Increased α2 Subunit–Associated AMPK Activity and PRKAG2 Cardiomyopathy

Ferhaan Ahmad, MD, PhD*; Michael Arad, MD*; Nicolas Musi, MD; Huamei He, MD, PhD; Cordula Wolf, MD; Dorothy Branco, BS; Antonio R. Perez-Atayde, MD; David Stapleton, PhD; Deeksha Bali, PhD; Yanqiu Xing, PhD; Rong Tian, MD, PhD; Laurie J. Goodyear, PhD; Charles I. Berul, MD; Joanne S. Ingwall, PhD; Christine E. Seidman, MD†; J.G. Seidman, PhD†

Background—AMP-activated protein kinase (AMPK) regulatory γ2 subunit (PRKAG2) mutations cause a human cardiomyopathy with cardiac hypertrophy, preexcitation, and glycogen deposition. PRKAG2 cardiomyopathy is recapitulated in transgenic mice overexpressing mutant PRKAG2 N488I in the heart (TGγ2N488I). AMPK is a heterotrimeric kinase consisting of 1 catalytic (α) and 2 regulatory (β and γ) subunits. Two α-subunit isoforms, α1 and α2, are expressed in the heart; however, the contribution of AMPK utilization of these subunits to PRKAG2 cardiomyopathy is unknown. Mice overexpressing a dominant-negative α2 subunit of AMPK (TGα2DN) provide a tool for selectively inhibiting α2, but not α1, subunit-associated AMPK activity.

Methods and Results—In compound-heterozygous TGγ2N488I/TGα2DN mice, AMPK activity associated with α2 but not α1 was decreased compared with TGγ2N488I. The TGα2DN transgene reduced the disease phenotype of TGγ2N488I, partially or completely normalizing the ECG, cardiac function, cardiac morphology, and exercise capacity in compound-heterozygous mice. TGγ2N488I hearts had normal resting levels of high-energy phosphates and could improve cardiac performance during exercise. Cardiac glycogen content decreased in TGγ2N488I mice after exercise stress, indicating availability of the stored glycogen for metabolic utilization. No differences in glycogen-metabolizing enzymes were observed.

Conclusions—The PRKAG2 N488I mutation causes inappropriate AMPK activation, which leads to glycogen accumulation and conduction system disease. The accumulated glycogen can serve as an energy source, and the animals have contractile reserve during exercise. Because the dominant-negative α2 subunit attenuates the mutant PRKAG2 phenotype, AMPK complexes containing the α2 rather than the α1 subunit are the primary mediators of the effects of PRKAG2 mutations. (Circulation. 2005;112:3140-3148.)

Key Words: cardiomyopathy ■ exercise ■ genetics ■ metabolism ■ Wolff-Parkinson-White syndrome

Mutations in AMP-activated protein kinase (AMPK) have been discovered to cause a glycogen storage cardiomyopathy that ultimately progresses to heart failure1–3 (for reviews, see Gollob4 and Gollob et al5). AMPK, a heterotrimeric protein composed of α, β, and γ subunits, is a metabolite-sensing protein kinase that is activated under conditions of energy depletion manifested by increased cellular AMP levels (reviewed in Hardie6). AMPK is able to phosphorylate >15 known target proteins and is thought to regulate energy metabolism by modulating a variety of metabolic activities, including glucose transport, stimulation of β-oxidation of fatty acids, inactivation of cholesterol synthesis, inhibition of creatine kinase, and transcriptional regulation of several genes.5–7 Mutations in the gene PRKAG2, encoding the γ2 subunit of AMPK, have been demonstrated to produce a distinct cardiomyopathy in

Clinical Perspective p 3148

Received March 25, 2005; revision received August 23, 2005; accepted September 12, 2005.
From the Department of Genetics (F.A., M.A., C.E.S., J.G.S.), Harvard Medical School and Howard Hughes Medical Institute, Boston, Mass; Cardiovascular Institute (F.A.), Department of Medicine, and Department of Human Genetics, University of Pittsburgh, Pittsburgh, Pa; Heart Institute (M.A.), Sheba Medical Center and Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel; Research Division (N.M., L.J.G.), Joslin Diabetes Center, Boston, Mass; NMR Laboratory for Physiological Chemistry (H.H., Y.X., R.T., J.S.I.), Cardiovascular Division, Department of Medicine, Brigham and Women’s Hospital, Boston, Mass; Department of Pathology (A.R.P.-A.), Children’s Hospital, Boston, Mass; Bio21 Molecular Science and Biotechnology Institute (D.S.), University of Melbourne, Melbourne, Australia; Pediatric Medical Genetics (D. Bali), Duke University Medical Center, Research Triangle Park, NC; and Cardiovascular Division (C.E.S.), Brigham and Women’s Hospital, Boston, Mass.
*The first 2 authors contributed equally to this work.
†The last 2 authors contributed equally to this work.
Guest Editor for this article was Roberto Bolli, MD.
Correspondence to J.G. Seidman, PhD. Department of Genetics, Harvard Medical School, 77 Ave Louis Pasteur, NRB 256, Boston, MA 02115. E-mail seidman@genetics.med.harvard.edu
© 2005 American Heart Association, Inc.

