AMP-activated protein kinase (AMPK) responds to impaired cellular energy status by stimulating substrate metabolism for ATP generation. Mutation of the γ2 regulatory subunit of AMPK in humans renders the kinase insensitive to energy status and causes glycogen storage cardiomyopathy via unknown mechanisms. Using transgenic mice expressing one of the mutant γ2 subunits (N488I) in the heart, we found that aberrant high activity of AMPK in the absence of energy deficit caused extensive remodeling of the substrate metabolism pathways to accommodate increases in both glucose uptake and fatty acid oxidation in the hearts of γ2 mutant mice via distinct, yet synergistic mechanisms resulting in selective fuel storage as glycogen. Increased glucose entry in the γ2 mutant mouse hearts was directed through the remodeled metabolic network toward glycogen synthesis and, at a substantially higher glycogen level, recycled through the glycogen pool to enter glycolysis. Thus, the metabolic consequences of chronic activation of AMPK in the absence of energy deficiency is distinct from those previously reported during stress conditions. These findings are of particular importance in considering AMPK as a target for the treatment of metabolic diseases.
Aberrant activation of AMP-activated protein kinase remodels metabolic network in favor of cardiac glycogen storage

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Introduction
AMP-activated protein kinase (AMPK) is a serine/threonine kinase that acts as a cellular energy sensor and a master regulator of metabolism in a variety of cell types including cardiac myocytes (1). AMPK responds to increases in the cellular AMP/ATP ratio, an ultrasensitive indicator of impaired energy status, and triggers multiple signaling cascades to restore energy balance by stimulating ATP-generating pathways while inhibiting ATP-consuming pathways (1–3). AMPK is a heterotrimeric protein consisting of a catalytic subunit (α) and 2 regulatory subunits (β and γ). Each subunit has 2–3 isoforms; all except γ3 are expressed in the heart. Mutations in the γ2 subunit (encoded by the PRKAG2 gene) cause human cardiomyopathy characterized by substantial myocardial glycogen accumulation, preexcitation syndrome, and cardiac hypertrophy (4–6). These characteristics closely resemble the cardiac manifestation of glycogen storage disease (7), which prompted us to focus on the mechanisms of glycogen accumulation in the cardiomyopathy caused by mutation of PRKAG2. Interestingly, mutations in the corresponding sites on the γ3 subunits of AMPK in skeletal muscle such as R200Q in pigs and R225Q in mice have also been shown to cause glycogen accumulation (8–10), suggesting an important role of altered glycogen metabolism in the phenotype caused by mutations of γ-AMPK.

In order to understand the pathogenesis of the cardiomyopathy caused by PRKAG2 mutation, transgenic mice with cardiac-specific overexpression of several mutant or WT γ2 subunits of AMPK have been generated. Transgenic mice expressing every mutant form but WT γ2-AMPK recapitulated the human disease phenotype, suggesting that mutation of, but not increased expression of, γ2 subunits caused the disease phenotype (11–13). Using the mouse model expressing the N488I mutant of γ2-AMPK in the heart (the γ2 mutant mouse), we have previously shown that the mutation causes aberrant activation of AMPK in the absence of energy deficit and that the disease phenotype can be rescued by introducing an inactive catalytic subunit of AMPK, which acts as a dominant-negative inhibitor of AMPK (14, 15).

Although the disease phenotype is known to be dependent on AMPK activity, the mechanisms by which altered AMPK activity cause cardiomyopathy remain unknown. Importantly, the metabolic consequence of high AMPK activity in the energetically intact heart is not understood, despite the established role of AMPK in promoting glucose and fatty acid use during energetic stress (16–20). In the present study, we found that altered AMPK activity under normal energetic status remodeled the cardiac metabolic network to establish and turn over a substantially enlarged glycogen pool, thereby causing a unique form of glycogen storage cardiomyopathy. Thus, contrary to the role of AMPK in stimulating substrate use during stress conditions, activation of AMPK in energetically normal hearts results in fuel storage.

Results
Increased glycogen content in hearts of γ2 mutant mice is associated with a higher rate of glucose uptake. In the hearts of γ2 mutant mice, there was a progressive increase in glycogen content that leveled off at 6–8

Nonstandard abbreviations used: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; 2-DG, 2-deoxyglucose; 2-DG-P, 2-DG-phosphate; G-6-P, glucose-6-phosphate; GS, glycogen synthase; γ2 mutant mouse, mouse expressing the N488I mutant of γ2-AMPK; PFK, phosphofructokinase; UDPG, UDP-linked glucose; UDPG-P, UDPlinked pyrophosphorylase; 2-TG mouse, transgenic mouse overexpressing WT γ2-AMPK.

