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ELEVATION OF CARDIAC GLYCOLYSIS REDUCES PYRUVATE
DEHYDROGENASE BUT INCREASES GLUCOSE OXIDATION

By

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B.S., Guangdong Medical College of China, 1999
M.S., University of Louisville, 2006

A Dissertaion
Submitted to the Faculty of the
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University of Louisville, School of Medicine
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May 2011

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ABSTRACT

ELEVATION OF CARDIAC GLYCOLYSIS REDUCES PYRUVATE DEHYDROGENASE BUT INCREASES GLUCOSE OXIDATION

Qianwen Wang

April 1, 2011

Heart failure is the most frequent cause of mortality in western countries. Currently, there is no cure treatment for heart failure and the long term survival rate following heart failure is poor, with one third of patients dying within a year of diagnosis. Thus, new therapeutic targets have to be developed.

Enhanced glycolysis is a very common phenomenon in the development of heart failure and maybe a target for drug development. However it is not know whether the increased glycolysis is a cause or an effect of heart failure. Also, metabolic modulators to increase glucose use by the heart have been used acutely in treatment in heart failure but the long term impact of increased glycolysis is not known. To understand whether chronically increased glycolysis specifically in the heart is beneficial or detrimental, glycolysis was chronically elevated by cardiac-specific overexpression of a modified, phosphatase-deficient 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase (PFK-2) in transgenic mice. PFK-2 controls the level of fructose-2, 6-bisphosphate (Fru-2, 6-P₂), an important regulator of phosphofructokinase and glycolysis. These transgenic mice were used to test two **hypotheses**: (1) Long term elevation of cardiac Fru-2, 6-P₂ will increase

glycolysis and alter glucose oxidation. (2) Chronically increased cardiac glycolysis will be detrimental to the heart.

To test these hypotheses we carried out three specific aims: Aim 1 was to produce transgenic mice with overexpression of phosphatase-deficient 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase (PFK-2). Aim 2 was to compare metabolites and glucose metabolism in transgenic and control samples using whole hearts, Langendorff perfused hearts and cultured adult cardiomyocytes. Aim 3 was to assess whether chronically increased glycolysis promotes cardiac fibrosis, hypertrophy or impaired function.

The results demonstrated a new line of transgenic mice called Mk, with cardiac expression of modified PFK2 and increased levels of Fru-2, 6-P₂. Mk hearts had elevated glycolysis that was less sensitive to inhibition by palmitate. Mk cardiomyocytes had increased glucose oxidation despite reduced pyruvate dehydrogenase complex (PDC) activity. PDC activity was decreased because of reduced protein levels of PDC subunit E1 α and because of increased PDC E1 α phosphorylation. Mk hearts had increased mitochondrial level of MCT-2 transporter protein and malate content. The increased malate content and elevated MCT2 expression suggested that anaplerosis pathways in transgenic hearts might explain the paradoxical finding of reduced PDC activity and elevated glucose oxidation.

Functional studies revealed that the elevation in glycolysis made transgenic cardiomyocytes highly resistant to contractile inhibition by hypoxia, *in vitro*. However, *in vivo* the transgene had no protective effects on ischemia-reperfusion injury. Furthermore, the transgenic hearts exhibited pathologic changes that included a 17% increase of the

heart weight-to-body weight ratio, greater cardiomyocyte length and increased cardiac fibrosis. Therefore, chronic elevation of glycolysis produced more pathological effects than protective effects on the heart.

TABLE OF CONTENTS

	PAGE
ACKNOWLEDGMENTS.....	iii
ABSTRACT.....	iv
LIST OF TABLES.....	viii
LIST OF FIGURES.....	ix
INTRODUCTION.....	1
METHODS AND MATERIALS.....	10
RESULTS.....	25
DISCUSSION.....	34
REFERENCES.....	99
CURRICULUM VITAE.....	107

LIST OF TABLES

TABLE	PAGE
1. Glycolytic intermediates concentration in FVB and Mk hearts.....	53
2. Mk hearts and myocytes are enlarged.....	89

LIST OF FIGURES

FIGURE	PAGE
1. A balance between glucose and fat consumption exists in the cardiomyocyte from normal heart.....	43
2. Alternative routes for carbon influx from glucose to the citric acid cycle (anaplerosis).....	45
3. Scheme for potential beneficial and detrimental effects of high glycolysis under myocardial ischemia.....	47
4. Transgenic hearts overexpress 6-phosphofructo-2kinase/fructose-2, 6-bisphosphatase (PFK-2) protein.....	49
5. Transgenic hearts have elevated fructose-2, 6-bisphosphate(Fru-2,6-P ₂).....	51
6. The kinase active PFK-2 transgene increases glycolysis in Langendorff perfused hearts.....	55
7. Effect of palmitate and insulin on glycolysis in FVB mice and Mk mice.....	57
8. The Mk transgene reduces palmitate oxidation in Langendorff-perfused hearts...	59
9. Levels of Fru-2, 6-P ₂ in FVB and Mk cardiomyocytes cultured for 24 hrs.....	61
10. Levels of Fru-1, 6-P ₂ in FVB and Mk cardiomyocytes cultured for 24 hrs.....	63
11. Measurent of glycolysis in Mk and FVB cardiomyocytes.....	65
12. Measurent of glucose oxidation in Mk and FVB cardiomyocytes.....	67
13. Active PDC and Total PDC activity are decreased in Mk transgenic	

cardiomyocytes.....	69
14. Active PDC and Total PDC activity are decreased in Mk transgenic hearts.....	71
15. PDC E1 α expression and phosphorylation in Mk transgenic and FVB control cardiomyocytes.....	73
16. PDC E1 α expression and phosphorylation in Mk transgenic and FVB control hearts.....	76
17. Increased amount of MCT-2 in isolated mitochondria from Mk transgenic hearts.	79
18. Malate content in whole heart.....	81
19. Oxaloacetate content in whole heart.....	83
20. Overexpression of kinase-active PFK-2 improves contractility under hypoxia.....	85
21. Myocardial infarct size (INF) was determined after 40 min of in vivo coronary occlusion and 24 h of reperfusion.....	87
22. Real time-PCR shows no elevation of BNP mRNA in MK transgenic mice.....	91
23. Real time-PCR shows no elevation of β -MHC mRNA in MK transgenic mice.....	93
24. H&E staining in Mk and FVB hearts.....	95
25. Fibrosis in Mk hearts.....	97

INTRODUCTION

Significance and Background

Heart failure, the most frequent cause of mortality in the western countries, is more prevalent than all cancers combined (71). Currently, no cure treatment for heart failure is available, and the long term survival rate following heart failure is poor, with one third of patients dying within a year of diagnosis (10). Thus, studies, focusing on identifying new therapeutic targets to prevent or reverse cardiac hypertrophy from the transition to heart failure, have to be developed.

In the development of heart failure, cardiac hypertrophy serves as an important risk factor and is explained largely by an increase in cardiac myocyte size due to pathological overload. In addition to structural changes in the myocardium, an altered utilization of cardiac metabolic substrate glucose is recognized as one of the biochemical hallmarks of the hypertrophied and failing heart (42). In the fetal heart, glucose is the primary cardiac substrate while fatty acid metabolism is reduced due to carnitine deficiency and delayed maturation of enzymes involved in fatty acid oxidation. In the healthy heart under resting conditions, the oxidation of fatty acid covers about 70% of the cardiac energy demand, with additional significant contributions from glucose and lactate (Figure 1). During the normal physiological growth of the heart including pregnancy-induced growth and exercise-induced cardiac hypertrophy, glucose utilization (includes glycolysis and glucose

oxidation) and fatty acid oxidation are enhanced. However, in the development of pathological cardiac hypertrophy and heart failure, fatty acid oxidation decreases, glycolysis increases while glucose oxidation varies (increases, decreases or no change) (10). Elevated glycolysis is considered to be an adaptive response to compensate for the reduced energy efficiency of the compromised heart (53). On the other hand, rather than being an adaptive response, elevation of cardiac glucose metabolism may be an example of the prominent reversion to fetal gene expression that occurs during heart failure (76). Decoupling of the rate of glucose oxidation from the rate of glycolysis has been demonstrated in experimental models of heart failure, and this is potentially detrimental to long term cardiac function (49). However it is not certain whether decoupling is a direct consequence of elevated glycolysis, or it occurs only in the context of heart failure.

Diabetes Reduces Glycolysis

Diabetes is one of the major risk factors in the development of heart failure, and diabetes has major effects on cardiac fuel metabolism. In the normal heart the rate of glycolysis is responsive to changing metabolic demand (8). In diabetes there is a marked reduction in the ability of the cardiac myocyte (75) to accelerate glycolytic rate due to decreased glycolytic capacity. This reduction in cardiac glucose usage is found both in the hearts of diabetic patients (4, 14, 80) and in experimental models of diabetes (75). Metabolic flux analysis (37) demonstrates that control of cardiac glycolysis is not limited to a single metabolic step; rather it is shared by several reactions, depending on ambient conditions. In the diabetic myocyte there is decreased activity in several critical steps of the glycolytic pathway ensuring that overall rate of glycolysis is impaired. Activity of glucose transporters (75), hexokinase (17), 6-phosphofructo-1-kinase (PFK) (55) and glyceraldehyde-3-phosphate dehydrogenase (19) all decline in diabetes. Mechanisms for reduced enzyme activity are multiple but include reduced gene expression for glucose transporters and hexokinase (12, 22) and elevated concentrations of metabolic inhibitors for hexokinase (15) glyceraldehyde-3-phosphate dehydrogenase and PFK (16).

In addition to reduced glucose usage, diabetic hearts also display an abnormally large reliance on fatty acid oxidation for energy. It has been proposed that this contributes to the development of diabetic cardiomyopathy (68). Fatty acids are the major source of fuel in the normal heart. But in diabetes this reliance is exaggerated. In part this is due to elevated circulating lipids. But it also appears to be a function of decreased glucose metabolism. Glucose metabolism produces increased intracellular levels of malonyl CoA

(5), a potent inhibitor of fatty acid conjugation to carnitine. This step is a major control point for movement of fatty acids into the mitochondria for oxidation. With reduced glucose metabolism, one of the normal braking systems for oxidation of lipids is lost.

Reduced Glycolysis and Diabetes Increase Vulnerability to Ischemic Damage

The reduced glycolytic activity of the diabetic heart is an important factor that predisposes to ischemic or hypoxic damage. In ischemic (54) hearts, dependence on glucose and glycolysis increases markedly. One of the protective responses of the heart is an increase in glycolysis (69, 74). In fact, stimulation of glycolysis with glucose and insulin has been used for many decades to protect patient hearts from ischemic or hypoxic damage (60, 61). In vitro experiments analyzing glycolysis and ischemia support this clinical practice. Studies in isolated perfused hearts reveal that manipulations to accelerate cardiac glucose use decrease ischemic damage (62, 83) while procedures that limit glycolysis tend to sensitize the heart to ischemia (31). In one of the genetic modification studies in this area, Tian et al., (78) found that cardiac specific knockout of the GLUT4 glucose transporter, a mutation that reduces cardiac glycolysis, sensitizes the heart to hypoxic damage. They proposed that the damage was similar to the damage seen in diabetic hearts. It is also established that one protective response of the heart to ischemic stress is a significant increase in glycolysis.

Diabetes reduces glycolysis, and studies of diabetic models reveal exacerbated ischemic damage that confirm the outcome found in diabetic patients. However, certain experimental variables can alter this outcome. The enhanced sensitivity of the heart is

most apparent when the diabetes is of long duration (66). Also, the diabetic deficit may be hidden if it is tested following complete blockage of flow. This is thought to be a function of lactate buildup in normal hearts (20, 38) which occurs during complete obstruction of flow. Diabetic hearts are protected from lactate build up by their lower rates of glycolysis (20, 38). Studies that use chronic models of diabetes and reduced flow, rather than zero flow ischemia find that diabetes increases ischemic damage (66).

The mechanism of glucose induced cardiac protection in ischemia has not been resolved. However, it is clear that glycolysis becomes the sole or primary source of ATP production in hypoxic hearts. Also, ATP derived from glycolysis seems to have a preferential role (85) in maintaining normal conductances for calcium, potassium and sodium ions, functions that are critical in maintaining cardiac myocytes viability during ischemia. Glycolytic enzymes are located in close proximity to the ATP dependent sarcoplasmic reticulum calcium pump (90) and the ATP regulated potassium channel(86). Active glycolysis has been shown to be critical in the activity of the sarcoplasmic calcium pump (90) and the ATP-sensitive potassium channel (86).

Reduced Glycolysis Predisposes to Cardiomyopathy

Genetic mutations, whether natural (3) or experimental (77), that impair glycolysis produce cardiac pathology. The most prominent finding in glucose transporter (GLUT4) knockout mice is cardiac hypertrophy (77). When additional knockout mice were made in which cre-loxP technology was used to confine the GLUT4 knockout to the heart, cardiac hypertrophy was still observed (1). These results indicate that chronic impaired

glucose metabolism promotes cardiac damage. Conversely it appears that enhanced glycolysis may prevent diabetic cardiomyopathy. A report by Belke et al., described the effect of overexpression of the GLUT4 glucose transporter on diabetic cardiomyopathy in (7). They found that db/db mice with a glucose transporter transgene had markedly improved cardiac contractility compared to nontransgenic db/db mice. However, conclusions based on this exciting result must be tempered by the fact that the glucose transporter transgene was active in many tissues. Cardiac protection may have been secondary to extra-cardiac actions of the transgene, such as reductions in blood glucose levels (23).

A number of findings from non-genetic studies support the hypothesis that altered glucose metabolism can contribute to cardiomyopathy. Apoptosis of cardiac myocytes, which is known to occur during heart failure (52), is inhibited by manipulations that increase glycolysis (50). Bishop and Altrud (11) reported 30 years ago that glycolytic metabolism is increased in cardiac hypertrophy and congestive heart failure. This may be an adaptive mechanism to take advantage of the higher yield of ATP from carbohydrate metabolism. Studies utilizing *in vitro* application of inhibitors of glucose metabolism, such as 2-deoxyglucose report weakened contractility, even under well-oxygenated conditions (36). Notably, the most prominent finding described was impaired diastolic relaxation, which is also the most prominent defect in diabetic cardiomyopathy (73).

