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Glucose-6-Phosphate Dehydrogenase Modulates Cytosolic Redox Status and Contractile Phenotype in Adult Cardiomyocytes

Mohit Jain, Daniel A. Brenner, Lei Cui, Chee Chew Lim, Bo Wang, David R. Pimentel, Stanley Koh, Douglas B. Sawyer, Jane A. Leopold, Diane E. Handy, Joseph Loscalzo, Carl S. Apstein, Ronglih Liao

Abstract—Reactive oxygen species (ROS)–mediated cell injury contributes to the pathophysiology of cardiovascular disease and myocardial dysfunction. Protection against ROS requires maintenance of endogenous thiol pools, most importantly, reduced glutathione (GSH), by NADPH. In cardiomyocytes, GSH resides in two separate cellular compartments: the mitochondria and cytosol. Although mitochondrial GSH is maintained largely by transhydrogenase and isocitrate dehydrogenase, the mechanisms responsible for sustaining cytosolic GSH remain unclear. Glucose-6-phosphate dehydrogenase (G6PD) functions as the first and rate-limiting enzyme in the pentose phosphate pathway, responsible for the generation of NADPH in a reaction coupled to the de novo production of cellular ribose. We hypothesized that G6PD is required to maintain cytosolic GSH levels and protect against ROS injury in cardiomyocytes. We found that in adult cardiomyocytes, G6PD activity is rapidly increased in response to cellular oxidative stress, with translocation of G6PD to the cell membrane. Furthermore, inhibition of G6PD depletes cytosolic GSH levels and subsequently results in cardiomyocyte contractile dysfunction through dysregulation of calcium homeostasis. Cardiomyocyte dysfunction was reversed through treatment with either a thiol-repleting agent (L-2-oxothiazolidine-4-carboxylic acid) or antioxidant treatment (Eukarion-134), but not with exogenous ribose. Finally, in a murine model of G6PD deficiency, we demonstrate the development of in vivo adverse structural remodeling and impaired contractile function over time. We, therefore, conclude that G6PD is a critical cytosolic antioxidant enzyme, essential for maintenance of cytosolic redox status in adult cardiomyocytes. Deficiency of G6PD may contribute to cardiac dysfunction through increased susceptibility to free radical injury and impairment of intracellular calcium transport. The full text of this article is available online at http://www.circresaha.org. (Circ Res. 2003;93:e9-e16.)

Key Words: glucose-6-phosphate dehydrogenase □ cardiomyocytes □ oxidant injury □ contractile dysfunction □ intracellular calcium

Reactive oxygen species (ROS)–mediated cell injury contributes to the pathophysiology of cardiovascular disease and myocardial dysfunction.1–3 These ROS originate from multiple sources, including cellular oxidase complexes and as mitochondrial byproducts of aerobic metabolism.3,4 To protect against ROS, cardiomyocytes contain a highly active cellular antioxidant defense. Central to the neutralization of ROS is the endogenous thiol, reduced glutathione (GSH), and glutathione cycling.5,6 GSH provides the reducing equivalents necessary for the conversion of hydrogen peroxide and lipid peroxides to water and lipid alcohols, respectively, thereby preventing degradation to highly toxic free radicals, including hydroxyl and peroxy radicals.7 GSH also plays an important role in protecting against oxidation of protein sulfhydryl groups.8 In the presence of ROS, the cellular redox state is altered such that GSH is rapidly converted to its oxidized form, GSSG. The maintenance of GSH pools, in turn, ultimately requires NADPH, an essential cofactor for regeneration of GSH from GSSG.6 In cardiomyocytes, GSH resides in two distinct and separate cellular compartments, the mitochondria and cytosol.5 Depletion of cytosolic GSH levels results in oxidative modification of cytosolic proteins and lipids, as well as polynucleotides. Whereas mitochondrial GSH is maintained largely by NADH interconversion to NADPH via transhydrogenase and by reduction of NADP+ to NADPH via isocitrate dehydrogenase (ICD), malic enzyme, and glutamate dehydrogenase, the mechanism responsible for sustaining cytosolic GSH in cardiomyocytes remains unclear.

