Aberrant activation of AMP-activated protein kinase remodels metabolic network in favor of cardiac glycogen storage

Ivan Luptak,1 Mei Shen,1 Huamei He,1 Michael F. Hirshman,2 Nicolas Musi,2 Laurie J. Goodyear,2 Jie Yan,1 Hiroko Wakimoto,3 Hiroyuki Morita,3 Michael Arad,3 Christine E. Seidman,3,4 J.G. Seidman,3 Joanne S. Ingwall,1 James A. Balschi,1 and Rong Tian1

1NMR Laboratory for Physiological Chemistry, Division of Cardiovascular Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts, USA. 2Metabolism Unit, Joslin Diabetes Center and Harvard Medical School, Boston, Massachusetts, USA. 3Department of Genetics, Harvard Medical School, Boston, Massachusetts, USA. 4Division of Cardiovascular Medicine, Brigham and Women’s Hospital, Boston, Massachusetts, USA.

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.

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...
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 glycos accumulation in isolated hearts perfused with nontransc-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 domi- nant-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 glyco- gen 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 isooform.

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 (P < 0.05; Figure 2A and B). The production of 13C-labeled lactate, originating 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 glyco-gen 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 signif- icant 13C-glucose incorporation in the hearts of 3-week-old WT mouse 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

![Figure 1](image1)

Figure 1
Glycogen content (A) and rate of glucose uptake (B) in hearts of WT, γ2 mutant, γ2 mutant crossed with mice overexpressing a dominant-negative catalytic subunit of AMPK (Mutant-DN), and γ2-TG mice at various ages. The rate of glucose uptake was assessed by a nontracer method using glucose analog 2-DG as described in Methods. Data are me-n= 4–6 per group. *P < 0.05 versus age-matched WT; #P < 0.05 versus age-matched mutant.

![Figure 2](image2)

Figure 2
Substrate use in the hearts of adult (7–10 weeks) WT and γ2 mutant mice. Myocardial oxygen consumption (MVO2; A) and relative contributions of each substrate to the oxidative metabolism (B) were measured in isolated mouse hearts after 45 minutes’ perfusion with 13C-labeled substrates. (C) 13C-lactate output of the heart provided an estimate of glycolytic flux of 13C-glucose that was not coupled with oxidation. (D) Phosphorylation of ACC (p-ACC) was determined in freeze-clamped cardiac tissue. Data are mean ± SEM (n = 5–6 per group). *P < 0.05 versus WT.
glycogen pool occurred in γ2 mutant mouse hearts that was greater during the rising phase of glycogen accumulation (P < 0.05, 3 weeks versus 7–10 weeks).

In hearts of 7- to 10-week-old WT mice, incorporation of 13C-glucose corresponded with the increase of myocardial total glycogen content: both reached a steady state with 1 hour of perfusion. In contrast, 13C-labeling of the glycogen pool in hearts of γ2 mutant mice 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 γ2 mutant mouse heart and that furthermore, there was substantially higher turnover of glycogen in these hearts. Thus, we remeasured 13C-lactate production and glycogen oxidation after 2 hours of perfusion with 13C-glucose, when the labeling of the glycogen pool reached steady state in the hearts of the γ2 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 γ2 mutant mice compared with WT mice (P < 0.05), while the combined contribution of glucose and glycogen to oxidative metabolism was not different between the 2 groups (Figure 3, C and D).

Comparison of results obtained after 45 minutes (Figure 2C) versus 2 hours (Figure 3C) of 13C-labeling showed that approximately 90% of lactate output from the γ2 mutant mouse hearts originated from glycogen. In hearts of WT mice, prolonging the duration of 13C-labeling did not significantly alter the results because the steady state was reached after 45 minutes of labeling. Together, these results suggested that glucose metabolism in the hearts of γ2 mutant mice was redirected in favor of the glycogen synthesis pathway and that the glycolytic flux in the hearts of adult γ2 mutant mice originated primarily from glycogen instead of exogenous glucose.