Therapeutic Implications

Traditional clinic treatment of heart failure involves management of risk factors and control of symptoms by using angiotensin converting enzyme inhibitors, angiotensin receptor blockers, beta blockers, diuretics, aldosterone antagonists and digitalis. However, these classic agents for treating heart failure have direct hemodynamic, inotropic or chronotropic effects, with significant potential for morbidity and mortality. Recently, metabolic modulators, such as trimetazidine, ranolazine, perhexiline and estomoxir, and other drugs have been developed as a new class of drugs that may act by shifting substrate utilization from fatty acid to glucose utilization, either directly through stimulation of glycolysis or indirectly through inhibition of fatty acid oxidation (63). Under normal physiological conditions, oxidation of fatty acid can yield higher ATP amounts compared with the oxidation of glucose (129 ATP per palmitate molecule versus 36 ATP per glucose molecule). However, in terms of oxygen cost, the oxidation of glucose is more efficient (ATP/O~3.1) than that of fatty acid (ATP/O~2.8). This means that switching from fatty acid utilization toward glucose oxidation is approximately 11% more economical in terms of oxygen cost in the heart (81). Therefore, the fact that metabolic modulators can shift the heart to use more glucose is thought to be beneficial. However, all these metabolic modulators are only acutely used in treatment in heart failure and the impact of increased glycolysis by this class of drugs on overall mortality for the long term use is still under investigation. (63) In order to reveal long term effects of increased cardiac glucose utilization on heart, a transgenic mice model (Mk) with

chronically cardiac specific increased glycolysis by overexpression of kinase active PFK-2 was established.

PFK-1 transgene and Transgenic mice

The control of cardiac glycolysis is shared by several reactions (37). One of the control reactions is carried out by 6-phosphofructo-1-kinase (PFK-1) (29, 55), which catalyzes the phosphorylation of fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate (Fru-1,6-P₂). F6P is in equilibrium with glucose-6-phosphate (G6P), 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 F6P, PFK-1 can also modulate several important reactions branching off of glycolysis. PFK-1 activity is tightly controlled and negatively regulated by products of fat metabolism, including ATP and citrate. The most important positive regulator of PFK-1 is fructose-2, 6-bisphosphate (Fru-2, 6-P₂). The bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase (PFK-2) catalyses the synthesis and degradation of Fru-2, 6-P₂. PFK-2 is, in turn, regulated by a complex network of kinases, phosphatases, and metabolites (59). In this study, we used a cardiac-specific transgene for a mutant form of liver PFK-2 to directly elevate cardiac Fru-2, 6-P₂ and increase glycolysis without inducing heart failure.

Hypothesis and Aims

To understand whether chronically increased glycolysis specifically in the heart is beneficial or detrimental, glycolysis was permanently elevated by cardiac-specific overexpression of a modified, phosphatase-deficient 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase (PFK-2) in transgenic mice. PFK-2 controls the level of fructose-2, 6-bisphosphate (Fru-2, 6-P₂), an important regulator of phosphofructokinase and glycolysis. These transgenic mice were used to test two **hypotheses**: (1) Long term elevation of cardiac Fru-2, 6-P₂ will increase glycolysis and alter glucose oxidation. (2) Chronically increased cardiac glycolysis will be detrimental to the heart.

To test these hypotheses the following specific aims were carried out:

Specific Aim 1: Produce transgenic mice with overexpression of phosphatase-deficient 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase (PFK-2).

Specific Aim 2: Compare metabolites and glucose metabolism in transgenic and control samples using whole hearts, Langendorff perfused hearts and cultured adult cardiomyocytes.

Specific Aim 3: Assess whether chronically increased glycolysis promotes cardiac fibrosis, hypertrophy or function.

METHODS AND MATERIALS

Animals

The bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2) controls levels of F-2,6-P₂ by catalyzing two opposing reactions: Fru6P + ATP → Fru-2,6-P₂ + ADP and Fru-2,6-P₂ → F6P + Pi. The development of the kinase active/bisphosphatase deficient PFK2 mutant has been previously described (38, 86). Cardiac specific expression was obtained by ligating a 1.6 kb KpnI/HindIII fragment of kinase active liver PFK2 (86) behind the α -myosin heavy chain (MHC) promoter(22). The transgene was designated Mk (for the MHC promoter and the kinase active PFK2 gene). Transgenic mice were produced on a FVB background and maintained as heterozygotes by breeding to FVB mice. Standard embryo microinjection procedures were used for producing transgenic animals. All procedures conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the USDA certified.

PFK-2 Expression Analysis

PFK-2 protein was analyzed by western blot analysis as previously reported (17). In brief, PFK-2 was extracted from fresh hearts by homogenization in buffer containing 20

mM N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES), 1 mM dithiothreitol, 100 mM KCl, 5 mM Ethylenediaminetetraacetic acid (EDTA), 5 mM Ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) (pH 7.8), 1.2 mM phenylmethylsulfonyl fluoride, 2.5 mg/liter leupeptin. After precipitation with 40% PEG, the pellet was dissolved in buffer containing 20 mM TES, 1 mM dithiothreitol, 100 mM KCl, 0.1 mM EDTA (pH 7.5), 0.5 mM phenylmethylsulfonyl fluoride, 2.5 mg/liter leupeptin. The concentration of extracted protein was measured by the BCA method (Pierce Chemical Co.). Equal amounts of extracted protein (100 μ g) were used for Western blots. Rabbit anti-rat liver PFK-2 serum was used as primary antibody at a 1:1,000 dilution. Bands were visualized using horseradish peroxidase-conjugated goat anti-rabbit secondary antibody and enhanced luminol-based chemiluminescent (ECL). (Amersham Life Science, Buckinghamshire, UK)

Measurement of Metabolites

For the assay of G6P, F6P and Fru-1,6-P₂, hearts were homogenized in 1 M ice-cold perchloric acid and centrifuged. The supernatants were neutralized with 2 M KHCO₃. The supernatant from neutralized tissue extracts was used for estimation of these metabolites by fluorometric method (45) .

Cardiac F-2,6-P₂ was extracted from fresh heart tissue in 10–20 volumes of 50 mM NaOH and kept at 80°C for 5 minutes. The extract was cooled and neutralized at 0°C by the addition of ice-cold 1 M acetic acid in the presence of 20 mM HEPES. After centrifugation at 8,000g for 10 minutes, supernatant was collected and assayed for Fru-2,6-P₂ by the PFK-1 activation method(80). For the assay of Fru-2, 6-P₂, the buffer

containing pH8.0 100mM TES, 6mM MgCl₂, 1mM EDTA, 0.1mM Dithiothreitol (DTT), 0.22mM β-Nicotinamide adenine dinucleotide (NADH), 100mM F-6-P, 10mg/ml P_{Pi}:PFK-1, aldolase, Glycero-3-phosphate Dehydrogenase (GDH), Triosephosphate isomerase (TIM). The reaction mixtures were kept at 30⁰C for 10 min, then read by spectrophotometer at 340nm wave length.

For the assay of glycogen snap frozen tissue was weighed, homogenized in 9 vol. of 30% KOH at 0°C and then heated at 70°C for 30 min. The glycogen was precipitated with absolute alcohol and saturated sodium sulfate and re-dissolved in 0.1 M acetate buffer, pH 4.7. Then 10 µl of homogenate glycogen was hydrolyzed to glucose by the addition of 50 ng amyloglucosidase (Boehringer-Mannheim) in 100 µl of 0.1 M acetate buffer. Glucose was then measured by fluorometric assay using hexokinase and glucose-6-phosphate dehydrogenase as we reported earlier (42).

Cardiac Perfusion

Langendorff perfusions were carried out as we previously described (41, 42). The heart was rapidly cannulated through the aorta and retrogradely perfused at 2 ml/min with Krebs-Henseleit buffer (KH) consisting of 120 mM NaCl, 20 mM NaHCO₃, 4.6 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgCl₂, 1.25 mM CaCl₂, 5 mM glucose. Throughout the perfusion KH buffer was continuously equilibrated with 95% O₂/5% CO₂ which 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). Perfusion pressure and contractility was monitored continuously during the perfusion, as described previously (41, 42).

Measurements of glycolysis, palmitate oxidation and lactate production in perfused hearts

Glycolysis was measured using [5-³H]glucose as the substrate as we have previously reported (7). For studying the effect of insulin, baseline glycolysis was determined for the first 30 min followed by 50 min in the presence of 200 μ U/ml insulin. Glycolysis in the presence of palmitate was measured by bringing the perfusate to 0.4mM palmitate (7) by addition of 1/20th volume of 8mM palmitate bound to 10% Albumin from bovine serum (BSA) in KH buffer.

Lactate production and glycolysis measurement with 5-³H-glucose were performed with perfusion buffer containing 5 mM glucose as previously described (17). Palmitate oxidation was measured by perfusion with 5 mM glucose plus 0.4 mM palmitate containing 0.75 μ Ci per ml [9, 10-³H] palmitate. Tritiated water produced from tritiated glucose or palmitate during cardiac perfusion was measured by diffusion and scintillation counting. Effluent from each time point of the perfusion 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 measured in each experiment using tritiated water. Lactate concentration was measured in 6-fold diluted effluent using Eaton Bioscience Inc. Kit-11112.

Culture of adult mouse cardiac myocytes

Cardiomyocytes from adult mouse hearts were isolated and cultured by modification of a previously published protocol (56). Briefly, mice were anesthetized with isoflurane and injected with heparin (1.7 IU/g body weight). The heart was rapidly removed and perfused through the ascending aorta for 4 min with perfusion buffer (120 mM NaCl, 15 mM KCl, 0.6mM KH₂PO₄, 0.6mM NaH₂PO₄, 1.2mM MgSO₄, 10mM HEPES, 30mM Taurine, 10mM 2,3-butanedione monoxime, and 5.5mM glucose, pH 6.95). After 4 min, the perfusion was switched to perfusion buffer supplemented with collagenase II 500U/ml perfusion buffer (Worthington, NJ) for 8-11 minutes. The ventricles were then teased into 10-12 small pieces in stop buffer (perfusion buffer with 10% bovine calf serum and 12.5μM calcium) and transferred to a 15ml conical tube. Tissue was further dissociated by pipetting and myocytes were allowed to sediment by gravity for 10 minutes. After removal of supernatant, the pellet was carefully suspended in stop buffer. Calcium was reintroduced in three steps at room temperature to reach a final concentration of 1.2 mM. Isolated ventricular myocytes were plated at a density of 50 rod-shaped myocytes per square millimeter on laminin-coated cover slips in 10cm² culture tubes (TPP, Switzerland) containing plating medium (MEM, 10% bovine calf serum, 10mM 2,3-butanedione monoxime, 100Units/ml penicillin, 2mM glutamine and 2mM NaATP), in 2% CO₂, 37°C. Following 1 hr incubation, the plating medium was removed and washed once. Myocytes were incubated in 2% CO₂, 37°C overnight in culture medium (MEM containing 10 ug/ml BSA, 1 unit/ml penicillin and 20 um glutamine with or without 0.4 mM palmitate prebound to 30% fatty acid-free BSA).

Measurement of glycolysis and glucose oxidation in cardiomyocytes

After 24 hours culture, medium was replaced with new medium containing [5-³H]glucose (0.25 μCi/ml) and [U-¹⁴C]glucose(0.15μCi/ml) in a final volume of 2 ml. For the no palmitate group, both the old culture medium and assay culture medium did not contain palmitate. For the 2 hour palmitate group, overnight culture medium did not contain palmitate and assay culture medium contained 0.4 mM palmitate. For 24 hour palmitate group, culture medium containing 0.4 mM palmitate and assay culture medium did not contain palmitate. For 24 plus 2 hour palmitate group, all culture medium contained 0.4mM palmitate. The culture tube was sealed with a rubber stopper and a plastic central well with a cellulose filter paper and incubated at 37°C for 2 hours. After the 2 hr assay incubation, the reaction was stopped by the injection of 70% perchloric acid (0.2 ml), which also liberates ¹⁴CO₂ dissolved in the cultured medium. 0.1 ml of 1M NaOH was injected into the central well containing filter paper for measuring glucose oxidation by quantitative collection of ¹⁴CO₂ released. The culture tube was held at room temperature overnight and the central well filter paper was taken for scintillation counting. Glycolysis was determined by measuring the production of ³H₂O from the [5-³H]glucose added to the media as previously described (82). The values determined for glycolysis and glucose oxidation were normalized to the number of cardiomyocytes to yield units of nmol/min/cardiomyocyte.

Measurement of Fru-2, 6-P₂ and Fru-1,6-P₂ in adult mouse cardiomyocytes

For the assay of Fru-2, 6-P₂, cardiomyocytes cultured for 24 hours were collected in 50mM NaOH (80 ul) and kept at 80 °C for 5 min. The cells were cooled and neutralized at 4 °C by the addition of ice-cold 1 M acetic acid in the presence of 20 mM HEPES. After centrifugation at 8,000 g for 10 min, the supernatant was collected and assayed for Fru-2, 6-P₂ by the PFK-1 activation method (80). For the assay of Fru-2, 6-P₂, the buffer containing pH8.0 100mM TES, 6mM MgCl₂, 1mM EDTA, 0.1mM DTT, 0.22mM NADH, 100mM F-6-P, 10mg/ml PPI:PFK-1, aldolase, Glycero-3-phosphate Dehydrogenase (GDH) and Triosephosphate isomerase (TIM). The reaction mixtures were kept at 30⁰C for 10 min, then read by spectrophotometer at 340nm wave length. For the assay of Fru-1, 6-P₂, cardiomyocytes were collected in 1M ice-cold perchloric acid (0.1 ml) and centrifuged. The assay was run as previously described (82).

Active and Total PDC measurement in adult mouse cardiomyocytes

Active PDH was measured as described essentially as described (92). After overnight culture medium was removed and cardiomyocytes in the flask/tube were inserted quickly into liquid nitrogen. Cells were collected in homogenization buffer (0.25 ml) containing 50 mM Hepes, pH 7.5, 0.2 mM KCl, 5 mM dithiothreitol, 0.1 mM Na-p-tosly-L-lysine chloromethyl ketone, 0.1 mmol/l trypsin inhibitor, 0.02 IU/ml aprotinin, 2% rat serum, and 0.25% (v/v) Triton X-100; then sonicated for 10 seconds by using 10% duty cycle and output control of 1 (Branson sonifier 450). Twenty µl of cell extracts and 20 ul of

reaction buffer (50 mM Hepes, pH 7.5, 1 mM MgCl₂, 3 mM NAD, 0.4 mM thiamine pyrophosphate, 0.4 mM CoA, 2 mM dithiothreitol, 0.1% Triton X-100, 7.5 U/ml lipoamide dehydrogenase, 1 mM pyruvate, and [1-¹⁴C] pyruvate (5 μCi/500ul) were added to a plastic cup in a 20 ml scintillation vials sealed with a rubber stopper attached to a plastic central well with a cellulose filter paper soaked in 1 N NaOH (0.1 ml) for collecting ¹⁴CO₂. The scintillation vials were incubated at 37°C for 20 min, the reaction was stopped by injecting 100 μl of 70% perchloric acid. After incubating at room temperature overnight, the central wells containing filter papers were taken for scintillation counting. Activity in pmol/min was normalized to cell number.