Glucose-6-phosphate dehydrogenase (G6PD) functions as the first and rate-limiting enzyme in the pentose phosphate pathway, responsible for the generation of NADPH in a reaction coupled to the oxidation of glucose-6-phosphate and...
de novo production of cellular ribose. In nonnucleated mammalian cells, most notably erythrocytes, G6PD is well-established in the protection against cytosol ROS, as deficiency of G6PD is associated with hemolytic disorders stemming from increased susceptibility of erythrocytes to oxidative stress.9,10 The role of G6PD in cardiomyocytes, however, remains highly controversial,11 largely owing to the high myocardial concentration of NADP⁺-dependent ICD, an alternative enzyme capable of NADPH generation.12 This ICD pool, however, remains confined to the mitochondria and, thus, has little if any effect on cytosolic reodox state. In this article, we demonstrate that G6PD is a critical cytosolic antioxidant enzyme in adult cardiomyocytes. G6PD activity is rapidly increased in response to cellular oxidative stress, with translocation of G6PD to the cell membrane. Furthermore, inhibition of G6PD depletes cytosolic GSH levels and subsequently results in cardiomyocyte contractile dysfunction through dysregulation of calcium homeostasis. Finally, in a murine model of G6PD deficiency, we demonstrate the development of in vivo adverse structural remodeling and contractile dysfunction over time.

Materials and Methods

Adult Cardiomyocyte Isolation, Culture, and Treatment

Adult ventricular cardiomyocytes were isolated from male Sprague-Dawley rats (weighing 250 to 300 g) obtained from Charles River Laboratories (Wilmington, Mass), as previously described.13 Cardiomyocytes were resuspended in 50 mL Dulbecco’s modified eagle medium (DMEM) (GIBCO), and layered over a bovine serum albumin (Sigma Chemical Co) solution to separate ventricular cardiomyocytes from nonmyocytes. For cell physiological or biochemical experiments, cells were plated onto laminin-coated coverslips or culture dishes, respectively. After 1 hour of culture, the medium was changed to ACC2 (2 mmol/L L-carnitine, 5 mmol/L creatine, 5 mmol/L taurocholic acid, 0.1% Fetal Bovine Serum) solution in DMEM.14 Cells were treated with increasing concentrations of dehydroepiandrosterone (DHA), a noncompetitive inhibitor of G6PD,14 in 0.1 mmol/L dimethylsulfoxide (DMSO), or DMSO vehicle alone (0 mmol/L DHA). DHA significantly affected cell morphology and survival at concentrations of 100 mmol/L and higher. Cell physiology experiments were, therefore, conducted with DHA concentrations ranging from 0 to 30 mmol/L, avoiding the confounding effect of cell death on experimental results and data interpretation. For ribose treatment, cardiomyocytes were incubated with 5 mmol/L d-ribose for 60 minutes before study. This concentration of ribose has previously been shown to augment nucleotide production in cultured cardiomyocytes.15 For G6PD gene transfer, cardiomyocytes were infected with recombinant adenovirus containing rat G6PD at an MOI of 10 for 24 hours, as previously described.10 For antioxidat experiments, cardiomyocytes were co-treated with either Eukarion-134 (50 mmol/L) (EUK), a superoxide-dismutase/catalase mimic,16 in 0.1% DMSO, or 1,2-oxothiolin-4-carboxylic acid (5 mmol/L) (OTC), a procysine glutathione-generating compound,18 in 0.1% EtOH. EUK was generously provided by Eukarion, Inc (Bedford, Mass). All other chemicals were obtained from Sigma Chemical Co.

