Superoxide production by NAD(P)H oxidase and mitochondria is increased in genetically obese and hyperglycemic rat heart and aorta before the development of cardiac dysfunction. The role of glucose-6-phosphate dehydrogenase-derived NADPH

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Type 2 diabetes affects a number of visceral organs, including the heart. Reduced diastolic function and left ventricular (LV) hypertrophy have been diagnosed in the absence of coronary heart disease in patients with diabetes, and heart failure is a leading cause of death among these patients (2). There is now solid evidence that, despite the elevated plasma glucose levels, the diabetic heart oxidizes less glucose and more fatty acid than the healthy heart (5). This altered cardiac energy metabolism is one of the causes of cardiac dysfunction and the development of diabetic cardiomyopathy. High fatty acid uptake and metabolism by the heart not only promotes accumulation of fatty acid intermediates and triglycerides, but it also increases oxygen demand and the generation of reactive oxygen species (ROS), leading to cardiac injury (2). In addition, hyperglycemia increases oxidative stress, inhibits synthesis of endothelium-derived nitric oxide (NO), and accelerates proliferation of vascular smooth muscle cells, leading to endothelial and vascular dysfunction, which is a main cause of cardiovascular complications in both diabetic animal models (11, 32, 59) and patients with diabetes (3, 11, 14, 15, 20–22, 25, 30). Although the mechanism underlying the increased production of ROS in the vasculature and the heart is still not completely understood, it has been proposed that the high rate of fatty acid oxidation increases mitochondrial membrane potential, which augments generation of ROS (2), and that overexpression of NAD(P)H oxidase isoforms (Nos-2 and Nos-4) in the vascular system and kidneys increases production of superoxide anion (O\textsubscript{2}•-) (13, 22). It has also been demonstrated that O\textsubscript{2}•- generated by Nos mediates oxidative damage to vascular tissue in diabetes (32, 48, 50).

Under physiological conditions, constitutively active Nos produces low levels of O\textsubscript{2}•- (28), which are removed by cellular antioxidants. However, under pathophysiological conditions protein kinase C (PKC)-dependent phosphorylation of p47\textsuperscript{phox}, a regulatory Nos subunit, leads to the activation of the enzyme following stimulation by factors such as angiotensin II, thrombin, and TNF-\alpha (28, 31), thereby enhancing O\textsubscript{2}•- generation. In addition, the O\textsubscript{2}•- generation catalyzed by Nos also depends on the availability of reducing equivalents. Therefore, we and others have suggested that NADPH generated by glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting enzyme in the pentose phosphate pathway (PPP), fuels Nos and sustains O\textsubscript{2}•- production in the heart (18, 60) and vasculature (17, 36). A small fraction of the total glucose in the cytosol of cardiomyocytes is oxidized to ribose by the PPP (57, 58), and we recently demonstrated that overexpression of G6PD augments NADPH production, which increases O\textsubscript{2}•- production by Nos in pacing-induced heart failure, an established model of dilated cardiomyopathy in dogs (18), and in ischemic cardiomyopathy.

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in humans (16). Likewise, several other investigators have reported carbohydrate and fatty acid metabolism to be profoundly altered in the nondiabetic failing heart (52). In particular, myocardial glucose uptake and oxidation are increased (12, 40) although the glycolytic pathway and the mitochondrial capacity to oxidize carbohydrates appear to be downregulated (29, 45). Notably, changes in cardiac metabolism occur early and precede the development of diabetic cardiomyopathy (2). In light of these observations, we hypothesized that, in the hyperglycemic/hyperinsulinemic heart, more glucose is oxidized through the PPP, making a larger amount of the electron donor NADPH available to fuel \( \text{O}_2 \)-generating enzymes. Consistent with our hypothesis, NADPH levels were found to be elevated in the endothelial cells of diabetic BB rats (37). Interestingly, it is well known that coronary arterial- and subendocardial endothelium-derived NO regulates myocardial mitochondrial respiration, substrate utilization, and contractility (7, 47, 52). In that regard, it has already been shown that endothelium-derived NO release is downregulated and \( \text{O}_2 \)-generation is upregulated in heart failure, which alters myocardial substrate utilization, eventually leading to cardiac dysfunction (46). Therefore, the aims of the present study were to determine 1) whether G6PD expression and activity are upregulated; 2) whether Nox is a source of \( \text{O}_2 \); and 3) whether Nox-generated \( \text{O}_2 \) causes cardiac dysfunction in young Zucker fa/fa rats, a type 2 diabetic model that resembles human diabetes.