Total PDC activity was measured as described by Liu et al. (87). Mg²⁺ and Ca²⁺ in this method were used to activate the intrinsic cardiomyocyte pyruvate dehydrogenase phosphatase activity which converts inactive PDC into active PDC (87, 92). For total PDC measurement 45 ul of cell extract from the above preparation were mixed and incubated with 5 ul buffer containing 1 mM CaCl₂, 1 mM of MgCl₂, and 5 mM dichloroacetate at 30°C for 30 min. The method for measuring total PDC activity was the same as used for measuring active PDC as described above.

Active and Total PDC measurement in adult mouse hearts

Mice were heparinized and anesthetized with isoflurane (3%). Hearts were rapidly cut from the vena cava and freeze-clamped in aluminum blocks pre-cooled in liquid nitrogen. Frozen myocardium was powdered under liquid nitrogen and half of the myocardium powder was used to measure PDC activity and half used for Western blot.

For analysis of the PDC activity, myocardium powder was homogenized for 10 seconds in 10 volumes of homogenization buffer using a 10% duty cycle and output control of 1 with a Branson sonifier 450. After centrifugation at 10,000 g, 4°C for 10 min, the supernatant was collected and assayed for active and total PDC activity as above described. PDC activity was normalized to protein content.

Isolation of mitochondria

Fresh heart tissue was excised and homogenized by a motor driven Teflon pestle (Wheaton scientific) in isolation buffer containing 220mM mannitol, 70 mM sucrose and 5mM MOPS, pH 7.4. The homogenate was centrifuged at 700g, 4°C for 10 min. The supernatant was moved to a new tube for centrifuging at 10,000 x g at 4°C for 10 min. The mitochondrial pellet was collected and washed again with isolation buffer.

Western blotting

Five or ten µg of total protein from freeze-clamped heart, cell lysates and mitochondrial protein were homogenized with lysis buffer containing 50 mmol/L Tris-HCl (pH 7.5), 5 mmol/L EDTA, 10 mmol/L EGTA, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 10 µg/mL pepstatin A, and 50 µg/mL phenylmethylsulfonyl fluoride. Tissue proteins were collected by centrifuging at 12,000 rpm at 4°C for 10 minutes. The protein concentration was determined, and the sample was then mixed with loading buffer (40

mmol/L Tris-HCl, pH 6.8, 1% SDS, 50 mmol/L dithiothreitol, 7.5% glycerol, 0.003% bromophenol blue) and heated at 95°C for 5 minutes and then subjected to electrophoresis on Bis-Tris 4-12% gels (Invitrogen) at 120 V. After electrophoresis of the gel, the proteins were transferred to a nitrocellulose membrane in transfer buffer containing 20 mmol/L Tris, 152 mmol/L glycine, and 20% methanol. The membranes were rinsed briefly in PBS and blocked in blocking buffer (5% milk and 0.5% BSA) at room temperature for 2 hours. The primary antibodies used were anti-PDH-E1 α (pSer232) rabbit pAb (Calbiochem), PDH-E1 α (pSer293) rabbit pAb (Calbiochem), PDH-E1 α (pSer300) rabbit pAb (Calbiochem), PDH-E1 α (E-23) rabbit polyclonal IgG (Santa Cruz Biotechnology), MCT2 (M-17) goat polyclonal IgG (Santa Cruz Biotechnology). The membranes were incubated with primary antibody overnight. On the second day, membranes were then washed 3 times with TBS-T containing 0.05% Tween-20 and reacted with secondary antibody for 1 hour. Antigen-antibody complexes were visualized by chemiluminescent signal using ECL (Amersham GE healthcare) and quantified using TotalLab TL100. Actin expression was used as a control. After detection, the membranes were stripped with stripping buffer (50 mmol/L Tris-HCl, pH 7.4, with 150 mmol/L NaCl and 0.1% β -mercaptoethanol) for 1 hour at room temperature.

Malate and Oxaloacetate measurement in adult mouse whole hearts

Hearts from transgenic and control groups were freeze-clamped. Frozen myocardium was powdered under liquid nitrogen and homogenized for 30 second in 2 volumes of ice-

cold 1M perchloric acid. The extract was centrifuged at 13,000 g, 4°C for 15 min. The supernatant was collected and was neutralized by the addition of 2M KHCO₃ (1.05 volumes of perchloric acid). After centrifugation at 13,000 g, 4°C for 10 min, the supernatant was collected and assayed for malate and oxaloacetate (45). For the malate assay, buffer consisted of 50mM 2-amino-2-methylpropanol buffer pH 9.9, 200uM NAD⁺, pH 9.9, 10mM glutamic acid , 3.5u/ml malate dehydrogenase, 0.4u/ml aspartate transaminase. The reaction mixtures were kept at room temperature for 5 min, then read by spectrophotometer at 340nm. For the oxaloacetate assay buffer of 50mM Tris-HCL buffer pH 8.1, 50mM NADH, 0.07u/ml malate dehydrogenase was used. The reaction mixtures were kept at room temperature for 5 min, then read by spectrophotometer at 340 nm wave length.

Murine *in vivo* myocardial ischemia-reperfusion and infarct size determination

Male FVB and Mk mice were subjected to *in vivo* left coronary artery occlusion and reperfusion as described previously(25, 30-33). Briefly, mice were anesthetized with intraperitoneal injections of ketamine hydrochloride (50 mg/kg) and sodium pentobarbital (50 mg/kg). The animals were then attached to a surgical board with their ventral side up. The mice were orally intubated with polyethylene (PE)-90 tubing connected to a Hugo Sachs mouse ventilator. The tidal volume and respiratory rate were set for each mouse based on body weight and according to standard mammalian allometric equations. The mice were supplemented with 100% oxygen via the ventilator side port. Throughout anesthesia, body temperature was monitored with a rectal thermometer and held constant

between 36-37 degrees Celsius with an infrared heating lamp. A median sternotomy was performed using an electric cautery, and the proximal left main coronary artery was visualized and completely occluded for 40 minutes with 7-0 silk suture mounted on a tapered needle (BV-1, Ethicon).

At 24 h of reperfusion, the mice were anesthetized as described previously, intubated, and connected to a mouse ventilator. A catheter (PE-10 tubing) was placed in the common carotid artery for Evans blue dye injection. A median sternotomy was performed, and the left main coronary artery was re-ligated in the same location as before. Evans blue dye (~1.2 ml of a 2% solution) was injected into the carotid artery catheter into the heart to delineate the ischemic zone from the nonischemic zone. The heart was rapidly excised and serially sectioned perpendicular to the long axis in 1-mm-thick sections, which were then incubated in 1.0% 2,3,5-triphenyltetrazolium chloride for five minutes at 37°C to demarcate the viable and nonviable myocardium within the risk zone. Each of the five 1-mm-thick myocardial slices was weighed. A blinded observer assessed the areas of infarct, risk, and nonischemic zone using computer-assisted planimetry (Image J, version 1.38x). All of the procedures for area at risk (AAR) and infarct size determination have been previously described (30-33).

Cardiomyocyte responses to hypoxia

Cardiomyocytes were isolated, and myocyte contractility was measured as previously described (90). For the oxygenated assay, myocytes were placed in an open chamber at

22°C in HEPES-buffered KH buffer [135 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 10 mM HEPES, 0.33 mM NaH₂PO₄, 10 mM glucose, 10 mM 2,3-butanedione monoxime (BDM), and 1.2 mM CaCl₂; pH 7.4] for 2 h. BDM was included in the contractility assay buffer because it preserves rod-shaped myocytes during metabolic inhibition (37). We (89) have previously described the use of BDM during hypoxic assays of myocyte contractility. For hypoxia, cardiomyocytes were perfused in the same buffer saturated with N₂ for 15 min and maintained and assayed in a sealed chamber for 2 h at 22°C. Myocytes were stimulated at 1 Hz using platinum electrodes attached to the chamber. The mechanical properties of ventricular myocytes were assessed using a video-based edge detection system (IonOptix, Milton, MA) as previously described (90). Cell shortening and re-lengthening were assessed using the following indexes: peak shortening, time to 90% re-lengthening, and maximal velocities of shortening and re-lengthening ($\pm dL/dt$).

Histological Study

Collagen accumulation in the heart sections was determined as described previously (2). Five μ m 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, as we previously described (17). The section was given a score of 0 for mild accumulation, 1 for moderate accumulation, and 2 for severe accumulation.

Quantitative RT-PCR

RNA expression of cardiac pathological markers brain natriuretic peptide (BNP) and β -MHC was measured by real-time quantitative PCR using an ABI 7300 instrument (Applied Biosystems, Foster City, CA) with a modified protocol (9). In brief, total RNA was purified from the frozen heart and reverse transcribed to cDNA. RNA-derived cDNA (10 ng) was mixed with Taqman universal PCR Master Mix and the appropriate primers and probe and run at 50°C for 2 min, 95°C for 10 min, and 95°C for 15 s followed by 60°C for 1 min for 40 cycles. The assay identification numbers for the specific primers and probe of each gene were Mm01255779_g1 for BNP and Mm00600555_m1 for β -MHC (Applied Biosystems). The results for β -MHC and BNP were normalized to ND4 mRNA level.

Statistical analysis

Two factor comparisons were analyzed by two-way ANOVA and Student Newman-Keuls post hoc test. Single comparisons were performed by paired or unpaired Student's t-test. The accepted level of significance was 0.05. Statistical analyses were performed with the program Sigma Stat.

RESULTS

Transgenic lines

Transgenic mice were generated with the transgene Mk, which contained a kinase-active/bisphosphatase deficient mutant of liver PFK-2 (85) regulated by the cardiac specific β -MHC promoter (22). Six lines of Mk transgenic mice were generated on a FVB background and were designated as lines Mk1 to Mk6. The founders of *lines 1, 2, 4, and 5* produced litters. Western blots with antibody made against liver PFK-2 (Fig. 4) demonstrated obvious overexpression of the transgene in Mk mouse hearts. To confirm the increase in PFK-2 activity, we measured the level of Fru-2, 6-P₂ in transgenic and control hearts. Cardiac Fru-2, 6-P₂ was elevated by three- to fourfold in all transgenic lines (Fig. 5). Mk1 and Mk2 mice were produced first and had indistinguishable phenotypes for gene expression and Fru-2, 6-P₂ levels, and they were used interchangeably in experiments concerning phenotype.

Cardiac metabolite concentrations

By modifying PFK activity, the Mk transgene is expected to alter the concentration of metabolites of the glycolytic pathway. Levels of several metabolites were measured in flash-frozen hearts from FVB and Mk transgenic mice that had been fasted for 3 h. Table 1 shows these results. G6P was reduced by ~1.6 fold in Mk transgenic hearts. Coincident with the reduction in G6P levels, glycogen was decreased by more than eightfold in Mk hearts. Two metabolites downstream of PFK, Fru-1, 6-P₂ and pyruvate, were increased in concentration; however, the increase did not reach statistical significance. Fru-1,6-P₂ was measured as the combined pool of Fru-1,6-P₂, dihydroxyacetone phosphate, and glyceraldehyde-3-phosphate since these metabolites are in equilibrium and are most accurately measured together (45).

Effect of kinase-active PFK-2 on cardiac metabolism

The effect of the Mk transgene on glycolysis was assessed in Langendroff-perfused hearts by measuring the metabolism of 5-tritiated glucose. The release of tritiated water measures the glycolytic steps through the triose phosphate isomerase reaction. The amount of lactate produced during the perfusion was also measured. As shown in Figure 6, in both the presence and absence of palmitate, the release of tritiated water and lactate were significantly elevated in Mk transgenic hearts compared with control hearts. The Mk effect on glycolysis was evident both before and after the addition of insulin. The

increase in glycolysis did not appear to be related to contractility or perfusion pressure since no significant differences were observed for contractility between transgenic and control hearts at any time point (data not shown). Also, in both groups, the perfusion pressure averaged 65 mm Hg while the flow rate was maintained at 2 ml/min. To determine if the transgene altered the effect of palmitate and insulin, the results for tritiated water release were replotted in Figure 5. Before the addition of insulin, we found palmitate to be a more potent inhibitor of glycolysis in FVB mice (55% inhibition) than in Mk mice (18% inhibition). In the presence of insulin, palmitate did not reduce glycolysis in FVB or Mk hearts. Our laboratory (17) has previously reported that a transgene that reduced cardiac Fru-2, 6-P₂ blunted the insulin response in perfused hearts. However, the present study (Figure 7, C and D) failed to support the converse: compared with FVB hearts, there was no increase in the insulin response in Mk hearts with increased Fru-2, 6-P₂. In the absence of palmitate, insulin increased glycolysis by 39% and 31%, respectively, in FVB and Mk hearts. In the presence of palmitate, the insulin response was markedly increased, by 230% of basal in FVB mice and by 93% of basal in Mk hearts. The very large insulin response in palmitate-perfused FVB hearts was due primarily to the potent inhibition of basal glycolysis by palmitate (Fig. 7A), which was eliminated by the addition of insulin. Folmes et al. (20) have also reported that insulin has a greater effect on glycolysis in the presence of palmitate. In addition to the Mk-induced increase in glycolysis, we found a 30–50% significant reduction in palmitate oxidation in Mk hearts that was true throughout the duration of the perfusion (Fig. 8A). Insulin produced a significant reduction ($P = 0.01$ by paired t -test) in palmitate oxidation in both FVB and Mk hearts. The average decrease produced by insulin was $32 \pm 1\%$ and $42 \pm 6\%$

for FVB and Mk hearts, respectively (Fig. 8B), which was not significantly different in the two types of hearts (FVB vs. Mk, $P = 0.13$).

Efficacy of the Mk transgene in cultured cardiomyocytes

To confirm that the Mk transgene in cultured cardiomyocytes retained the activity we found in whole heart, the products of PFK1 and PFK2 were measured. As shown in Figure 9 and Figure 10, Fru-2, 6-P₂ was 2.8 fold higher in Mk myocytes compared to FVB myocytes and Fru-1, 6-P₂ was increased by 1.7 fold. This demonstrates that the transgene was still effective in 24 hr cultured cardiomyocytes.

Glycolysis, glucose oxidation and effect of palmitate on glycolysis and glucose oxidation in cultured adult mouse cardiac myocytes

Glycolysis and glucose oxidation were measured in cardiomyocytes to test the effect of elevated Fru 2,6P₂ on cardiomyocyte metabolism and to assess how it interacted with the effect of palmitate. Figure 11 shows the results for glycolysis. Mk myocytes had significantly higher rates of glycolysis than FVB myocytes in the presence or absence of palmitate and palmitate had similar inhibitory effects on glycolysis in transgenic and control myocytes. To reduce glycolysis required the 24 hour pre-assay exposure to palmitate, no inhibition was seen if palmitate was present only during the 2 hour assay. Figure 12 shows the glucose oxidation rates measured in the same myocyte cultures. Mk

myocytes had higher rates of glucose oxidation than FVB myocytes except under the No Palmitate condition when palmitate was absent before and during the oxidation assay. Palmitate inhibited glucose oxidation in transgenic and control myocytes under all treatment conditions. Unlike glycolysis, palmitate inhibited glucose oxidation even if it was not present during the 24 hr culture period.

