Cardiac Expression of Kinase-deficient 6-Phosphofructo-2-kinase/ Fructose-2,6-bisphosphatase Inhibits Glycolysis, Promotes Hypertrophy, Impairs Myocyte Function, and Reduces Insulin Sensitivity*

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Glycolysis is important to cardiac metabolism and reduced glycolysis may contribute to diabetic cardiomyopathy. To understand its role independent of diabetes or hypoxic injury, we modulated glycolysis by cardiac-specific overexpression of kinase-deficient 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (kd-PFK-2). PFK-2 controls the level of fructose 2,6-bisphosphate (Fru-2,6-P2), an important regulator of glycolysis. Transgenic mice had over 2-fold reduced levels of Fru-2,6-P2. Heart weight/body weight ratio indicated mild hypertrophy. Sirius red staining for collagen was significantly increased. We observed a 2-fold elevation in glucose 6-phosphate and fructose 6-phosphate levels, whereas fructose 1,6-bisphosphate was reduced 2-fold. Pathways branching off of glycolysis above phosphofructokinase were activated as indicated by over 2-fold elevated UDP-N-acetylglycosamine and glycogen. The kd-PFK-2 transgene significantly inhibited glycolysis in perfused hearts. Insulin stimulation of metabolism and Akt phosphorylation were sharply reduced. In addition, contractility of isolated cardiomyocytes was impaired during basal and hypoxic incubations. The present study shows that cardiac overexpression of kinase-deficient PFK-2 reduces cardiac glycolysis that produced negative consequences to the heart including hypertrophy, fibrosis, and reduced cardiomyocyte function. In addition, metabolic and signaling responses to insulin were significantly decreased.

An important role of glycolysis in heart failure has been appreciated for over 35 years because Bishop and Altrud (1) reported that glycolytic metabolism is increased in cardiac hypertrophy and congestive heart failure. Glycolysis is also critical to cardiac survival in hypoxia (2), because glycolysis is the sole source of ATP production in the absence of oxygen. In diabetes there is a chronic reduction in cardiac glycolytic capacity (3, 4). Diabetics are prone to heart failure (5) and they suffer more damage following an infarction (6). It has been proposed that reduced cardiac glycolysis contributes to the heart failure and cardiac damage often suffered by diabetics (7, 8).

The significance of cardiac glycolysis has led to careful analysis of its regulation. Metabolic flux analysis (9) demonstrates that control is shared by several reactions. One of the most critical reactions is carried out by 6-phosphofructo-1-kinase (PFK-1) (10, 11), which catalyzes the phosphorylation of fructose 6-phosphate (Fru-6-P) to fructose 1,6-bisphosphate (Fru-1,6-P2). Fru-6-P is in equilibrium with glucose 6-phosphate (Glc-6-P) and these two sugars initiate glycogen synthesis, the hexosamine pathway, and the hexose monophosphate shunt. Thus PFK-1 not only has an important role in regulating glycolysis but by controlling the metabolism of Fru-6-P, PFK-1 can also modulate several important reactions branching off of glycolysis. PFK-1 activity is tightly controlled and the most important positive regulator is fructose 2,6-bisphosphate (Fru-2,6-P2). Until recently the sole function of Fru-2,6-P2 was thought to be regulation of PFK-1. However, Wu et al. (12) has recently provided evidence that liver Fru-2,6-P2 may directly or indirectly modulate gene expression and insulin signaling. The bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2) catalyzes the synthesis and degradation of Fru-2,6-P2. PFK-2 is in turn regulated by a complex network of kinases, phosphatases, and metabolites (13). In this study we have used a cardiac-specific transgene for a mutant form of PFK-2 to directly reduce cardiac Fru-2,6-P2 and cardiac glycolysis. Our results demonstrate that impaired glycolysis produces several indicators of cardiac pathology, which are reminiscent of the cardiomyopathy seen in diabetes. In addition, reduced Fru-2,6-P2 produces a state of cardiac insulin resistance.