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Reference
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weeks of age (Figure 1A). We assessed the rate of glucose uptake in hearts of γ2 mutant mice during the rising phase (19 days) and the plateau phase (49 days) of glycogen accumulation in isolated hearts perfused with nontracer 2-deoxyglucose (2-DG) using 13C nuclear magnetic resonance (NMR) spectroscopy. The γ2 mutant mouse hearts showed a 2-fold increase in the insulin-independent glucose uptake rate at both ages compared with nontransgenic WT controls (P < 0.05) and a 1.5-fold increase compared with hearts of transgenic mice overexpressing WT γ2-AMPK (γ2-TG; P < 0.05; Figure 1B). Crossing the γ2 mutant mice with mice overexpressing a dominant-negative catalytic subunit of AMPK, which served to inhibit the AMPK activity in γ2 mutant mouse hearts (14), blocked the increases in glucose uptake and glycogen content (P < 0.05; Figure 1, A and B). Thus, excessive accumulation of glycogen in the hearts of γ2 mutant mice is associated with increased glucose uptake, and the alterations are dependent on AMPK activity and not accounted for by overexpression of the γ2 isoform.

Increased fatty acid oxidation and decreased carbohydrate use in hearts of γ2 mutant mice. To track the metabolic fate of increased glucose influx, we assessed the use of multiple exogenous substrates by mitochondrial oxidative metabolism in hearts of adult (7–10 weeks) γ2 mutant and WT mice that were perfused with 13C-labeled substrates using 13C NMR isotopomer analysis. At similar rates of myocardial oxygen consumption, the contribution of fatty acids to oxidative metabolism was 30% higher, while the contribution from carbohydrate (glucose and lactate) was more than 2-fold lower, in hearts of γ2 mutant mice compared with WT mice (Figure 2, A and B). The production of 13C-labeled lactate, originated from 13C-labeled glucose, was markedly decreased in γ2 mutant mouse hearts, suggesting a decreased glycolytic flux from exogenous glucose (P < 0.05; Figure 2C). Phosphorylation of acetyl-CoA carboxylase (ACC), an endogenous substrate of AMPK, was significantly increased (Figure 2D). Because cardiac ACC (ACCβ) catalyzes the production of malonyl-CoA, a potent inhibitor of carnitine-palmitoyl transferase 1, phosphorylation and inactivation of ACCβ is consistent with the increased fatty acid oxidation observed in γ2 mutant mouse hearts.

Increased glucose entry in hearts of γ2 mutant mice is directed toward glycogen. Because increased glucose uptake in the hearts of γ2 mutant mice was not associated with increased use via glycolysis or oxidation, we next tested the possibility that increased glucose entry was directed toward glycogen synthesis. We perfused the hearts of 3-week-old and 7- to 10-week-old mice with 13C-labeled glucose and measured the time-dependent accumulation of 13C-glycogen (Figure 3A). These 2 ages were chosen to represent the rising and peak phases of glycogen accumulation based on results shown in Figure 1A. The rate of 13C-glucose incorporation into the glycogen pool was 0.088 ± 0.009 and 0.067 ± 0.001 µmol/g/min in the hearts of γ2 mutant mice at 3 weeks and 7–10 weeks, respectively. This was roughly 5 and 4 times higher, respectively, than that in hearts of WT mice at 7–10 weeks (0.018 ± 0.002 µmol/g/min; P < 0.05). We were unable to detect significant 13C-glucose incorporation in the hearts of 3-week-old WT mice or γ2-TG mice; it is likely that we reached the lower limit of the instrument’s sensitivity, due in part to the low rate of incorporation in these 2 groups and in part to the small heart size (50–60 mg), since NMR signals are proportionate to heart mass. Nevertheless, we were able to show that in contrast to a very low to undetectable flux in WT or γ2-TG hearts, a robust flux of exogenous glucose toward the
To approximately 90% of lactate output from the combined contribution of glucose and glycogen to oxidative metabolism and glucose oxidation after 2 hours of perfusion with glucose and glycogen to glycolysis and oxidation. Using this approach, the hearts of the 2 mutant mouse hearts did not reach steady state until 2 hours of perfusion, despite minimal increases in total glycogen content during the second hour (Figure 3B). These results suggested that exogenous glucose was preferentially directed toward glycogen synthesis in the 2 mutant mouse heart and that furthermore, there was substantially higher turnover of glycogen in these hearts. Thus, we remeasured 13C-lactate production and glucose oxidation after 2 hours of perfusion with 13C-glucose, when the labeling of the glycogen pool reached steady state in the hearts of the 2 mutant mice. Measurements taken at this time point determined the combined contribution from exogenous glucose and glycogen to glycolysis and oxidation. Using this approach, we found that 13C-lactate production was 40% higher in hearts of 2 mutant mice compared with WT mice (P < 0.05; Figure 4C) (24). Furthermore, we found that UDPG-PPL activity was increased 5-fold in 2 mutant mouse hearts (P < 0.05) and that this increase was not affected by phosphorylation treatment (Figure 4D); the latter finding suggests that modification by phosphorylation was unlikely. These results collectively suggest that transcriptional mechanisms contribute to the upregulation of UDPG-PPL activity caused by mutation of 2-AMPK.