PDC activity in Mk hearts and cardiomyocytes

Pyruvate dehydrogenase complex (PDC) activity is generally rate limiting for mitochondrial glucose oxidation. PDC activity was measured to determine if it could account for the increase found in cardiomyocyte glucose oxidation. Unexpectedly, both active PDC (PDCa) and total PDC activity (PDCt) were significantly ($P < 0.01$) reduced in Mk compared to FVB cardiomyocytes (Figure 13). Mk PDCa activity was reduced by 59% without palmitate and 37% with palmitate. Mk PDCt activity was reduced by 46% without palmitate and 52% with palmitate.

To be sure that the Mk decrease in PDCa and PDCt activity was not an artifact of cardiomyocytes isolation or culture conditions, PDCa and PDCt was measured in whole hearts. As shown in Figure 14 PDCa activity and PDCt activity were significantly ($P < 0.01$) reduced in Mk transgenic hearts compared to FVB hearts. The magnitude of the whole heart Mk reduction was approximately 20% for PDCa and PDCt.

PDC E1 α in cardiomyocytes and whole hearts

Western blots were used to measure the amount and phosphorylation state of PDC subunit E1 α (Figure 15A) in cardiomyocytes. Consistent with the reduction PDC activity, the abundance of PDC E1 α was reduced by 52% in Mk cardiomyocytes. The activity of PDC is acutely controlled by reversible phosphorylation of 3 serine residues on the E1 α subunit. Western blots using antibodies specific for different phosphorylation sites on PDC E1 α revealed a two-fold increase in E1 α P Ser 293 but no apparent change in the levels of E1 α P Ser 232 or P Ser 300. However, when levels of phosphorylated forms of PDC E1 α were normalized to total E1 α protein both P Ser 293 and P Ser 300 were significantly increased compared to FVB (Figure 15C).

Western blots of whole heart protein produced similar, though more modest differences between Mk and FVB samples (Figure 16). In Mk heart E1 α protein was significantly reduced and E1 α P Ser 293 was significantly increased. Normalized to the amount of E1 α content, P Ser 293 and P Ser 232 were significantly elevated in Mk heart.

Alternative pathways for glucose oxidation

Elevated glucose oxidation despite reduced PDC activity indicated that Mk myocytes must have other means for entry of glycolytic products into the mitochondria and citric acid cycle. One proposed mechanism, independent of PDC, for lactate or pyruvate entry into mitochondria is via the monocarboxylate transporter (MCT). MCTs have been identified on mitochondria of many tissues including heart (12, 24). Our unpublished

gene array comparison of Mk and FVB hearts revealed a 70% increase in MCT2 expression. Based on this suggestive data we isolated mitochondria from Mk and FVB hearts and performed a Western blot for MCT2 (Figure 17). The level of MCT-2 in Mk mitochondria was significantly increased by 2.15 fold over FVB cardiac mitochondria.

Multiple anaplerotic pathways exist for glucose oxidation including conversion of pyruvate to malate via malic enzyme and conversion of pyruvate to oxaloacetate via pyruvate carboxylase. As shown in Figure 18 malate content was significantly increased by 84% in Mk hearts, whereas oxaloacetate was the same in FVB and Mk hearts (Figure 19). Since Pound et al., (65) showed that malate is increased in hearts with elevated anaplerosis via malic enzyme our results are at least suggestive of increased anaplerosis via malate in Mk hearts.

Myocyte Contractility in Hypoxia

To determine the effect of increased glycolysis on cardiomyocyte contractility and response to hypoxia in Mk transgenic hearts, myocytes were isolated from Mk and FVB hearts and incubated with oxygenated or nitrogen bubbled Krebs buffer. Myocyte contractility was measured using video based edge detection system and the results are shown in figure 20. Under normoxic condition there was no significant difference in contractility between Mk and FVB myocytes, as assessed by $\pm dl/dt$ and % peak shortening. However, under hypoxic condition, Mk myocytes showed significantly increased contractility compared with FVB control myocytes for all three parameters. In FVB myocytes hypoxia reduced all of the variables of contractility by twenty to over

thirty percent, but in Mk myocytes hypoxia had a zero to ten percent effect. These results indicate that the Mk transgene provided almost complete protection from hypoxia-induced contractile deficits in our culture conditions.

Myocardial infarct size

In order to establish whether the cytoprotective effects associated with Mk transgenesis in isolated myocytes extended to the *in vivo* setting, we subjected FVB and Mk mice to experimental myocardial infarction. As shown in Figure 21, the areas-at-risk for infarction with respect to the left ventricular size (AAR/LV) were not significantly different between FVB (46.3 +/- 3.3%) and Mk (49.3 +/- 6.3%) groups. Infarct size expressed relative to the AAR (INF/AAR) was also not different between FVB (28.7 +/- 5.1%) and Mk (26.2 +/- 4.8%). Likewise, the INF expressed relative to the entire LV (INF/LV) was not different between FVB (13.5 +/- 2.8%) and Mk (12.8 +/- 2.8%).

Cardiac Hypertrophy in Transgenic Hearts

Despite the fact that the Mk transgene was beneficial to hypoxic cardiomyocyte function we noted an increase in cardiac size. We found that heart weight and heart to body weight ratio were significantly increased by ~17% compared to FVB control mice, whereas body weights were similar between Mk mice and FVB mice (Table 2). To determine if this corresponded to a change in cardiomyocyte size we also compared isolated cardiomyocyte length (right column of Table 2), measured using video based

edge detection. Cardiomyocyte length in Mk transgenic hearts was significantly longer than that in FVB hearts. The cardiac hypertrophy raised the possibility that elevated glycolysis was detrimental to the heart. Expression of two markers of cardiac stress, β -*MHC* and BNP were unchanged (Figure 22 and 23) and H&E staining (Figure 24) of Mk hearts appeared normal. However, sirius red staining for fibrosis appeared to be consistently increased in Mk hearts (Figure 25A) and this was confirmed by semi-quantitative analysis (Figure 25B).

DISCUSSION

Lipid metabolism provides most energy for cardiac contraction but under some pathological conditions such as hypertrophy and ischemia-reperfusion (2), glucose metabolism increases. It is not clear whether this increase in glucose metabolism is a beneficial, adaptive response of the heart or a detrimental, maladaptive response. To avoid the effect of cardiac pathology investigators have employed overexpression of glucose transporters in the heart to increase glycolysis. Belke et al (7) found that overexpression of human GLUT4 increased glycolysis but this did not increase glucose oxidation. On the other hand cardiac overexpression of GLUT1 (43, 46) increased both glycolysis and glucose oxidation.

Mk transgenic mice

In the current experiments, we developed transgenic mice designed to have a specific and permanent increase in cardiac glycolysis, independent of hypertrophy or ischemia. Multiple lines of mice were produced with a cardiac specific, bisphosphatase deficient mutant of the enzyme PFK2, designated transgene Mk. The transgene produced a three to four fold increase in cardiac levels of Fru-2, 6-P₂, which is a potent stimulator of the glycolytic regulatory enzyme, PFK1. In this study, we show multiple effects of the transgene. It increased glycolysis and glucose oxidation but reduced total and active PDC activity. Despite depletion of available PDC, coupling between glycolysis and glucose oxidation was maintained possibly via anaplerosis through malate. The Mk transgene

reduced palmitate oxidation, and made glycolysis less susceptible to inhibition by palmitate. The transgene markedly improved myocyte contractility during hypoxia, but it did not alter infarct size in response to ischemia in vivo. Despite some beneficial actions, the Mk transgene promoted hypertrophy and cardiac fibrosis.

For effects produced by the Mk transgene to provide relevant information about the role of Fru-2, 6-P₂ in cardiac pathology, it is important that the increase in Fru-2, 6-P₂ concentration in Mk transgenic hearts be similar to the concentrations achieved in known conditions of cardiac pathology. Pressure over-load hypertrophy induces a 2-6 fold increase in Fru-2, 6-P₂ (6, 51). Anoxia or ischemia results in a 2-3 fold elevation in cardiac Fru-2, 6-P₂ content (49). And stimulation of isolated cardiomyocytes with insulin produces a 4-fold increase in Fru-2, 6-P₂ content (39). Thus the three to four fold increase in Fru-2, 6-P₂ seen in our transgenic mice is similar to the increases produced by insulin, hypertrophy, anoxia or ischemia in the heart (6, 39, 49, 51)

Mk effects on glycolytic metabolism in vivo

An elevation in Fru-2, 6-P₂ levels should stimulate activity of PFK-1. If this is true, glycolytic metabolites upstream of PFK-1 should be reduced and metabolites downstream of PFK-1 should be increased in transgenic hearts. Our data showed that the concentration of Glc-6-P was significantly reduced by 1.6 fold. This reduction was likely the result of increased disposal of Fru-6-P by greater flux through PFK-1, creating a drain on Glc-6-P concentrations. Glycogen, the production of which branches off upstream of PFK-1, was diminished almost nine fold in transgenic hearts. The large magnitude of the glycogen effect may be due to the fact that Glc-6-P has two roles in the control of

glycogen content: Glc-6-P is a substrate for glycogen production and Glc-6-P is a positive regulator of glycogen synthase (54). In addition the increased PFK-1 activity promotes greater use of glycogen to fuel glycolysis. The expected increase in metabolites downstream of PFK1 was not as clear as the decrease in upstream metabolites. The concentrations of Fru-1, 6-P₂ and pyruvate increased 50 - 70 %, but the changes were not statistically significant. This smaller downstream effect may reflect cardiac adaptation to increased PFK-1 flux. This could be due to greater lactate disposal, as we saw in perfused transgenic hearts, or to greater mitochondrial oxidation of pyruvate obtained from glucose (32, 33, 35, 37, 54).

The conversion of F6P to Fru-1, 6-P₂ by PFK is one of the rate-limiting, regulated steps of cardiac glycolysis. Therefore, we anticipated an increase of glycolytic flux in perfused Mk hearts. Even in the absence of lipid, glycolytic flux in Langendorff perfused Mk hearts was consistently 20% higher than in nontransgenic hearts. This was not secondary to elevated contractility or to altered coronary vascular resistance since we detected no effect of the transgene on these variables. The most likely cause was the increased activity of PFK. These results support a significant role for Fru-2,6-P₂ even in the absence of lipid. The addition of 0.4 mM palmitate to the perfusate produced an apparent two-fold increase in the impact of the transgene on glycolytic flux. This was because palmitate depressed glycolysis less in Mk hearts than in FVB control hearts. Fatty acids inhibit glycolysis in large part by inhibiting PFK via the PFK inhibitors citrate and ATP. Fru-2, 6-P₂ antagonizes this inhibition (27). In addition, we found that palmitate oxidation was about twofold lower in Mk hearts; consequently, palmitate perfusion produced less citrate and ATP in Mk hearts than in FVB hearts. Therefore, we

suggest that a combination of elevated Fru-2, 6-P₂ and reduced production of citrate and ATP minimized the effect of fatty acids to inhibit glycolysis in Mk hearts.

Mk effects on glycolysis and glucose oxidation in cultured cardiomyocytes

To investigate *in vitro* metabolism changes in Mk mice, primary cultured adult cardiomyocytes were used. The level of Fru-2,6-P₂ was increased by 2.8 fold in Mk cardiomyocytes compared to FVB myocytes, which was consistent with increased Fru-2,6-P₂ in Mk whole heart and in concert, the level of Fru-1,6-P₂ was also significantly increased by 73% in Mk cardiomyocytes.

With increased Fru-2, 6-P₂, glycolysis in Mk cardiomyocytes increased by 40% in the both the presence and absence of palmitate and glucose oxidation was significantly increased by 37%-62% in Mk myocytes in the presence of palmitate. In the absence of palmitate, glucose oxidation had no significant difference between MK group and FVB group. The Randle cycle illustrates that there is a competition between glucose and palmitate for their oxidation. Overexpression of GLUT1 transgene (43, 46) increased glucose oxidation and decreased fatty acid oxidation. Cardiac specific overexpressed human GLUT4 (7) in mouse led to increase glycolysis but not in glucose or fatty acid oxidation.

In hypertrophied heart, the coupling of glycolysis and glucose oxidation becomes disrupted by a not fully delineated mechanism (40). However, increased glycolysis was coupled with increased glucose oxidation in our Mk cardiomyocytes. We expected an increase in PDC activity in our Mk transgenic hearts, because PDC, an important

regulator for glucose oxidation, links glycolysis to TCA cycle by catalyzing the irreversible oxidative decarboxylation of pyruvate to acetyl-CoA, CO₂ and NADH. However, unexpectedly, both active and total PDC activities were decreased. One human study (23) shows that activity of PDC was decreased in muscle, heart and liver but was normal in skin fibroblasts. Furthermore, other studies support the finding of changes of PDH activity and content. A mild hypertrophied heart induced by five weeks of pressure overload exhibits a decrease of PDC activity (70) and a failing heart with higher glucose oxidation and lower fatty acid oxidation induced by 3 weeks of pacing shows a reduction of PDH-E2 mRNA and protein, although with normal PDC activity (65). Currently there are no clear mechanisms to explain the decrease PDC activity. However, an islet study demonstrated that accumulation of long chain acyl-CoA can inhibit PDC activity (44). In Mk hearts, both fatty acid oxidation and NADPH content (data not shown here) were reduced, suggesting long chain acyl-CoA may be accumulated in Mk heart to inhibit PDC activity. Metabolic acidosis show pyruvate oxidation in both liver and human skeletal muscle during exercise by decreasing activity of PDC (26, 77). Reduced PDC activity results from alterations in the content of PDC substrates, acetyl-CoA, NADH and H⁺, leading to greater relative activity of PDK (26). In our Mk hearts, lactate production was about 2 fold higher compared to control group, suggesting lactic acidosis may stimulate PDK activity contributing to decrease of PDC activity by phosphorylation or accelerating PDH protein degradation. Other factors e.g. HNE (28) and TNF- α (91) could also be possible reasons involved in the pathogenesis of decreased PDC activity in Mk hearts.