MATERIALS AND METHODS

Development of Transgenic Mice

The bifunctional enzyme PFK-2 controls levels of Fru-2,6-P2 by catalyzing two opposing reactions: Fru-6-P + ATP → Fru-2,6-P2 + ADP and Fru-2,6-P2 → Fru-6-P + P+. Development and in vivo analysis of the kinase-deficient PFK-2 (kd-PFK-2) mutant has been previously de-
**Cardiac Expression of Kinase-deficient PFK-2**

**Analysis of Transgene Expression**

Cardiac Fru-2,6-P$_2$ Content—Fru-2,6-P$_2$ was extracted from fresh heart tissue in 10–20 volumes of 50 mM NaOH and kept at 80 °C for 5 min. The extract was cooled and neutralized at 0 °C by addition of ice-cold 1 M acetic acid in the presence of 20 mM HEPES. After centrifugation at 8,000 × g for 10 min, supernatant was collected and assayed for Fru-2,6-P$_2$. The 6-phosphofructo-1-kinase activation method (17) was used for estimation of glucose 6-phosphate, fructose 6-phosphate, and fructose 1,6-bisphosphate from neutralized tissue extracts.

**Metabolic Intermediates**

Metabolic intermediates were measured in each experiment using tritiated water. Lactate concentration was determined by diffusion. A 400-ml sample of cardiac effluent and 25 µl of 0.6 M HCl were added to 1.5-ml tubes that were placed inside 20-ml scintillation vials containing 2 ml of water. Vials were incubated for 72 h at 37 °C. The inside tube was removed and scintillate was added for counting. Effluent from each time point was assayed in duplicate.

**Cardiac Perfusion**

Langendorff perfusions were carried out as previously described by us (18, 22). The heart was removed from the chest and the aorta was cannulated to the perfusion apparatus. The heart was retrogradely perfused at 2 ml/min with Krebs-Henseleit buffer consisting of 120 mM NaCl, 20 mM NaHCO$_3$, 4.6 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgCl$_2$, 1.25 mM CaCl$_2$, 5 mM glucose. Throughout the perfusion Krebs-Henseleit buffer was continuously equilibrated with 95% O$_2$, 5% CO$_2$ that maintained a pH of 7.4 and temperature was maintained at 37 °C. The heart was paced throughout the procedure at 6 Hz (6 V, 3 ms). For studies on the effect of insulin, baseline glycolysis was determined for the first 30 min followed by 50 min in the presence of 200 micromolar/ml of insulin. Perfusion pressure was maintained at 2 ml/min. Perfusion pressure and contractility was monitored continuously during the perfusion.

**Measurements of Glycolysis and Lactate Production**

Glycolysis was measured using [5-^3H]glucose as the substrate as we have previously reported (22). Lactate production was measured as we have described (18). Tritiated water produced from [5-^3H]glucose during perfusion was determined by diffusion. A 400-µl sample of cardiac effluent and 25 µl of 0.6 M HCl were added to 1.5-ml tubes that were placed inside 20-ml scintillation vials containing 2 ml of water. Vials were incubated for 72 h at 37 °C. The inside tube was removed and scintillate was added for counting. Effluent from each time point was assayed in duplicate. For each experiment, background counts were determined by performing the same equilibration on perfusion buffer that had not passed through the heart. Diffusion efficiency was also measured in each experiment using tritiated water. Lactate concentration was measured in 6-fold diluted effluent using Sigma kit 826-B (18).

**Histological Study**

Collagen accumulation in the heart sections was determined as described previously (24). 5-µm heart sections were placed on slides, deparaffinized, and incubated with a saturated solution of picric acid containing 0.1% Sirius red for staining collagen and 0.1% Fast Green, which stains noncollagen proteins. Sections were kept in the dark and incubated for 30 min. They were then rinsed with distilled water, dehydrated, and mounted with permount. The sections were visualized, photographed, and the interstitial fibrosis was quantified by a blind observer using a scale of 0–2 based on the severity of the collagen accumulation. The section was given a score of 0 for mild accumulation, 1 for moderate accumulation, and 2 for severe accumulation.
Cardiomyocyte Assay

Cardiomyocytes were isolated and myocyte contractility was measured as we have described (25). For the oxygenated assay, myocytes were placed in an open chamber in HEPES-buffered Krebs buffer for 2 h. For hypoxia, cardiomyocytes were perfused with N2-saturated Krebs buffer for 15 min and maintained and assayed in a sealed chamber for 2 h. Myocytes were stimulated at 1 Hz using platinum electrodes attached to the chamber. Mechanical properties of ventricular myocytes were assessed using a video-based edge-detection system (IonOptix, Milton, MA) according to the previous description (25). Cell shortening and relengthening were assessed using the following indexes: peak shortening, time to 90% relengthening (TR90), and maximal velocities of shortening and relengthening (±dL/dt).

