Distinct early signaling events resulting from the expression of the PRKAG2 R302Q mutant of AMPK contribute to increased myocardial glycogen

Folmes et al. The PRKAG2 R302Q mutation and myocardial glycogen

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ABSTRACT

Background: Humans with an R302Q mutation in AMPKγ2 (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 AMPKγ2 R302Q. While considerable information is known regarding the consequences of harboring the γ2 R302Q mutation, little is known about the early signaling events that contribute to the development of this cardiomyopathy.

Methods and Results: In order to distinguish the direct effects of γ2 R302Q expression from later compensatory alterations in signaling, we utilized transgenic mice expressing either the wildtype AMPKγ2 subunit (TGγ2 WT) or the mutated form (TGγ2 R302Q), in combination with acute expression of these proteins in neonatal rat cardiomyocytes. While acute expression of γ2 R302Q induces AMPK activation and upregulation of glycogen synthase (GS) and AS160, with an associated increase in glycogen content, AMPK activity, GS activity and AS160 expression are reduced in hearts from TGγ2 R302Q mice, likely in response to the existing 37-fold increase in glycogen. Interestingly, γ2 WT expression has similar, yet less dramatic effects than γ2 R302Q expression in both cardiomyocytes and hearts. Conclusions: Utilizing acute and chronic models of γ2 R302Q expression, we have differentiated the direct effects of the AMPKγ2 R302Q mutation from eventual compensatory modifications. Our data suggest that expression of γ2 R302Q 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 PRKAG2 mutations on cardiac metabolic signaling events.

Keywords: glycogen; molecular biology; signal transduction; AMPK
INTRODUCTION

AMP-activated protein kinase (AMPK) is a highly conserved kinase that is responsible for controlling cellular energy homeostasis (see 1 for review). 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/mobilization\(^6,8\); all of which likely contribute to maintaining adequate ATP supply that is necessary for normal cardiac function. In non-cardiac cells, AMPK plays important roles in the regulation of many pathways including gluconeogenesis, fatty acid synthesis, lipolysis, whole-body metabolism (reviewed in \(^9\)) and appetite\(^10\). As a result, there has been considerable interest in developing therapies that modulate AMPK activity for the treatment of obesity and diabetes\(^11\) especially given the fact that the anti-diabetic drug metformin has been shown to increase AMPK activity\(^12\). Moreover, pharmacological activation of AMPK has also been proposed as a treatment for limiting myocardial ischemia/reperfusion injury\(^13,14\). However, given the existence of naturally occurring gain- and loss-of-function mutations in AMPK and their association with certain cardiac abnormalities\(^15-17\), it is imperative that we first gain fundamental insight into the role(s) that AMPK play in the heart prior to pursuing these therapeutic strategies. Studying and understanding these naturally occurring mutations will greatly assist in this endeavor.

AMPK consists of a catalytic subunit (\(\alpha\)) and two regulatory subunits (\(\beta, \gamma\))\(^16,19\). The \(\gamma\) subunit of AMPK has been shown to bind AMP\(^20\), which ultimately enhances phosphorylation and activation of the \(\alpha\) subunit to increase AMPK activity. A number of mutations within the AMPK\(\gamma2\) gene (PRKAG2) have been shown to produce a glycogen storage cardiomyopathy distinguished by ventricular pre-excitation, 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\textsuperscript{16, 17}, faster depolarization of the
ventricular myocardium and resulting tachycardia\textsuperscript{21}. This cardiac phenotype in humans can be
cau\textsuperscript{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 $\gamma_2$ subunit\textsuperscript{15}.

In transgenic mice overexpressing either the $\gamma_2$R302Q mutation, the $\gamma_2$N488I mutation or
the $\gamma_2$R531G mutation, the resulting phenotypes include significant glycogen accumulation
within the cardiomyocyte and dramatic cardiac hypertrophy\textsuperscript{22-24}. This phenotype has been
attributed to alterations in AMPK activity resulting from the mutations. However, the $\gamma_2$R302Q
and $\gamma_2$R531G mutations have been reported to inhibit AMPK activity, whereas the $\gamma_2$N488I
mutation results in AMPK activation\textsuperscript{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\textsuperscript{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 $\gamma_2$N488I mutation
fluctuates in response to the levels of glycogen accumulation\textsuperscript{25}. Therefore, based on these
complex interactions between AMPK and glycogen accumulation in the young as well as the
adult heart, the objective of this investigation was to distinguish the acute, direct effects of the
$\gamma_2$R302Q mutation from the chronic, potentially compensatory effects on AMPK activity as well
as 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 as well as 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 as well as ischemia/reperfusion injury.