**METHODS**

All drugs and salts were purchased from either Sigma (St. Louis, MO) or Calbiochem (San Diego, CA). The stock solutions of cis-N-(2-phenylcyclopentyl)azacyclopentec-1-en-2-amine HCl (MDL 12330A), chelerythrine, 6-aminoaminonitidamide (6-AN), and dihydroepiandrosterone (DHEA) were made in dimethyl sulfoxide (Sigma), and final 1:1,000 dilutions in buffered physiological salt solution were used in the study.

**Animal model.** Our Institutional Animal Use Committee approved all protocols and surgical procedures, which were in accordance with National Institutes of Health (NIH) and American Physiological Society guidelines. To test our hypothesis, experiments were performed with adult male Zucker fa/fa rats, a model of hyperglycemia, and type 2 diabetes, purchased from Charles River Laboratories (Wilmington, MA). All rats were exposed to a 12-h:12-h light-dark cycle and allowed free access to standard rat food and water. Animals were anesthetized with Nembutal (10 mg/kg), and the chest cavity was opened. The heart was quickly removed, and Langendorff perfusion was established. Each heart was perfused retrogradely with modified Krebs-Henseleit solution containing (in mM) 116.0 NaCl, 25.0 NaHCO3, 2.5 CaCl2, 1.2 MgSO4, 4.7 KCl, 1.2 KH2PO4, and 5.5 glucose (pH 7.4) at a constant flow rate (12 ml/min) without recirculation. The perfusate was warmed to 38°C and oxygenated with 95% \( \text{O}_2 \) and 5% \( \text{CO}_2 \) gas mixture to maintain a PO of 400 mmHg during the entire experimental period. The perfused hearts were cut in small pieces and homogenized as described in individual protocols and used for biochemical analyses. In some experiments, the heart was perfused with drugs for 30 min at 37°C and then removed for biochemical analyses.

**Measurement of glucose and insulin levels.** Blood glucose levels were determined using standard glucose strips with a glucometer (Abbott Laboratory), and insulin levels were quantified by an ELISA kit purchased from Cayman Chemical.

**Measurement of \( \text{O}_2^- \) and \( \text{O}_2^+ \) levels.** Measurement of \( \text{O}_2^- \) and \( \text{O}_2^+ \) levels were determined using previously described protocols (18). Briefly, tissue homogenates (50 µl) were incubated at 37°C in 20 mM MOPS buffer (pH 7.4) and 250 mM sucrose for 10 min in absence or presence of drugs, and \( \text{O}_2^+ \) was detected based on lucigenin (5 µM) chemiluminescence. Some experiments examined \( \text{O}_2^- \) production in the presence of a NADPH regenerating system [glucose-6-phosphate (200 µM) plus NADP+ (200 µM)] to evaluate the relative roles of NADPH.

**Measurement of superoxide dismutase activity.** Superoxide dismutase (SOD) activity was assessed by measuring the dismutation of \( \text{O}_2^- \) generated by xanthine oxidase and hypoxanthine in a convenient 96-well format using a kit from Cayman Chemical.

**Measurement of glutathione peroxidase and glutathione reductase activities.** Glutathione (GSH) peroxidase (GSH-Px) and GSH reductase activities were measured using a kit from Cayman Chemical. The oxidation of NADPH to NADP+ was accompanied by a decrease in absorbance at 340 nm (A340). The rate of the A340 reduction was directly proportional to the activities of both GSH-Px and GSH reductase in the sample.

**Measurement of GSH.** Total GSH levels were measured using a GSH reductase-based recycling method.

**Measurement of triglycerides and free fatty acid.** Triglyceride and free fatty acid levels in serum collected from Zucker lean and fa/fa rats were determined using kits purchased from Teco Diagnostics (Anaheim, CA).