We next measured the activity of glycogen synthase (GS) at increasing concentrations of glucose-6-phosphate (G-6-P), the allosteric activator of the enzyme (Figure 5A). At 9 days, GS activity was not different in hearts of WT and 2 mutant mice over the entire range of G-6-P concentrations. At 19 and 49 days, the relationship of GS activity to G-6-P concentration was significantly altered in the 2 mutant mouse hearts (P < 0.05, interaction between G-6-P concentration and genotype on GS activity; 2-way repeated-measurements of GS activity to G-6-P concentration was significantly altered in the hearts of 2 mutant mice aged 3 weeks and 7–10 weeks). In hearts of 7- to 10-week-old WT mice, incorporation of 13C-glucose during 2 hours’ perfusion in the hearts of WT, 2-TG, and 2 mutant mice aged 3 weeks and 7–10 weeks. Linear fit was applied to the rising phase for each group. Shown are the slopes of 3-week-old 2 mutant mouse (0.088 ± 0.009 μmol/g/min; r², 0.995), 7- to 10-week-old WT mice (0.018 ± 0.002 μmol/g/min; r², 0.916), and 7- to 10-week-old 2 mutant mouse (0.067 ± 0.001 μmol/g/min; r², 0.995). No significant slopes were found in 3-week-old WT hearts or 2-TG hearts. (A) Time-dependent labeling of glycogen with 13C-pyruvate, derived from glucose and glycogen, to oxidative metabolism measured at the end of 2-hour perfusion (D). Data are mean ± SEM (n = 5–7 per group). *P < 0.05 versus age-matched WT.
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altered energy homeostasis redirected the fluxes of the cardiac meta-

In this study, we showed that altered AMPK activity in the absence of
glycogen synthesis at this stage was consistent with the transition
in the physiological range of G-6-P concentration (about 0.2 mM in
hearts of 7-week-old mutants compared with those of WT mice at 19 and 49 days.
The progressive increase in GS phosphorylation during this period
suggests differential phosphorylation of GS in the hearts of γ2 mutant mice compared with those of WT mice at 19 and 49 days.
The role in the phosphorylation and activation of phosphofructokinase-2
— a known target of AMPK for regulating fatty acid oxidation in muscle.
While these metabolic changes were not unexpected in hearts with
increased AMPK activity, their consequences in γ2 mutant mouse hearts were distinct from those of AMPK activation during energetic
stress. We showed that simultaneous increases in glucose uptake and fatty acid oxidation in the hearts of γ2 mutant mice with normal
energy status yielded a glucose-sparing effect that contributed to glycogen synthesis (Figure 6). It has previously been shown that
increased glycogen synthesis occurs in the heart when enhanced oxidation of non-glucose substrate is associated with sustained glucose uptake rate under certain experimental conditions (25–27). This is
because increased glucose entry and decreased glucose use for ATP production result in elevated intracellular G-6-P concentration,
which serves as the source for glycogen synthesis and at the same
time as a powerful allosteric stimulator of GS. Consistent with our
present observations, prior studies in skeletal muscle have shown
that chronic pharmacological activation of AMPK leads to glycogen
storage (28, 29). A recent study using transgenic mice overexpressing a mutant γ1-AMPK also showed increased AMPK activity and higher glycogen content in the muscle (30). In contrast, overexpression of the WT γ2-AMPK in the heart that does not change AMPK activity
causes a minimal disease phenotype (11–13, 15). These results collectively suggest that alterations of AMPK activity, rather than the
isoform composition of the γ subunit, are primarily responsible for the glycogen storage phenotype.