Immunoblotting and Subcellular Fractionation

Total G6PD protein levels were determined by standard Western blot. Cultured cardiomyocytes were lysed in Triton X-100 lysis buffer (Cell Signaling) with protease inhibitors (2 mmol/L leupeptin, 1 mmol/L PMSF). The lysate was sonicated and centrifuged at 16 000g for 15 minutes at 4°C. The supernatant was collected, and protein concentration measured according to the method of Lowry.19 Samples were run on 12% Tris-Glycine precast gels (BioWhitaker) and transferred to PVDF membranes. Equal protein loading was confirmed by Ponceau staining. After blocking in 5% non-fat milk, PVDF membranes were probed with rabbit anti-rat G6PD IgG (kindly provided by Drs Richard A. Cohen and Reiko Matsui, Boston, Mass) followed by HRP conjugated anti-rabbit secondary antibody (Pierce Chemical Co). Protein levels were detected by chemiluminescence (Pierce Chemical Co).

Subcellular fractionation of cardiomyocytes was conducted as previously described.20 Cardiomyocytes were homogenized in 4°C HES buffer (10 mmol/L HEPES, 250 mmol/L Sucrose, 1.0 mmol/L EDTA, pH 7.4) with protease inhibitors. Homogenates were centrifuged at 700 g for 10 minutes at 4°C. The supernatant was collected and centrifuged at 10 000g for 25 minutes. The resulting supernatant was added to the original pellet and designated the membrane/cytosolic fraction. This fraction was sonicated and again centrifuged at 700 g. The supernatant was collected (pellet discarded) and centrifuged at 100 000g for 60 minutes at 4°C. The pellet was resuspended in Triton X-100 lysis buffer and designated as the membrane fraction. The supernatant was diluted with Triton X-100 lysis buffer and designated as the cytosolic fraction. Protein concentration was determined in both membrane and cytosolic fractions and protein levels of G6PD determined via Western blot, as described above.

Measurement of Cardiomyocyte Cell Shortening and Intracellular Calcium

Cell shortening and intracellular calcium transients ([Ca²⁺]i), transients) were recorded in cardiomyocytes, as previously described.13,21,22 Cultured cardiomyocytes were incubated with membrane permeant fura-2 (1 μmol/L) (Molecular Probes) and probenecid (500 μmol/L), to prevent leakage of fura-2 from cells. Cardiomyocytes were perfused with 1.2 mmol/L CaCl₂ Tyrode solution at 37°C and electrically paced at 300 beats per minute via platinum wires. Cell shortening/relengthening, and [Ca²⁺]i transients were measured using video edge detection and fluorescence measurements of the Fura-2 ratio, respectively (SoftEdge Acquisition System and IonWizard, IonOptix Inc).13,21,22 Percent cell shortening (% CS) was calculated as diastolic cell length minus systolic cell length normalized to the diastolic cell length. The rate of cardiomyocyte contraction (−dL/dt) and rate of cardiomyocyte relaxation (+dL/dt) were calculated by commercially available acquisition software (IonWizard, IonOptix Inc). The time constant (τ), a cell length–independent measure of cardiomyocyte relaxation, was calculated as previously described.22 [Ca²⁺]i was calculated from fura-2 fluorescence as previously described,22 and [Ca²⁺]i transients were analyzed in a similar fashion to cell shortening experiments. Each value presented for the cardiomyocyte contractility or calcium experiments represents 6 to 10 independent experiments (at each concentration of DHA) with 5 to 20 cell measurements per experiment.

Biochemical Assays

Biochemical assays were performed on mitochondrial-free cardiomyocyte homogenates. Mitochondrial free-lysates were prepared as previously described.23 Cardiomyocytes were homogenized in 4°C HES buffer with protease inhibitors (as described above). Homogenates were centrifuged at 16 000g for 15 minutes at 4°C. The supernatant was collected and used for assays. The absence of mitochondria in the supernatant was confirmed through Western blotting for mitochondrial proteins, including cytochrome C and manganese superoxide dismutase.

