *$P<0.05$ versus NTG or AdGFP; and #$P<0.05$ versus TGγ_2WT or Adγ_2WT.

hearts (Figure 4A). Consistent with this, inhibitory phosphorylation of GS was significantly increased compared with both NTG and TGγ_2WT hearts (Figure 4B and 4C), and GS protein levels were significantly reduced compared with TGγ_2WT hearts (Figure 4B and 4D). This inhibition of GS is likely a compensatory mechanism resulting from the profound glycogen accumulation in these hearts. As observed with other parameters, GS activity and phosphorylation in TGγ_2WT hearts were intermediate between NTG and TGγ_2R302Q hearts (Figure 4A and 4C, respectively). In addition to changes in GS activity and phosphorylation, glycogen phosphorylase (GP) activity was slightly but significantly reduced in TGγ_2R302Q hearts compared with NTG and TGγ_2WT hearts, indicating a potential impairment in glycogen mobilization (Figure 4E). Paradoxically, cardiomyocytes acutely expressing γ_2R302Q exhibited no change in GS activity (Figure 4F) or phosphorylation (Figure 4G and 4H); however, GS expression was significantly increased compared with controls (Figure 4G and 4I). In addition, GP activity was unchanged in cardiomyocytes expressing either γ_2R302Q or γ_2WT compared with control (Figure 4J). Although we do not provide evidence explaining why activities of GS and GP were unchanged in cardiomyocytes expressing either γ_2R302Q or γ_2WT, it is possible that the assay in cardiomyocytes is not sensitive enough to detect subtle changes in GS and GP activities that may be occurring in these cells.

Glucose Handling

Because the majority of glucose provided for glycogen synthesis comes from an increase in glucose uptake or a shunting of glucose away from glycolysis, we investigated glycolysis and glucose uptake in our models. Glycolytic metabolism of exogenous glucose was not altered in either the transgenic mice (G. Lopaschuk, personal communication) or in the isolated cardiomyocytes expressing the γ_2R302Q mutation (Figure 5A). Given the increase in glycogen levels and unchanged rates of glycolysis in the cardiomyocytes expressing the γ_2R302Q mutation, the calculated glucose uptake in cells expressing γ_2R302Q was 1.76-fold higher than controls (Figure 5B). In addition, although activation of Akt was not changed (data not shown), phosphorylation of the Akt substrate of 160 kDa (AS160) was significantly increased in cardiomyocytes expressing γ_2R302Q compared with control (Figure 5C). Interestingly, total protein levels of AS160 were also increased in cardiomyocytes expressing γ_2R302Q compared with controls (Figure 5D). Although this increase in total protein levels of AS160 prevented a significant increase in the P-AS160/AS160 ratio (data not shown), the upregulation of both P-AS160 and total AS160 may be