MATERIALS AND 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 NIH animal care guidelines.

Materials- Primary antibodies against phospho-Akt (Ser 473), Akt, phospho-AMPKα (Thr 172), AMPKα, AMPKγ2, AMPKδ2, phospho-ACC (Ser 79), phospho-Ser/Thr Akt substrate, AS160, GS and phospho-p70S6K (Thr 389) were purchased from Cell Signaling Technology. The primary antibody against phospho-GS (Ser641/5) was purchased from Novus Biologicals. The primary antibodies against actin and peroxidase labeled streptavidin as well as secondary antibodies were obtained from Santa Cruz Biotechnology. Radiolabeled substrates were purchased from Perkin Elmer Life Sciences, Inc.

Transgenic Mice- Transgenic mice with cardiomyocyte-restricted expression of the wildtype (TGγ2WT) and the mutant human PRKAG2 gene (TGγ2R302Q; substitution of glutamine for arginine at residue 302) were generated with the cardiac specific promoter α-myosin heavy chain as we have previously described. Male and female mice were weighed prior to euthanasia (whole body weight). Hearts were extracted from 2-5 month-old mice, rinsed in ice cold PBS, and total heart weight was determined. Hearts from 7 day-old and 2-5 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\textsuperscript{26}.

**Cardiomyocyte Infection**- Newborn (1-3 day-old, male and female) rat hearts were isolated and neonatal rat cardiomyocytes were isolated and cultured as we have previously described\textsuperscript{27}. Neonatal rat cardiomyocytes were infected with GFP, $\gamma_2$WT or $\gamma_2$R302Q expressing adenoviruses (AdGFP, Ad$\gamma_2$WT, Ad$\gamma_2$R302Q, respectively) at a multiplicity of infection of 20. Forty-eight hours post-infection, cells were harvested as described\textsuperscript{28}.

**Cell Surface Area Measurements**- The cardiomyocyte cell surface area measurements were obtained using ImagePro Plus software (MediaCybernetics).

**[3H]Phenylalanine Incorporation**- 1 Ci/mL $[^3]$Hphenylalanine was added to isolated neonatal rat cardiomyocytes for 24 h and incorporation was determined as previously described\textsuperscript{29}.

**Measurement of NFAT activity**- To determine NFAT transcriptional activity, cardiomyocytes were cultured as described above and infected with adenoviruses harboring either GFP (AdGFP) or the NFAT-Luc-Promoter (AdNFAT; Seven Hills Bioreagents) and assayed for luciferase activity as we have previously described\textsuperscript{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\textsuperscript{31}.
**Immunoblot Analysis**- Boiled samples of heart or isolated cardiomyocytes homogenates were subjected to SDS-PAGE in gels containing 5-10% acrylamide and transferred to nitrocellulose and immunoblotted as previously described\(^32\).

**Histology**- One-micrometer sections of human heart endomyocardial biopsy samples fixed in 10% formalin were prepared and stained with hematoxylin and eosin (H&E) stain, as previously described\(^24\). 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 previously described\(^16\). Glycogen was measured as μmol glucosyl units/gram wet weight of tissue or cells.

**Measurement of Glycogen Synthase and Phosphorylase Activity**- Activity was measured in cardiomyocyte homogenates essentially as previously described\(^34, 35\).

**Measurement of Glycolytic Rates in Isolated Cardiomyocytes**- Rates of glycolysis were measured using 5-[\(^3\)H] D-glucose as described in Folmes et al\(^36\).

**Statistical Analysis**- All data are presented as mean ± standard error of the mean (S.E.M.). For comparison of three groups, analysis of variance followed by the Bonferroni multiple comparisons test was used for the determination of statistical analysis. For comparison to AdGFP (set at an arbitrary value of 1), a one-sample t-test to a hypothetical mean was
employed. For comparison of two groups, a two-tailed t-test was utilized. A value of p<0.05 was considered significant.