Circulation is available at http://www.circulationaha.org DOI: 10.1161/CIRCULATIONAHA.105.550806
hominan families characterized by ventricular hypertrophy, ventricular preexcitation, and progressive conduction system disease.\textsuperscript{1–3,8} Mutations recognized thus far are Exon5:InsLeu, His142Arg, Arg302Gln, Thr400Asn, Tyr487His, Asn488Ile, and Arg531Gly. The cardiomyopathy associated with \textit{PRKAG2} mutations is not associated with the myocyte and myofibrillar disarray and cardiac fibrosis characteristic of mutations in genes encoding sarcomeric proteins. Rather, cardiac hypertrophy results from formation within the myocytes of vacuoles filled with glycogen-associated granules.\textsuperscript{1,9} \textit{PRKAG2}-mediated cardiomyopathy, unlike other glycogen storage disorders, is characterized by cardiac glycogen accumulation in the absence of other remarkable clinical features, such as skeletal myopathy. The mechanisms by which these \textit{PRKAG2} missense mutations mediate cardiomyopathy and the accessibility of the accumulated glycogen as an energy source remain uncertain. Both cell and murine models of \textit{PRKAG2} cardiomyopathy have been described.\textsuperscript{1,9–11} Several in vitro studies suggest that \textit{PRKAG2} cardiomyopathy is caused by increased AMPK activity.\textsuperscript{1} Introduction of the Thr400Asn and Asn488Ile mutations into the yeast homolog of the \textit{PRKAG2} subunit, resulted in constitutive activation of the Snf1/Snf4 kinase.\textsuperscript{1} A similar Arg70Gln mutation in the \textit{y} subunit introduced into COS7 cells and pulmonary fibroblasts caused markedly increased AMPK activity, associated with increased phosphorylation of the \textit{y} subunit Thr172 and increased phosphorylation of 1 of its major substrates, acetyl coenzyme A carboxylase.\textsuperscript{5} However, introduction of several mutant \textit{PRKAG2s} into mammalian CCL13 cells caused either a decrease or no change in AMPK activity.\textsuperscript{10,11} Furthermore, recent studies by Sidhu and colleagues\textsuperscript{12} have suggested that the \textit{PRKAG2} Arg302Gln missense mutation inactivates AMPK and thereby causes glycogen accumulation and cardiac hypertrophy.

\textit{TG}\textsubscript{y}\textsubscript{N488I} mice provide a model of \textit{PRKAG2} cardiomyopathy by which they express a transgene encoding the Asn488Ile (N488I) mutation, which is expressed only in the heart under control of the \textit{\alpha}-myosin heavy-chain promoter.\textsuperscript{13} \textit{TG}\textsubscript{y}\textsubscript{N488I} hearts demonstrate elevated AMPK activity, massive glycogen deposition in cardiac myocytes up to 30-fold above normal, dramatic left ventricular (LV) hypertrophy, ventricular preexcitation, and sinus node dysfunction. Disruption of the annulus fibrosus by glycogen-filled myocytes is the anatomic substrate for preexcitation.\textsuperscript{13,14} \textit{TG}\textsubscript{y}\textsubscript{N488I} extracts have markedly elevated AMPK activity,\textsuperscript{13} and this increased activity is associated with both the \textit{\alpha}1 and \textit{\alpha}2 subunits.

\textit{TG}\textsubscript{y}\textsubscript{N488I} mice provide a unique tool for dissecting the mechanisms leading to \textit{PRKAG2} cardiomyopathy. We demonstrate, using a novel murine stress-echocardiography procedure, that although energy metabolism remains unchanged in the resting \textit{TG}\textsubscript{y}\textsubscript{N488I} heart, the accumulated glycogen in the \textit{TG}\textsubscript{y}\textsubscript{N488I} heart can be mobilized and metabolized during exercise. Furthermore, we use a genetic approach to confirm the model that \textit{PRKAG2} cardiomyopathy results from hyperactive AMPK, hypothesizing that if the \textit{PRKAG2} missense mutations function by inactivating AMPK, then dominant-negative \textit{\alpha}2-subunit mutations\textsuperscript{15} would not alter the phenotype of transgenic mice. Because the transgenic allele T\textit{G}\textsubscript{2}D\textsubscript{010}, which expresses a dominant-negative form of the AMPK \textit{\alpha}2 catalytic subunit, inactivates AMPK containing the \textit{\alpha}2 but not the \textit{\alpha}1 subunit,\textsuperscript{15} we are able to define the relative contributions of AMPK containing these \textit{\alpha} subunits. We conclude that \textit{PRKAG2} cardiomyopathy results from overactive AMPK associated primarily with the \textit{\alpha}2 rather than the \textit{\alpha}1 subunit.