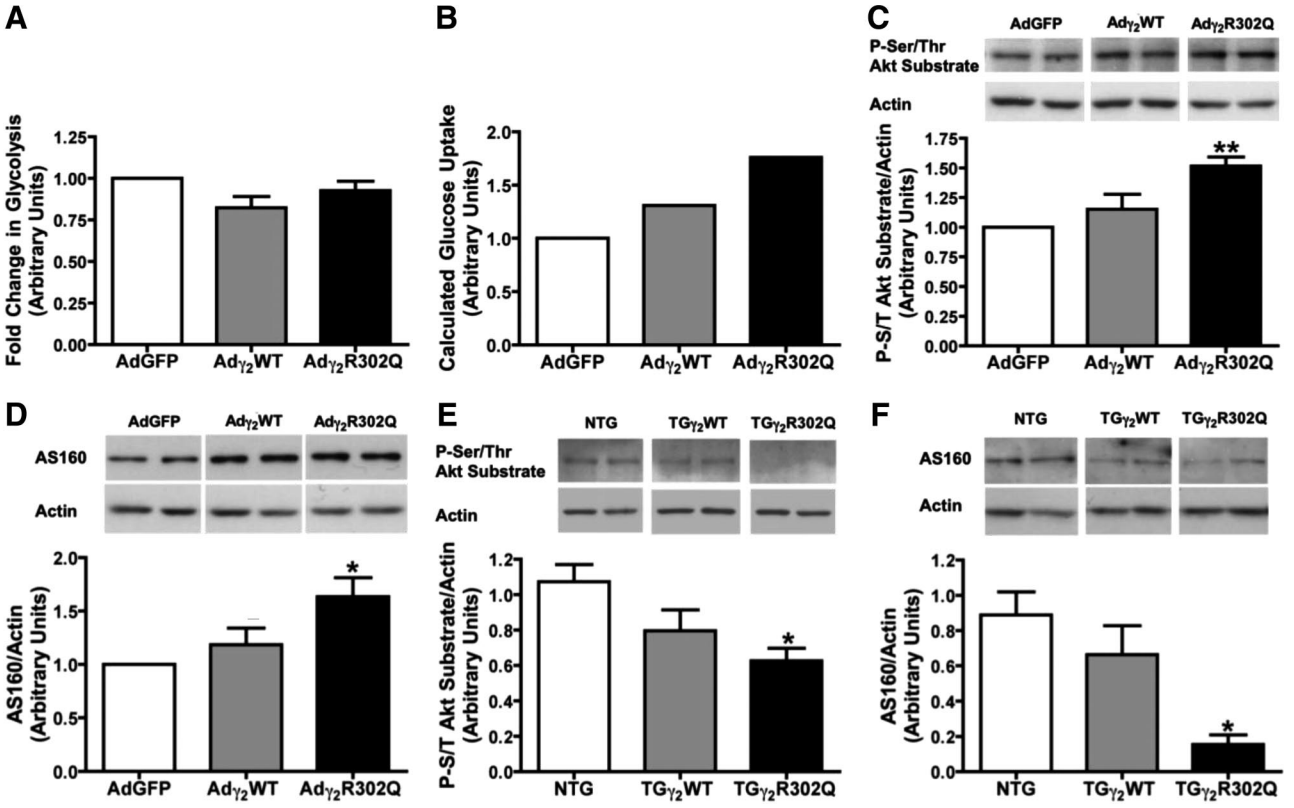

Figure 5. Mechanisms involved in the regulation of glucose uptake and metabolism are differentially regulated by acute and chronic expression of γ_2R302Q. Glycolysis was unchanged in isolated neonatal rat cardiomyocytes expressing GFP, γ_2WT, or γ_2R302Q (A). Glucose uptake (calculated based on numbers from glycolysis and glycogen) was increased in γ_2R302Q-expressing cells (B). AS160 phosphorylation (C) and expression (D) were increased in cell lysates from γ_2R302Q-expressing cardiomyocytes, as measured by antiphospho ser/thr Akt substrate, anti-AS160, and antiactin antibodies. AS160 phosphorylation (E) and expression (F) were decreased in TGγ_2R302Q heart homogenates, as measured by antiphospho ser/thr Akt substrate, anti-AS160, and antiactin antibodies. *$P<0.05$ versus NTG or AdGFP or ** $P<0.01$ versus NTG or AdGFP.

sufficient to promote glucose uptake especially since AS160 plays an important role in GLUT4 translocation to the membrane and increased glucose transport.[39] Despite this rationale, there did not seem to be an increase in GLUT4 in the plasma membrane in γ_2R302Q-expressing cardiomyocytes as compared with controls (data not shown). In contrast to these findings in cardiomyocytes acutely expressing γ_2R302Q, Akt phosphorylation was decreased (Figure 2C) and both P-AS160 and total AS160 expression were significantly reduced in the TGγ_2R302Q hearts compared with NTG hearts (Figure 5E and 5F, respectively), potentially as a chronic compensatory mechanism to prevent further glucose uptake and consequent glycogen accumulation.

Discussion

In this study, we focused on the acute effects of expression of both γ_2WT and γ_2R302Q in the control of AMPK phosphorylation and activity and compared these effects to the long-term, potentially compensatory effects of transgenic overexpression of these same proteins. In contrast to hearts from transgenic mice expressing the γ_2N488I mutation, hearts from TGγ_2R302Q mice display reduced AMPK activity as compared with hearts from wild-type mice, which was attributed to decreases in both AMPK α_1 and α_2 subunit activity. However, consistent with our hypothesis that acute expression of the γ_2 mutant may have differential effects compared with chronic expression, acute expression of the γ_2R302Q mutation in hearts from 7-day-old transgenic mice and in isolated cardiomyocytes resulted in a significant activation of AMPK. This initial increase in AMPK activity is consistent with that of mice with a γ_2N488I mutation, where AMPK activity was increased in hearts from 7-day-old transgenic mice but not in hearts from older transgenic mice.[25] As our isolated cardiomyocyte data show that the γ_2R302Q mutation is an activating mutation, the data obtained from the mouse models suggest that there is likely an inhibitory feedback mechanism that is responsible for decreased AMPK activity in the adult mouse heart expressing the γ_2R302Q mutation. Although we do not provide evidence for this, we propose that the profound glycogen deposition in hearts from TGγ_2R302Q mice may be responsible for decreased myocardial AMPK activity in these mice as suggested previously.[25]