**Western blot analysis.** Homogenates were prepared from frozen tissues, and the total protein content was measured as described previously, after which 35-µg samples were subjected to SDS-PAGE. Western blot analyses were then performed using rabbit polyclonal anti-G6PD (Sigma Chemical); mouse monoclonal anti-Nox-1, anti-Nox-2, and anti-p67phox (Transduction Laboratory, San Jose, CA); goat polyclonal anti-Nox-4 and anti-p47phox; and mouse monoclonal anti-α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA). Bands were visualized by autoradiography and quantified using densitometry. Densitometric values for specific proteins were normalized to α-tubulin and expressed as a ratio of the two proteins.

**Measurement of G6PD activity.** G6PD activity was measured in myocardial homogenates by following the reduction of NADP+ to NADPH (17). NADPH fluorescence was detected as 460 nm emission excited at 340 nm using a Flx800 microplate fluorescence detector (BioTek Instruments, Winoski, VT).

**Measurement of NADPH levels.** Frozen tissues were homogenized in an extraction medium containing NaOH (0.02 N) and cysteine (0.5 mM) at 0°C. The extracts were then heated at 60°C for 10 min and neutralized with 2 ml of 0.25 M glycylglycine buffer (pH 7.6), as described previously. NADPH levels were then estimated by determining NADPH fluorescence (460 nm emission excited at 340 nm) using a previously described recycling method (35).

**Isometric force generation in isolated rat aorta.** Aortic rings were prepared as previously described (19) with minor modifications. The rings were mounted on wire hooks attached to force displacement transducers (V99-50; Coulbourn Instruments, Allentown, PA) for measurement of changes in isometric force, which were recorded on a polygraph (S13-02; Coulbourn Instruments). Resting passive force was adjusted to a previously determined optimum (1 g, determined based on the maximum response to 123 mM KCl), and vessels were equilibrated for 1 h in muscle baths containing Earle’s balanced salt solution gassed with 21% \( \text{O}_2 \)-5% \( \text{CO}_2 \)-74% \( \text{N}_2 \). To assess endothelial function, rings with intact endothelium were precontracted with phenylephrine (1 µM) and, after a stable contraction was reached, the rings were dilated with either acetylcholine or nitroglycerin, an NO donor. All values for vasodilation were expressed as percent changes from the precontracted force.
Blood chemistry in Zucker lean and fa/fa rats

Table 1. Blood chemistry in Zucker lean and fa/fa rats

<table>
<thead>
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<th>Lean</th>
<th>Zucker fa/fa</th>
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<tr>
<td>Glucose, mM</td>
<td>7.1±0.6</td>
<td>19.0±0.7*</td>
</tr>
<tr>
<td>Insulin, ng/mL</td>
<td>0.67±0.27</td>
<td>3.04±0.22*</td>
</tr>
<tr>
<td>Free fatty acid, mM</td>
<td>0.63±0.33</td>
<td>3.87±0.10*</td>
</tr>
<tr>
<td>Triglycerides, mg/mL</td>
<td>0.34±0.07</td>
<td>5.21±1.15*</td>
</tr>
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Values are means ± SE; n = 6 lean and 6 Zucker fa/fa rats. *P < 0.05.

Echocardiography. Rats underwent echocardiography after sedation with pentobarbital sodium (25 mg/kg body wt ip), which did not induce a significant change in heart rate. The measurements were made using a 13-MHz linear transducer (Acuson, Mountain View, CA), and images were obtained in the parasternal long and short axes. LV chamber dimensions and wall thickness were measured in a papillary muscle plane, perpendicular to the LV in M-mode recordings. LV chamber volumes were assessed in a two-dimensional parasternal view using the cylinder-ellipse Bull’s formula

\[ V = \frac{\pi}{6} \cdot A \cdot L \]

where A is the short-axis area and L is the long-axis length. Stroke volume (SV) was measured as the difference between LV end-diastolic volume (EDV) and the LV end-systolic volume (ESV). Ejection fraction (EF) was calculated as \( EF = \frac{SV}{EDV} \times EDV \). Cardiac output (CO) was calculated as \( CO = SV \times HR \).

Statistical analysis. Values are means ± SE. Comparisons between groups were made using Student’s t-test or ANOVA with Scheffé’s post hoc test for multiple comparisons. Values of \( P < 0.05 \) were considered significant.

RESULTS

Young Zucker fa/fa rats are obese, hyperglycemic, and hyperinsulinemic. Zucker fa/fa (9–11 wk old) rats were hyperglycemic and hyperinsulinemic and showed elevated blood triglyceride and free fatty acid levels, compared with lean animals (Table 1). They were somewhat obese (Table 2).

