Increased Myocardial Dysfunction After Ischemia-Reperfusion in Mice Lacking Glucose-6-Phosphate Dehydrogenase

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Background—Free radical injury contributes to cardiac dysfunction during ischemia-reperfusion. Detoxification of free radicals requires maintenance of reduced glutathione (GSH) by NADPH. The principal mechanism responsible for generating NADPH and maintaining GSH during periods of myocardial ischemia-reperfusion remains unknown. Glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting enzyme in the pentose phosphate pathway, generates NADPH in a reaction linked to the de novo production of ribose. We therefore hypothesized that G6PD is essential for maintaining GSH levels and protecting the heart during ischemia-reperfusion injury.

Methods and Results—Susceptibility to myocardial ischemia-reperfusion injury was determined in Langendorff-perfused hearts isolated from wild-type mice (WT) and mice lacking G6PD (G6PD def) (20% of WT myocardial G6PD activity). During global zero-flow ischemia, cardiac function was similar between WT and G6PD def hearts. On reperfusion, however, cardiac relaxation and contractile performance were greatly impaired in G6PD def myocardium, as demonstrated by elevated end-diastolic pressures and decreased percent recovery of developed pressure relative to WT hearts. Contractile dysfunction in G6PD def hearts was associated with depletion of total glutathione stores and impaired generation of GSH from its oxidized form. Increased ischemia-reperfusion injury in G6PD def hearts was reversed by treatment with the antioxidant MnTMPyP but unaffected by supplementation of ribose stores.

Conclusions—These results demonstrate that G6PD is an essential myocardial antioxidant enzyme, required for maintaining cellular glutathione levels and protecting against oxidative stress-induced cardiac dysfunction during ischemia-reperfusion. (Circulation. 2004;109:898-903.)

Key Words: ischemia ■ reperfusion ■ glucose ■ free radicals ■ antioxidants

Oxidative stress secondary to free radical generation is an important mechanism of cardiac dysfunction during reperfusion of ischemic myocardium.1–3 Detoxification of free radicals generated during ischemia-reperfusion is mediated primarily by a highly active intracellular antioxidant defense system, and targeted ablation of antioxidant enzymes predisposes the heart to ischemia-reperfusion injury.4 Central to the neutralization of free radicals are endogenous thiol-containing compounds, in particular, the cysteine-containing tripeptide reduced glutathione (GSH).5,6 GSH allows for the conversion of deleterious hydrogen peroxide and lipid peroxides to water and alcohols, respectively.6 Depletion of cardiac GSH exacerbates myocardial ischemia-reperfusion injury,7–9 whereas exogenous supplementation with GSH protects against such injury.7,10,11 Generation of GSH from its oxidized form, GSSG, and subsequent maintenance of intracellular GSH pools require reducing equivalents in the form of the pyridine nucleotide NADPH.4 Although the importance of NADPH in the cardiac antioxidant system is well established, the mechanisms responsible for generating NADPH during ischemia-reperfusion remain unknown.

Glucose-6-phosphate dehydrogenase (G6PD), the first and rate-limiting enzyme in the pentose phosphate pathway, generates NADPH through a NADP+-reduction reaction linked to the oxidation of glucose-6-phosphate and the de novo production of cellular ribose. We have previously shown that G6PD modulates redox status in cardiomyocytes.12 We therefore hypothesized that G6PD is essential for maintaining myocardial redox homeostasis and protecting the heart from oxidative injury during ischemia-reperfusion. In the present report, we demonstrate that G6PD activity increases rapidly during ischemia-reperfusion in isolated hearts. Furthermore, mice lacking myocardial G6PD exhibit greater sensitivity to ischemia-reperfusion-induced contractile and diastolic dysfunction, associated with depletion of intracellular thiols and loss of redox homeostasis. Finally, increased susceptibility to ischemia-reperfusion injury in G6PD-deficient hearts is found to be primarily a result of increased
cellular oxidative stress and independent of de novo ribose production.