**Mutation of cardiac γ2-AMPK remodels the glycogen metabolism.** To further understand the mechanisms responsible for directing exogenous glucose toward the glycogen pathway, we assessed the regulation of the key enzymes involved in glycogen synthesis in the hearts of WT and γ2 mutant mice. A key step in the glycogen synthesis pathway is the addition of UDP-linked glucose (UDPG) to glycogenin. The generation of UDPG is catalyzed by UDPG pyrophosphorylase (UDPG-PPL). Northern and Western blotting analyses showed significant increases in the mRNA and protein levels of UDPG-PPL in the hearts of γ2 mutant mice compared with WT mice (Figure 4, A and B). The increase in protein expression was first observed at 1 week, which was coincident with the full activation of the α-myosin heavy chain promoter that drove the mutant Prkag2 gene (21–23). The increase was attenuated by crossing the γ2 mutant mice with mice overexpressing a dominant-negative catalytic subunit of AMPK (Figure 4C). The increased expression of UDPG-PPL in the hearts of γ2 mutant mice was unlikely attributable to increased glucose entry, because the UDPG-PPL level was downregulated in transgenic hearts with elevated glucose uptake rate due to overexpression of the insulin-independent glucose transporter 1 (P < 0.05; Figure 4C) (24). Furthermore, we found that UDPG-PPL activity was increased 5-fold in γ2 mutant mouse hearts (P < 0.05) and that this increase was not affected by phosphatase 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 γ2-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 γ2 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 γ2 mutant mouse hearts (P < 0.05, interaction between G-6-P concentration and genotype on GS activity; 2-way repeated-meas-
Discussion
In this study, we showed that altered AMPK activity in the absence of altered energy homeostasis redirected the fluxes of the cardiac met}

Figure 4
UDPG-PPL expression and activity in the hearts of γ2 mutant mice. (A) Representative Western blot showing age-dependent changes in the protein amount of UDPG-PPL from 5 days to 8 weeks in hearts of γ2 mutant (Mut) compared with age-matched WT mice. (B) Representative Northern blot showing that the mRNA level of UDPG-PPL in hearts of 4-week-old γ2 mutant mice was 1.7 times higher than that of age-matched WT mice. (C) Representative Western blot of hearts from 7- to 10-week-old mice and protein levels of UDPG-PPL in hearts of WT mice, γ2 mutant mice, γ2 mutant mice crossed with mice overexpressing a dominant-negative catalytic subunit of AMPK, and transgenic mice expressing insulin-independent glucose transporter 1 (GLUT1-TG). (D) UDPG-PPL activity measured in adult hearts (7–10 weeks) before and after treatment with phosphatase. Data are mean ± SEM (n = 4–6 per group). *P < 0.05 versus age-matched WT; †P < 0.05 versus age-matched mutant.

sures ANOVA). The γ2 mutant mouse hearts showed a gradual increase in total GS activity accompanied by decreased sensitivity to G-6-P concentration. The G-6-P concentration–independent activity of GS was unaltered in at 9 and 19 days in γ2 mutant mouse hearts, but was markedly reduced at 49 days (Figure 5A). Western blotting showed a marked band shift for GS protein (Figure 5B) that was eliminated by phosphatase treatment (data not shown), suggesting differential phosphorylation of GS in the hearts of γ2 mutant mice compared with those of WT mice at 19 and 49 days. The progressive increase in GS phosphorylation during this period corresponded with the alterations in the relationship between GS activity and G-6-P concentration. The 2-fold increase in total GS activity in hearts of 49-day-old γ2 mutant mice compared with age-matched WT mouse hearts (P < 0.05) was associated with a similar increase in GS protein (Figure 5B). In the hearts of 19-day-old γ2 mutant mice, a 2-fold increase in cardiac G-6-P level was observed compared with age-matched WT mice, which served as an effective stimulus to drive glycogen synthesis, as evidenced by the ability of the heart to maintain normal UDPG content despite significant upregulation of UDPG-PPL (Figure 5, C and D). In contrast, a higher G-6-P concentration and increased total GS protein in the hearts of 7-week-old γ2 mutant mice failed to enhance GS activity in the physiological range of G-6-P concentration (about 0.2 mM in normal hearts), which led to accumulation of UDPG by more than 2-fold at this age (P < 0.05; Figure 5, A–D). The evidence of falling glycogen synthesis at this stage was consistent with the transition from active glycogen accumulation to the plateau phase.