G6PD expression and activity is upregulated in Zucker fa/fa rat hearts. It is well known that cardiac glucose uptake and metabolism is altered in type 2 diabetes. However, the fate of glucose shunted through the PPP under conditions of hyperglycemia is not well studied. Because blood triglycerides and free fatty acids are elevated in Zucker fa/fa rats (Table 1), and fatty acid metabolism is increased in the type 2 diabetic heart (2), we hypothesized that glucose metabolism through the PPP, which is the major source of NADPH production in cardiac myocytes, should concomitantly increase to support and sustain the increased lipid metabolism. To test that idea, we compared the expression and the activity of G6PD, the rate-limiting enzyme in the PPP, in Zucker fa/fa and lean rats. Western blot analysis showed that G6PD expression was significantly increased in hearts from fa/fa rats, compared with lean rats (Fig. 1, A and B). In addition, G6PD activity in myocardial homogenates from obese rats was more than twice that in samples from age-matched lean rats (Fig. 1C). NADPH, a by-product of the G6PD-catalyzed reaction, and 6-phosphogluconate, an intermediate product, were also significantly increased in fa/fa rats (Fig. 1, D and E).

G6PD is activated by PKC in Zucker fa/fa rats. G6PD is inhibited by cAMP-dependent kinases in aortic endothelial cells cultured in high glucose medium and in kidneys from streptozotocin-treated rats (54, 55). Conversely, it is activated by PKC in rat renal cortical cells (53) and by Src and PKC in failing human hearts (16). We, therefore, speculated that various signaling molecules in which activities are altered in diabetes, such as PKC, as well as insulin receptor-dependent mediators, such as phosphatidylinositol 3-kinase (PI3-kinase) and Src kinase, may modulate G6PD activity in the hearts of Zucker fa/fa rats. We found that G6PD activity was inhibited by chelerythrine, a PKC inhibitor, in fa/fa hearts (Fig. 1F), as well as by calphostin C (100 nM; 0.005 ± 0.0001 nmol·min⁻¹·mg⁻¹ protein), another PKC inhibitor. Interestingly, inhibition of PI3-kinase and Src kinases using LY-294002 (10⁻⁶ M) and 4-amino-5-(4-chlorophenyl)-7-(r-butyl)pyrazole[3,4-d]pyrimidine (PP2; 10 μM), respectively, did not inhibit cardiac G6PD activity in the fa/fa rats (untreated control, 0.016 ± 0.005 nmol·min⁻¹·mg⁻¹ protein; LY-294002, 0.018 ± 0.002 nmol·min⁻¹·mg⁻¹ protein; and PP2, 0.038 ± 0.014 nmol·min⁻¹·mg⁻¹ protein).

Oxidative stress is increased in Zucker fa/fa rat hearts. We assessed oxidative stress by estimating \( O_2^- \) and \( H_2O_2 \) production in hearts from Zucker fa/fa and lean rats. We found that levels of \( O_2^- \) were actually about twofold higher in fa/fa than in lean rats (Fig. 2A), whereas \( H_2O_2 \) levels were not different (Fig. 2B). To confirm that the observed elevation in \( O_2^- \) production was not an artifact, homogenates were pretreated with polyethylene glycol SOD (peg-SOD; 300 U/ml) to scavenge the \( O_2^- \) produced in the myocardium of fa/fa rats. As expected, peg-SOD treatment significantly reduced \( O_2^- \) levels by 60–80% (\( P < 0.05 \)), compared with the untreated controls.

Antioxidant levels are reduced in Zucker fa/fa rat hearts. We also found that the activity of antioxidant enzymes was altered in Zucker fa/fa rats. The activities of both SOD and GSH-Px were lower in the fa/fa than in lean rats (Fig. 2, C and D). GSH reductase activity, which is dependent on G6PD-derived NADPH, was also significantly lower in fa/fa hearts (Fig. 2E). Estimation of GSH levels revealed that the GSSG-to-GSH ratio was markedly reduced (Fig. 2F).