\textbf{Methods}

\textbf{Mice}

Transgenic mice overexpressing human full-length, wild-type (\textit{TG}\textsubscript{2}D\textsubscript{010}) and N488I (\textit{TG}\textsubscript{y}\textsubscript{N488I}) \textit{PRKAG2} have been described.\textsuperscript{13} In brief, a T\textrightarrow{}A substitution was introduced at nucleotide 1553 in the human \textit{PRKAG2} cDNA to alter codon 488 from Asn\textright{}Ile, corresponding to a known human mutation. The transgene is expressed under control of the strong cardiac-specific \textit{\alpha}-myosin heavy-chain promoter. Transgenic mice overexpressing a dominant-negative \textit{\alpha}2 subunit of AMPK (T\textit{G}\textsubscript{2}D\textsubscript{010}) under control of the same \textit{\alpha}-myosin heavy-chain promoter were generated as previously reported.\textsuperscript{15} The Asp\rightarrow{}Ala substitution at codon 157 in rat \textit{PRKAA2} cDNA creates mutant \textit{\alpha}2 subunits, which bind to \textit{\alpha}2 subunits, producing enzymatically inactive complexes. The \textit{\alpha}1 subunit contains the YPYDVPDYA (HA, a synthetic peptide) tag. All mouse studies were performed in accordance with protocols approved by the institutional animal care and use committee at Harvard Medical School.

\textbf{AMPK Activity}

Resting mice were humanely killed by cervical dislocation without anesthesia, and the heart tissue was immediately frozen in LN\textsubscript{2}. Freeze-clamped heart samples were homogenized, and lysates were used for AMPK activity assays as described.\textsuperscript{16} Protein (200 \mu g) was immunoprecipitated with antibodies recognizing amino acids 339 to 358 of rat AMP \textit{\alpha}1 or 352 to 366 of \textit{\alpha}2. The kinase reaction was performed, in the presence of 0.2 mmol/L AMP, with synthetic substrate for AMP-activated protein kinase (SAMS) peptide as a substrate, and AMPK activity was measured as picomoles phosphate incorporated per milligram protein per minute.

\textbf{Glycogen Enzyme Activity Assays}

Hearts from wild-type, \textit{TG}\textsubscript{2}D\textsubscript{010}, and \textit{TG}\textsubscript{y}\textsubscript{N488I} mice were excised. Brancher enzyme or 1,4-\alpha-glucan branching enzyme [1,4-\alpha-D-glucan:1,4-\alpha-D-glucan 6-\alpha-D-(1,4-\alpha-D-glucan)transferase; EC 2.4.1.18] and glycogen phosphorylase (1,4-\alpha-glucan:phosphate \textit{\alpha}-D-glucosyltransferase; EC 2.4.1.38) were measured by free phosphate levels with inorganic phosphorus reagent (Roche Diagnostics).\textsuperscript{17,18} Debrancher enzyme or 4-\alpha-glucanotransferase (4-\alpha-D-glucanotransferase 1,4-\alpha-D-glucan:4-\alpha-D-glucosyltransferase; EC 2.4.1.28) and glycogen phosphorylase-\lambda (ATP:phosphorylase-\beta-phosphotransferase; EC 2.7.1.38) were measured by release of free glucose from Roche glucose reagent according to a standard protocol.\textsuperscript{17,19,20}

\textbf{Immunobots}

Antibodies recognizing \textit{\alpha}1, \textit{\alpha}2, and \gamma2 subunits of AMPK were produced as previously described.\textsuperscript{15,21,22} A lamin B antibody was used as a loading control (sc-6217, Santa Cruz).

\textbf{Energetics Assays}

Isolated, Langendorff-perfused hearts were freeze-clamped, and myocardial levels of adenine nucleotides and phosphocreatine were determined by a high-performance liquid chromatography method as reported previously.\textsuperscript{21,23} 1\textsuperscript{1}P nuclear magnetic resonance (NMR) spectra were obtained as previously described\textsuperscript{24} at 161.94 MHz with a GE-400 wide-bore spectrometer. Hearts were placed in a 10-mm
Cardiac Glycogen and Lactate Levels

Glycogen content was determined by perchloric acid extraction and amyloglucosidase digestion, followed by determination of glucose levels with a glucose oxidase kit (Sigma-Aldrich).25 Lactate levels were determined according to Sigma technical bulletin 826-UV with the use of NAD, glycine, and lactate dehydrogenase.

Exercise Stress Testing

To assess exercise capacity, mice were placed on a treadmill with an adjustable belt speed and incline and electric shock bars. Mice were subjected to an endurance protocol as previously described.26 Each mouse was encouraged to run to the limit of its capacity on this protocol, and total exercise duration was recorded.

Stress Echocardiography

The treadmill exercise protocol was modified to bring mice rapidly to maximum exercise capacity. Exercise was initiated at a treadmill incline of 15° and a speed of 10 m/min. The speed was increased by 2.5 m/min every 3 minutes until each mouse reached its maximum capacity and appeared exhausted. Mice were acclimated to the stress protocol by 1 practice exercise session 1 day before stress echocardiography. Echocardiography was performed with an Agilent Sonos 4500 ultrasound machine and a 6- to 15-MHz linear-array transducer as previously described,23 with the exception that no anesthesia was used and that immediate postexercise echocardiography was limited to M-mode views. LV end-diastolic and end-systolic diameters and wall thicknesses were obtained from M-mode tracings from measurements averaged from 3 separate cardiac cycles. LV fractional shortening (in percent) was derived from the equation fractional shortening = 100 × (LVIDd − LVIDs) / LVIDd, where LVIDd is LV internal dimension during diastole and LVIDs is LV internal dimension during systole.