Glucose-6-Phosphate Dehydrogenase Activity

Glucose-6-phosphate dehydrogenase activity was determined in mitochondrial-free cardiomyocyte homogenates according to the methods of Tian and colleagues.24 Protein concentration was determined according to the method of Lowry.19
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from frozen embryos. G6PDdef 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.28 Male hemizygous G6PDdef mice and wild-type littermates (WT) were used in this study. All animal studies strictly adhered to the regulations of Boston University Animal Care Committee and the National Society for Medical Research.

Intracellular ROS
Intracellular ROS was assessed using the ROS-sensitive fluorophore, dichlorofluorescein diacetate (DCF) (Molecular Probes). Oxidation of DCF by intracellular ROS results in the formation of a fluorescent compound.26 Cardiomyocytes were incubated with 20 μmol/L DCF for 30 minutes and DCF fluorescence visualized and quantified using epi-fluorescent microscopy and video imaging (BIOQUANT, version 2.5).

G6PD-Deficient Mice
G6PD-deficient mice (G6PDdef),27 were rederived in our laboratories from frozen embryos. G6PDdef 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.28 Male hemizygous G6PDdef mice and wild-type littermates (WT) were used in this study. All animal studies strictly adhered to the regulations of Boston University Animal Care Committee and the National Society for Medical Research.

Transthoracic Echocardiography
Transthoracic echocardiograms were serially obtained in conscious mice, as previously described,21,29 using an Acuson Sequoia C-256 echocardiograph machine and a 15-MHz probe. The heart was imaged in the two-dimensional parasternal short-axis view, and an M-mode measurement was recorded at the mid-ventricle at the level of the papillary muscles. Heart rate, septal wall thickness, and end-diastolic and end-systolic dimensions were measured from the M-mode image using analysis software (Acuson, Sequoia). Fractional shortening was defined as the end-diastolic dimension minus the end-systolic dimension normalized for the end-diastolic dimension, and was used as an index of cardiac contractile function.

Statistical Analysis
Statistical significance was evaluated by one-way analysis of variance (ANOVA). A post hoc test of least significant differences was used to determine differences among groups. All data are expressed as mean±SEM. A value of \( P<0.05 \) was considered statistically significant.

Glutathione Levels
Total (GS\(_2\)), reduced (GSH), and oxidized (GSSG) glutathione concentrations were determined in mitochondrial-free cardiomyocyte homogenates using a commercially available kit (Cayman Chemical) based on an enzymatic recycling method described by Tietze and coworkers.25 All assays were run in triplicate and averaged to obtain a mean value per sample.

Results
Activation of G6PD and Membrane Translocation With Oxidative Stress
To determine whether G6PD activity is responsive to oxidative stress in cardiomyocytes, G6PD activity was measured after exposure to hydrogen peroxide. As shown in Figure 1A, in cardiomyocytes, hydrogen peroxide resulted in a dose-dependent increase in G6PD activity, with a 3-fold induction at 200 μmol/L. In addition, cardiomyocyte G6PD activity was increased with oxidative stress in a time-dependent fashion (Figure 1B), with rapid induction within 15 minutes. We further determined G6PD protein levels in response to oxidative stress. As shown in Figure 1C, total protein levels of G6PD were unchanged with hydrogen peroxide treatment over the 30-minute time period; however, G6PD exhibited a rapid translocation from the cytosol to the cell membrane, as demonstrated by cellular fractionation, correlating with the increase in enzyme activity. These results suggest that G6PD is rapidly activated in response to cellular oxidative stress in cardiomyocytes, with corresponding translocation to the cellular membrane.