Immunoblot for Akt

Hearts were frozen in liquid nitrogen after the basal period or following the period of insulin perfusion. Tissue extracts were prepared in lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The lysates were centrifuged and protein concentration of the supernatants was measured by the BCA method (Pierce). Proteins equivalent to 20 µg were analyzed by Western blot as explained previously (26, 27). In brief, protein samples were separated on 4–20% gradient SDS-PAGE gels and transferred onto polyvinylidene difluoride membrane. The membrane was blocked with 5% nonfat milk and incubated overnight with phospho-Akt (Ser-473) antibody or Akt antibody from Cell Signaling. The membranes were washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody and bands were visualized using ECL (Amersham Biosciences).

Statistical Analysis

Comparisons of repeated measurements were performed by two-way repeated measures ANOVA and Tukey post-hoc test. Single comparisons were performed by Student’s t test. The accepted level of significance was 0.05.

RESULTS

Transgenic Lines—A transgene, designated Mb was constructed for cardiac expression of a kinase-deficient mutant of PFK-2 (kd-PFK-2). Eight transgenic lines were produced and these were designated as lines Mb 1–8. Founders of lines 1, 4, 5, and 6–8 produced litters.

The Northern blot shown in Fig. 1A demonstrated that the transgene product was produced primarily in the heart and was absent in nontransgenic FVB mice. Overexpression of kd-PFK-2 protein was confirmed by Western blot with PFK-2 specific antibody (Fig. 1B). Preliminary assays (not shown) indicated that

TABLE I
Concentrations of glycolytic metabolites in FVB and Mb7 hearts

| Glucose 6-phosphate | Fructose 6-phosphate | Fructose 2,6-bisphosphate | Fructose 1,6-bisphosphate | Glycogen | UDP-GlcNAc |
|---------------------|----------------------|---------------------------|---------------------------|----------|------------|
| nmol/g              | nmol/g               | nmol/g                    | nmol/g                    | µmol/g   | nmol/µg protein |
| FVB                 | 148.7 ± 17.0         | 32.6 ± 6.0                | 3.41 ± 0.24               | 498.5 ± 62.6 | 18.6 ± 4.19 | 0.98 ± 0.05 |
| Mb7                 | 293.8 ± 45.2a        | 97.9 ± 14.4b              | 1.39 ± 0.12c              | 261.8 ± 62.6a | 50.2 ± 9.23c | 1.96 ± 0.02c |

a p < 0.05.
b p < 0.01.
c p < 0.001.

Fig. 3. The kd-PFK-2 transgene reduces cardiac glycolysis and the stimulatory effect of insulin. Glycolysis was measured by consumption of [5-3H]glucose (A) and lactate release (B). The lower graphs show the percent increase in glycolysis (C) and lactate release (D) produced by 50 min stimulation with insulin. Values shown are mean ± S.E. for FVB (12) and Mb7 (14) mice. Panels A and B were analyzed by two-way repeated measures ANOVA followed by Tukey post-hoc test. Panels C and D were analyzed by Student’s t test. *, p < 0.05; **, p < 0.002; #, p < 0.02.
transgenic lines Mb1, -4, -6, and -7 had levels of Fru-2,6-P2, which were similar to one another and substantially lower than that of non-transgenic control mice. Lines Mb1 and Mb7 were expanded most rapidly and were used as subjects for subsequent assays. Hearts from Mb1 and Mb7 mice had only 40% as much Fru-2,6-P2 as measured in control FVB hearts (Fig. 2). Because line Mb7 appeared to have higher kd-PFK-2 protein expression, it was used for all subsequent studies.