RESULTS

**AMPK activity and subunit expression**- In order to confirm that hearts from TGG2R302Q mice demonstrated reduced AMPK activity as previously published24, whole heart homogenates were assayed for AMPK activity. Although hearts from both \( \gamma_2 \) wildtype overexpressing transgenic mice (TGG2WT) and \( \gamma_2 \) mutant overexpressing transgenic mice (TGG2R302Q) displayed decreases in AMPK activity compared to non-transgenic (NTG) hearts, AMPK activity in TGG2R302Q hearts was also significantly decreased compared to TGG2WT hearts (Fig. 1A).

Consistent with the reductions in total AMPK activity, \( \alpha_1 \) and \( \alpha_2 \) activities were both reduced to approximately 50\% of NTG levels in TGG2WT hearts, while the activities of both isoforms in TGG2R302Q hearts were decreased to approximately 35\% (data not shown). These data indicate that there are no specific effects of the mutation on either one of the two catalytic isoforms of AMPK. In agreement with an overall decrease in AMPK activity, AMPK\( \alpha \) phosphorylation at Thr172, a surrogate marker of the in vivo activation status of AMPK, was significantly reduced in hearts from TGG2R302Q mice compared to NTG and TGG2WT (Fig. 1B).

Interestingly, the \( \gamma_2 \) subunit was more highly expressed in the TGG2WT mouse hearts as compared to the TGG2R302Q mouse hearts, while \( \gamma_2 \) was not detected in NTG hearts at this exposure (Fig. 1C). While the expression of all other subunits was unchanged (data not shown), expression of \( \beta_2 \) was also increased in both TGG2WT and TGG2R302Q mouse hearts as compared to NTG hearts (Fig. 1D).

In contrast to the findings in hearts from transgenic mice, acute adenoviral-mediated expression of the \( \gamma_2 \) mutant (AdG2R302Q) in isolated cardiomyocytes resulted in significantly...
higher AMPK activity (Fig. 1E), with a trend to increase phosphorylation of the AMPKα subunit (Fig. 1F) compared to control GFP-expressing cardiomyocytes (AdGFP). This increase in AMPK activity was also observed in hearts from 7-day old TGγ2R302Q mice (Fig. 1G). Consistent with activation of AMPK, phosphorylation of acetyl CoA carboxylase (ACC), a downstream target of AMPK, was also significantly increased in γ2R302Q expressing cardiomyocytes compared to control (1.63 ± 0.15 fold increase, p<0.05 vs. 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 γ2WT subunit was present at higher levels than the γ2R302Q subunit in adeno-virally transduced cardiomyocytes (Fig. 1H), while expression of the β2 subunit was increased in both groups compared to GFP control (Fig. 1I), suggesting that increased γ2 levels may have a specific effect on β2 expression and/or stability.

The development of cardiac hypertrophy. TGγ2R302Q mice exhibited significant cardiac hypertrophy as indicated by the increase in heart weight (HW) (Fig. 2A) compared to NTG and TGγ2WT mice without a significant change in body weight (BW) (34.2 ± 2.7 g, 27.6 ± 1.0 g, 35.2 ± 3.0 g for NTG, TGγ2WT and TGγ2R302Q, respectively, p=N.S.). Although many different myocardial cell types may contribute to increased HW/BW, we show a direct increase in cardiomyocyte size in TGγ2R302Q mice as compared to both NTG and TGγ2WT controls (Fig. 2B). Interestingly, TGγ2WT mice also developed a significant increase in HW and an increase in cardiomyocyte cell surface area compared to NTG controls (Fig. 2A and 2B, respectively), suggesting that expression of the non-mutated form of the γ2 subunit also has a modest effect on cardiomyocyte growth. As we have previously shown that AMPK is a negative regulator of hypertrophy29 and that inhibition of AMPK signaling may lead to a permissive environment for development of hypertrophy37, 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 was significantly decreased in the TGγ2R302Q hypertrophic hearts compared to NTG hearts (Fig. 2C and 2D, respectively), suggesting that protein synthesis is not increased in adult TGγ2WT or TGγ2R302Q mouse hearts. In addition, acute expression of either the γ2WT or the γ2R302Q subunit in isolated cardiomyocytes did not result in increased cardiomyocyte size (Fig. 2E), accelerated protein synthesis (basal or phenylephrine-stimulated; Fig. 2F), or stimulation of pro-hypertrophic growth pathways (Fig. 2G), suggesting that alterations in AMPK activity by these mutations do not promote cardiomyocyte cell growth via conventional mechanisms.