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
research article

(PFK-2), leading to increased synthesis of fructose-2,6-bisphosphate, a potent stimulator of the rate-limiting enzyme for the glycolytic pathway, PFK-1 (18, 31). However, PFK-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, PFK-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 PFK-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 PFK-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 synergetic 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 bepanized 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 hearts of WT or γ2 mutant mice was used to calculate 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.
research article

(40). The rate of 2-DG uptake was expressed as the slope of time-dependent changes in 2-DG-P concentration.

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, [1-13C]fatty acid and [3-13C]lactate were used to determine the relative contributions of fatty acid and lactate to acetyl-CoA. Identical experiments with enriched [U-13C]glucose and [2,4-13C]β-hydroxybutyrate determined 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 isotope enrichment 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 C-1 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 cysteine pH 7.4 (final concentration, 5 mg tissue/ml). Aliquots were removed for assays of protein content using BSA as the standard (46). Tissue of glucose released from glycogen with a glucose assay kit (catalog no. GAHK-20; Sigma-Aldrich). An alkaline extraction procedure was used to determine glycogen and exogenous glucose in the tissue (49). Cardiac content of G-6-P and UDPG was determined in freeze-clamped tissue by the methods of Lowry and Passonneau (50). All reagents were purchased from Sigma-Aldrich and were at least analytical grade.

The level of mRNA for UDPG-PPL in the heart was determined by Northern blotting. Cardiac tissue lysates were used for immunoblotting with antibodies against ACC (Cell Signaling Technology) and phosphorylated ACC (Upstate USA Inc.), GS (Chemicon International), and UDPG-PPL (kindly provided by J.C. Lawrence, University of Virginia School of Medicine, Charlottesville, Virginia, USA).

Statistics. Data are presented as mean ± SEM. One-way ANOVA was performed for multiple-group comparisons, and unpaired Student’s t test was used for comparisons between 2 groups. Two-way repeated-measures ANOVA was used to compare the time-dependent changes and the dose-response relationships obtained during 13C-glycogen labeling experiments and GS activity assays, respectively. For the dose-response relationship between G-6-P concentration and GS activity, 2-way repeated-measures ANOVA was used to determine whether the GS activity is affected by G-6-P 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.

Acknowledgments
This work is supported by NIH grants AR45670 and DK68626 (to L.J. Goodyear), HL52320 (to J.S. Ingwall), HL46033 (to J.A. Balschi), and HL67970 and HL59246 (to R. Tian), as well as by grants from the National Heart, Lung, and Blood Institute, NIH (to J.G. Kemp). R. Tian is an Established Investigator of the American Heart Association. I. Luptak was a recipient of the American Heart Association postdoctoral fellowship.

Received for publication October 16, 2006, and accepted in revised form February 13, 2007.

Address correspondence to: Rong Tian, NMR Laboratory, Division of Cardiovascular Medicine, Brigham and Women’s Hospital, 221 Longwood Avenue, Room 252, Boston, Massachusetts 02115, USA. Phone: (617) 732-6994; Fax: (617) 732-6990; E-mail: ritian@rics.bwh.harvard.edu.

13. Kudo, N., Barr, A.J., Barr, R.L., Desai, S., and Lopaschuk, G.D. 1995. High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in malonyl-CoA lev-


research article