Inhibition of G6PD Alters Cardiomyocyte Redox Status
To assess the role of G6PD in modulation of cytosolic redox status in cardiomyocytes, cells were treated with the noncompetitive G6PD inhibitor, DHA, and cytosolic glutathione levels determined. DHA inhibited cardiomyocyte G6PD activity in a dose-dependent manner (Figure 2A), with approximately 40% inhibition of G6PD activity at 30 μmol/L and 60% inhibition at 100 μmol/L. Inhibition of G6PD with DHA resulted in a decrease in both total glutathione and GSH levels in a concentration-dependent manner (Figures 2B and 2C), with nearly complete depletion of cytosolic total glutathione and GSH at 100 μmol/L. Furthermore, the ratio of GSH to GSSG, a sensitive marker of cytosolic redox status, declined with increasing concentration of DHA, and fell to
approximately 30% of control values at 100 μmol/L DHA. Corresponding with the depletion of cytosolic glutathione, intracellular reactive oxygen species increased greater than two-fold with 100 μmol/L DHA treatment, as assessed by DCF fluorescence (24.2±1.8 Control versus 56.2±2.9 100 μmol/L DHA arbitrary units of fluorescence/cell; *P<0.01) (Figure 2E). These data suggest that G6PD is essential for maintenance of cytosolic glutathione levels and redox balance in cardiomyocytes.

Inhibition of G6PD Activity Impairs Cardiomyocyte Function
We subsequently determined the effects of G6PD inhibition and altered redox state on cardiomyocyte contractility. End diastolic cell length was unaffected by treatment with DHA (0, 3, 10, and 30 μmol/L DHA: 106±8, 102±4, 100±6, and 101±5 μm; P=NS). As shown in Figure 3A, cardiomyocytes exhibited a decrease in percent cell shortening (%CS) in a concentration-dependent manner with DHA treatment, with a 60% reduction in %CS at 30 μmol/L DHA. A similar attenuation of contractile function was also observed in the online data supplement at http://www.circresaha.org. Cardiomyocyte relaxation was also impaired in a graded manner in DHA-treated cells, resulting in a prolonged time constant (τ) for relaxation (Figure 3B), and a slowed maximum rate of cellular relaxation (+dL/dt) (online Figure 1B). Adenoviral-mediated overexpression of recombinant G6PD restored cellular G6PD activity to control values (data not shown) and restored cardiomyocyte shortening and relaxation to control values (Figures 3A and 3B, online Figures 1A and 1B), suggesting that DHA-induced contractile dysfunction was secondary to inhibition of G6PD. Transfection with control adenovirus containing an empty vector did not alter cellular G6PD levels or cardiomyocyte function (data not shown). Impairment of cardiomyocyte shortening and relaxation was also unaffected by co-treatment with exogenous ribose (Figures 4A and 4B, online Figures 2A and 2B), suggesting that cellular dysfunction in G6PD inhibited cells was not secondary to depletion of cellular ribose, the end product of the pentose phosphate pathway. In contrast, cardiomyocyte dysfunction was completely reversed by co-treatment with the superoxide-dismutase/catalase mimetic, Eukarion-134 (Figures 4A and 4B, online Figures 2A and 2B). Similarly, coinoculation with the procysteine glutathione-generating compound, L-2-oxothiazolidine-4-carboxylic acid, protected against cardiomyocyte contractile dysfunction (Figures 4A and 4B, online Figures 2A and 2B), suggesting that cardiomyocyte dysfunction in G6PD-inhibited cells was secondary to impairment in cellular redox state and depletion of endogenous thiols.

Inhibition of G6PD Impairs Intracellular Calcium Transport
To determine the mechanism by which redox imbalance and oxidative stress induces contractile dysfunction in G6PD-inhibited cardiomyocytes, intracellular calcium transients ([Ca^{2+}], transients) were determined in paced cells using the calcium-sensitive fluorescent probe, Fura-2. As shown in Figure 5A, DHA resulted in a concentration-dependent decrease in [Ca^{2+}], transient amplitude during cardiomyocyte contraction. Furthermore, inhibition of G6PD with DHA also...
impaired removal of \([\text{Ca}^{2+}]\), during cellular relaxation (Figure 5B). Importantly, impairment of both calcium release and calcium reuptake in cardiomyocytes closely correlated with contractile dysfunction (Figures 3A and 3B) and were restored to control values with normalization of G6PD activity through G6PD gene transfer (Figures 5A and 5B), but were unaffected by transfection with empty viral vector controls (data not shown). In addition, dysfunctional calcium homeostasis, as observed with cardiomyocyte contractile function, was unaffected through treatment with ribose, but returned to control values with antioxidant treatment and restoration of cellular thiols (Figures 6A and 6B). Representative tracings of cardiomyocyte shortening and corresponding \([\text{Ca}^{2+}]\) transients are demonstrated in Figure 7. These results suggest that dysregulation of calcium homeostasis may be accountable for contractile dysfunction in G6PD inhibited cardiomyocytes.