Table 1. Characteristics of Wild-Type (WT), TGα2DN, TGγ2DNββ, and TGγ2DNββ/TGα2DN Mouse Hearts

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Wildtype</th>
<th>TG-γ2488I</th>
<th>TGα2DN</th>
<th>TGγ2DNββ</th>
<th>TG-γ2488I</th>
<th>TGα2DN</th>
<th>TG-γ2488I/TGα2DN</th>
<th>Pγ2488I vs WT</th>
<th>Pγ2488I/Tα2DN vs γ2488I</th>
<th>Pγ2488I/Tα2DN vs WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Lamin B**

Figure 1. Western blots of wild-type, TGα2DN, TGγ2DNββ, and TGγ2DNββ/TGα2DN cardiac extracts reacted with AMPK α1, α2, and γ2 subunit– and lamin B–specific antibodies. γ2-subunit polypeptide expression is dramatically increased in TGγ2DNββ hearts and also increased in mice expressing the α2DN allele (TGγ2DNββ/TGα2DN). TGα2DN and TGγ2DNββ/TGα2DN mice express the higher-molecular-weight α2 subunit encoded by the α2DN allele rather than the normal α2 allele expressed in wild-type and TGγ2DNββ hearts. α1-subunit levels are indistinguishable in all 4 mouse hearts. Anti-lamin B antibody was used to correct for differences in the amount of total protein extract. Abbreviations are as defined in text.
shortening=[(LV end-diastolic diameter−LV end-systolic diameter)/LV end-diastolic diameter]×100. Echocardiography was completed in unanesthetized animals within 5 minutes before exercise and within 30 seconds after peak exercise.

**Static and Continuous Ambulatory (Holter) ECG**
ECG was performed as previously described.14,27 Limb-lead ECGs were recorded to detect evidence of ventricular preexcitation, namely, shortened PR intervals and delta waves. In a separate set of experiments, a telemetry device was implanted to allow continuous ambulatory ECG (Holter monitoring) and assessment of the chronotropic response to exercise. Mice were exercised to maximum capacity according to the exercise stress testing protocol described for stress echocardiography and were monitored for another 10 minutes during recovery.

**Data Analysis**
All data are presented as mean±SD, except as noted in the figure legends. Comparisons between groups were made with Student’s t test for pairwise comparisons and ANOVA for multiple comparisons.

**Results**

**Inhibiting AMPK in TGγ2<sup>N488I</sup> Mice With α2 Dominant-Negative Transgene**
We have previously presented evidence suggesting that the N488I mutation in the γ2 subunit results in increased AMPK activity.1,13 Xing et al15 have described a transgenic mouse, designated TGα2<sup>DN</sup>, that expresses a dominant-negative form of the α2 subunit of AMPK. TGα2<sup>DN</sup> mice have significantly reduced α2 activity but normal α1 activity.15 By mating TGα2<sup>DN</sup> mice with TGγ2<sup>N488I</sup> transgenic mice, mice carrying both the TGγ2<sup>N488I</sup> transgene and the TGα2<sup>DN</sup> transgene,15 designated TGγ2<sup>N488I/TGα2<sup>DN</sup></sup>, were obtained. Levels of TGγ2<sup>N488I</sup> polypeptide were the same in TGγ2<sup>N488I</sup> and TGγ2<sup>N488I/TGα2<sup>DN</sup></sup> hearts (Figure 1C). However, the nonfunctional TGα2<sup>DN</sup> peptide, which migrates as a higher-molecular-weight polypeptide owing to its HA tag, completely replaced the lower-molecular-weight, endogenous, wild-type α2 subunit (Figure 1B). The α1-subunit levels in
Wild-type, TG\(\gamma_{2}^{N488I}\), TG\(\alpha_{2}^{DN}\), and TG\(\gamma_{2}^{N488I}/\alpha_{2}^{DN}\) hearts were not significantly different (Figure 1A), as suggested previously.\(^{15}\)

We assessed the amount of AMPK activity associated with the \(\alpha_{1}\) and \(\alpha_{2}\) subunits in 18-day-old transgenic mice (Table 1). TG\(\gamma_{2}^{N488I}/\alpha_{2}^{DN}\) mice demonstrated a significant reduction in AMP-stimulated, \(\alpha_{2}\) subunit-associated AMPK activity, to \(\approx 37\%\) of that seen in TG\(\gamma_{2}^{N488I}\) mice (Table 1) but no significant reduction in \(\alpha_{1}\)-associated AMPK activity compared with TG\(\gamma_{2}^{N488I}\) mice. Consequently, we proceeded to phenotypic characterization of these mice to define the roles of the \(\alpha_{1}\) and \(\alpha_{2}\) subunit–associated AMPK activities.