An unexpected finding of this study was the decrease of glycolysis from exogenous glucose in the γ2 mutant mouse hearts, because activation of AMPK is normally associated with increased myocardial glycolysis. AMPK has previously been shown to play an important role in the phosphorylation and activation of phosphofructokinase-2
(PKF-2), leading to increased synthesis of fructose-2,6-bisphosphate, a potent stimulator of the rate-limiting enzyme for the glycolytic pathway, PKF-1 (18, 31). However, PKF-1 activity is also regulated by other stimulators (e.g., ADP, AMP, and inorganic phosphate) and inhibitors (e.g., ATP, H⁺, and citrate). Under normal conditions, PKF-1 is minimally stimulated because of high concentrations of inhibitors and low concentrations of stimulators in vivo. During stress, intracellular concentrations of these regulators change coordinately, releasing the inhibition of PKF-1 and allowing glycolysis to increase in the heart (31). Aberrant AMPK activity in the hearts of γ2 mutant mice, accompanied by no changes in other regulators of PKF-1 (14, 15), is likely less effective in stimulating glycolysis. Moreover, the lack of stimuli and/or demands for increasing glycolytic ATP production in the hearts of γ2 mutant mice is combined with the active diversion of exogenous glucose toward the glycogen synthesis pathway, due to upregulation of UDPG-PPL, and thus jointly reduces the flux of exogenous glucose to glycolysis.

In addition to increased intracellular G-6-P concentration, our results suggest that increased expression and activity of UDPG-PPL in the hearts of γ2 mutant mice contribute to the “pulling” of glucose toward glycogen. In the presence of a high G-6-P concentration, an enhanced UDPG-PPL reaction served a dual role of diverting intracellular glucose toward UDPG as well as driving glycogen synthesis by increasing substrate supply via a feed-forward mechanism (Figure 6). Of note, the upregulation of UDPG-PPL (encoded by the Ugp2 gene) was an early event in the γ2 mutant mouse heart that preceded the marked rise of myocardial glycogen content. We also showed that increased UDPG-PPL expression was independent of increased glucose uptake, because increasing glucose uptake by overexpressing glucose transporter 1 downregulated UDPG-PPL expression. Consistent with this finding, increased Ugp2 mRNA levels have also been shown in the skeletal muscle of mice (R225Q) and pigs (R200Q) bearing mutations at corresponding sites of γ3-AMPK and presenting a glycogen storage phenotype (32, 33). These findings collectively suggest that upregulation of UDPG-PPL is a primary response to AMPK mutation rather than an adaptive response to altered glucose metabolism. Furthermore, our observations suggest what we believe to be a novel role of AMPK in muscle glycogen metabolism via transcriptional regulation of UDPG-PPL.

It has previously been shown that acute activation of AMPK causes phosphorylation of GS at site 2, resulting in decreased GS activity in skeletal muscles (34). However, in apparent conflict with this observation, long-term activation of AMPK increases glycogen content in skeletal muscle of normal rats and improves glycogen synthesis in insulin-resistant muscle (35, 36). Because our analyses of substrate metabolism were performed in the presence of insulin, they cannot address the potential alterations of insulin signaling in the hearts.

Figure 5
GS activity and its regulation in γ2 mutant mouse hearts. (A) GS activity in cardiac tissue of WT and γ2 mutant mice measured at G-6-P concentrations of 0.0625–7.5 mM (shown in log scale) with saturating UDPG (4.5 mM). (B) Representative Western blot of the GS protein and average quantification of the total amount of GS protein in hearts of WT and γ2 mutant (Mut) mice at 3 time points. Bars denote relative slow- and fast-migrating fractions of the GS. Shifts of bands in gel electrophoresis and its subsequent elimination by phosphatase treatment (not shown) suggest altered phosphorylation of the GS in the hearts of γ2 mutant mice. (C and D) Tissue content of G-6-P (C) and UDPG (D) in the hearts of γ2 mutant mice compared with those of age-matched WT mice during the period of glycogen accumulation. Data are mean ± SEM (n = 5–8 per group). *P < 0.05 versus age-matched WT.
of γ2 mutant mice. Nonetheless, we found a significant increase in the rate of insulin-independent glucose uptake in γ2 mutant mouse hearts. It has been speculated that AMPK-mediated increases in glucose transport, after sufficiently elevating intracellular G-6-P concentrations, are able to stimulate glycogen synthesis by overcoming the inhibitory phosphorylation of GS in the skeletal muscle (28, 29, 34, 37). Our present results support such a mechanism in the heart. It is likely that increased G-6-P is particularly important for the accumulation of glycogen in the hearts of young γ2 mutant mice when GS activity is minimally altered. Furthermore, we suggest that elevated expression of GS in the hearts of adult γ2 mutant mice also contributes to counteract the inhibition of GS activity and, hence, sustain glycogen synthesis in the later stage.