The paradoxical findings between PDC activity and glucose oxidation in these studies suggested that PDC did not play a primary role or directly relate with alteration of

glucose oxidation. PDC is responsible for the pyruvate decarboxylation which accounted for about 42% of citrate. Since entry of pyruvate into the TCA cycle through PDC is disrupted in Mk hearts, alternative routes for pyruvate influx to the TCA cycle have been proposed. Isolated perfused heart experiments (62) showed that pyruvate can be carboxylated to form malate or oxaloacetate (OAA) via malic enzyme or pyruvate carboxylase (Figure 2). In Mk hearts, malate content increased by 84%, although the OAA content was not changed between Mk and FVB. MCT-2, a pyruvate and lactate transporter was increased by about 70% in mRNA level and elevated by 2 fold in protein level in mitochondria, suggesting an increased flux of pyruvate into the mitochondria for OAA production through pyruvate carboxylase and an increased flux of malate via malic enzyme into mitochondria. High rate of citric acid cycle flux with low concentration of intermediates (55) may explain the lack of change of OAA content despite the increase of MCT-2 protein in mitochondria. Furthermore, in isolated rat heart, studies show that malic enzyme but not pyruvate carboxylase predominates in the heart (62). Thus, elevation of malate content by about 84% in Mk transgenic hearts might indicate a major anaplerosis pathway for increasing citric acid cycle flux, although both malic enzyme and PC are constitutive processes in the heart.

Functional and morphological effects of the Mk transgene

Basal contractility was unchanged in Mk hearts when assayed either during Langendorff perfusion or in isolated cardiomyocytes. This is consistent with results we have obtained with two other transgenic models of increased or decreased glycolysis (17, 42). The results from all of these transgenic lines have shown that altering glycolysis for

many months does not change myocyte contractility when assayed in oxygenated conditions. During hypoxia, glycolysis becomes more important to myocyte function. Consistent with this, cultured Mk myocytes were much less susceptible to hypoxia. In fact, under hypoxic conditions that significantly impaired control myocytes in our assays, we saw no detectable decline in Mk cardiomyocyte contractility. Resistance to hypoxia is the opposite of what we previously found in myocytes with impaired glycolytic capacity (17). This confirms the essential role of glycolysis during hypoxic conditions. However, isolated myocyte experiments do not allow for extrapolation to *in vivo* conditions. *In vivo*, multiple fuel substrates are available, in contrast to the sole substrate of glucose used for *in vitro* myocyte culture. Also, excess lactate (Figure 3) from increased glycolysis in Mk hearts may accumulate in poorly perfused myocardium of ischemic intact hearts (68). In addition, fibrosis and hypertrophy in Mk mice could increase cardiac work during *in vivo* ischemia, and the reduced glycogen content (Table 1) of Mk hearts reduced carbohydrate reserves. Therefore, it was not a complete surprise that the protection from hypoxia in isolated myocytes was not replicated *in vivo*, where infarct damage following ischemia- reperfusion was similar in transgenic and control hearts. A more complete analysis of ischemia resistance in Mk hearts should be undertaken using low-flow and stop-flow perfusion of isolated hearts to distinguish what components contribute to resistance to hypoxia of cardiomyocytes in culture but not *in vivo*.

Despite increased glycolysis by which the Mk transgene can protect cardiomyocytes against hypoxia, we found there is an increase in cardiac size. Heart weight, heart weight-to-body weight ratio, and cardiomyocyte length were all significantly elevated in Mk transgenic mice, demonstrating hypertrophy. To test if this mild hypertrophy indicated

cardiac pathology, we examined two other signs of pathology: upregulation of “fetal state cardiac genes” (53, 54) and induction of fibrosis. There was no significant effect of the Mk transgene on the expression of β -MHC and BNP. Since these genes are upregulated by stress, this did not support the conclusion that Mk hearts were undergoing severe pathological stress (63). On the other hand, we found that cardiac fibrosis was increased, and fibrosis is a cardiac stress marker (9). Overall, the weight of the evidence, fibrosis and hypertrophy imply that the Mk transgene induces a mild state of pathology. By constitutively elevating Fru-2,6-P₂, the Mk transgene chronically elevated glycolysis and blocked the normal mechanisms controlling carbohydrate usage. The increased glycolysis of chronic heart failure may ultimately be maladaptive. It is striking that our present transgene, which elevated Fru-2, 6-P₂, and our previous transgene (17), which reduced Fru-2, 6-P₂, both produced mild hypertrophy and fibrosis. This demonstrates the sensitivity of the heart to disruption of normal metabolic control.

Relevance to diabetic cardiomyopathy

In diabetes the metabolism of the heart becomes much more dependent on fatty acids and simultaneously the rate of glycolysis and glucose oxidation goes down. This change in fuel usage may be one of the causes of diabetic cardiomyopathy. Our Mk mice represent the opposite situation to diabetes: Glycolysis and glucose oxidation are elevated and fat usage is reduced. Since the Mk mice had mild cardiac pathology this result suggests that a reversal of diabetic cardiac fuel usage would not necessarily be good for the heart. Both diabetic cardiomyopathy and Mk cardiac pathology suggest that

maintaining the metabolic flexibility of the heart to use the most appropriate or available fuel substrate is essential to avoiding cardiac dysfunction.

Summary

In summary, we produced mice with chronic and stable elevation of cardiac Fru-2, 6-P₂. This resulted in significant changes in cardiac metabolite concentrations, increased glycolysis and glucose oxidation, reduced palmitate oxidation, elevation of malate content and decreased PDC activity without uncoupling the glycolysis and glucose oxidation and protection of isolated myocytes from hypoxia. Increased malate content suggested that increased anaplerosis pathway in Mk hearts solved the paradoxical finding between reduced PDC activity and elevated glucose oxidation. This increased anaplerosis pathway could be associated with the pathological changes of hypertrophy with cardiac fibrosis in Mk heart. Finally, chronic elevation of glycolysis produced detrimental effects, suggesting that the elevation of glycolysis in failing hearts could be injurious to an already compromised heart.

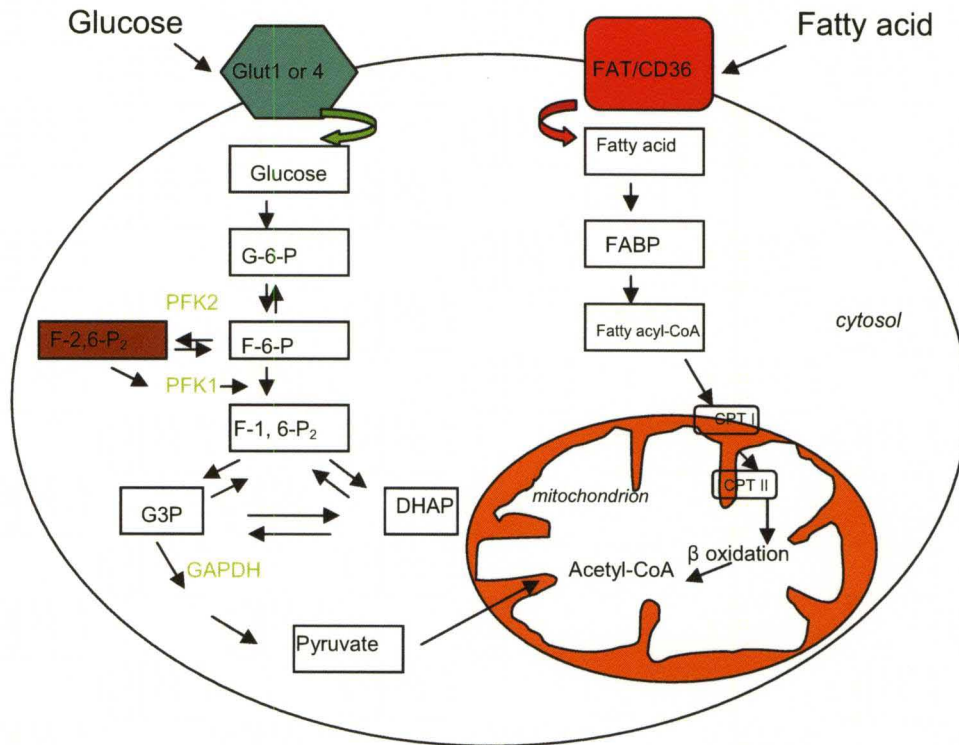


Figure 1: A balance between glucose and fat consumption exists in the cardiomyocyte from normal heart. Glucose is transported into cytosol by glucose transporter 1 and 4 and provides 30% of energy for heart through glycolysis and glucose oxidation as shown in the left pathway. Fatty acid is transported into cytosol by transporters FAT/CD36 etc. for fatty acid oxidation and provides 70% energy for heart as shown in the right pathway.

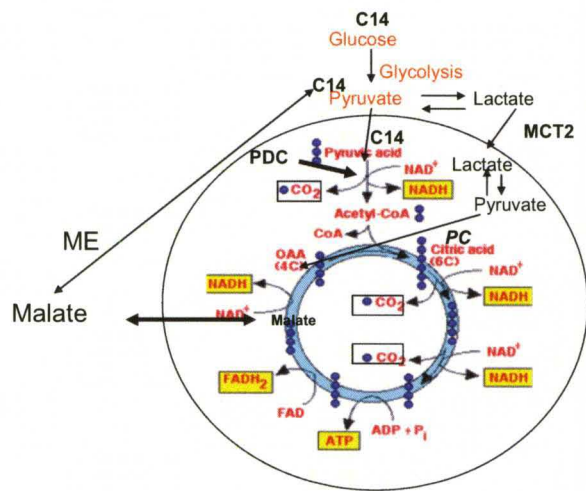


Figure 2: Alternative routes for pyruvate influx from glucose to the citric acid cycle (anaplerosis). The major route for pyruvate to enter the citric acid cycle is by conversion to acetyl-CoA by the pyruvate dehydrogenase complex (PDC). There are also two anaplerotic pathways shown for entry of pyruvate into the citric acid cycle. Cytosolic pyruvate carboxylation to malate via malic enzyme (ME) or mitochondrial carboxylation to oxaloacetate via pyruvate carboxylase (PC). MCT-2 can transport pyruvate or lactate into the mitochondria for further oxidation.

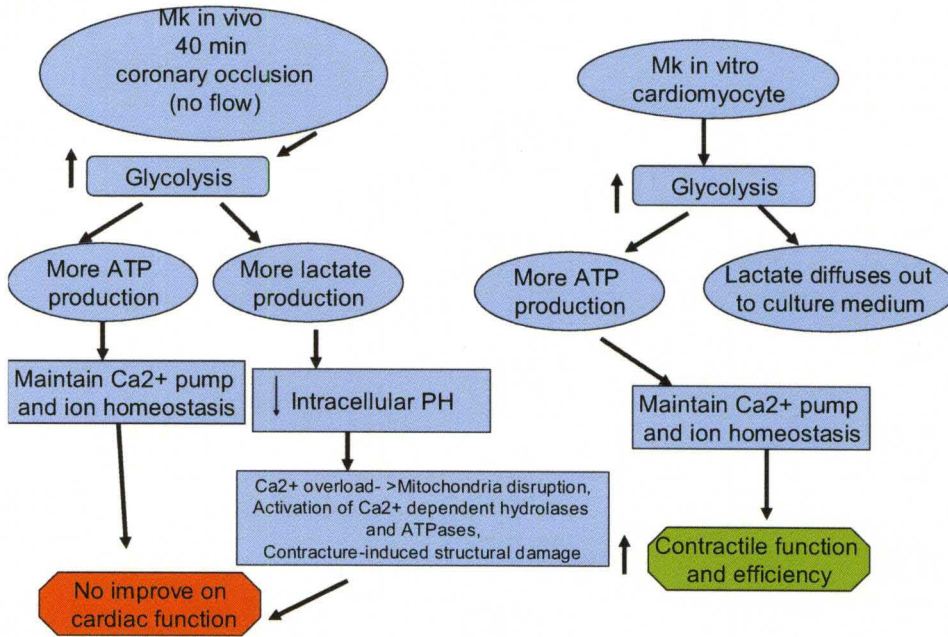
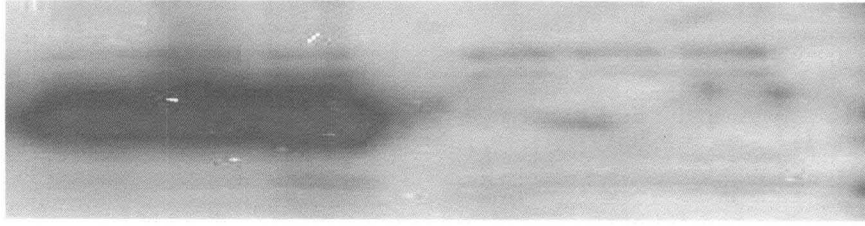


Figure 3: Scheme for potential beneficial and detrimental effects of high glycolysis under myocardial Ischemia.



Mk

FVB

Figure 4. Transgenic hearts overexpress 6-phosphofructo-2kinase/fructose-2,6-bisphosphatase (PFK-2) protein For the Western blot, cardiac protein was analyzed using rabbit anti-rat liver PFK-2 antibody. Extracts from 3 Mk1 hearts are shown. Similar results were obtained in all Mk transgenic lines.

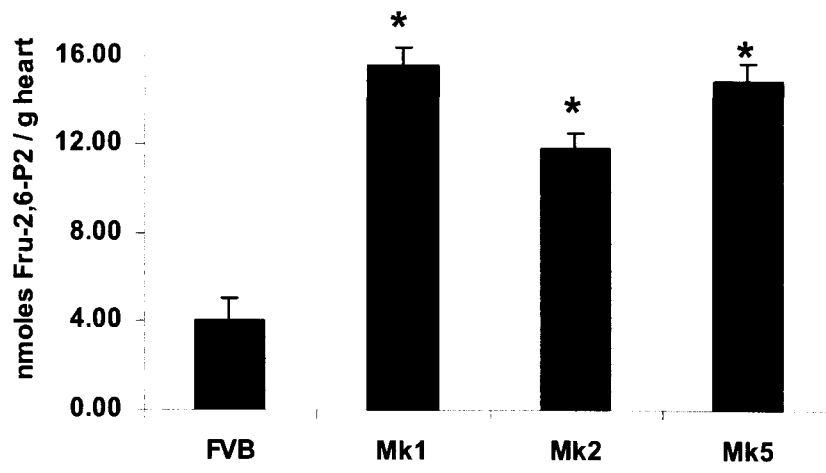


Figure 5. Transgenic hearts have elevated fructose-2,6-bisphosphate(Fru-2,6-P₂).

For the measurement of Fru-2, 6-P₂, hearts were extracted with 50 mM NaOH and Fru-2, 6-P₂ was estimated using pyrophosphate-dependent fructose-6-phosphate 1-phosphotransferase. Transgenic Fru-2, 6-P₂ values were significantly higher than those in FVB hearts (**P* < 0.01 by ANOVA). No significant differences were obtained within Mk groups. The numbers of animals used were 4 FVB, 4 Mk1, 4 Mk2, and 3 Mk5 mice.