Metabolic Concentration—Decreased Fru-2,6-P2 would be expected to reduce PFK activity and have a significant impact on concentrations of cardiac metabolites. Levels of several relevant metabolites were measured in freeze-clamped hearts of FVB and Mb7 transgenic mice maintained in free fed conditions. These results are shown in Table I. G6P and Fru-6-P levels were elevated more than 2-fold, whereas the fructose 1,6-bisphosphate level was 2-fold lower in transgenic mice. Coincident with the elevation in the G6P-6-P levels, we observed that glyceroneogenesis was increased by more than 2-fold in the Mb7 transgenic mice. The principal end product of the hexosamine pathway is UDP-GlcNAc. As shown in Table I, we found a 2-fold elevation in UDP-GlcNAc levels. This corresponds to the increased accumulation of Fru-6-P, which may drive the hexosamine pathway.

Effect of Kd-PFK-2 on Cardiac Glucose Metabolism and Insulin Response—The effect of the Mb transgene on cardiac glycolysis was assessed in Langendorff-perfused hearts by measuring consumption of 5-tritiated glucose and production of lactate. 3H2O is released from the metabolism of [5-3H]glucose by triose-phosphate isomerase and enolase steps of the glycolytic pathway (28). As shown in Fig. 3 both 3H2O metabolized from [5-3H]glucose and lactate release were significantly reduced in Mb7 transgenic hearts. Perfusion pressure was constant at 65 mm Hg and throughout the experiment flow was stable and equal in Mb7 and FVB hearts. We saw a reduction in contractility in the Mb7 mice hearts of about 20% (data not shown) but this difference did not reach significance despite evaluation in more than 10 mice per group. After addition of insulin to the perfusate the difference in metabolism between transgenic and control hearts became even greater, because of a clearly diminished response to insulin in transgenic hearts (Fig. 3, C and D). At the end of the 50-min perfusion with insulin, 5-tritiated glucose consumption was increased by 70% in FVB hearts but only by 34% in transgenic hearts (Fig. 3C). Similarly, lactate output was stimulated by 90% in FVB hearts but only by 25% in Mb7 hearts (Fig. 3D). The differences in insulin response were significant (p < 0.02).

To determine whether the decline in insulin action extended beyond glucose metabolism, we measured insulin-stimulated phosphorylation of Akt. Hearts were perfused with or without insulin, extracted, and protein was analyzed by Western blotting. The results are shown in Fig. 4. In the absence of insulin there was no significant difference in the expression levels of Akt nor any difference seen in the phosphorylated form of Akt. Following insulin perfusion serine 473 phosphorylation of Akt was increased by 230 ± 54% in FVB hearts but only by 63 ± 18% in Mb7 transgenic hearts. Two-way ANOVA indicated that the insulin-stimulated increase was significant for FVB (p < 0.01) but did not reach significance for Mb7 hearts (p = 0.14). These results demonstrated that the Kd-PFK-2 protein reduced insulin action in pathways other than glucose metabolism.

Pathology in Transgenic Hearts—The hearts of Mb7 mice of both sexes showed a small but significant increase in heart weight of ~10% as shown in Table II. However, body weights were not different from control FVB mice. The heart weight/body weight ratio was significantly increased in both male and female Mb7 mice. The increased heart weight was accompanied by a significant increase in cardiac fibrosis in Mb7 mice as shown in Fig. 5.

To assess the direct effect of impaired glycolysis on cardiomyocyte contractility, myocytes were isolated from Mb7 and FVB hearts and incubated with oxygenated or nitrogen-bubbled Krebs buffer. Myocyte contractility was measured and the results are shown in Fig. 6. Mb7 myocytes showed significantly reduced contractility compared with FVB control myocytes as assessed by +dl/dt, −dl/dt, and % peak shortening both under normoxic and hypoxic conditions. In addition, Mb7 myocytes required more time to relengthen (TR90) than FVB myocytes.