**Glycogen deposition**- Humans with the γ2R302Q mutation develop PRKAG2 syndrome, a characteristic of which is excessive glycogen accumulation within the cardiomyocytes. Previous studies have shown that large vacuoles detected with H&E staining of cardiomyocytes are representative of areas of glycogen deposition. Consistent with this study and positive PAS staining in TGγ2R302Q mouse hearts, large vacuoles and distended cardiomyocytes, indicative of glycogen accumulation, were evident in the atria of a patient with the γ2R302Q mutation (Fig. 3A) but were absent in the atria of a patient without this mutation (Fig. 3B). In agreement with these data and previous results, TGγ2R302Q mouse hearts displayed a significant 37-fold increase in glycogen compared to NTG hearts (Fig. 3C). In addition, TGγ2WT hearts had a 10-fold increase in glycogen compared to NTG hearts, but still had significantly lower glycogen levels than TGγ2R302Q hearts (Fig. 3C). In agreement with these findings, acute expression of either γ2WT or the γ2R302Q mutation in isolated cardiomyocytes also significantly increased glycogen content (70% and 270% vs. control, respectively; Fig. 3D), suggesting that cellular alterations induced by the expression of either the WT or the mutant form of the γ2 subunit can both promote glycogen accumulation.
**Glycogen turnover**- In order to determine the mechanism by which glycogen accumulation occurs, we examined the enzymes involved in glycogen synthesis and degradation. In TG\(_{\gamma_2}\)R302Q hearts, activity of glycogen synthase (GS), the rate-limiting enzyme in glycogen synthesis, was significantly decreased compared to NTG hearts (Fig. 4A). Consistent with this, inhibitory phosphorylation of GS was significantly increased compared to both NTG and TG\(_{\gamma_2}\)WT hearts (Fig. 4B and C) and GS protein levels were significantly reduced compared to TG\(_{\gamma_2}\)WT hearts (Fig. 4B and D). 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 TG\(_{\gamma_2}\)WT hearts were intermediate between NTG and TG\(_{\gamma_2}\)R302Q hearts (Fig. 4A and C, respectively). In addition to changes in glycogen synthase activity and phosphorylation, glycogen phosphorylase (GP) activity was slightly but significantly reduced in TG\(_{\gamma_2}\)R302Q hearts compared to NTG and TG\(_{\gamma_2}\)WT hearts, indicating a potential impairment in glycogen mobilization (Fig. 4E). Paradoxically, cardiomyocytes acutely expressing \(\gamma_2\)R302Q exhibited no change in GS activity (Fig. 4F) or phosphorylation (Fig. 4G and H), yet GS expression was significantly increased compared to controls (Fig. 4G and I). In addition, GP activity was unchanged in cardiomyocytes expressing either \(\gamma_2\)R302Q or \(\gamma_2\)WT compared to control (Fig. 4J). Although we do not provide evidence explaining why activities of GS and GP were unchanged in cardiomyocytes expressing either \(\gamma_2\)R302Q or \(\gamma_2\)WT, 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**- Since the vast 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 (personal correspondence, Dr. G.
Lopaschuk) or in the isolated cardiomyocytes expressing the \( \gamma_2 \)R302Q mutation (Fig. 5A).

Given the increase in glycogen levels and unchanged rates of glycolysis in the cardiomyocytes expressing the \( \gamma_2 \)R302Q mutation, the calculated glucose uptake in cells expressing \( \gamma_2 \)R302Q was 1.76-fold higher than controls (Fig. 5B). In addition, while activation of Akt was not changed (data not shown), phosphorylation of the Akt substrate of 160 kDa (AS160) was significantly increased in cardiomyocytes expressing \( \gamma_2 \)R302Q compared to control (Fig. 5C). Interestingly, total protein levels of AS160 were also increased in cardiomyocytes expressing \( \gamma_2 \)R302Q compared to controls (Fig. 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 sufficient to promote glucose uptake since AS160 plays an important role in GLUT4 translocation to the membrane and increased glucose transport\(^{39}\). Despite this rationale, there did not appear to be an increase in GLUT4 in the plasma membrane in \( \gamma_2 \)R302Q-expressing cardiomyocytes as compared to controls (data not shown). In contrast to these findings in cardiomyocytes acutely expressing \( \gamma_2 \)R302Q, Akt phosphorylation was decreased (Fig. 2C) and both P-AS160 as well as total AS160 expression were significantly reduced in the TG\( \gamma_2 \)R302Q hearts compared to NTG hearts (Fig. 5E and F, respectively), potentially as a chronic compensatory mechanism to prevent further glucose uptake and consequent glycogen accumulation.