Although the γ_2R302Q mouse model used in this study differs from other mouse models expressing γ_2 mutations in terms of AMPK activity,[22] there are still consistencies between the models. For example, the TGγ_2R302Q mice also developed significant myocardial hypertrophy, which is in agreement with another transgenic mouse model expressing a mutated form of the γ_2 protein.[22] However, given that

isolated cardiomyocytes acutely expressing the γ_2R302Q mutation did not display evidence of growth, NFAT activation, or increased protein synthesis, our data suggest that the hypertrophy observed in the transgenic mice likely occurs by means of nonconventional mechanisms. In support of this, the phosphorylation status of Akt and p70S6K, which are normally increased in conventional hypertrophy, were reduced in the hearts of TGγ_2R302Q mice. However, consistent with glycogen accumulation inducing myocardial growth through nonconventional means,[17] hearts from TGγ_2R302Q mice displayed a significant 37-fold increase in glycogen compared with NTG hearts. Because humans possessing the γ_2R302Q mutation also have profound glycogen accumulation, our study supports the notion that glycogen deposition and associated water accumulation may be the underlying cause of the cardiac hypertrophy also observed in a subgroup of this patient population.[15]

An interesting finding in this study was that expression of the γ_2WT protein also induced a cardiac phenotype in transgenic mice that was less severe than that observed in TGγ_2R302Q mice. Indeed, hearts from TGγ_2WT displayed a 50% reduction in AMPK activity, an 84% increase in HW, and a 10-fold increase in glycogen compared with NTG mice, indicating that a component of the cardiac phenotype observed in the TGγ_2R302Q mice may be related to simple overexpression of the γ_2 subunit. Although we cannot explain why expression of the γ_2WT subunit results in similar, albeit less marked changes compared with expression of the γ_2R302Q mutant, the intermediate phenotype may be because of the much higher expression level of the γ_2 subunit in TGγ_2WT hearts. While it would be reasonable to assume that the elevated level of γ_2WT protein in the mouse heart is due to variations in genomic incorporation of the γ_2WT transgene compared with γ_2R302Q, this is likely not the case given that the same expression profile is observed with epichromosomal expression of the γ_2 subunits using adenoviral delivery. Although these data suggest that the stability of the γ_2 protein may be influenced by the R302Q mutation, we still cannot explain how expression of the γ_2WT protein also produces profound glycogen deposition. However, while the expression levels of the majority of the other subunits of AMPK were unaltered, protein expression of the AMPKβ_2 subunit was also significantly increased in hearts and in cardiomyocytes expressing either the WT or the R302Q mutant forms of γ_2. As previous work using AMPK $\alpha_2-/-$ mice demonstrate a correlation between β_2 expression (which possesses a glycogen binding domain) and glycogen levels,[40] increased expression of the β^2 subunit in this study may contribute to the glycogen accumulation in hearts and in isolated cardiomyocytes.

To better characterize the mechanisms involved in glycogen accumulation in isolated cardiomyocytes and transgenic mice expressing γ_2WT and the γ_2R302Q mutation, we first examined the glycogen synthesis pathway. Although GS protein levels were increased in isolated cardiomyocytes acutely expressing γ_2R302Q, GS protein content and activity were significantly reduced in TGγ_2R302Q hearts, likely in response to massive glycogen accumulation. Surprisingly, despite the 10-fold increase in glycogen content, GS expression and activity were not reduced in TGγ_2WT hearts. In fact, GS protein content was significantly increased compared with NTG, suggesting that a $>$10-fold increase in glycogen is required before any compensatory mechanisms are initiated. As the glucose necessary for glycogen synthesis must come from either increased glucose uptake or decreased glycolysis, we also measured these parameters in isolated cardiomyocytes. The rates of glycolytic metabolism of exogenous glucose were not reduced in isolated cardiomyocytes acutely expressing the γ_2R302Q mutation. Interestingly, cardiomyocytes expressing the γ_2R302Q mutation also do not have increased GLUT4 protein in the plasma membrane nor were we were able to detect increased glucose uptake compared with controls. Although we cannot explain this lack of effect, it may be because of the sensitivity of the glucose uptake method used in this study. Indeed, both calculated glucose uptake as well as expression and phosphorylation of AS160, an enzyme involved in GLUT4 translocation to the plasma membrane,[39] are increased in isolated cardiomyocytes acutely expressing γ_2R302Q compared with control, suggesting that glucose uptake is likely elevated in these cells. As a result, we propose that acute expression of the γ_2R302Q mutation results in an activation of the AMPK holoenzyme and an upregulation of both AS160 phosphorylation/expression and GS expression, resulting in increased glucose uptake and glycogen synthesis. Conversely, long-term expression of this mutation in transgenic mice results in an inactivation of AMPK, a downregulation of Akt phosphorylation, decreased AS160 phosphorylation/expression, and an inhibition of GS, potentially as a compensatory mechanism to prevent further deleterious accumulation of glycogen. Whether the observed effects are a result of activation and/or inhibition of various transcription factors is currently being investigated.