DISCUSSION

Changes in glucose metabolism accompany cardiac hypertrophy but it is not known if this change in metabolism is an adaptive or maladaptive response. To examine directly the role of glucose metabolism in long term cardiac function we developed transgenic mice with reduced cardiac glycolysis. Multiple lines of mice were produced with the cardiac-specific, kinase-deficient PFK-2 transgene Mb, which markedly reduced cardiac levels of Fru-2,6-P2 a potent regulator of PFK-1. This resulted in significant changes in cardiac function, some of which were expected and others unexpected. As anticipated, glycolysis was reduced and anticipated changes occurred in the concentration of glycolytic intermediates and metabolites of pathways that branch off from glycolysis. The transgene produced cardiac pathology as demonstrated by increased cardiac weight, increased fibrosis, and decreased cardiomyocyte con-
tractility. Unexpectedly the transgene markedly decreased car-
diac sensitivity to insulin. Cardiac metabolites and pathways upstream of PFK-1 were
up-regulated in transgenic hearts because of inhibition of
PFK-1. The concentrations of Glc-6-P and Fru-6-P were in-
creased 2- and 3-fold, respectively. Glc-6-P is the first substrate
for glycogen production and Glc-6-P stimulates glycogen syn-
thesis (30). These combined effects resulted in almost a 3-fold
increase in glycogen content of transgenic hearts. Fru-6-P is
the first substrate of the hexosamine pathway and the end
product of this pathway, UDP-GlcNAc, was increased 2-fold.
These changes in side pathway products were probably not a
result of nonspecific effects of the transgene nor to the overall
reduction in the glycolytic flux induced by the Mb transgene.
We say that because we previously observed similar increases
in cardiac glycogen content when we overexpressed yeast hex-
okinase in the heart (18). Hexokinase, like the kd-PFK-2 pro-
tein, also produced a 2-fold increase in UDP-GlcNAc levels.
These results show that important pathways that branch from glycolysis are more
directly affected by changes in the concentration of glycolytic
intermediates than they are by changes in glycolytic flux.

Diabetes is the major clinical condition in which there is a
chronic inhibition of cardiac glycolysis (4). In diabetes glycoly-
sis is reduced because insulin stimulates many steps in the
pathway of glucose metabolism including glucose transport,
hexokinase, PFK, and glyceraldehyde-3-phosphate dehydro-
genase. Our transgenic mice also had a permanent reduction in
glycolysis and they exhibited most of the characteristics of
diabetic cardiomyopathy. Mild hypertrophy (31) and increased
fibrosis (32) are common findings in diabetic hearts. These
were seen in our transgenic mice as a > 5% increase in heart
to body weight in males and females, respectively. Fibrosis was
evident as clearly increased Sirius red staining. These affects
were not simply a function of cardiac overexpression of a trans-
gene, because we have not seen these pathologies with any of
the other cardiac-specific transgenes we have described (18, 22,
33–35). Similar hypertrophy has been reported for cardiac se-
lective deletion of GLUT4, which also reduced glycolysis (8).

In addition to altered morphology, diabetic hearts (31) and

| Line | Sex | n | Body wt | Heart wt | HW/BW |
|------|-----|---|---------|----------|-------|
| FVB  | Male| 21| 28.3 ± 0.7| 126.7 ± 3.6| 4.48 ± 0.08 |
| Mb7  | Male| 17| 29.4 ± 0.5| 138.5 ± 2.4∗| 4.74 ± 0.09b |
| FVB  | Female| 20| 24.7 ± 0.8| 110.2 ± 3.5| 4.49 ± 0.13 |
| Mb7  | Female| 13| 23.9 ± 0.5| 123.7 ± 5.4a| 5.18 ± 0.21a |

∗p < 0.01 for Mb7 versus FVB.

†p < 0.05 for Mb7 versus FVB.

FIG. 5. Collagen accumulation in FVB and Mb7 hearts. Sirius
red staining was visualized at ×40 and photographed. A, repre-
sentative collagen staining for FVB and Mb7 mice is shown. B, scoring of collagen
content in 14 photographs taken from FVB (3) and Mb7 (4) mouse
hearts is shown. Staining was rated by a blind observer on a scale of
0–2, where 0 indicates mild, 1 increased, and 2 severe interstitial
accumulation of collagen. The values shown are mean ± S.E. and were
analyzed by Student’s t test (∗, p < 0.01).