Cardiac function and morphology were assessed in TG\(\gamma_{2}^{N488I}\), TG\(\alpha_{2}^{DN}\), and TG\(\gamma_{2}^{N488I}/\alpha_{2}^{DN}\) mice. At age 5 weeks, heart-body mass ratios were indistinguishable between wild-type and TG\(\alpha_{2}^{DN}\) mice, whereas TG\(\gamma_{2}^{N488I}\) mice demonstrated massive cardiac hypertrophy (Table 1). In contrast, age-matched TG\(\gamma_{2}^{N488I}/\alpha_{2}^{DN}\) mice had near-normal heart-body mass ratios (Table 1). The heart-body mass ratio in TG\(\gamma_{2}^{N488I}/\alpha_{2}^{DN}\) mice remained mildly elevated, at a level similar to that found in TG\(\gamma_{2}^{DN}\) mice (6.9 \pm 1.0 versus 7.2 \pm 0.3).\(^{13}\) Echocardiographic studies performed in 8-week-old animals demonstrated a similar significant reduction in LV wall thickness in TG\(\gamma_{2}^{N488I}/\alpha_{2}^{DN}\) hearts compared with TG\(\gamma_{2}^{N488I}\) hearts (Table 1; 1.17 versus 1.54 mm; \(P = 0.01\)). These differences persisted at age 20 weeks (Table 1).

The reduction in cardiac hypertrophy in TG\(\gamma_{2}^{N488I}/\alpha_{2}^{DN}\) mice was accompanied by an 8-fold decrease in glycogen content compared with TG\(\gamma_{2}^{N488I}\) hearts (Table 1). Both light and electron microscopy confirmed the disappearance of glycogen-laden vacuoles, the pathological hallmark of PRKAG2 cardiomyopathy (Figure 2).

TG\(\gamma_{2}^{N488I}\) mice have ventricular preexcitation, manifested by a short PR interval and delta waves on ECG.\(^{13,14}\) The PR interval was 24% longer in TG\(\gamma_{2}^{N488I}/\alpha_{2}^{DN}\) mice relative to TG\(\gamma_{2}^{N488I}\) mice (\(P = 0.02\)), representing normal atrioventricular conduction without ventricular preexcitation (Figure 3 and Table 1). As we previously reported, TG\(\gamma_{2}^{N488I}\) mice develop LV dilatation and systolic dysfunction, phenomena not observed in wild-type and TG\(\gamma_{2}^{DN}\) mice.\(^{13}\) However, TG\(\gamma_{2}^{N488I}/\alpha_{2}^{DN}\) mice demonstrated completely normal LV end-diastolic diameters and fractional shortening at 20 weeks of age (Table 1).

The maximal exercise capacity of wild-type, TG\(\gamma_{2}^{DN}\), TG\(\gamma_{2}^{N488I}\), and TG\(\gamma_{2}^{N488I}/\alpha_{2}^{DN}\) mice was assessed to determine the consequences of cardiac morphological and metabolic changes. Wild-type, TG\(\gamma_{2}^{DN}\), TG\(\alpha_{2}^{DN}\), and TG\(\gamma_{2}^{N488I}/\alpha_{2}^{DN}\) mice demonstrated similar exercise capacities (Figure 4A and data not shown). However, TG\(\gamma_{2}^{N488I}\) mice demonstrated an exercise capacity approximately half that of wild-type mice (32.4 \pm 3.6 versus 58.1 \pm 6.0 minutes, \(P = 0.001\); Figure 4A). This exercise deficit was completely eliminated in double-transgenic TG\(\gamma_{2}^{N488I}/\alpha_{2}^{DN}\) mice.

**Energy Reserve in TG\(\gamma_{2}^{N488I}\) Mice**

To determine the physiological basis underlying impaired exercise capacity in TG\(\gamma_{2}^{N488I}\) mice, we measured resting cardiac levels of phosphocreatine by NMR and of ATP, ADP, and AMP by high-performance liquid chromatography and assessed chronotropic and contractile responses to exercise. Because defects in glycogen metabolism, as well as cardiomyopathies secondary to mutations in genes encoding sarcromeric proteins, have been found to exhibit altered energetics,\(^{24,28,29}\) we hypothesized that TG\(\gamma_{2}^{N488I}\) mice might have abnormal energy stores. However, we detected no significant differences in phosphocreatine, ATP, ADP, and AMP levels among wild-type, TG\(\gamma_{2}^{DN}\), and TG\(\gamma_{2}^{N488I}\) in isolated, perfused hearts from 5-week-old mice (Table 2).

On stress echocardiography, TG\(\gamma_{2}^{N488I}\) mice had lower baseline cardiac contractility (Table 3). However, TG\(\gamma_{2}^{DN}\) mice had an enhanced inotropic response to exercise, increased fractional shortening, and decreased LV end-systolic dimension, thereby suggesting adequate contractile reserve. Contrasted with wild-type mice, TG\(\gamma_{2}^{N488I}\) mice demonstrated significantly reduced heart rates at baseline, during exercise, and during
were humanely killed within 10 seconds after completion of 
from resting and exercised mice (Table 4). Exercised mice 
shown.