Adverse Structural Remodeling and Contractile Dysfunction in G6PD-Deficient Mice

Although our results suggest that inhibition of G6PD may alter redox homeostasis and impair contractile function in isolated cardiomyocytes, it is uncertain whether G6PD deficiency results in the development of contractile dysfunction in vivo. In vivo cardiac structure and function were, therefore, serially evaluated in male hemizygous mutant mice (G6PD\textsuperscript{def}) and wild-type (WT) littermate counterparts. Hearts from G6PD\textsuperscript{def} mice exhibited approximately 20% of WT G6PD activity (6.2±0.3 G6PD\textsuperscript{def} versus 30.8±1.8 WT AU/mg protein; *P<0.01). Young WT and G6PD\textsuperscript{def} mice (3 months) exhibited similar cardiac structural and contractile phenotypes (Table). G6PD\textsuperscript{def} mice, however, exhibited progressive adverse structural remodeling and cardiac dysfunction over time, marked by increased ventricular septal wall thickening and end-systolic chamber dimensions, as well as a significant decrease in fractional shortening relative to age-matched WT controls at 9 months of age. These results suggest that G6PD deficiency may modulate cardiac structure and contractile function in vivo.

Discussion

The exquisite sensitivity of myocardium to even ambient levels of oxidative injury is well established.\textsuperscript{30,31} This article demonstrates the necessity of G6PD as a critical component of the cellular antioxidant system in adult cardiomyocytes, required for maintaining cytosolic redox homeostasis and contractile function in adult cardiomyocytes, both in vitro and in vivo.
G6PD and Cardiomyocyte Redox State

Cardiomyocyte defense against oxidative stress is mediated primarily through neutralization of deleterious ROS via reducing equivalents in the form of intracellular thiols. In cardiomyocytes, we demonstrate that acute exposure to the oxidant hydrogen peroxide resulted in rapid upregulation of G6PD activity. Importantly, this increase in G6PD activity was not translationally regulated, as has been suggested to occur with more chronic stress response. Rather, G6PD activation was associated with translocation from its cytosolic position to its active site on the cellular membrane. This translocation allows for entering glucose to shunt rapidly to the pentose phosphate pathway, thereby providing substrate for G6PD for NADPH generation. In fact, previous reports have demonstrated an increase in pentose phosphate pathway flux after exposure to oxidative stressors. Similar regulation of G6PD activity through cellular translocation has been shown to occur in neutrophils, in which acute increases in G6PD activity regulate cytosolic NADPH-dependent oxidase complexes. Furthermore, in cardiomyocytes, inhibition of G6PD resulted in loss of redox homeostasis and an increase in intracellular oxidative stress, evidenced by a decrease in total glutathione, reduced glutathione, and the ratio of reduced-to-oxidized glutathione, as well as a marked increase in cytosolic ROS. Importantly, G6PD activity correlated directly with levels of cytosolic reduced glutathione. Taken together, these data suggest that G6PD is essential for maintenance of cytosolic glutathione stores and subsequent protection against cellular ROS.

These data differ significantly from previous reports, which have suggested an almost nonexistent role for G6PD in...
Inhibition of G6PD and Cardiomyocyte Function

The functional consequences of impaired cytosolic redox status in cardiomyocytes with inhibition of G6PD included dysfunction of cellular contraction and relaxation, secondary to impairment in intracellular calcium transport. Interestingly, the progressive development of contractile dysfunction, accompanied by global structural changes, was also observed in vivo in an animal model of G6PD deficiency. Rescue of cardiomyocyte function in vitro with a glutathione-generating compound or antioxidant treatment, along with the absence of any effect with ribose treatment, strongly supports the hypothesis that cellular dysfunction secondary to G6PD inhibition occurred as a result of depletion of cytosolic thiols and increased oxidative stress, rather than as a result of decreased end-product generation by the pentose phosphate pathway. Importantly, the effects of G6PD inhibition with DHA were reversed with reconstitution of G6PD activity through adenoviral gene transfer, suggesting that nonspecific effects of DHA were not responsible for our observations.