**DISCUSSION**

In the present study we focused on the acute effects of expression of both \( \gamma_2 \)WT and \( \gamma_2 \)R302Q 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 \( \gamma_2 \)N488I mutation, hearts
from TG\(_{\gamma_2}\)R302Q mice display reduced AMPK activity as compared to hearts from wildtype mice, which was attributed to decreases in both AMPK \(\alpha_1\) and \(\alpha_2\) subunit activity. However, consistent with our hypothesis that acute expression of the \(\gamma_2\) mutant may have differential effects compared to chronic expression, acute expression of the \(\gamma_2\)R302Q 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 \(\gamma_2\)N488I 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 \(\gamma_2\)R302Q 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 \(\gamma_2\)R302Q mutation. Although we do not provide evidence for this, we propose that the profound glycogen deposition in hearts from TG\(_{\gamma_2}\)R302Q mice may be responsible for decreased myocardial AMPK activity in these mice as previously suggested\(^{25}\).

Although the \(\gamma_2\)R302Q mouse model used in this study differs from other mouse models expressing \(\gamma_2\) mutations in terms of AMPK activity\(^{22}\), there are still consistencies between the models. For example, the TG\(_{\gamma_2}\)R302Q mice also developed significant myocardial hypertrophy, which is in agreement with another transgenic mouse model expressing a mutated form of the \(\gamma_2\) protein\(^{22}\). However, given that isolated cardiomyocytes acutely expressing the \(\gamma_2\)R302Q 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 via non-conventional 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 TG\(_{\gamma_2}\)R302Q mice. However, consistent with glycogen accumulation inducing myocardial growth
via non-conventional means\textsuperscript{17}, hearts from TG\textsubscript{\(\gamma_2\)R302Q} mice displayed a significant 37-fold increase in glycogen compared to NTG hearts. Since humans possessing the \(\gamma_2\)R302Q 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\textsupscript{15}.

An interesting finding in this study was that expression of the \(\gamma_2\)WT protein also induced a cardiac phenotype in transgenic mice that was less severe than that observed in TG\textsubscript{\(\gamma_2\)R302Q} mice. Indeed, hearts from TG\textsubscript{\(\gamma_2\)WT} displayed a 50 % reduction in AMPK activity, an 84 % increase in heart weight and a 10-fold increase in glycogen compared to NTG mice, indicating that a component of the cardiac phenotype observed in the TG\textsubscript{\(\gamma_2\)R302Q} mice may be related to simple overexpression of the \(\gamma_2\) subunit. Although we cannot explain why expression of the \(\gamma_2\)WT subunit results in similar, albeit less dramatic changes compared to expression of the \(\gamma_2\)R302Q mutant, the intermediate phenotype may be due to the much higher expression level of the \(\gamma_2\) subunit in TG\textsubscript{\(\gamma_2\)WT} hearts. While it would be reasonable to assume that the elevated level of \(\gamma_2\)WT protein in the mouse heart is due to variations in genomic incorporation of the \(\gamma_2\)WT transgene compared to \(\gamma_2\)R302Q, this is likely not the case given that the same expression profile is observed with epichromosomal expression of the \(\gamma_2\) subunits using adenoviral delivery. Although these data suggest that the stability of the \(\gamma_2\) protein may be influenced by the R302Q mutation, we still cannot explain how expression of the \(\gamma_2\)WT 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 AMPK\textsubscript{\(\beta_2\)} subunit was also significantly increased in hearts and in cardiomyocytes expressing either the WT or the R302Q mutant forms of \(\gamma_2\). As previous work using AMPK \(\alpha_2^{-/-}\) mice demonstrate a
correlation between β2 expression (which possesses a glycogen binding domain) and glycogen levels, increased expression of the β2 subunit in the present study may contribute to the glycogen accumulation in hearts as well as in isolated cardiomyocytes.