A unique feature of the glycogen storage disease phenotype caused by mutations of γ2-AMPK is that the heart sustains its ability to use glycogen. We have previously shown that cardiac glycogen in the hearts of γ2 mutant mice can be used effectively in vivo to support increased energy demand during exercise and to offset decreased energy supply during substrate deprivation in isolated perfused hearts (14, 15). A recent study by Davies et al. found decreased branching of glycogen particles in transgenic hearts expressing a different mutant γ2-AMPK, R531G, although the glycogen branching enzyme activity was not examined (12). We previously found normal activity of glycogen branching enzyme in N488I mutant mouse hearts (14). Furthermore, activities of the key enzymes involved in glycogenolysis, such as debranching enzyme, glycogen phosphorylase, and glycogen phosphorylase kinase, are unaltered in the N488I mutant mouse heart (14). These observations show that glycogen storage in the γ2 mutant mouse heart is not attributable to a defective pathway for glycogenolysis as seen in majority of glycogen storage disease. It does not, however, exclude the possibility that glycogen breakdown is partially inhibited in the hearts of γ2 mutant mice during the phase of active glycogen accumulation. Nevertheless, our results support the model that glycogen storage in the hearts of γ2 mutant mice results from enhanced glycogen synthesis that exceeds the rate of glycogen breakdown. Furthermore, we suggest that the ultimate plateau of glycogen accumulation is set by the balance between decreased GS activity and increased contribution of glycogen to glycolytic flux. It has previously been shown that glycogen loading inhibits muscle GS activity by feedback mechanisms independent of AMPK or GS kinase (34, 38, 39). In the present study, we found greater inhibition of GS activity in the physiological range of G-6-P concentration in the hearts of γ2 mutant mice with peak glycogen content (7 weeks) compared with those during the phase of glycogen accumulation (2.5 weeks). Unlike the phase of active glycogen accumulation, a high G-6-P concentration and increased amount of GS protein in the hearts of 7-week-old γ2 mutant mice failed to maintain a normal UDPG level, suggesting that the rate of glycogen synthesis becomes restricted at this stage. At the same time, glycolytic flux in the hearts of adult γ2 mutant mice (90% originated from the glycogen pool) exceeded that of age-matched WT mouse hearts. Thus, the stabilization of glycogen pool size represents the dynamic balance of declined influx and active efflux of glycogen to the glycolytic pathway.

In summary, we have demonstrated that the N488I mutation of γ2-AMPK modified substrate metabolism at multiple pathways via a variety of mechanisms leading to glycogen storage and eventually an active turnover of a large glycogen store. The synergistic nature of these modifications in promoting glycogen accumulation is a crucial factor in the pathogenesis of cardiac glycogen storage. Remarkably, such an extensively remodeled metabolic network is capable of maintaining energetic homeostasis in the hearts of γ2 mutant mice and supporting increased energy demand during increases in workload (14). However, the tightly protected energy homeostasis in the γ2 mutant mouse heart, inherent to the role of AMPK as a signaling intermediary linking energy demand to substrate metabolism, comes at the expense of developing glycogen storage cardiomyopathy. The metabolic phenotype demonstrated here not only provides the mechanisms for the pathogenesis of cardiomyopathy caused by Prkag2 mutations but also cautions against the use of nonselective AMPK activation in the absence of increased energy demand as a therapeutic strategy.

Methods

Animals. All animal experiments were approved by the Harvard Medical Area Standing Committee on Animals (Boston, Massachusetts, USA). All mice used were on an FVB background and studied at ages ranging 1.5–20 weeks as specified in each individual experiment. Transgenic mice were matched with WT littermates as controls.