It is well established that pure free radical generating compounds, including hydrogen peroxide, alter both systolic and diastolic myocardial function, although the mechanisms of injury remain less clear. Highly reactive free radicals may potentially impair cellular metabolism, damage DNA, alter membrane integrity, and disrupt ionic balance, with the principle targets of damage highly dependent on cell type and the nature of the oxidative stress imposed. Contractile dysfunction in cardiomyocytes with G6PD inhibition was closely accompanied by an impairment of calcium release during contraction and slowed calcium reuptake during relaxation. Free radical–mediated disruption of intracellular calcium mobilization has previously been shown to occur. Thus, this would suggest that disruption of intracellular calcium transport might be the primary mechanism by which oxidative stress impaired cardiomyocyte contractility in cells with inhibition of G6PD. Despite slowed calcium reuptake, no change in diastolic calcium levels was observed with inhibition of G6PD, in contrast to previous studies in cardiomyocytes with direct exposure to hydrogen peroxide. This disparity may be related to both the means of free radical generation (antioxidant inhibition versus direct ROS exposure) and the degree of oxidative stress imposed. Impaired calcium handling in cardiomyocytes with inhibition of G6PD may be the result of decreased expression and/or direct posttranslational modification of calcium-handling proteins in human erythrocytes with genetic deficiency of G6PD, oxidative modification of the calcium ATPase has also been proposed as a major mechanism promoting ion imbalance and hemolysis. Alterations of calcium handling proteins secondary to increased oxidative stress are, therefore, likely responsible for the impairment of calcium mobilization and the decrease in cell contractility observed in G6PD-inhibited cardiomyocytes.

Free radicals contribute to the progression of many forms of cardiovascular disease. The role of G6PD as an essential antioxidant enzyme in the heart has not heretofore been established. These results suggest that G6PD is essential for maintaining cytosolic redox homeostasis and protecting against free radical injury in the myocardium and suggest that deficiency of G6PD may contribute to cardiac dysfunction through impairment of calcium homeostasis.

Acknowledgments

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Online Supplement Figure Legend

**Online Figure 1**: Inhibition of G6PD impairs contractile function in cardiomyocytes. Maximum rate of cell shortening (-dL/dt) (**Figure 1A**) and maximum rate of cell relengthening (+dL/dt) (**Figure 1B**) in cardiomyocytes paced at 300 bpm following 12-hour incubation with dehydroepiandrosterone (DHA) (0-30 µmol/L), and/or 24 hour incubation with adenovirus containing recombinant rat G6PD (Ad-G6PD) at an MOI of 10. *: p<0.05 vs 0 µmol/L DHA; †: p<0.05 vs 3 µmol/L DHA; ‡: p<0.05 vs 30 µmol/L DHA.

**Online Figure 2**: Rescue of contractile function in G6PD inhibited cardiomyocytes with glutathione-generating or antioxidant treatment. Maximum rate of cell shortening (-dL/dt) (**Figure 2A**) and maximum rate of cell relengthening (+dL/dt) (**Figure 2B**) in cardiomyocytes paced at 300 bpm following treatment with vehicle or 30 µmol/L dehydroepiandrosterone (DHA), 5 mmol/L D-ribose (ribose), 50 µmol/L Eukarion-134 (EUK), or 5 mmol/L L-2-oxothiazolidine-4-carboxylic acid (OTC). *: p<0.05 vs 0 µmol/L DHA; ‡: p<0.05 vs 30 µmol/L DHA.