TABLE 1	Glc-6-P (nmol/g)	Fru-1, 6-P ₂ (nmol/g)	Fru-2, 6-P ₂ (nmol/g)	Pyruvate (nmol/g)	Glycogen (umol/g)
FVB	225±67	277±93	5.12±0.88	133±44	2.4±1.94
Mk	140±17	356±108	10.4±1.34	227±110	0.27±0.22
P value	0.01	0.25	0.01	0.15	0.01

Table 1: Glycolytic intermediates concentration in FVB and Mk hearts. Values are means \pm SE for at least 5 mice in each group after a 3 hr fast. P values were determined by students t-test. G6P was reduced by \sim 1.6 fold in Mk transgenic hearts ($p < 0.01$). Coincident with the reduction in G6P levels, glycogen was decreased by more than eightfold in Mk hearts ($p < 0.01$). Two metabolites downstream of PFK, Fru-1, 6-P₂ and pyruvate, were increased in concentration; however, the increase did not reach statistical significance ($p = 0.25$ for Fru-1, 6-P₂ and $p = 0.15$ for pyruvate).

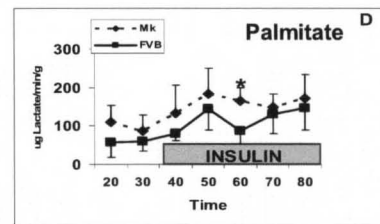
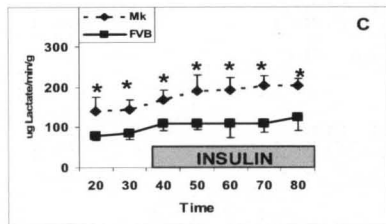
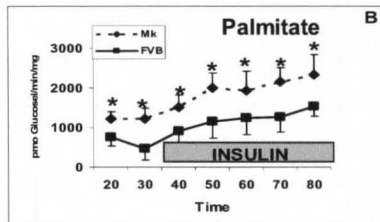
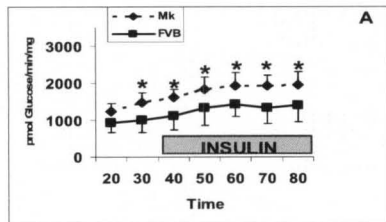


Figure 6: The kinase active PFK-2 transgene increases glycolysis in Langendorff perfused hearts. *A* and *C*: glycolysis (*A*) and lactate release (*C*) without palmitate. *B* and *D*: glycolysis (*B*) and lactate release (*D*) in the presence of 0.4 mM palmitate. The shaded box shows the addition of 200 uU/ml insulin. Values for FVB and Mk hearts were compared by two-way ANOVA ($*P < 0.05$). Values are means \pm SE for 5 mice/time point.

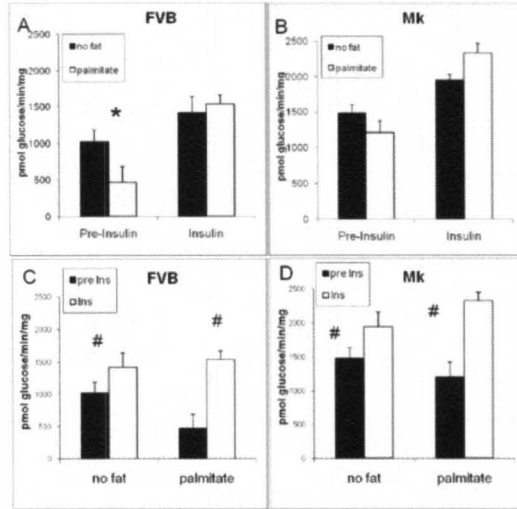


Figure 7. Effect of palmitate and insulin on glycolysis in FVB mice and Mk mice.

Preinsulin glycolysis was reduced by 55% ($*P < 0.02$) in FVB mice (*A*) but only by 18% in Mk mice (*B*; $P < 0.24$). In the presence of insulin, palmitate did not reduce glycolysis in FVB or Mk hearts. *C* and *D*: insulin elevated glycolysis in FVB (*C*) and Mk (*D*) hearts with or without palmitate ($\#P < 0.02$, preinsulin vs. insulin by paired *t*-test). The relative effect of insulin was greater in the presence of palmitate because insulin decreased palmitate inhibition of glycolysis. $n = 5$ FVB and 5 Mk hearts. Data are from Fig. 4 at 30 and 80 min.

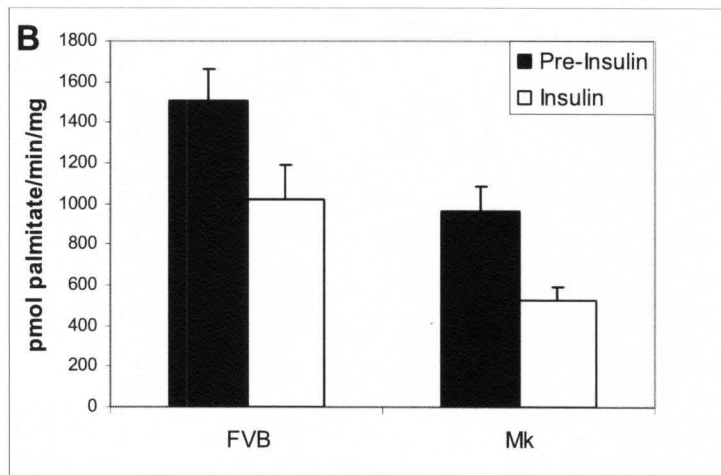
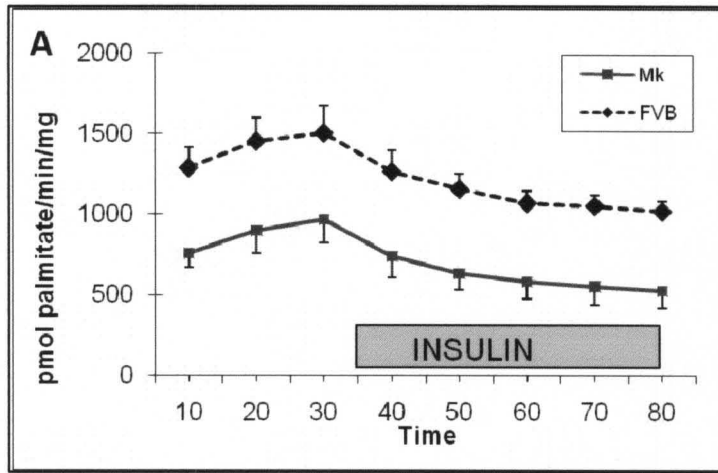


Figure 8. The Mk transgene reduces palmitate oxidation in Langendorff-perfused hearts. *A*: FVB palmitate oxidation values were greater than Mk values at all time points ($P < 0.01$ by two-way ANOVA, $n = 5$ FVB and 7 Mk hearts). *B*: effect of insulin on palmitate oxidation by replotting the 30- and 80-min results from *A*. Insulin reduced palmitate oxidation by 32% in FVB hearts and by 42% in Mk hearts ($\#P < 0.01$ by paired *t*-test).

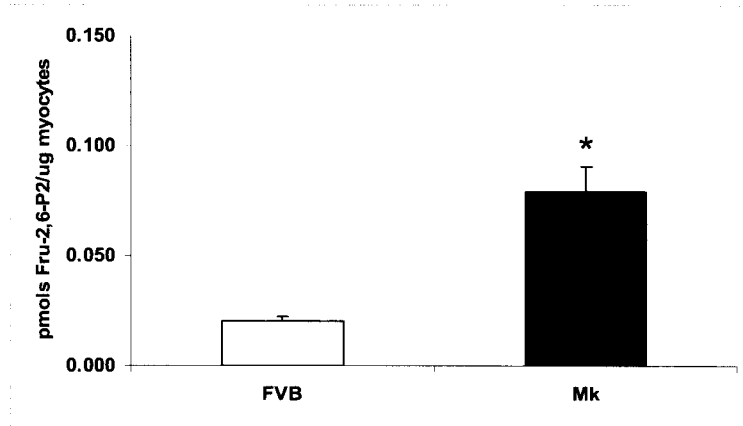


Figure 9: Levels of Fru-2, 6-P₂ in FVB and Mk cardiomyocytes cultured for 24 hrs.

The * in both panels indicates that each metabolite is higher in Mk cardiomyocytes

(P<0.01 by t-test). Values are the mean and standard error of the mean (SEM) for at 8 to

12 assays.

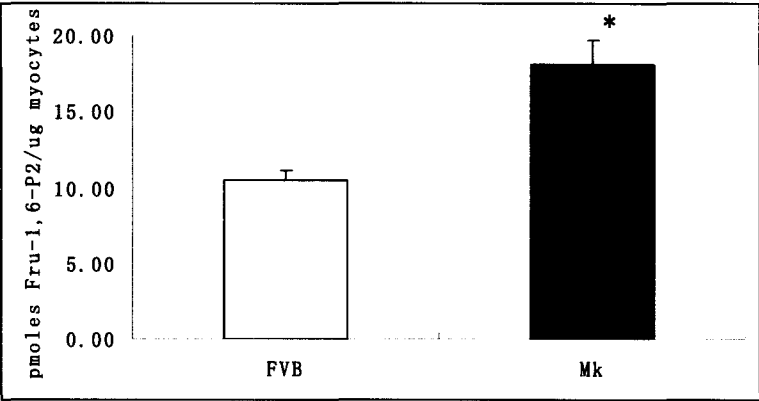


Figure 10: Levels of Fru-1,6-P₂ in FVB and Mk cardiomyocytes cultured for 24 hrs. The * in both panels indicates that each metabolite is higher in Mk cardiomyocytes (P<0.01 by t-test). Values are the mean and standard error of the mean (SEM) for 12 assays.

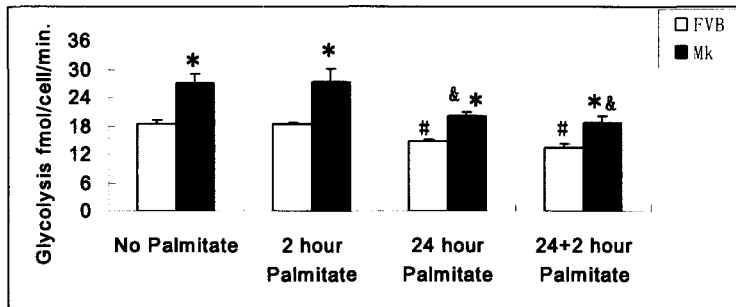


Figure 11: Measurement of glycolysis in Mk and FVB cardiomyocytes. All myocytes were cultured for 24 hrs and then assayed for metabolism during the next 2 hrs. Palmitate (0.4mM) was present during the 24 hr culture period and/or the 2 hr assay period as indicated on the X axis. * indicates that Mk glycolysis values were significantly greater than FVB values measured under the same conditions ($P < 0.01$). # indicates that for FVB myocytes glycolysis values were significantly lower than values obtained from FVB myocytes cultured and assayed under the No Palmitate condition (Panels A). The symbol & indicates that for the different palmitate conditions Mk glycolysis values were significantly lower than values obtained from Mk myocytes cultured and assayed under the No Palmitate condition ($P < 0.05$). The number of animals used was 7 FVB and 6 Mk. Statistics were performed by two-way ANOVA. Vertical bars are SEM.

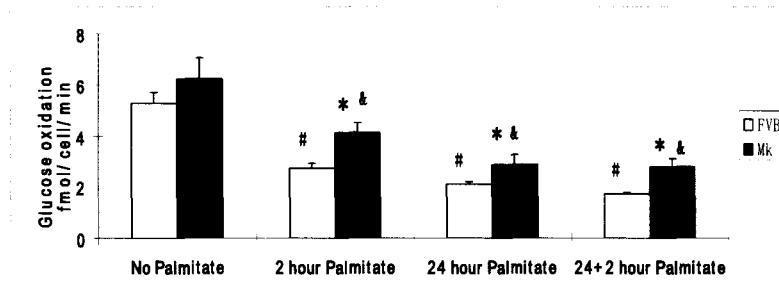


Figure 12: Measurement of glucose oxidation in Mk and FVB cardiomyocytes. All myocytes were cultured for 24 hrs and then assayed for metabolism during the next 2 hrs. Palmitate (0.4mM) was present during the 24 hr culture period and/or the 2 hr assay period as indicated on the X axis. * indicates that Mk glucose oxidation values were significantly greater than FVB values measured under the same conditions ($P < 0.05$). # indicates that for FVB myocytes glucose oxidation values were significantly lower than values obtained from FVB myocytes cultured and assayed under the No Palmitate condition ($P < 0.01$). The symbol & indicates that for the different palmitate conditions Mk glucose oxidation values were significantly lower than values obtained from Mk myocytes cultured and assayed under the No Palmitate condition ($P < 0.05$). The number of animals used was 7 FVB and 6 Mk. Statistics were performed by two-way ANOVA. Vertical bars are SEM.

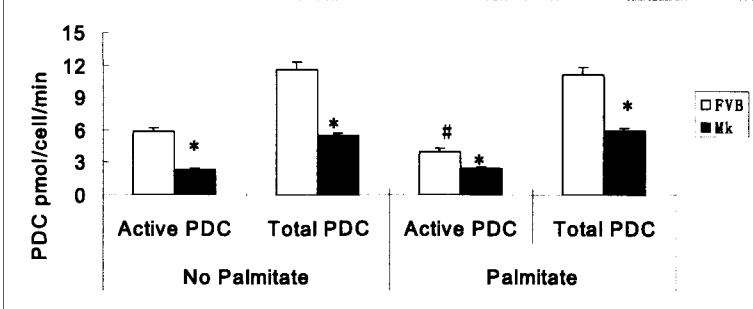


Figure 13: Active PDC and Total PDC activity are decreased in Mk transgenic cardiomyocytes. PDC activity was measured after 24hr culture with or without 0.4mM palmitate. Active and total PDC were measured as described in Methods and activity was normalized to the number of cells in each culture. * indicates Mk was lower than FVB for the same assay and culture conditions ($P < 0.01$). # indicates that for FVB myocytes active PDC was lower for the palmitate group than the no palmitate group ($P < 0.01$). The number of animals used was 10 for FVB and 10 for Mk. Statistics were obtained by two-way ANOVA using Mk and palmitate as factors. Vertical bars are SEM.