**Methods**

**G6PD-Deficient Animals**

G6PD-deficient mice (G6PD<sup>M</sup>)<sup>13</sup> were rederived in our laboratories from frozen embryos. G6PD<sup>het</sup> mice carry a mutation at the 5′ untranslated sequence of the X-linked G6PD gene and, through a splicing defect, exhibit decreased G6PD protein expression and enzyme activity.<sup>14</sup> Mice were genotyped by use of a 2-step procedure to detect a mutation in the DdeI site in the G6PD gene, as previously described.<sup>15</sup> Briefly, DNA was prepared from animal tails, and a 269-bp fragment of the G6PD gene was amplified by polymerase chain reaction (PCR) using the forward primer GGAAAACCTGCTGTCGGCTAC and reverse primer TCAGCTCCGCTCTCTCTCTG. The PCR fragment was subsequently digested with the DdeI restriction endonuclease (New England BioLabs), and the restriction digestion products were separated on a 2.5% agarose gel. DdeI enzyme produced bands of 55 and 214 bp in wild-type (WT) mice but did not cleave the mutant G6PD sequence in G6PD<sup>het</sup> mice. Hearts from male hemizygous G6PD<sup>het</sup> and female homozygous G6PD<sup>def</sup> mice exhibited a similar degree of G6PD expression and activity and were therefore grouped and compared with WT littermate controls in this study. All animal studies adhered strictly to the regulations of the Boston University Animal Care Committee and the National Society for Medical Research.

**Isolated Heart Perfusion Studies**

Hearts were isolated from mice and perfused in the Langendorff mode as previously described.<sup>16,17</sup> Briefly, animals were injected intraperitoneally with heparin (10 000 U/kg) and anesthetized with a mixture of ketamine (150 mg/kg) and xylazine (15 mg/kg). The thorax was rapidly opened and the heart excised and arrested in ice-cold saline. A short perfusion cannula was inserted into the aortic root to initiate retrograde perfusion. The hearts were perfused with Krebs-Henseleit buffer (in mmol/L: NaCl 118, KCl 4.7, CaCl<sub>2</sub> 1.8, KH<sub>2</sub>PO<sub>4</sub> 1.2, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 24.0, and glucose 10.0) equilibrated with 5% O<sub>2</sub>/5% CO<sub>2</sub> to yield a pH of 7.4. A thin cannula was pierced through the apex of the left ventricle (LV) to vent theesian drainage. A ventricular balloon, composed of polyvinyl chloride film and connected to a polyethylene tube, was inserted into the LV through the mitral valve via an incision in the left atrium. The balloon was connected to a pressure transducer (Statham P23Db, Gould) for recording of LV pressures. The balloon was inflated with saline to adjust the end-diastolic pressure (EDP) to 10 mm Hg, and the balloon volume was held constant for the duration of the experiment. Hearts were paced (Grass Instruments) with platinum wires placed on the epicardial surface of the right ventricle. Coronary perfusion pressure was held constant during the duration of the experiment at 80 mm Hg. An inline ultrasonic flow probe (Transonics Systems Inc) was positioned immediately above the aortic cannula to measure coronary flow. Systolic pressure, EDP, and coronary flow were collected online by use of a commercially available data acquisition system (PowerLab ADInstruments). Developed pressure (the difference between systolic and diastolic pressures) and EDP were used as indices of contractile and diastolic function, respectively.

During 20 minutes of baseline stabilization, all hearts were maintained at 37°C and paced at 7 Hz. Hearts were subsequently subjected to 15 minutes of zero-flow ischemia followed by 30 minutes of reperfusion.

For ribose treatment, animals received 100 mg/kg IV of D-ribose (Sigma Chemical Co) in sterile water, a dosage previously determined to supplement myocardial ribose stores.<sup>18</sup> Sixty minutes after treatment, hearts were isolated from animals and subjected to ischemia-reperfusion injury as described above. For antioxidant treatment, isolated hearts were perfused with the superoxide dismutase/catalase mimetic Mn(III)tetrakis(1-methyl-4-pyridyl) por-

**Figure 1.** Activation of G6PD during ischemia-reperfusion in isolated hearts. A, G6PD activity and B, protein levels by Western blot at baseline and after ischemia-reperfusion (Post-IR) in isolated hearts (protocol as in Methods). All data represent an average of 4 to 6 individual experiments. *P<0.05 vs baseline. AU, arbitrary units.

phyrin pentachloride (MnTMPyP)<sup>19,20</sup> (Calbiochem) at a concentration of 50 μmol/L during both baseline and reperfusion periods.