Nox and mitochondria are major sources of \( O_2^- \) in Zucker lean and fa/fa rat hearts. Although studies have shown that \( O_2^- \) is elevated in diabetes, the source and mechanism of \( O_2^- \) generation are still unclear. To assess the extent to which Nox contributes to the elevated \( O_2^- \) levels seen in type 2 diabetes,
we estimated $O_2^-$ production in homogenates of hearts harvested from Zucker lean and fa/fa rats. As mentioned above, $O_2^-$ levels were significantly higher in hearts from fa/fa rats than in those from lean rats (Fig. 2A). Moreover, gp91$^{ds-tat}$ (50 μM), a Nox inhibitor, as well as rotenone (50 μM) and antimycin (10 μM), inhibitors of mitochondrial respiratory chain complexes I and III, respectively, inhibited $O_2^-$ generation in Zucker lean and fa/fa rat hearts (Fig. 3A). Lower concentration of rotenone (1 μM) inhibited [not significant (NS)] $O_2^-$ generation by 30–35% compared with untreated controls. Scrambled gp91$^{ds-tat}$ peptide (50 μM), a negative control for gp91$^{ds-tat}$, did not reduce $O_2^-$ levels (data not shown).

Nox expression is not altered in Zucker fa/fa rat heart. To clarify the mechanism underlying the increased $O_2^-$ generation in the Zucker fa/fa heart, we tested whether the expression of any of the Nox subunits was altered. Using Western blot analysis, we estimated the levels of Nox-2, Nox-4, p47$^{phox}$, and p67$^{phox}$ in homogenates of heart from Zucker lean and fa/fa rats but found no significant differences between their expressions in the two groups (Fig. 3, B and C).

Nox is activated by PKC in Zucker fa/fa rats. Phosphorylation of p47$^{phox}$ and p67$^{phox}$ by PKC triggers their translocation to the membrane, where they activate Nox-2, a Nox isoform present in phagocytes and the vasculature (27). We, therefore, speculated that PKC-dependent signaling may be involved in activating Nox and increasing $O_2^-$ production in Zucker fa/fa hearts. Consistent with that idea, we found that the PKC inhibitor, chelerythrine or calphostin C, reduced levels of $O_2^-$ in homogenates of Zucker fa/fa heart but not homogenates of lean heart (Fig. 4A). PI3-kinase and Src kinase modulate Nox activity in the heart and vascular tissue (16, 27, 51); their inhibition using LY-294002 and PP2, respectively, significantly reduced $O_2^-$ generation in Zucker fa/fa hearts (untreated control, 405.2 ± 86.2 AU/mg protein; LY-294002, 255.1 ± 19.5 AU/mg protein; and PP2, 237.5 ± 15.6 AU/mg protein; P < 0.05).

Nox and G6PD act in concert to increase $O_2^-$ production in diabetic tissues. We and others have suggested that G6PD-derived NADPH fuels $O_2^-$ production (16, 18, 36). G6PD is the major source of NADPH in the cardiac cells, and Nox requires NADPH for activity. Together, these observations prompted us to ask whether Nox and G6PD function in a coordinated manner to maintain elevated $O_2^-$ in Zucker fa/fa rats. To test that idea, we incubated tissues in the absence and presence of 6-AN (5 mM), a competitive inhibitor of G6PD, or DHEA (100 μM), a noncompetitive inhibitor (Fig. 4B). We found that $O_2^-$ levels were significantly reduced by 67–78% (P < 0.05) in the presence of either 6-AN or DHEA.

$O_2^-$ generation in Zucker lean and fa/fa rat hearts is NADPH dependent. Nox-catalyzed $O_2^-$ generation generally requires either NADPH or NADH to serve as a cofactor. To investigate which was responsible for potentiating $O_2^-$ generation in diabetic tissues, we estimated $O_2^-$ levels in the presence of either...
NADPH (100 μM) or NADH (100 μM). Our results suggest that, in both lean and Zucker fa/fa hearts, O$_2^-$ production is significantly potentiated by NADPH (lean, 658.4 ± 29.9 AU/mg protein; and fa/fa, 669.8 ± 103.8 AU/mg protein) but not by NADH (lean, 251.5 ± 12.6 AU/mg protein; and fa/fa, 294.8 ± 30.0 AU/mg protein).

