N488I Mutation of the γ2-Subunit Results in Bidirectional Changes in AMP-Activated Protein Kinase Activity

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Abstract—Mutations in the human gene encoding the nucleotide-binding region in the γ2-subunit of AMP-activated protein kinase (AMPK) cause cardiomyopathy with preexcitation syndrome. Mutant AMPK showed reduced binding affinity to nucleotides in vitro raising the possibility that altered regulation of AMPK activity by AMP/ATP could contribute to the disease phenotype. In this study, we determined the sensitivity of AMPK activity to AMP/ATP in the beating hearts using transgenic mice expressing a mutant (N488I, γ2-mutant) or wild-type γ2-subunit (γ2-TG). The [ATP] and [AMP] were unaltered in all hearts but the AMPK activity was increased by 2.5-fold in γ2-mutant hearts freeze-clamped at normal AMP/ATP compared with nontransgenic (WT) or γ2-TG. The increased basal AMPK activity was caused by increased Thr-172 phosphorylation of the α-subunit (p-AMPK, by 4-fold) at normal [ATP] and was not changed by reducing glycogen content by 60% in the γ2-mutant hearts. A reversal of AMP/ATP, caused by ATP degradation, increased p-AMPK by 7-fold in WT but caused no change in γ2-mutant hearts. These results demonstrate that the mutation renders AMPK insensitive to the inhibitory and stimulatory effects of the regulatory nucleotides ATP and AMP, respectively, suggesting that the pathogenesis of the human disease may not be attributable to a simple loss- or gain-of-function. (Circ Res. 2005;97:323-328.)

Key Words: AMP-activated protein kinase  ■ heart  ■ magnetic resonance spectroscopy  ■ mouse mutant

Mutations in the human gene encoding the γ2 subunit of the AMP-activated protein kinase (AMPK) cause ventricular preexcitation (Wolff-Parkinson-White syndrome) and cardiac hypertrophy.1–3 Mechanisms by which these mutations cause the cardiac disease phenotype are unclear. AMPK is a heterotrimeric protein consisting of a catalytic subunit (α) and 2 regulatory subunits (β and γ). The activity of AMPK is regulated by the ratio of cellular AMP and ATP, which renders this kinase an intracellular energy sensor.4,5 Regulation of AMPK activity involves dual mechanisms: (1) The binding of AMP or ATP allosterically regulates kinase activity; and (2), AMP/ATP modulates the phosphorylation of the α-subunit.1–6 For both mechanisms, high [AMP] stimulates AMPK activity whereas high [ATP] inhibits the activity. A recent study has demonstrated that the nucleotide-binding module of the AMPK is located on the γ subunit, consisting of 2 pairs of cystathionine β-synthase sequences, and each pair of the cystathionine β-synthase domain binds 1 molecule of AMP or ATP in a mutually exclusive mode.7 The AMPK protein shows a significantly higher binding affinity (roughly 4-fold) for AMP than for ATP in vitro,7 consistent with the observation that AMPK is sensitive to changes in cellular [AMP]. Because the in vivo [ATP] is in several orders of magnitude higher than free [AMP], the very low AMP/ATP allows the kinase to remain in largely inactive state under normal conditions.8 The higher affinity for AMP, however, allows a robust response when a small change occurs in AMP/ATP making the AMPK signaling a highly efficient stress response pathway.

AMPK bearing the human mutations of the γ2-subunit demonstrate a decreased binding affinity to AMP and ATP and reduced activation by AMP in vitro.7 These findings suggest that mutations in the γ2-subunit disrupt the allosteric regulation of AMPK activity thus raising the possibility that mutations on the γ2-subunit cause cardiac disease by altering the regulation of AMPK activity.8,9 Interestingly, recent studies using transgenic mice overexpressing the γ2-subunit bearing 2 of the human mutations in the heart showed consistent disease phenotype but contradictory changes in AMPK activity in excised cardiac tissue.10,11 These findings prompted us to determine the effects of γ2-mutation on the regulation of AMPK activity in the beating heart where the relative concentrations of ATP and AMP are substantially different than the in vitro assay conditions. Furthermore, we also determined the relationship between AMP/ATP and the phosphorylation of α-AMPK by the upstream kinase, a...
predominant mechanism for AMPK activation, which has not been examined for the mutant AMPK in the intact heart. We found that the N488I mutation of the γ2-subunit resulted in increased phosphorylation of Thr172 on the α subunit and increased kinase activity under normal conditions whereas the response to ATP depletion was abrogated. Thus, the change in AMPK activity caused by the mutation on the γ subunit is likely dependent on the relative concentrations of AMP and ATP in the heart that regulates the phosphorylation status of the kinase.