**Biochemical Assays**

Frozen hearts were homogenized and centrifuged at 16 000g for 15 minutes at 4°C, and the supernatant was collected and used for assays. G6PD activity was determined in homogenates as previously described.<sup>15</sup> Protein concentration was determined according to the method of Lowry et al.<sup>21</sup> GS<sub>T</sub>, GSH, and GSSG concentrations were determined in homogenates by use of a commercially available kit (Cayman Chemical) as previously described.<sup>15</sup> All assays were run in triplicate and averaged to obtain a mean value per sample.

**G6PD Immunoblotting**

Total G6PD protein levels were determined by standard Western blot. Frozen hearts were homogenized in Triton X-100 lysis buffer (Cell Signaling) with protease inhibitors (2 μmol/L leupeptin, 1 mmol/L PMSF). The lysate was centrifuged at 16 000g for 15 minutes at 4°C. The supernatant was collected, and protein concentration was measured according to the method of Lowry et al.<sup>21</sup> Samples were run on 12% Tris-glycine precast gels (BioWhittaker) and transferred to polyvinylidene difluoride membranes. Equal protein loading was confirmed by Ponceau staining. After blocking in 5% nonfat milk, polyvinylidene difluoride membranes were probed with rabbit anti-rat G6PD IgG (Bethyl Laboratories Inc) followed by horseradish peroxidase–conjugated anti-rabbit secondary antibody (Pierce Chemical Co). Protein levels were detected by chemiluminescence (Pierce Chemical Co) and quantified by densitometry.

**Statistical Analysis**

Statistical significance was evaluated by 1-way or 2-way ANOVA. A post hoc test of least significant differences was used to determined differences among groups. All data are expressed as mean±SEM. A probability value of P<0.05 was considered statistically significant.

**Results**

**Activation of G6PD During Ischemia-Reperfusion**

To determine whether myocardial G6PD was activated during reperfusion of ischemic myocardium, G6PD activity and protein levels were determined in hearts after ischemia-reperfusion injury. As shown in Figure 1A, ischemia-reperfusion resulted in a 50% increase in myocardial G6PD activity. Increased G6PD activity, however, was not associated with increased expression of G6PD (Figure 1B). These
data suggest that G6PD is rapidly activated in response to myocardial ischemia-reperfusion injury, independent of cellular G6PD protein levels.

**Deficiency of G6PD Increases Susceptibility to Ischemia-Reperfusion Injury**

To determine the role of G6PD in the myocardial response to ischemia-reperfusion, we used G6PD-def mice. G6PD-def mice carry a mutation at the 5′ untranslated sequence of the X-linked G6PD gene and, through a splicing defect, exhibit decreased G6PD expression.14 WT and G6PD-def genotypes were confirmed in mice via PCR analysis (Figure 2A). As shown in Figure 2B, hearts from G6PD-def mice exhibited decreased expression of G6PD with a corresponding 80% reduction in myocardial G6PD activity (Figure 2C) relative to WT hearts.

WT and G6PD-def hearts were subsequently subjected to ischemia-reperfusion injury. During global zero-flow ischemia, EDP rose in both WT and G6PD-def hearts, with comparable values at peak contracture (WT versus G6PD-def, 74.2±3.6 versus 78.8±5.9 mm Hg, P=NS) and at end-ischemia (WT versus G6PD-def, 69.3±3.1 versus 75.8±4.0 mm Hg, P=NS) (Figure 3A). Over the reperfusion period, however, cardiac relaxation was significantly impaired in G6PD-def hearts, with increased EDP relative to WT hearts (Figure 3A), most pronounced at end reperfusion (WT versus G6PD-def, 33.2±3.4 versus 51.3±3.2 mm Hg, P<0.01). Similarly, contractile performance was also impaired in G6PD-def hearts during reperfusion, with blunted recovery of developed pressure (Figure 3B). Although WT hearts recovered to 60.9±5.0% of baseline developed pressure, G6PD-def hearts recovered to only 40.5±5.7% (P<0.01). Although G6PD-def hearts exhibited greater contractile and diastolic dysfunction during ischemia-reperfusion, coronary flow was similar between WT and G6PD-def hearts at baseline (WT versus G6PD-def, 3.5±0.4 versus 3.7±0.3 mL/min, P=NS) and during the reperfusion period (WT versus G6PD-def, 2.4±0.3 versus 2.3±0.3 mL/min, P=NS) (Figure 3C), suggesting a similar degree of reperfusion-induced vasoconstriction between groups. These data suggest that G6PD is essential in mediating myocardial susceptibility to ischemia-reperfusion injury.