Cardiac function is unchanged in Zucker fa/fa rats. Because it has been proposed that increased ROS generation underlies the cardiac dysfunction and cardiomyopathy stemming from altered glucose and fatty acid metabolism in diabetic hearts, we used echocardiography to evaluate LV structure and function in 9- to 11-wk-old Zucker lean and fa/fa rats (Fig. 5). The data summarized in Table 2 suggest that there was no significant LV dysfunction or failure (unchanged LV EF and CO), despite the greater ROS production in the fa/fa rats. On the other hand, end-diastolic and end-systolic diameters were increased by 13% and 19% (NS), respectively, in fa/fa rats, whereas the end-diastolic and end-systolic posterior wall thicknesses were decreased by 25% and 14% (P < 0.05), respectively. There is thus a tendency for hearts from Zucker fa/fa rats to be somewhat dilated.

Nox and G6PD are also responsible for O$_2^-$ production in aorta. Although it is clear that augmented oxidative stress impairs vascular function in diabetes, the source of ROS in the vasculature remains uncertain. Because expression of Nox-2 is upregulated in the aortas of streptozotocin-treated rats (22), we...
carried out a set of experiments to determine whether $O_2^\bullet$ generation was catalyzed by Nox in aorta and whether that activity was dependent on G6PD-derived NADPH. We found that both G6PD activity (Fig. 6A) and NADPH levels (Fig. 6B) were significantly increased in aortas from Zucker fa/fa rats, as was $O_2^\bullet$ production (Fig. 6C). Notably, inhibition of NADPH oxidase using gp91\textsuperscript{ds-tat} (50 μM) or inhibition of G6PD using 6-AN (5 mM) significantly reduced $O_2^\bullet$ production (Fig. 6C).

**Endothelial function is severely impaired in Zucker fa/fa rat aorta.** We assessed acetylcholine-induced relaxation of aorta isolated from healthy and diabetic animals, as a marker for endothelial function. Although there were no differences between phenylephrine-induced contraction of Zucker lean and fa/fa rat aortas (Fig. 6D), subsequent endothelium-dependent relaxation was completely abolished in the fa/fa rats and was restored by peg-SOD or gp91\textsuperscript{ds-tat} (Fig. 6E). By contrast, there was no significant difference in nitroglycerin-induced relaxations of precontracted Zucker lean and fa/fa aortic rings (Fig. 6F).

**DISCUSSION**

The salient findings of this study are that, in obese Zucker fa/fa rats, 1) cardiac G6PD activity is increased, 2) increases in Nox-catalyzed $O_2^\bullet$ generation are dependent on G6PD activity, 3) activation of both G6PD and Nox is regulated by PKC, 4) increased Nox-derived $O_2^\bullet$ severely impairs endothelial function, and 5) G6PD activity and Nox- and mitochondrial respiratory chain-derived $O_2^\bullet$ are increased before the development of cardiac dysfunction. Collectively, these findings suggest that activation of the PPP and ROS formation may play key roles in triggering cardiac dysfunction and idiopathic cardiomyopathy in type 2 diabetes.

The Zucker fa/fa rat is a type 2 diabetes model characterized by obesity, insulin resistance, and diabetes mellitus. Due to a nonfunctional leptin receptor gene, food consumption is increased in these animals, and they develop numerous pathological conditions, including hyperglycemia, obesity, moderate hypertension, and neuropathy, by the time they are 8–12 wk old (14). In this model, G6PD activity and $O_2^\bullet$ production are upregulated in cardiac tissue before the development of diabetes and the associated cardiac dysfunction.

In type 2 diabetes, glucose uptake and metabolism are markedly altered in various organs. However, there is conflicting evidence as to the role of G6PD in diabetes, and the fate of glucose metabolized via the PPP remains unclear. For instance, G6PD activity is inhibited and NADPH levels are diminished in islet cells (1), Leydig cells (8), the kidney (54), and lens (23) in type 1 diabetic rat models and in the liver (9), mononuclear leukocytes (38), and erythrocytes (26) of patients with chronic diabetes. By contrast, NADPH levels are increased in endothelial cells isolated from spontaneously diabetic (insulin-independent) BB rats (37), and G6PD is overexpressed in obese diabetic (db/db, ob/ob, and diet-induced obesity) mice (41, 42). Bearing in mind these contrasting observations, we assessed the expression and activity of G6PD in the hearts of Zucker lean and fa/fa rats. Consistent with earlier studies (4), we found that G6PD activity is much higher in the liver and lungs than in the heart and aorta of lean animals (not shown). Moreover, G6PD expression was significantly increased in the fa/fa heart, in accordance with the significantly increased G6PD activity observed in the hearts of Zucker fa/fa rats. Although the
mechanisms underlying the increased G6PD expression and activity in fa/fa rats are unclear, our results suggest that inhibition of PKC activity, which is increased in diabetes (6), led to inactivation of G6PD.