To better characterize the mechanisms involved in glycogen accumulation in isolated cardiomyocytes and transgenic mice expressing γ2WT and the γ2R302Q mutation we first examined the glycogen synthesis pathway. While GS protein levels were increased in isolated cardiomyocytes acutely expressing γ2R302Q, GS protein content and activity were significantly reduced in TGγ2R302Q 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 TGγ2WT hearts. In fact, GS protein content was significantly increased compared to NTG, suggesting that a greater than 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 γ2R302Q mutation. Interestingly, cardiomyocytes expressing the γ2R302Q mutation also did not have increased GLUT4 protein in the plasma membrane nor were we able to detect increased glucose uptake compared to controls. Although we cannot explain this lack of effect, it may be due to 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, are increased in isolated cardiomyocytes acutely expressing γ2R302Q compared to control, suggesting that glucose uptake is likely elevated in these cells. As a result, we propose that acute expression of the γ2R302Q mutation results in an activation of the AMPK holoenzyme as well as 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.

Lastly, as our data have shown that AMPK activity is increased to a similar extent in isolated cardiomyocytes expressing either the $\gamma_2$R302Q mutation or $\gamma_2$WT, yet glycogen content is significantly higher in $\gamma_2$R302Q-expressing cells, it is tempting to speculate that glycogen levels and not alterations in AMPK activity per se may be responsible for the WPW phenotype. In fact, while the TG$\gamma_2$WT mice do display a less dramatic 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 pre-excitation. What appears to be a necessary component of WPW in the $\gamma_2$ mutation models is profound glycogen accumulation, independent of AMPK activity, as originally hypothesized$^{41}$. Indeed, despite variable effects of the three different mutations (i.e. $\gamma_2$R302Q, $\gamma_2$N488I, and $\gamma_2$R531G) 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 WPW as opposed to AMPK activity per se. In addition, the phenotype observed in the TG$\gamma_2$WT mice highlights the confounding factors associated with the existing transgenic mice expressing cardiomyocyte-restricted AMPK$\gamma_2$ 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 γ2R302Q mutation with respect to the effect that it has on AMPK activity. 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 and the potential cardiac side effects that may be associated with these agents.

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REFERENCES


FIGURE LEGENDS

Figure 1. AMPK activity is differentially affected by acute and chronic expression of γ2R302Q. Whole heart homogenates (n=6,7) from adult TGγ2WT and TGγ2R302Q mice display decreased AMPK activity (pmol/min/mg protein) compared to NTG mice (A) whereas AMPK activity is increased in cell lysates (n=4) from adenovirally transduced cardiomyocytes expressing γ2R302Q (Adγ2R302Q) (E) as well as in heart homogenates from 7-day old TGγ2R302Q mice (G). Phosphorylation of AMPKα(Thr172) is reduced in TGγ2R302Q heart homogenates (B) and unchanged in cell lysates from cardiomyocytes expressing γ2R302Q (F), as measured by immunoblot analysis using anti-phospho-AMPKα(Thr 172) and anti-total-AMPKα antibodies. Expression of γ2 is increased in whole heart homogenates (C) as well as in cell lysates from cardiomyocytes expressing γ2WT (H), while γ2 is increased in heart homogenates (D) and cell lysates from cardiomyocytes expressing either γ2R302Q or γ2WT (I), as measured using anti-AMPKγ2, anti-AMPKβ2 and anti-actin antibodies. ** p<0.01 vs. NTG or AdGFP; ** p<0.01 vs. NTG or AdGFP, ** p<0.01 vs. TGγ2WT or Adγ2WT, * p<0.05 vs. NTG or AdGFP, # p<0.05 vs. TGγ2WT or Adγ2WT.