Materials and Methods

Animal Model

Transgenic mice with cardiac-specific overexpression of a mutant human γ2 AMPK subunit (N488I) were used for the study (γ2-mutant). As previously reported, this mutation was identified in a patient family with preecitration syndrome and cardiac hypertrophy, and the transgenic-mouse model successfully recapitulated the human disease phenotype.3,10 All mice were studied at the age of 8 to 10 weeks. Because the overexpression of the mutant γ2-subunit was expected to displace the native γ1-subunit, transgenic mice overexpressing the wild-type human γ2-subunit (γ2-TG) were generated as controls in addition to the nontransgenic mice (WT). As shown in Figure 1, γ1-subunit was completely replaced by the overexpression of the γ2-subunit in both γ2-mutant and γ2-TG hearts. Thus, γ2-TG group served as the control in this study for the isoform change in the γ-subunit of AMPK.

Isolated Heart Perfusion and 31P NMR Spectroscopy

Mice were heparinized and anesthetized with sodium pentobarbital (100 mg/kg i.p.) and hearts were quickly removed. Hearts were perfused in the Langendorff mode with phosphate-free Krebs-Henseleit buffer containing (in mmol/L) NaCl 118, NaHCO3, 25, KCl 5.3, CaCl2 2.5, MgSO4 1.2, EDTA 0.5, glucose 5, and pyruvate 5 at 37°C as previously described.12 All hearts were stabilized for 25 minutes at a constant perfusion pressure of 80 mm Hg. A baseline 31P NMR spectrum (208 scans) was collected for all hearts after stabilization.

In one protocol, hearts were freeze-clamped after the baseline NMR measurement and the tissue were immediately processed for biochemical assays. In another protocol, no-flow global ischemia was applied to the heart to deplete two-thirds of the ATP content, monitored by 31P NMR spectroscopy. This stress had been shown to result in near maximal activation of AMPK in perfused hearts.13 The myocardial ATP content was measured by HPLC using cardiac tissue freeze-clamped at baseline perfusion. The HPLC results were converted to [ATP] by assuming an intracellular water content of 0.48 mL/g and a protein content of 0.15 g/g of blotted wet tissue.14 The average baseline [ATP] thus obtained was used to calibrate the ATP peak area of the baseline 31P NMR spectra for each group. Concentrations of other metabolites were calculated using the ratio of their peak areas to the ATP peak area.15 Intracellular free [AMP] was calculated using the equilibrium expressions of the creatine kinase and adenylate kinase reactions as previously described.6,16 The total AMP content (free and bound) was measured by HPLC in hearts freeze-clamped at baseline and after ischemia.

Myocardial glycogen content was determined by measuring the amount of glucose released from glycogen with a Sigma assay kit. An alkaline extraction procedure was used to separate glycogen and exogenous glucose in the tissue.15

Data Analysis and Statistics

All data are presented as the mean±SEM. One-way ANOVA was performed for multiple group comparisons and unpaired t-test was used for 2 group comparisons. The changes in the AMPK activity in response to increasing [AMP] in the assay buffer were compared across the groups using 2-way ANOVA. For all comparisons, a value of P<0.05 was considered to be significant.

Results

Myocardial Energetic Status and AMPK Activity During Normal Perfusion

Using 31P NMR spectroscopy of isolated perfused hearts, we found concentrations of phosphocreatine (PCr), ATP, and the free AMP were unaltered in either γ2-mutant (n = 17) or γ2-TG (n = 5) hearts compared with WT (n = 10) at normal perfusion (Figure 2A). These results showed that the myocardial energetic status was normal in γ2-mutant hearts, and AMP/ATP ratio, the primary determinant of AMPK activity, was unaltered. However, there was a 2.5-fold increase in the α2-AMPK activity in γ2-mutant hearts freeze-clamped at such energetic status (Figure 2B). This increase was unlikely caused by the predominance of γ2-subunit in the AMPK complex because the kinase activity was unchanged in γ2-TG. The α1-AMPK activity showed considerable variations and no significant differences were observed among the 3 groups. The larger variation in α1-AMPK activity among the groups is likely caused by a significant contribution of nonmyocyte α1-
AMPK (≈50%) in cardiac tissue homogenate \(^{18}\) (Figure 2C) because the \(\alpha_1\)-AMPK from nonmyocyte origin does not carry the mutant \(\gamma_2\)-subunit.