**Depletion of Cellular Glutathione in G6PD-def Hearts After Ischemia-Reperfusion Injury**

To determine whether increased ischemia-reperfusion injury in G6PD-def hearts was associated with depletion of cellular thiols, GSx, GSH, GSSG, and the GSH/GSSG ratio, a
sensitive marker of cellular redox state, were determined in frozen WT and G6PD def hearts at baseline and after ischemia-reperfusion (Figure 4). In WT hearts at baseline, the vast majority of cellular glutathione was in the reduced state, with a GSH/GSSG ratio greater than 300 (Figure 4D) and with less than 0.3% of GST existing as GSSG. Notably, before ischemia-reperfusion, GST, GSH, GSSG, and the GSH/GSSG ratio (Figure 4) were comparable between WT and G6PD def hearts, signifying no baseline oxidative deficit in G6PD def animals. With ischemia-reperfusion, however, G6PD def hearts exhibited increased susceptibility to cellular thiol depletion and redox imbalance relative to WT counterparts. In G6PD def hearts at end-reperfusion, myocardial GST (Figure 4A) and GSH (Figure 4B) were decreased to 30% of WT values, with a marked increase in GSSG (Figure 4C) and a corresponding decline in the GSH/GSSG ratio (Figures 4D). Although in WT hearts after ischemia-reperfusion, GSSG composed 0.5±0.1% of GST, in G6PD def hearts, GSSG was 32.9±13.0% of GST (P=0.01). These data suggest that deficiency of G6PD exacerbates cellular loss of total thiols and limits regeneration of GSH from its oxidized form during ischemia-reperfusion injury.

**Rescue of Ischemia-Reperfusion Injury in G6PD def Hearts by Antioxidant Treatment**

We subsequently determined whether increased susceptibility to ischemia-reperfusion injury in G6PD def hearts was secondary to an impairment in cellular redox state or secondary to a decrease in cellular ribose stores. WT and G6PD def hearts were therefore treated with the superoxide dismutase/catalase mimetic MnTMPyP or animals injected with exogenous ribose, and response to ischemia-reperfusion was examined. Neither MnTMPyP nor ribose treatment altered EDP or contractile function during ischemic conditions (data not shown). Although G6PD functions as the first and rate-limiting enzyme in the de novo production of cellular ribose, supplementation of myocardial ribose stores did not alter ischemia-reperfusion injury in either WT or G6PD def hearts (Figure 5). In contrast, during reperfusion, treatment with MnTMPyP restored both diastolic function (Figure 5A) and contractile function (Figure 5B) in G6PD def hearts to WT values. These data suggest that depletion of cellular thiols and exacerbated oxidative stress, rather than impaired de novo production of ribose, are accountable for cardiac dysfunction in G6PD def hearts during ischemia-reperfusion.

**Discussion**

In this report, we demonstrate the novel finding that G6PD, the first and rate-limiting enzyme in the pentose phosphate pathway, is essential in maintaining redox homeostasis and protecting against oxidative injury during ischemia.
reperfusion in the heart. In response to myocardial ischemia-reperfusion, G6PD is rapidly activated without a change in G6PD protein levels. Mice lacking G6PD exhibited increased cardiac dysfunction during myocardial ischemia-reperfusion, associated with depletion of intracellular glutathione and increased oxidative stress.

**Increased G6PD Activity During Myocardial Ischemia-Reperfusion**

With ischemia-reperfusion in isolated hearts, a rapid increase in myocardial G6PD activity was observed. A similar increase in G6PD activity has been shown to occur in myocardial tissue during exposure to increased oxidative load, suggesting that free radical stress during ischemia-reperfusion may be the stimulus for this phenomenon. The increase in myocardial G6PD activity during ischemia-reperfusion was not associated with a concordant change in G6PD protein levels, consistent with the acute time course. By contrast, more chronic oxidative stressors have been associated with induction of G6PD expression. It is therefore likely that during ischemia-reperfusion, G6PD activity is acutely regulated via a posttranslational mechanism. Potential mechanisms may include intracellular translocation, direct oxidative modification, removal of inactivating oligosaccharide groups, and association with intracellular structural elements/stress proteins, all of which have previously been proposed to be important posttranslational regulators of G6PD activity.