Isolated heart perfusion and 31P NMR spectroscopy. Mice were heparinized and anesthetized with sodium pentobarbital (100 mg/kg i.p.), and their hearts were quickly removed. Hearts were perfused in the Langendorff mode with phosphate-free Krebs-Henseleit buffer containing 118 mM NaCl, 25 mM NaHCO₃, 5.3 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 0.5 mM EDTA, 5 mM glucose, and 5 mM pyruvate at 37.5°C as previously described (40). All hearts were stabilized for 25 minutes at a constant perfusion pressure of 80 mmHg. A baseline 31P NMR spectrum (208 scans) was collected for all hearts after stabilization. To determine the rate of glucose uptake, hearts were perfused with a buffer in which glucose was replaced with 5 mM of the glucose analog 2-DG. Five consecutive 8-minute 31P NMR spectra were collected for determination of the time-dependent accumulation of 2-DG-phosphate (2-DG-P). The rate of glucose uptake was estimated by the slope of the fitted line as previously described (19, 41). During 2-DG perfusion, 1.2 mM KH₂PO₄ and 5 mM pyruvate were supplied to replenish the intracellular inorganic phosphate pool and to maintain ATP synthesis. The 31P NMR resonance areas corresponding to ATP, phosphocreatine (PCr), inorganic phosphate (Pi), and 2-DG-P were fitted to Lorentzian functions and corrected for saturation (ATP, 1.0; PCr, 1.2; Pi, 1.15; 2-DG-P, 1.8).

The mean value of ATP concentration previously measured by HPLC for the hearts of WT or γ2 mutant mice was used to calibrate the ATP peak area of the baseline 31P NMR spectrum (14). Concentrations of other metabolites were calculated using the ratio of their peak areas to the ATP peak area.
13C NMR spectroscopy and isotopomer analysis. Isolated mouse hearts were perfused with Krebs buffer containing the following substrates: 5.5 mM glucose; 0.4 mM mixed long chain fatty acids (bound to 1% albumin) of 60% palmitic acid, 8% palmitoleic acid, 24% oleic acid, and 8% linoleic acid; 0.38 mM DL-β-hydroxybutyrate; 1.2 mM lactate; and 50 μM/ml insulin. Myocardial oxygen consumption was determined by measuring the coronary flow rate and the oxygen pressure difference between perfusate and effluent from the pulmonary outflow tract (42). Hearts were perfused for 45 minutes with all substrates, among which 2 were 13C enriched for each perfusion study as previously described (43). In one series, [U-13C]fatty acid and [3-13C]lactate were used to determine the relative contributions of fatty acid and lactate to acetyl-CoA. Identical experiments were performed with enriched [U-13C]glucose and [2,4,6-13C]β-hydroxybutyrate to determine the relative contributions of these 2 substrates. Proton-decoupled 13C NMR (9.4T, 102.8 MHz) spectra of cardiac tissue extracts were acquired using a 3-mm NMR probe (Varian Medical Systems). The contributions of each labeled substrate and the total of unlabeled substrates to the oxidative metabolism were determined using the 13C isotopomer peaks areas of the C3 and C4 of glutamate by modeling the tricarboxylic acid cycle fluxes as previously described (44, 45). The contribution from unlabeled endogenous substrates was determined as the difference from 100%.

In a separate cohort of hearts, dynamic incorporation of [1-13C]glucose into the C1 resonance of glycogen was assessed. 13C NMR spectra of the perfused adult hearts (7–10 weeks old) were averaged for 8-minute intervals until the 13C glycogen peak area reached a steady state (approximately 2 hours). The spectra were calibrated using an external standard of [1-13C]glucose. Due to their much smaller heart size, the spectra of 3-week-old mouse hearts were averaged for 24-minute intervals during the 2-hour perfusion. Hearts were freeze-clamped at the end of perfusion for biochemical assays and 13C isotopomer analysis as described above.

Biochemical assays and Northern and Western blotting. Ventricular tissue (approximately 10 mg) was homogenized for 10 seconds at 4°C in potassium phosphate buffer containing 1 mMol/l EDTA and 1 mMol/l β-mercaptoethanol, pH 7.4 (final concentration, 5 mg tissue/ml). Aliquots were removed for assays of protein content using BSA as the standard (46). Tissue activities of UDPG-PPL were determined before and after treatment with phosphatase as previously described (47). GS activity was determined as previously described with increasing concentrations of G-6-P and UDPG was determined in freeze-clamped tissue by the tenfold of G-6-P and UDPG activity assays, respectively. For the dose-response relationship of concentration, genotype, or interaction of concentration and genotype (i.e., does concentration have the same effect on both genotypes?). For all comparisons, a value of P < 0.05 was considered to be significant.

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