Because the increased \(\alpha_2\)-AMPK activity in \(\gamma_2\)-mutant group (shown in Figure 2B) was observed when a saturating [AMP] (200 \(\mu\)mol/L) was used in the assay buffer, we subsequently assessed the allosteric stimulation of AMPK activity by varying [AMP] in the assay buffer from 0 to 200 \(\mu\)mol/L. As shown in Figure 3, \(\alpha_2\)-AMPK activity increased 8-fold in WT but only 2-fold in \(\gamma_2\)-mutant hearts with the increasing [AMP] \((P<0.05)\). The increase in \(\alpha_2\)-AMPK activity was similar for \(\gamma_2\)-TG and WT. These results were consistent with the recombinant protein studies showing reduced allosteric stimulation by AMP in \(\gamma_2\)-mutant AMPK. Furthermore, they suggest that mechanism(s) other than allosteric regulation contribute to the higher basal activity of \(\alpha_2\)-AMPK in the \(\gamma_2\)-mutant hearts. Less changes in \(\alpha_1\)-AMPK activity was found (1.5 to 2-fold) by varying [AMP] in the assay buffer, and there was no difference among the groups (Figure 3).

Using Western blotting, we found the total amount of AMPK was unchanged in \(\gamma_2\)-mutant or \(\gamma_2\)-TG hearts. Interestingly, the phosphorylation of Thr-172 on the \(\alpha\)-AMPK increased by 4-fold in \(\gamma_2\)-mutant hearts compared with WT, and there was no difference between \(\gamma_2\)-TG and WT hearts (Figure 4). Thus, the increased AMPK activity in \(\gamma_2\)-mutant hearts was likely attributable to increased phosphorylation of the \(\alpha\)-AMPK.

Figure 2. A, Concentrations of PCr, ATP, and AMP in WT (dark), \(\gamma_2\)-TG (hatched), and \(\gamma_2\)-mutant hearts (white). B, Isoform-specific AMPK activity measured in cardiac tissue of the above-mentioned 3 groups using 0.2 mmol/L ATP and AMP in the assay buffer \((n=5\text{ to }6\text{ for each group})\). Data are shown as mean±SE, *\(P<0.05\) vs WT. C, Representative Western blots of \(\alpha_1\)- and \(\alpha_2\)-AMPK protein in the heart tissue vs isolated cardiac myocytes (left), and average values for \(\alpha_1\)-AMPK (right, \(n=3\)); *\(P<0.05\) vs heart tissue.

Figure 3. Isoform-specific AMPK activity assayed with 0–200 \(\mu\)mol/L AMP in the assay buffer for WT (open circle, \(n=5\)), \(\gamma_2\)-TG (black circle, \(n=6\)), and \(\gamma_2\)-mutant (black triangle, \(n=6\)) groups. Results (mean±SE) are presented as percentage changes over the activity at 0 \(\mu\)mol/L AMP for each group, *\(P<0.05\) vs WT by 2-way ANOVA.
In contrast, Thr-172 phosphorylation was the same in γ2-mutant hearts despite the 17-fold increase in AMP/ATP (Figure 5). These results suggest that N488I mutation renders the phosphorylation of α-AMPK insensitive to changes in AMP or ATP, resulting in the loss of energy sensor function.

**AMPK Activity and the Glycogen Content**

Because glycerone content in γ2-mutant hearts is markedly increased and a glycerone-binding domain has been identified in the β-subunit of AMPK, it has been speculated that increased glycogen accumulation may alter AMPK activity in vivo. In the present study, we were able to force glycogen utilization in isolated perfused γ2-mutant hearts yielding 60% reduction of glycogen content without significantly decreasing [ATP] (−12±7%) or increasing the [AMP] in these hearts (Figure 6A and 6B). These results demonstrated that the glycogen pool in the γ2-mutant hearts was not sequestered from the normal metabolic pathway and could be mobilized. Nevertheless, neither the Thr-172 phosphorylation nor the AMPK activity was changed in γ2-mutant hearts with reduced glycogen content (Figure 6C and 6D).