**Exacerbated Ischemia-Reperfusion Injury in G6PD<sup>def</sup> Mice**

When subjected to ischemia-reperfusion, hearts isolated from G6PD<sup>def</sup> mice exhibited impairment in both diastolic and contractile function relative to WT mice. Notably, cardiac dysfunction in G6PD<sup>def</sup> hearts was similar to that of WT counterparts during ischemia and was exacerbated only during the reperfusion period. This finding is concordant with G6PD functioning as an essential antioxidant enzyme, as studies directly measuring free radicals in myocardial tissue have demonstrated only a minimal amount of reactive oxygen or nitrogen species generation occurring during ischemia, with a significant free radical burst on reperfusion. Furthermore, increased susceptibility to ischemia-reperfusion injury in G6PD<sup>def</sup> animals was similar to the dysfunction observed in mice lacking other previously established antioxidant enzymes, including glutathione peroxidase, superoxide dismutase, and heme-oxygenase, using comparable models of severe zero-flow ischemia and reperfusion in isolated hearts, further supporting an antioxidant role for G6PD. Interestingly, although G6PD has been shown to regulate endothelial cell nitric oxide bioavailability, no difference in coronary flow during ischemia-reperfusion was seen in mice lacking G6PD, further implicating a cardiomyocyte-specific pathogenesis for the increased ischemia-reperfusion injury observed in G6PD<sup>def</sup> mice.

G6PD deficiency, the most common genetic enzyme disorder, is estimated to affect more than 400 million people worldwide. Although G6PD deficiency has long been established to predispose red blood cells to dietary and pharmacological oxidative stressors and more recently has been associated with an increased incidence of hypertension and diabetes mellitus, it is uncertain whether deficiency of G6PD increases cardiac dysfunction in humans with cardiovascular disease or in other physiologically relevant models of ischemia-reperfusion, although such studies are currently ongoing.

**Depletion of Cellular Glutathione in G6PD<sup>def</sup> Mice During Ischemia-Reperfusion**

Associated with an increase in cardiac dysfunction, G6PD<sup>def</sup> hearts exhibited a loss of redox homeostasis during ischemia-reperfusion, marked by both depletion of GS<sub>T</sub> and impaired regeneration of GSH from its oxidized form. Depletion of GS<sub>T</sub> may represent a loss of intracellular thiols from either passive membrane leakage from damaged cells, formation of higher oxidative states of glutathione, active transport of GSSG from cells, and/or binding of oxidized thiols to intracellular peptides, all of which occur in cardiomyocytes during oxidative stress. Depletion of GSH pools and accumulation of GSSG in G6PD<sup>def</sup> hearts after ischemia-reperfusion suggest that G6PD is essential for providing the reducing equivalents required to maintain glutathione in its reduced state. Interestingly, at baseline before ischemia-reperfusion, G6PD<sup>def</sup> hearts exhibited no deficit in cellular glutathione relative to WT hearts, suggesting either that the residual 20% G6PD activity in G6PD<sup>def</sup> hearts was adequate to maintain redox status during baseline conditions or that G6PD may be essential to maintain glutathione levels only during periods of increased oxidative stress.

Impaired diastolic and contractile function in G6PD<sup>def</sup> hearts is similar to the cardiac dysfunction that has previously been shown to occur during ischemia-reperfusion with depletion of cellular glutathione or with inhibition of glutathione cycling. This suggests that G6PD mediates susceptibility to ischemia-reperfusion injury through maintenance of cellular glutathione and protection against oxidative stress. This conclusion is further supported by rescue of G6PD<sup>def</sup> hearts by the antioxidant MnTMPyP. Importantly, supplementing myocardial ribose stores did not alter the cardiacoresponse to ischemia-reperfusion in G6PD<sup>def</sup> hearts, suggesting that levels of endogenous ribose, the end product of the pentose phosphate pathway, did not influence cardiac function in G6PD<sup>def</sup> hearts.

The importance of antioxidant enzymes in protecting against myocardial ischemia-reperfusion injury has been well established previously. This report demonstrates that G6PD also exists as an essential antioxidant enzyme required for maintaining cellular glutathione levels and protecting against oxidative stress–induced cardiac dysfunction during ischemia-reperfusion.

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