<table>
<thead>
<tr>
<th>Exercise Duration (min)</th>
<th>WT</th>
<th>TGwt</th>
<th>TG2N488I</th>
<th>TG2N488I/TGα2DN</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>20</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>30</td>
<td>70</td>
<td>70</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>40</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>60</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>70</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>80</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>90</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

**Figure 4.** A, Adult wild-type, TG2wt, TG2N488I, and TG2N488I/TGα2DN mice were exercised on a treadmill with stepwise increments in incline and speed. Maximum exercise duration for each individual mouse and the mean±SD for each group are shown. P vs wild type, 0.001 for TG2N488I and 0.302 for TG2N488I/TGα2DN mice. B, Adult wild-type (●, n=6) and TG2N488I (●, n=6) mice were implanted with ambulatory ECG telemetry devices and exercised. Heart rate responses at baseline, at each stage of exercise, and after recovery (minutes from the end of exercise) are shown as mean±SE. P=0.001 for differences between wild-type and TG2N488I mice. Abbreviations are as defined in text.

recovery (Figure 4B; P=0.004). Heart rates of wild-type, 
TG2wt, TGα2DN, and TG2N488I/TGα2DN mice were not signifi- 
cantly different (data not shown). Therefore, decreased exercise 
performance in TG2N488I mice appears to be attributable to the 
impairment in cardiac contractility and lower heart rates at 
baseline and during exercise, but not to defective energetics.

To determine whether the increased glycogen in the car- 
diac myocytes of TG2N488I mice could be metabolized, we 
measured cardiac tissue glycogen and lactate levels in hearts 
from resting and exercised mice (Table 4). Exercised mice 
were humanely killed within 10 minutes after completion of 
maximal exercise. Wild-type and TG2wt mice demonstrated 
significant (at least 40%) decreases in glycogen levels with 
exercise. Glycogen levels decreased by ≈25% in TG2N488I 
hearts, which represented a very large absolute quantity of 
glycogen. Concomitantly, more lactate was produced, consis- 
tent with the model that stored glycogen was utilized as an 
energy source during exercise.

**Glycogen Metabolism in TG2N488I Hearts**

In an attempt to define the mechanism leading to glycogen 
accumulation in TG2N488I hearts, we assayed the activity of 
4 enzymes involved in either glycogen synthesis or glyco- 
genolysis, which are known to cause human glycogen storage 
cardiomyopathies when defective. No significant differences 
were detected in the activities of branching enzyme, de- 
brancher enzyme, glycogen phosphorylase, and glycogenphosphorylase kinase in 5-week-old TG2N488I and wild-type 
hearts (Table 2).

**Discussion**

TG2N488I mice provide a useful tool for gaining further 
insights into the mechanisms by which mutations in the γ2 
subunit of AMPK cause cardiac glycogen accumulation, 
preexcitation, cardiomyopathy, and heart failure. Here we 
provide evidence that (1) PRKAG2 cardiomyopathy results 
from activation of AMPK, which is mediated primarily via 
α2 subunit-associated AMPK, and (2) there is no cardiac 
energy depletion in PRKAG2 cardiomyopathy at rest, and the 
presence of contractile reserve in response to exercise chal- 
lenge is observed on stress exercise echocardiography. 
Despite the pivotal role of AMPK in maintaining energy 
metabolism, inappropriate activation of AMPK has minimal 
effects on resting cardiac high-energy phosphate levels.

Murine models of hypertrophic cardiomyopathy resulting 
from mutations in genes encoding sarcomeric proteins have 
demonstrated impaired cellular energetics. In contrast, 
TG2N488I mice exhibit normal resting cardiac cellular ener- 
getics (Table 2). Thus, although AMPK is a central regulator 
of cellular energy levels, the N488I mutation does not perturb 
resting cellular energy levels in the heart. Presumably, ho- 
meostatic mechanisms preserve normal energetics, at least in 
the absence of stress. The functional impairment observed in 
these mice is likely the result of glycogen deposition in the 
heart rather than energy depletion. The normal resting cellular 
energetics observed in TG2N488I mice also underscores the 
fact that the mechanism of cardiac hypertrophy and cardio- 
mypathy resulting from this mutation is distinct from the 
hypertrophic cardiomyopathy caused by sarcomere protein 
gene mutations.

Stress echocardiography allows direct assessment of car- 
diatic function in response to exercise challenge and is a 
standard technique for evaluating myocardial ischemia in 
humans with coronary heart disease. Although this test has 
been found to have prognostic value in patients with hyper- 
trrophic or dilated cardiomyopathy, with impaired contractile 
response to stress connoting a poorer prognosis, few if any 
patients with PRKAG2 missense mutations have undergone 
this test. To evaluate further cardiac energetics during exer- 
cise in TG2N488I mice, we developed a stress echocardiogra- 
phy protocol for assessing cardiac function in mice. Stressed 
TG2N488I mice demonstrated marked improvement in cardiac 
function, as fractional shortening increased from 31% to 46% 
(P<0.0001; Table 3). The improved cardiac function of 
exercised TG2N488I mice may have resulted from any 1 of
several mechanisms, including increased glycogen utilization. In contrast, fractional shortening was maximal at rest in wild-type and TGγ2N488I mice and did not improve further with exercise.