**Discussion**

In this study, we demonstrate that the N488I mutation in the γ2-subunit of AMPK causes increased AMPK activity in the beating mouse heart under normal cellular energetic state. Increased AMPK activity is likely caused by increased phosphorylation of Thr-172 on the α-subunit. Furthermore, we show that increased phosphorylation and hence the activity of the mutant AMPK under normal [ATP] was associated with blunted stress response during ATP depletion, suggesting the energy-sensing function is defective in this mutant AMPK.

Previous studies showed that several disease mutations in the γ2-subunit of AMPK lowered its affinity for both AMP and ATP. This change led to impaired AMPK activation by high AMP/ATP via allosteric regulation in vitro. It is worth noting that AMPK activity assay was commonly performed at a [ATP] 10 to 50 times lower than [ATP] of the heart, this resulted in a substantially higher AMP/ATP in the assay buffer. Whereas such a condition is ideal for determining the stimulatory effect of AMP on AMPK activity, it does not allow us to test whether the decreased affinity for ATP in the mutant γ2-subunit impairs the inactivation of AMPK by high [ATP]. To our knowledge, the present study is the first to

### Myocardial Content of ATP and AMP

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<th>Normal perfusion</th>
<th>ATP (mmol/L)</th>
<th>Free AMP (µmol/L)</th>
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All data are presented as mean±SE; *P<0.05 vs normal perfusion. The free [AMP] could not be calculated after ATP depletion because of the complete depletion of PCr by ischemia.

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**Figure 4.** Phosphorylation of Thr172 on the α-AMPK (p-AMPK) in WT (dark bar), γ2-TG (hatched bar), and γ2-mutant hearts (white bar) freeze-clamped at baseline conditions (n=5 to 7 per group). The total amount of α-AMPK was unchanged as shown in the representative blot. Data are presented as mean±SE. *P<0.05 vs WT baseline.

**B**

The Relationship Between α-AMPK Phosphorylation and AMP/ATP

To determine whether increased phosphorylation of α-AMPK in γ2-mutant hearts at normal AMP/ATP was caused by an altered sensitivity to the cellular nucleotide concentrations, we sought to reverse AMP/ATP by subjecting hearts to ischemia that led to ATP depletion and AMP accumulation. Table shows the myocardial content of ATP and AMP at baseline and after ATP depletion measured by 31P NMR spectroscopy and HPLC. Similar to our prior reports, the total myocardial AMP, assessed by HPLC, was ≈2% of ATP in hearts with normal perfusion whereas the free AMP, calculated using the equilibrium expression of the adenylate kinase reaction, was several orders of magnitude lower than ATP. Thus, the AMP/ATP in the intact hearts, assessed by either approach, is markedly lower than the ratio in the assay buffer used for AMPK activity measurements in vitro. In freeze-clamped hearts with 30% of normal ATP, the ratio of total AMP to ATP increased by 15- and 17-fold for WT and γ2-mutant hearts, respectively. This change in AMP/ATP caused a 7-fold increase in Thr-172 phosphorylation in WT.

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**Table.**

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<th>Based on Measurements by 31P NMR Spectroscopy</th>
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<td>ATP (mmol/L)</td>
<td>Free AMP (µmol/L)</td>
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All data are presented as mean±SE; *P<0.05 vs normal perfusion. The free [AMP] could not be calculated after ATP depletion because of the complete depletion of PCr by ischemia.
simultaneously document the energy status and the AMPK activity in hearts that express a mutant γ2-subunit of AMPK. In this study, we show that one of the disease mutations (N488I) increases AMPK activity even though the allosteric response to increasing AMP/ATP is reduced. Our results also show that increased AMPK activity results from increased phosphorylation of Thr-172 on the catalytic subunit (α-subunit) under normal in vivo AMP/ATP, i.e., at a high [ATP]. These findings corroborate a most recent study showing that another disease-causing mutation on the γ2-subunit of AMPK (R531Q and R531G) caused increases in phosphorylation of the α-AMPK as well as the kinase activity, although that study did not determine the AMP/ATP under their study conditions.20