FIG. 6. Contractile properties of ventricular myocytes isolated
from FVB and Mb7 hearts. A, % peak shortening is reduced in Mb7
transgenic mice both under normoxic and hypoxic conditions. B, maxi-
mal velocities of cell shortening (+dL/dt) and relengthening (~dL/dt)
are reduced by induction of hypoxia. C, TR90 in Mb7 transgenic mice is
increased both under normoxic and hypoxic conditions. The values
shown are mean ± S.E., n = 120 cells/group, six mice/group. The values
were analyzed by two-way ANOVA followed by Bonferroni post-hoc test.
∗, p < 0.01 compared with FVB. All the hypoxic values were signifi-
cantly different from normoxic values, except TR90.
cardiomyocytes from diabetic animals (22) display reduced contractility and greater sensitivity to hypoxia. Cardiomyocytes from transgenic Mb7 mice showed clearly reduced contractility and the degree of impairment was similar to what we have observed in Type I and Type 2 diabetic models (36). In addition to reduced contractility, transgenic myocytes exhibited delayed relaxation, an essential characteristic of diabetic hearts (37) and cardiomyocytes (38). Diabetic hearts are also more sensitive to ischemia (39). Impaired glycolysis contributes to this sensitivity because glycolysis is the only remaining source of ATP production in a hypoxic environment. We found that Mb7 myocytes had reduced function during hypoxia compared with FVB myocytes. However, because their function was already reduced before hypoxia it is not clear if this indicates a true increase in hypoxic sensitivity. These results indicate that merely by reducing glycolysis, even without hyperglycemia, we can produce a model with all or most of the characteristics of diabetic cardiomyopathy. This is consistent with the findings of Belke et al. (40) that overexpression of GLUT4 can protect from diabetic cardiomyopathy. All of these reports suggest that impaired glycolysis contributes to the cardiac pathology common in diabetics.

Insulin resistance was an unexpected but an obvious effect of expression of the kd-PFK-2 protein. Resistance was apparent as a 50–80% reduction in glycolytic response to insulin. It was also seen in a marked reduction in phosphorylation of Akt produced by insulin stimulation. Fru-2,6-P₂ has not been shown to have a direct effect on cardiac insulin signaling, perhaps because Fru-2,6-P₂ has not been directly altered before. However, Wu et al. (12) has recently shown that manipulation of liver Fru-2,6-P₂ content produces corresponding changes in the level of phosphorylated Akt. Because Akt phosphorylation is a prerequisite to the metabolic actions of insulin this may explain the resistance to insulin for glycolysis. The primary cause of insulin resistance and reduced Akt phosphorylation is not clear. One demonstrated mechanism is increased hexosamine flux (41, 42), which was indicated in this model by the 2-fold increase in levels of UDP-GlcNAc. Hexosamines are currently believed to exert their effects by increasing the levels of enzymatic O-linked glycosylation of cytosolic and nuclear proteins by N-acetylgalactosamine (43–45). For example, it has been recently demonstrated that elevated glycosylation by O-linked GlcNAc can inhibit insulin-dependent activation of Akt (43). Thus one possible explanation for insulin resistance in Mb7 hearts stems from the backup of glycolysis at PFK-1, leading to increased flux through the hexosamine pathway. Other direct or indirect effects of Fru-2,6-P₂ may contribute to the loss of insulin sensitivity.

In conclusion, we have shown in the present study that decreasing cardiac Fru-2,6-P₂ content produces significant changes in cardiac metabolism and pathology. In perfused hearts glycolysis and insulin sensitivity were decreased. In vivo transgenic hearts showed an elevation of glycolytic substrates upstream of PFK-1, which led to elevation of glycolygen and UDP-GlcNAc. The elevation in UDP-GlcNAc may have contributed to the development of insulin resistance. Also we found that reduced glycolysis was accompanied by mild hypertrophy and accumulation of collagen. In combination with impaired cardiomyocyte contractility these results indicate that a deficit in glycolysis is an important component of diabetic cardiomyopathy.
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