Hypothesizing that improved cardiac function could result from increased glycogen utilization, we investigated whether the excess stored glycogen in TGγ2N488I mice could be mobilized during exercise. TGγ2N488I mice demonstrated a decrease in cardiac glycogen levels with exercise, indicating that the excess glycogen in their hearts could be mobilized and metabolized (Table 4). TGγ2N488I mice also demonstrated an increase in cardiac lactate, presumably from increased glycogen metabolism. The large decrease in glycogen levels was even more remarkable, considering that TGγ2N488I mice have a markedly reduced exercise tolerance. These results raise the possibility that mobilization of cardiac glycogen allows improved cardiac function.

We and others have previously suggested that PRKAG2 cardiomyopathy results from inappropriate activation of AMPK.1,9,13 However, other investigators have proposed other models, including a biphasic response manifested by increased resting activity but impaired responsiveness to metabolic stress.10–12,31 We have used a genetic approach to determine whether the principal effect of the N488I mutation is to increase or decrease AMPK activity. If the phenotypic effects of this mutation result from an increase in AMPK α2-subunit activity, then they should be attenuated or abolished in the presence of a dominant-negative AMPK α2 subunit. The features of double-transgenic mice carrying both mutations (TGγ2N488I/TGα2ΔDN) are consistent with the model that PRKAG2 with the N488I mutation inappropriately activates the AMPK α2 subunit. In TGγ2N488I/TGα2ΔDN mice, normal catalytic α2 subunits were replaced by an inactive form of the α2 subunit (Figure 1). As a result, TGγ2N488I/TGα2ΔDN mice had a significant reduction in α2-, but not α1-, subunit activity, and exhibited normal or near-normal cardiac function, cardiac morphology, and conduction system function (Table 1 and Figures 2 through 4). Hence, reducing AMPK-associated α2-subunit activity restores cardiac physiology to nearly normal, consistent with the model that in the presence of the N488I γ2 subunit, the AMPK α2 subunit is inappropriately active. Moreover, despite the 2-fold increase in α1 subunit–associated AMPK activity observed in TGγ2N488I mice,13 cardiac hypertrophy was almost completely

### Table 2. High-Energy Phosphate Levels and Glycogen Metabolic Enzyme Activity in Wild-Type (WT), TGγ2WT, and TGγ2N488I Mice

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>WT (mmol/L (n))</th>
<th>TGγ2WT (mmol/L (n))</th>
<th>TGγ2N488I (mmol/L (n))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphocreatine, mmol/L</td>
<td>18.6±1.3 (11)</td>
<td>19.6±1.9 (9)</td>
<td>21.8±2.2 (10)</td>
</tr>
<tr>
<td>ATP, mmol/L</td>
<td>11.0±0.5 (4)</td>
<td>10.4±0.2 (4)</td>
<td>11.2±0.2 (5)</td>
</tr>
<tr>
<td>ADP, mmol/L</td>
<td>1.6±0.2 (4)</td>
<td>1.7±0.3 (4)</td>
<td>2.0±0.2 (5)</td>
</tr>
<tr>
<td>AMP, mmol/L</td>
<td>0.26±0.11 (4)</td>
<td>0.24±0.07 (4)</td>
<td>0.22±0.04 (5)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glycogen metabolic enzymes, μmol/g tissue per min</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Branching enzyme</td>
<td>22±7 (2)</td>
<td>33±14 (2)</td>
<td>31±8 (2)</td>
</tr>
<tr>
<td>Debranching enzyme</td>
<td>0.36±0.06 (2)</td>
<td>0.32±0.06 (2)</td>
<td>0.29±0.01 (2)</td>
</tr>
<tr>
<td>Glycogen phosphorylase</td>
<td>9.3±0.3 (2)</td>
<td>9.3±2.2 (2)</td>
<td>8.3±3.3 (2)</td>
</tr>
<tr>
<td>Glycogen phosphorylase kinase</td>
<td>0.13±0.02 (2)</td>
<td>0.09±0.00 (2)</td>
<td>0.13±0.04 (2)</td>
</tr>
</tbody>
</table>

n indicates No. of mice used in each assay. No differences were found in phosphocreatine as measured by 31P NMR; in ATP, ADP, and AMP levels as measured by high-performance liquid chromatography; or in activities of 4 enzymes involved in glycogen synthesis or degradation. Enzyme activities are reported as P>0.05 for all comparisons between WT, γ2WT, and γ2N488I mice.