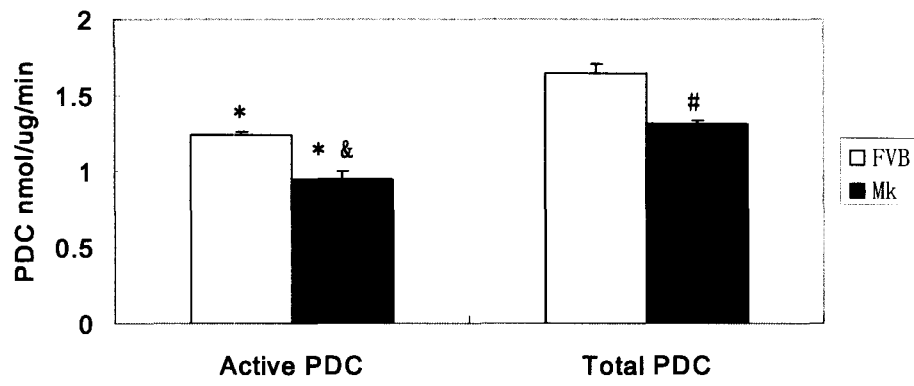
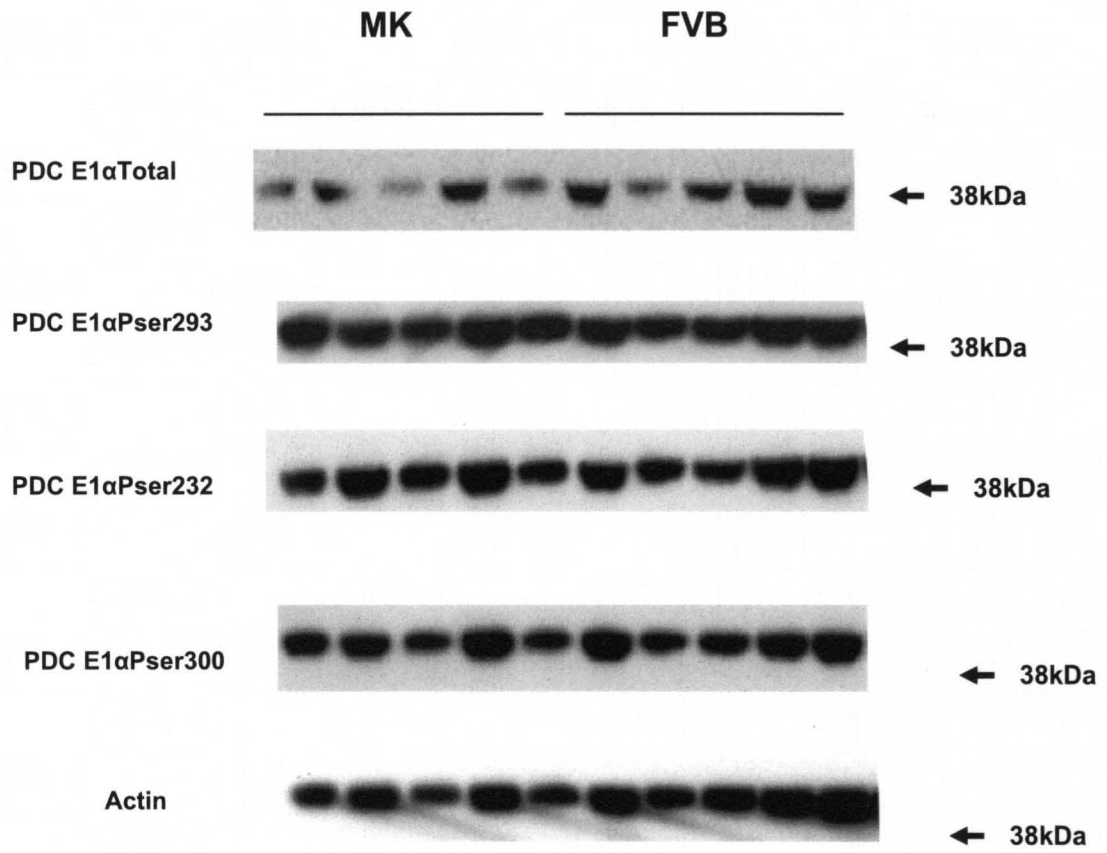


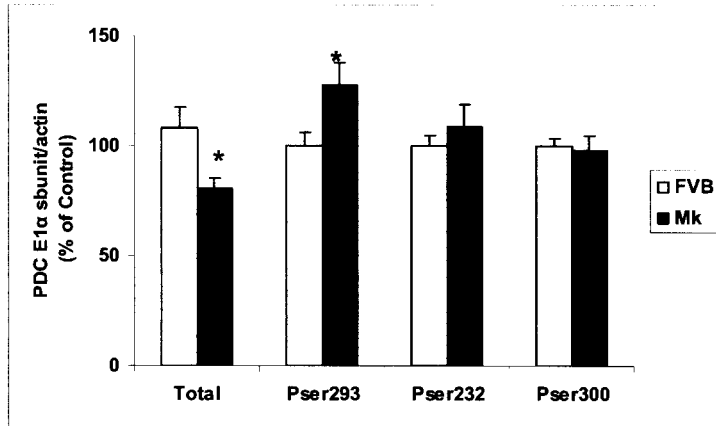
Figure 14: Active PDC and Total PDC activity are decreased in Mk transgenic hearts. Active and total PDC were measured as described in Methods and activity was normalized to the number of cells in each culture. * indicates Mk was lower than FVB for the same assay conditions ($P < 0.01$). The number of animals used was 4 for FVB and 4 for Mk. Statistics were calculated by t-test. Vertical bars are SEM.

(A)

Cardiac myocyte



(B)



(C)

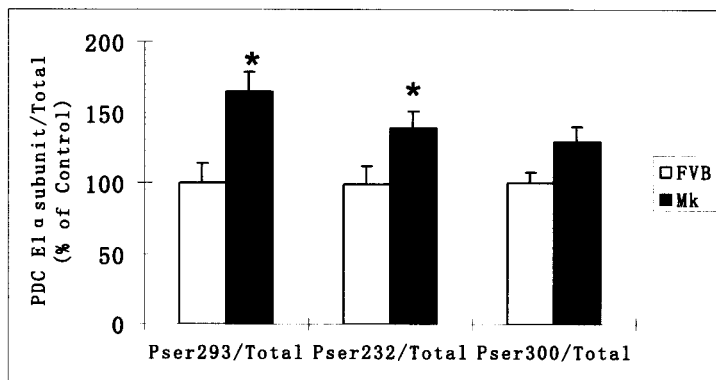
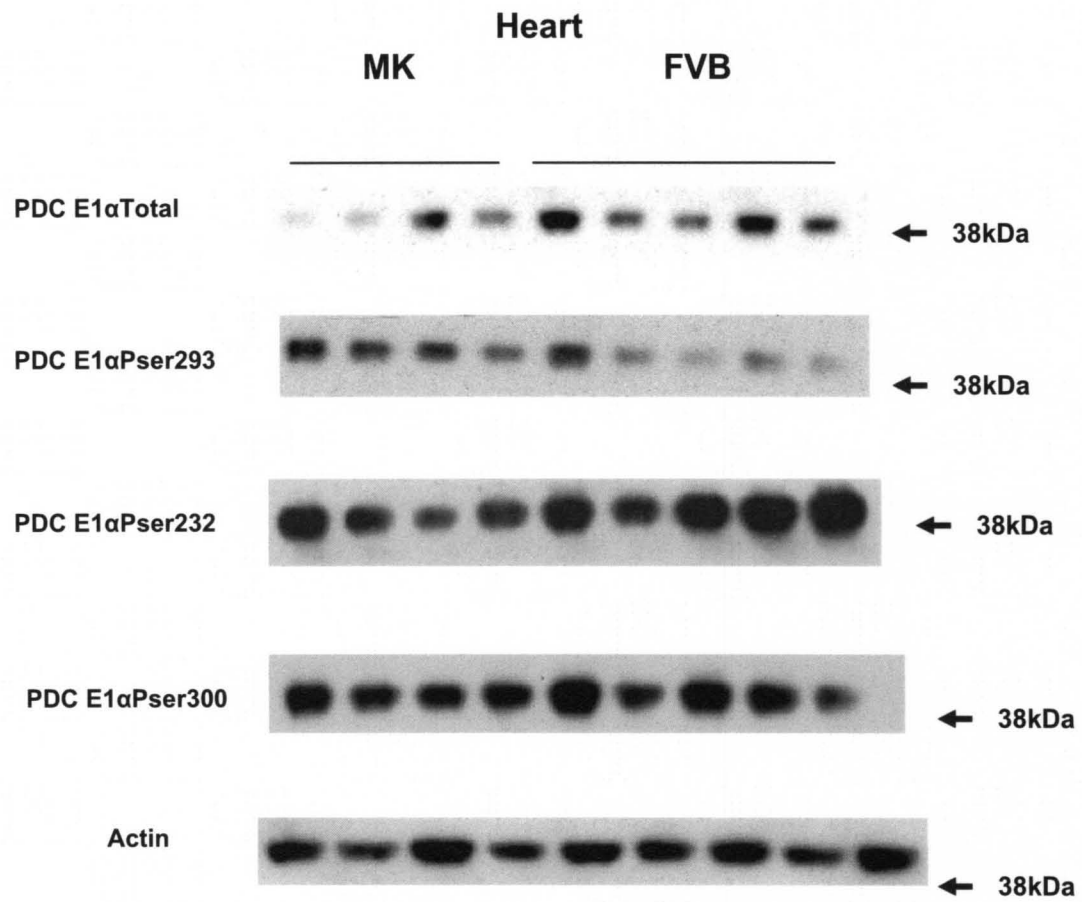
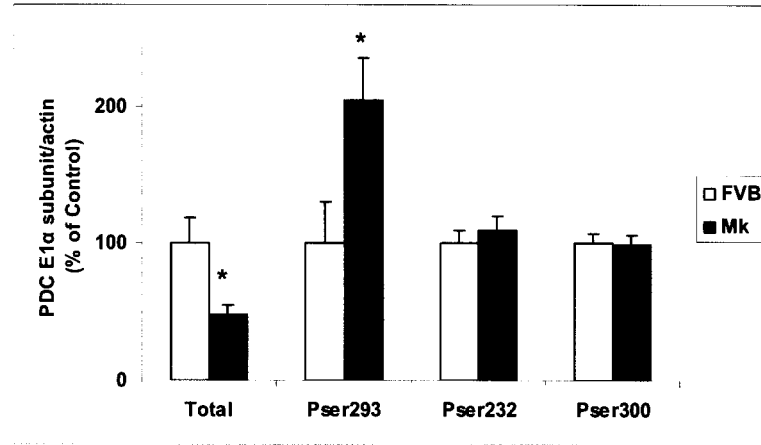


Figure 15: PDC E1 α expression and phosphorylation in Mk transgenic and FVB control cardiomyocytes. (A) Western blots for the indicated antibodies. Each lane contains protein from cardiomyocytes isolated from a different Mk or FVB heart. (B) Densitometric analysis of protein subunit levels normalized to actin and expressed as % of average FVB. (C) Ratio of each PSer normalized to the amount of PDC E1 α for that sample. * indicates that Mk was significantly different from FVB for the same variable. n=5 for FVB and 7 for Mk.

(A)



(B)



(C)

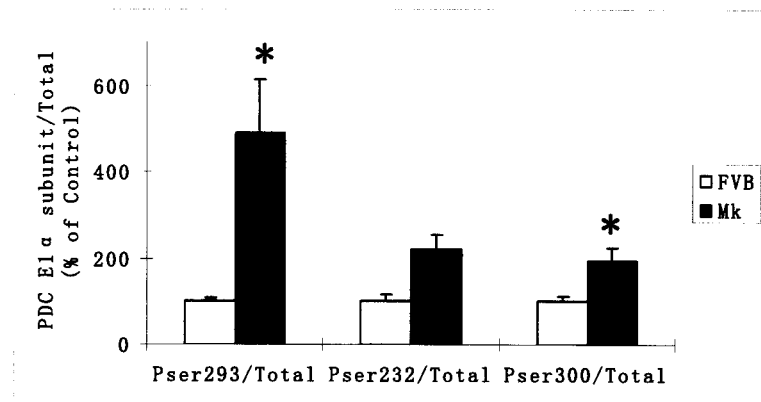
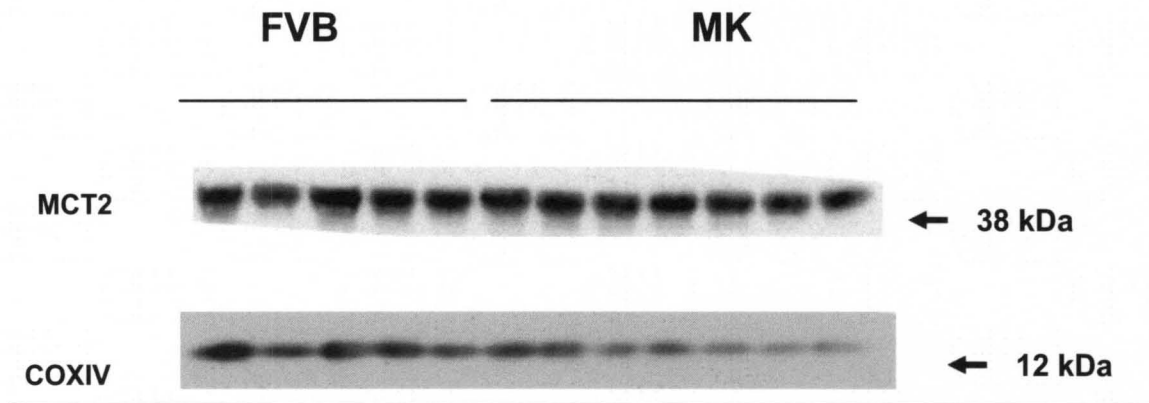


Figure 16: PDC E1 α expression and phosphorylation in Mk transgenic and FVB control hearts. (A) Western blots for the indicated antibodies. Each lane contains protein from a different Mk or FVB freeze clamped heart. (B) Densitometric analysis of protein subunit levels normalized to actin and expressed as % of average FVB. (C) Ratio of each PSer normalized to the amount of PDC E1 α for that heart. * indicates that Mk was significantly different from FVB for the same variable. n=5 hearts per group.

(A)



(B)

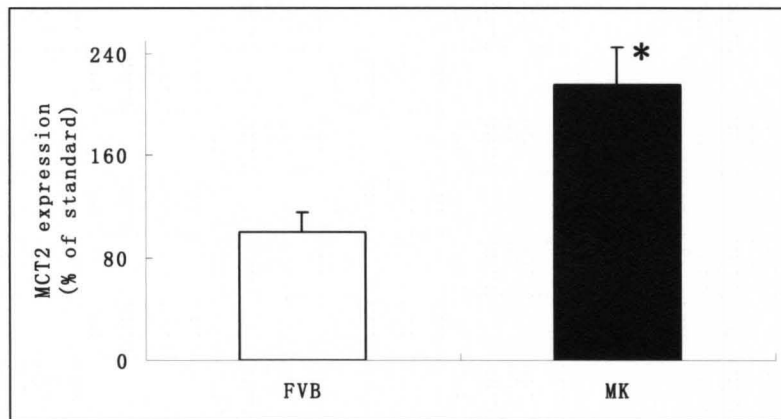


Figure 17: Increased amount of MCT-2 in isolated mitochondria from Mk transgenic hearts. (A) Immunoblots for MCT2 and COXIV loading control: each lane contains mitochondrial protein from a different heart. Arrows indicate positions of MW markers MCT2 is 44 kDa and COXIV is 17 kDa. (B) Densitometric analysis of MCT2 normalized to COXIV expressed in % of the average FVB value. *: Mk is significantly different from FVB ($P < 0.01$). $n = 5$ hearts per group.