Figure 2. Cardiac hypertrophy is induced in transgenic mice with chronic expression of γ2WT and γ2R302Q but is absent in isolated cardiomyocytes acutely expressing γ2WT and γ2R302Q. Heart weight (A) and ventricular myocyte cell surface area (99-125 cells/group isolated from 3 hearts in each group) (B) were increased in adult TGγ2WT and TGγ2R302Q mice compared to NTG mice. Isolated neonatal rat cardiomyocytes transduced with adenoviruses expressing GFP, γ2WT, or γ2R302Q (AdGFP, Adγ2WT, Adγ2R302Q, respectively) (238-255 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 TGγ2R302Q mice, as measured by anti-phospho-Akt(Ser473), anti-phospho-p70S6K(Thr389) and anti-actin antibodies (C and D). Basal or phenylephrine-induced protein synthesis was not affected by increased γ2WT or γ2R302Q expression in neonatal rat cardiomyocytes, as measured by [3H]Phenylalanine incorporation (F). Co-infection of neonatal rat cardiomyocytes with the NFAT-luciferase reporter gene (AdNFAT) and AdGFP, Adγ2WT, or Adγ2R302Q resulted in impaired NFAT transcriptional activity in cardiomyocytes expressing γ2WT or γ2R302Q (G).

*** p<0.001 vs. NTG or AdGFP; ### p<0.001 vs. TG

Figure 3. Glycogen levels are increased with acute and chronic expression of γ2WT and γ2R302Q. H&E staining of a human heart endomyocardial biopsy sample from a patient with the γ2R302Q mutation (A) shows evidence of glycogen accumulation (arrow points to a vacuolated myocyte indicative of glycogen deposition) compared to a control patient with no γ2R302Q mutation (B). Glycogen was measured from frozen powdered mouse heart ventricles from adult NTG, TGγ2WT and TGγ2R302Q mice and from AdGFP, Adγ2WT, and Adγ2R302Q infected cardiomyocytes. Myocardial glycogen was increased in TGγ2WT and TGγ2R302Q hearts (μmol glucosyl units/gram wet weight of tissue, n=3,4) and in Adγ2WT and Adγ2R302Q infected cardiomyocytes (standardized to AdGFP, n=7,8) (C and D). *** p<0.001 vs. NTG or AdGFP; ** p<0.01 vs. NTG or AdGFP, ## p<0.01 vs. TGγ2WT or Adγ2WT, * p<0.05 vs. NTG or AdGFP; # p<0.05 vs. TGγ2WT or Adγ2WT.

Figure 4. Glycogen synthase and glycogen phosphorylase are differentially regulated by acute and chronic expression of γ2R302Q. Glycogen synthase (GS) and glycogen phosphorylase (GP) activities, measured as percent of total activity using 15 mmol/l glucose-6-
phosphate or 200 mmol/L glycogen, respectively, were decreased in TGγ2R302Q hearts (A and E). GS protein expression was decreased (B, middle panel), while inhibitory phosphorylation was increased (B, upper panel) in TGγ2R302Q hearts, as measured using anti-phospho GS (Ser641/645), anti-GS and anti-actin 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, γ2WT or γ2R302Q. GS protein expression was increased in cell lysates from neonatal rat cardiomyocyte expressing γ2R302Q (G, middle panel), while GS phosphorylation was unchanged, (G, upper panel) as measured using anti-phospho GS (Ser641/645), anti-GS and anti-actin antibodies. Densitometry of phospho-GS/GS and GS/actin ratios are shown (H and I, respectively). * p<0.001 vs. TGγ2WT or Adγ2WT; * p<0.05 vs. NTG or AdGFP; # p<0.05 vs. TGγ2WT or Adγ2WT.

**Figure 5.** Mechanisms involved in the regulation of glucose uptake and metabolism are differentially regulated by acute and chronic expression of γ2R302Q. Glycolysis was unchanged in isolated neonatal rat cardiomyocytes expressing GFP, γ2WT or γ2R302Q (A). Glucose uptake (calculated based on numbers from glycolysis and glycogen) was increased in γ2R302Q-expressing cells (B). AS160 phosphorylation (C) and expression (D) were increased in cell lysates from γ2R302Q-expressing cardiomyocytes, as measured by anti-phospho ser/thr Akt substrate, anti-AS160 and anti-actin antibodies. AS160 phosphorylation (E) and expression (F) were decreased in TGγ2R302Q heart homogenates, as measured by anti-phospho ser/thr Akt substrate, anti-AS160 and anti-actin antibodies. * p<0.05 vs. NTG or AdGFP, ** p<0.01 vs. NTG or AdGFP.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Distinct early signaling events resulting from the expression of the PRKAG2 R302Q mutant of AMPK contribute to increased myocardial glycogen

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