Finally, as our data have shown that AMPK activity is increased to a similar extent in isolated cardiomyocytes expressing either the γ_2R302Q mutation or the γ_2WT while glycogen content is significantly higher in γ_2R302Q-expressing cells, it is tempting to speculate that glycogen levels and not alterations in AMPK activity per se may be responsible for the Wolff-Parkinson-White phenotype. In fact, although the TGγ_2WT mice do display a less marked phenotype, this study highlights that inhibition of AMPK activity, a 10-fold increase in glycogen, and modest hypertrophic growth are not sufficient for the development of preexcitation. What seems to be a necessary component of Wolff-Parkinson-White in the γ_2 mutation models is profound glycogen accumulation, independent of AMPK activity, as originally hypothesized.[41] Indeed, despite variable effects of the 3 different mutations (ie, γ_2R302Q, γ_2N488I, and γ_2R531G) on AMPK activity during different stages of life, what is consistent in all mouse models is the extreme glycogen deposition observed in the cardiomyocyte.[17,23] Taken together, these data suggest that excessive glycogen accumulation is the major contributor to the cause of Wolff-Parkinson-White syndrome as opposed to AMPK activity per se. In addition, the phenotype observed in the TGγ_2WT mice highlights the confounding factors associated with the existing transgenic mice expressing cardiomyocyte-restricted AMPKγ_2 mutations. As such, it is becoming increasingly evident that a knock-in mouse is the ideal

and necessary model to use to study the cardiac effects of these mutations.

In conclusion, our results serve to highlight the complexity of AMPK signaling in the development of glycogen storage cardiomyopathy and help to resolve the controversy surrounding the γ_2R302Q mutation with respect to the effect that it has on AMPK activity.[24] As such, these findings offer valuable insights into the early and late signaling mechanisms that underlie the excessive glycogen deposition associated with PRKAG2 cardiomyopathy. These findings are particularly relevant given the recent advancements made in the identification of AMPK activators,[11] and the potential cardiac side effects that may be associated with these agents.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Mutations in the *PRKAG2* gene encoding for the γ_2 subunit of the energy-sensing kinase, AMP-activated protein kinase (AMPK), produce a glycogen storage cardiomyopathy characterized by ventricular preexcitation, atrial arrhythmias, progressive conduction system disease, and in certain cases, cardiac hypertrophy. This constellation of cardiac abnormalities occurs in humans with an Arg302Gln mutation in the AMPK γ_2 subunit (γ_2R302Q) and in transgenic mice with cardiomyocyte-restricted expression of the same mutation. Although earlier reports indicated that this mutation inactivates AMPK, we provide evidence that the γ_2R302Q mutation results in the activation of AMPK in neonatal cardiomyocytes. This activation of AMPK in the early developmental period contributes to enhanced glucose uptake and glycogen synthesis and the eventual increase in glycogen accumulation. These data describing early signaling events induced by the γ_2R302Q mutation suggest that the reduced AMPK activity observed in the hearts of adult γ_2R302Q transgenic mice is a compensatory response to the significant elevation of myocardial glycogen that develops over time. Consistent with this negative feedback inhibition of AMPK, glucose uptake and glycogen synthesis are both suppressed in these glycogen-filled adult cardiomyocytes. Taken together, our study provides insight into the cellular mechanisms that underlie the excessive glycogen deposition associated with PRKAG2 cardiomyopathy. The information gleaned from this study may be useful in considering future pharmacological intervention in the early stages of the disease that may attenuate severe clinical progression.