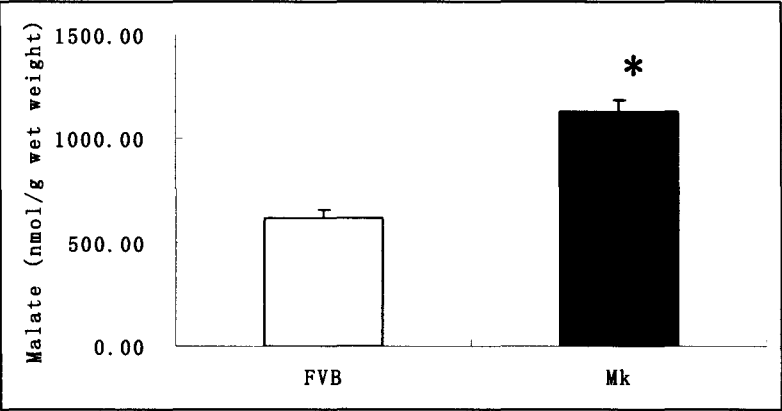


Figure 18. Malate content in whole heart. Metabolites were measured in perchloric acid extracts of whole heart as described in Methods. * indicates that malate content was significantly elevated in Mk heart. Hearts from 7 Mk mice and 5 FVB mice were assayed.

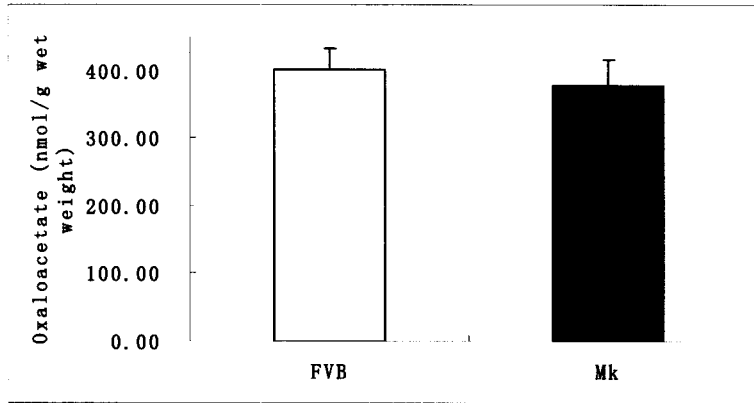


Figure 19. Oxaloacetate content in whole heart. Metabolites were measured in perchloric acid extracts of whole heart as described in Methods. Hearts from 7 M_k mice and 5 FVB mice were assayed.

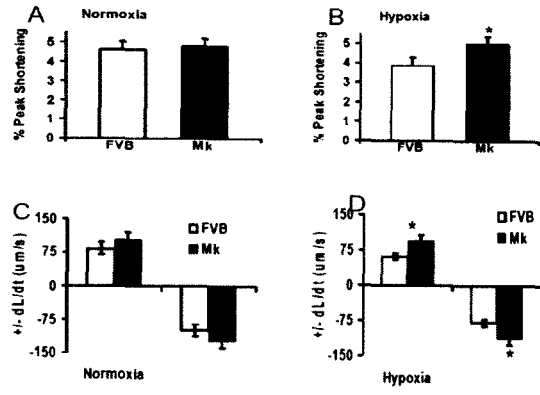


Figure 20: Overexpression of kinase-active PFK-2 improves contractility under hypoxia. Cardiomyocytes were isolated and incubated under normoxic or hypoxic conditions. The contractile properties of ventricular myocytes from FVB control and Mk transgenic mouse hearts was measured by video-based edge detection. Graphs show the percentage of shortening under normoxic (*A*) and hypoxic (*B*) conditions as well as the maximal velocities of cell shortening and relengthening ($\pm dL/dt$) under normoxia (*C*) and hypoxia (*D*). Values are means \pm SE for 60–80 cells from 4 mice/group. * $P < 0.02$, Mk vs. FVB by Student's *t*-test. n=5 hearts per group.

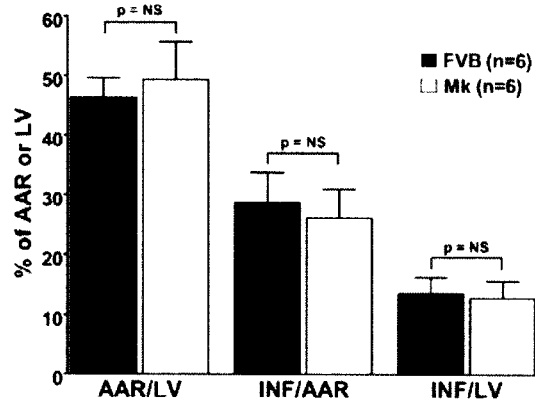


Figure.21. Myocardial infarct size (INF) was determined after 40 min of in vivo coronary occlusion and 24 h of reperfusion. The area at risk (AAR) with respect to the left ventricle (LV) was not significantly different between FVB wild-type and Mk transgenic mice, indicating consistent execution of the surgical protocol between the two groups. The amount of nonviable myocardium, according to triphenyltetrazolium negativity, was also similar between the two groups. This was true whether INF was expressed relative to the AAR (INF/AAR) or to the entire LV (INF/LV). $P =$ not significant (NS) for all comparisons. $n = 6$ mice/group.

Table 2	sex	age	body wt (gm)	heart wt (mg)	hw/bw	myocyte length µm
FVB	m	138 ± 2	29.9 ± 0.6	132.7 ± 3.3	4.44 ± 0.07	126 ± 3
Mk	m	131 ± 4	29.8 ± 0.6	155.3 ± 2.5*	5.21 ± 0.08 *	151 ± 3 *

Table 2. Mk hearts and myocytes are enlarged Values are means \pm SE; n =13 male Mk mice and 27 male FVB mice. Myocyte length was the average of 52 myocytes from 4 mice/group. *P < 0.001 by Student's t-test.

BNP

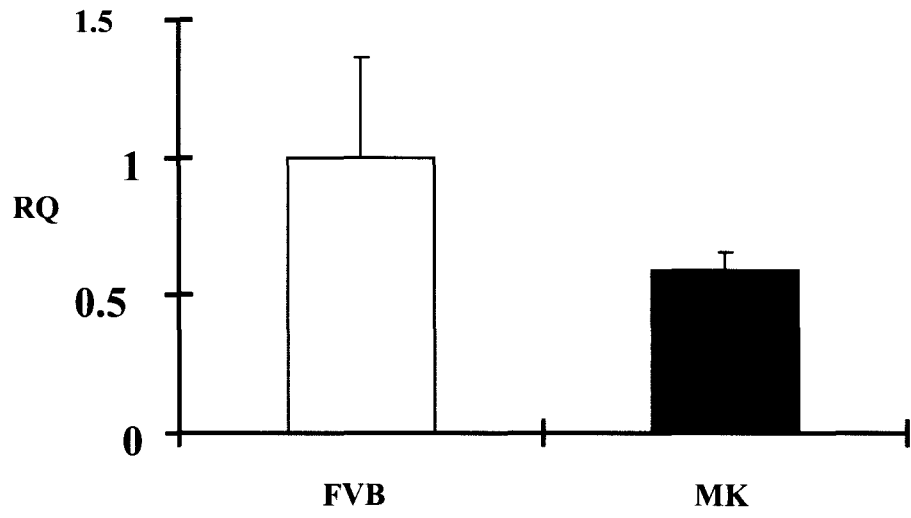


Figure 22: Real time-PCR shows no elevation of BNP mRNA in MK transgenic mice. Values are the mean \pm SE for 4 mice per group. Values were not significantly different by students t-test.

β -MHC

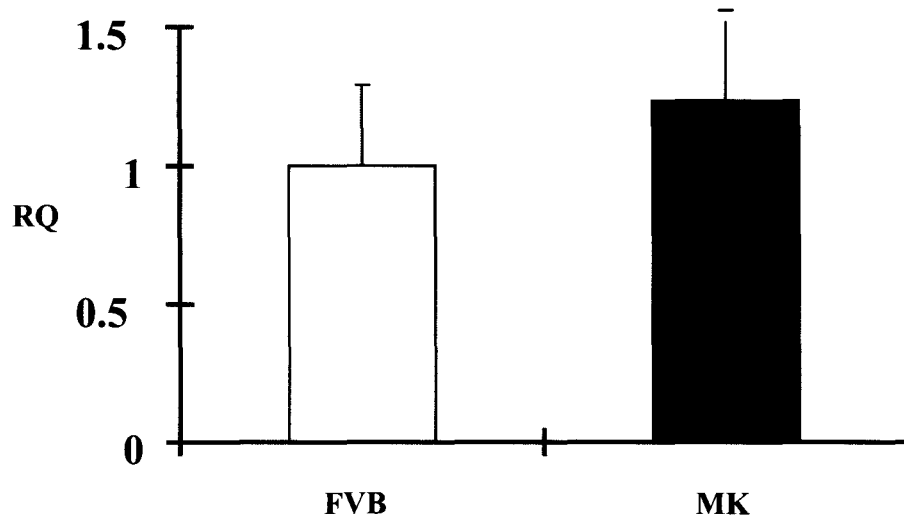
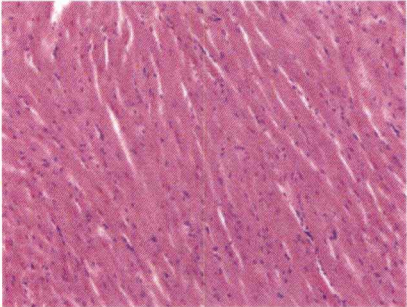


Figure 23: Real time-PCR shows no elevation of β -MHC mRNA in MK transgenic mice. Values are the mean \pm SE for 4 mice per group. Values were not significantly different by students t-test.

H&E staining

FVB



Mk

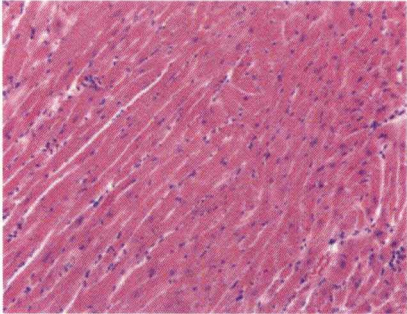
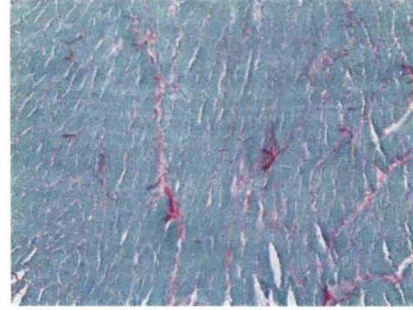
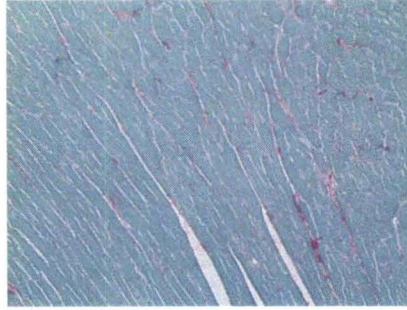


Figure 24: H&E staining in Mk and FVB hearts. Cardiac morphology was visualized by H&E staining. The above picture shows representative staining of FVB(3) and Mk(3) hearts.

A

FVB

Mk



B

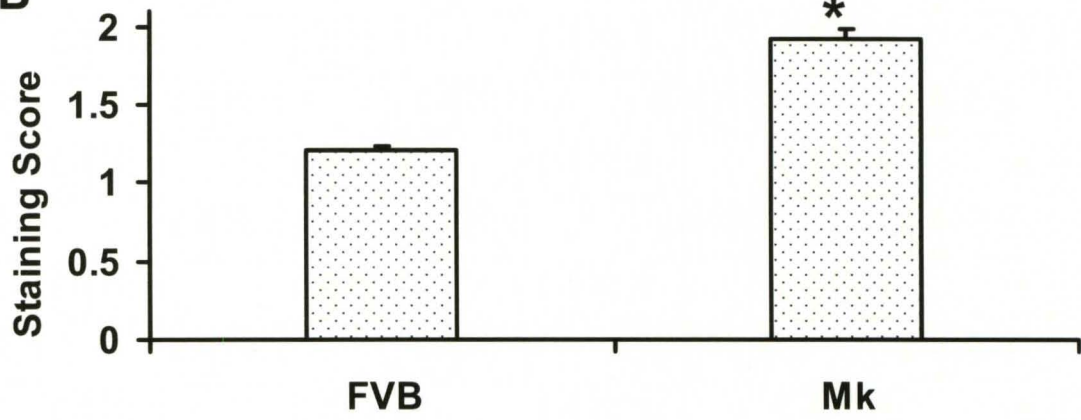


Figure 25: Fibrosis in Mk hearts. Collagen accumulation was visualized by sirius red staining at x40. A, shows representative staining of FVB and Mk hearts. B shows average (\pm SE) score for sirius red staining from 60 photographs taken from FVB (3) and Mk (3) mouse hearts. Staining was rated by a blind observer on a scale of 0-2, where 0 indicates mild, 1 increased and 2 severe interstitial accumulation of collagen. The values shown are means \pm SE and were analyzed by student's t-test (*P < 0.01).

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Peer reviewed Publications:

1. **Wang Q**, Donthi RV, Wang J, Lange AJ, Watson LJ, Jones SP, Epstein PN. Cardiac phosphatase-deficient 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase increases glycolysis, hypertrophy, and myocyte resistance to hypoxia. *Am J Physiol Heart Circ Physiol*. 2008 Jun;294 (6):H2889-97. Epub 2008 May 2.

2. Wang J, Song Y, **Wang Q**, Kralik PM, Epstein PN. Causes and characteristics of diabetic cardiomyopathy.
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Manuscripts in preparation:

1. **Qianwen Wang** , Jianxun Wang , and Paul N. Epstein Cardiomyocyte overexpression of kinase-active PFK-2 increases glucose oxidation despite decreased abundance and activity of pyruvate dehydrogenase complex
2. Jianxun Wang , **Qianwen Wang** , Lewis J. Watson , Steven P. Jones and Paul N. Epstein Cardiac overexpression of mitochondrial 8-oxoguanine DNA glycosylase 1 protects against cardiac fibrosis following transaortic constriction
3. Jianxun Wang , **Qianwen Wang** , Lewis J. Watson , Steven P. Jones and Paul N. Decreased glycolysis increases susceptibility to cardiac hypertrophy and heart failure
4. Jianxun Wang , **Qianwen Wang** , Lewis J. Watson , Steven P. Jones and Paul N. Increased glycolysis increases susceptibility to cardiac hypertrophy and heart failure