### Table 3. Stress Echocardiography in 8-Week-Old Wild-Type (WT), TGγ2WT, and TGγ2N488I Mice

<table>
<thead>
<tr>
<th>Measure</th>
<th>WT</th>
<th>TGγ2WT</th>
<th>TGγ2N488I</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of mice</td>
<td>7</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>LVEDD before exercise, mm</td>
<td>2.31±0.35</td>
<td>2.69±0.12</td>
<td>3.81±0.75</td>
</tr>
<tr>
<td>LVEDD after exercise, mm</td>
<td>2.25±0.26</td>
<td>2.31±0.20</td>
<td>3.51±0.81</td>
</tr>
<tr>
<td>P LVEDD, before vs after exercise</td>
<td>0.6</td>
<td>0.06</td>
<td>0.05</td>
</tr>
<tr>
<td>Fractional shortening before exercise, %</td>
<td>75±3</td>
<td>73±9</td>
<td>31±15</td>
</tr>
<tr>
<td>Fractional shortening after exercise, %</td>
<td>71±7</td>
<td>72±3</td>
<td>46±10</td>
</tr>
<tr>
<td>P fractional shortening, before vs after exercise</td>
<td>0.1</td>
<td>0.7</td>
<td>0.0009</td>
</tr>
<tr>
<td>Heart rate before exercise, beats/min</td>
<td>683±32</td>
<td>651±36</td>
<td>602±76</td>
</tr>
<tr>
<td>Heart rate after exercise, beats/min</td>
<td>606±26</td>
<td>680±75</td>
<td>549±81</td>
</tr>
<tr>
<td>P Heart rate, before vs after exercise</td>
<td>0.008</td>
<td>0.5</td>
<td>0.1</td>
</tr>
</tbody>
</table>

LVEDD indicates end-diastolic diameter. Other abbreviations are as defined in ext. TGγ2N488I mice were able to improve chamber size and contractility, as assessed by fractional shortening, during exercise.
reversed in the presence of a dominant-negative α2 subunit, indicating that hypertrophy is mediated largely through the effects of mutations in α2-subunit activity. Although the mechanism is uncertain, these data suggest that in vivo α2-associated AMPK and α1-associated AMPK have different biological targets. This target specificity may be determined directly by the α subunit or by a preference of the α subunit for a particular β or γ isoform, which may be responsible for target specificity.

Although inappropriately active α2 subunit–associated AMPK activity is responsible for the phenotype of this cardiomyopathy, the mechanism of glycogen accumulation is still unclear. The activities of branching enzyme, debranching enzyme, glycogen phosphorylase, and glycogen phosphorylase kinase are unchanged in adult TGγ2N488I hearts (Table 2). These enzymes are involved in the synthesis or degradation of glycogen, and deficiencies in each of these enzymes cause glycogen storage disease. However, regulation of 1 or more glycogen metabolic enzyme activities must be altered in PRKAG2 cardiomyopathy. PRKAG2 cardiomyopathy in TGγ2N488I mice provides a unique opportunity to investigate the role of AMPK and glycogen in regulating cardiac energy metabolism. Eventually, cardiac AMPK targets and the mechanisms leading from AMPK activation to glycogen storage will be defined. Identification of these targets, coupled with an understanding of the physiological pathways by which glycogen accumulates in cardiac myocytes, will provide novel therapeutic targets for a variety of conditions associated with defective cardiac energetics.

Acknowledgments

This work was supported by the Howard Hughes Medical Institute (F.A., M.A., C.E.S., J.G.S.), the Canadian Institutes of Health Research (F.A.), the National Institutes of Health (HL 67970 to R.T. and HL 52320 and HL 63985 to J.S.I.), the American Heart Association (R.T.), and the American Diabetes Association (N.M., L.G.).

References


**CLINICAL PERSPECTIVE**

We demonstrate that PRKAG2 cardiomyopathy results from activated AMP-dependent protein kinase (AMPK) and that most of the increased AMPK activity is mediated by α2-subunit associated AMPK. Transgenic mice over-expressing mutant γ2 AMPK subunit (PRKAG2) N488I (TGγ2N488I) mimic human PRKAG2 cardiomyopathy characterized by cardiac hypertrophy, pre-excitation and glycogen accumulation. Normally, most cardiac AMPK activity is associated with the α1 subunit, however in TGγ2N488I hearts approximately 50% of AMPK activity is associated with α1-subunit containing AMPK and 50% with α2-subunit AMPK. To determine which AMPK isoform is responsible for the cardiac phenotypes we selectively inhibited α2-, but not α1-, subunit associated AMPK activity by introducing a dominant negative form of α2 subunit into TGγ2N488I hearts. PRKAG2 cardiomyopathy was markedly attenuated by dominant negative α2 subunit, concomitant with a significant decrease in α2-associated AMPK activity, confirming that inappropriate AMPK activation causes disease. Further, glycogen stores found in PRKAG2N488I hearts were not associated with energy deficiency and in fact represent a dynamic pool of glycogen, which can be mobilized during metabolic stress. These results suggest that: (1) pharmacological inhibition of α2 associated AMPK should prevent PRKAG2 cardiomyopathy, and (2) increased AMPK activity may increase cardiac tolerance to metabolic stress.
Increased α2 Subunit–Associated AMPK Activity and PRKAG2 Cardiomyopathy
Ferhaan Ahmad, Michael Arad, Nicolas Musi, Huamei He, Cordula Wolf, Dorothy Branco, Antonio R. Perez-Atayde, David Stapleton, Deeksha Bali, Yanqiu Xing, Rong Tian, Laurie J. Goodyear, Charles I. Berul, Joanne S. Ingwall, Christine E. Seidman and J.G. Seidman

*Circulation.* 2005;112:3140-3148; originally published online November 7, 2005; doi: 10.1161/CIRCULATIONAHA.105.550806
*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/112/20/3140

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation* is online at:
http://circ.ahajournals.org/subscriptions/