Phosphorylation of Thr-172 on the α-subunit is required for increased AMPK activity and is likely the primary mechanism for AMPK activation in vivo.5,21 It has been proposed that the binding of AMP to the AMPK makes it a better substrate whereas the binding of ATP makes it a poor substrate for the upstream kinase.4 This notion is supported by the fact that a recently identified upstream kinase, LKB-1, is by itself insensitive to changes in AMP/ATP.22 Thus, the decreased affinity to the regulatory nucleotides (AMP and ATP), found in mutant γ2-AMPK, can lead to either reduced activation of AMPK (when [AMP] is high) or failure to maintain low AMPK activity (when [ATP] is high). In the present study, increased AMPK activity in the mutant hearts at normal [ATP] reflects a loss of the inhibitory effect by high ATP. This is consistent with the biochemical study showing decreased binding affinity of mutant γ2 subunit for ATP.7,20 During ischemia, when [ATP] is depleted and the stimulatory effect of AMP prevails, γ2-mutant hearts show a reduced response compared with the WT hearts, consistent with a decreased sensitivity to AMP. Thus, decreased binding affinity to the regulatory nucleotides caused by the mutation of the γ2 subunit can lead to bidirectional changes of AMPK activity depending on the relative cellular [AMP] and [ATP] at the time of sampling.

Although our study did not directly address whether it is the increased basal AMPK activity or the blunted activation of AMPK during the stress that contributes to the development of cardiac disease phenotype, several possibilities can be discussed. We have previously reported that crossing the γ2-mutant mice with mice overexpressing a dominant-

Figure 5. Representative blots and average values for phosphorylation of Thr172 on the α-AMPK (p-AMPK) in WT and γ2-mutant hearts freeze-clamped at normal [ATP] and after ATP depletion (n=4 to 5 per group). See Table for the total AMP/ATP in the heart under each condition. Data are presented as mean±SE.

*P<0.05 vs WT baseline; †P<0.05 vs WT low-ATP.

Figure 6. The effects of increased glycogen content on AMPK activity. A, Myocardial glycogen content in WT (dark), γ2-mutant hearts (white), and γ2-mutant hearts subjected to glycogen depletion protocol (hatched, n=5 to 7 per group). B through D, [AMP], phosphorylation of Thr172 on the α-AMPK (p-AMPK), and isoform-specific AMPK activity, respectively, in the above-mentioned groups of hearts. Data are shown as mean±SE.

*P<0.05 vs WT; †P<0.05 vs γ2-mutant.
negative catalytic subunit (α2-DN-AMPK) rescues the disease phenotype, suggesting that increased AMPK activity likely accounts for the disease development with this mutation. Intriguingly, a recent study found that a mouse model overexpressing γ2-AMPK with R302Q mutation developed similar disease phenotype, yet, showed decreased AMPK activity in cardiac tissue. The relationship between AMPK activity and in vivo AMP/ATP in that mutation has not been reported. Because hearts expressing α2-DN-AMPK showed a substantial loss of AMPK activity but did not show phenotypes of cardiac hypertrophy or preexcitation, it is unlikely that a simple reduction of AMPK activity is sufficient to account for the disease phenotype caused by mutations in the γ2-AMPK. On the other hand, widely used antidiabetic drug metformin is known to increase AMPK activity but not known to cause a similar cardiac complication as caused by mutations in γ2-AMPK. It is though, possible that inappropriate activation of AMPK at a specific stage during the development is critical for the development of the cardiac disease in hearts bearing the mutant γ2-AMPK.

Finally, we found that altered regulation of AMPK activity in γ2-mutant heart was not affected by reducing myocardial glycogen accumulation. Because the glycogen content in the γ2-mutant hearts is still 2-fold higher than WT after the 60% reduction in glycogen content, we cannot exclude the possibility that AMPK activity can be modulated by the glycogen content near or lower than normal physiological level, such as in skeletal muscle during exercise. In summary, we found that basal AMPK activity was increased whereas its response to severe stress was reduced in mouse hearts expressing a mutant γ2-AMPK. These changes occurred as the results of altered Thr-172 phosphorylation of α-AMPK in mutant hearts in response to changes in AMP/ATP under (patho)physiological conditions. These results demonstrate that altered sensitivity to AMP and ATP in mutant AMPK may lead to bi-directional changes in the kinase activity in the heart. Therefore, the pathogenesis of cardiac disease in patients with mutations of γ2-AMPK may involve mechanisms beyond a simple loss- or gain-of-function of this kinase.

Acknowledgments

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