Recent studies have shown that G6PD is overexpressed in adipocytes from obese (including db/db, ob/ob, and diet-induced obesity) mice, in adipocytes and stromal-vascular cells from diabetic db/db mice, and in adipocytes cultured under high glucose conditions. This overexpression of G6PD is one of the causes of defective hormonal release (e.g., adiponectin and leptin) from adipose tissue, dyslipidemia, and insulin resistance (41, 42). Moreover, it is well established that such hormonal dysregulation, or a defect in the leptin receptor-associated signaling pathway, such as that in the Zucker fa/fa rat model, not only induces obesity but also evokes changes in cardiac glucose metabolism (34).

The major biological function of G6PD/PPP is generation of NADPH, an important cofactor for many enzymatic reactions that facilitates fatty acid synthesis and NO and GSH production. Since the elevated generation of NADPH facilitates generation of GSH by GSH reductase, this suggests that, by elevating GSH levels in cardiac myocytes, activation of G6PD and elevation of NADPH could protect the heart from oxidative damage caused by ROS. In contrast, we found that the activities of SOD, GSH-Px, GSH reductase, and the GSSG-to-GSH ratio were all lower in hearts from Zucker fa/fa than lean rats, although levels of G6PD-derived NADPH were higher. This led us to examine the role of the G6PD-derived NADPH in exacerbating oxidative stress. We found that NADPH increased \( \text{O}_2^- \) production and that, conversely, inhibition of G6PD significantly reduced \( \text{O}_2^- \) production in both lean and fa/fa rat hearts. We and others have shown that increased G6PD-derived NADPH fuels Nox activity to sustain \( \text{O}_2^- \) production in the bovine and mouse vasculature (17, 36) and in failing dog and human hearts (16, 18). It has been suggested, moreover, that inhibition of G6PD protects rat hearts from ischemia-reperfusion injury caused by oxidative stress (60). These observations together with the results in Fig. 4 suggest that endogenous G6PD-derived NADPH fuels Nox and is an important cofactor for regulating Nox activity.

Overexpression of G6PD in 3T3-L1 adipocytes cultured in high glucose medium increases oxidative stress by inducing expression of Nox-2, p22\(^{phox}\), and p40\(^{phox}\) subunits (41); however, we found that Nox-2, Nox-4, p67\(^{phox}\), and p47\(^{phox}\) were not overexpressed in hearts from Zucker fa/fa rats. Nevertheless, our finding that Nox inhibition significantly attenuates \( \text{O}_2^- \) production in myocardial homogenates from Zucker fa/fa rats suggests that increased G6PD-derived NADPH fuels Nox activity in myocardial homogenates from Zucker fa/fa rats. Nevertheless, our finding that Nox inhibition significantly attenuates \( \text{O}_2^- \) production in myocardial homogenates from Zucker fa/fa rats suggests that increased G6PD-derived NADPH fuels Nox activity is, at least in part, responsible for the increased \( \text{O}_2^- \) production in fa/fa hearts. The concomitant upregulation of G6PD activity and \( \text{O}_2^- \) generation in the heart provides novel evidence that G6PD activity functions to compensate for the increased NADPH consumption by Nox to sustain NADPH levels in the Zucker fa/fa heart. We also found that inhibition of mitochondrial respiratory complexes I and III reduced cardiac \( \text{O}_2^- \) levels. These complexes are known to be a potential source of ROS in failing (decompensated) dog and human hearts (16, 24), and our results provide new evidence that mitochondria are a source of cardiac oxidative stress.

Fig. 6. Nox and G6PD are also responsible for \( \text{O}_2^- \) production and endothelial dysfunction in aorta. Graphs represent G6PD activity (A) and NADPH levels (B) in aortas from Zucker lean (n = 6) and fa/fa (n = 6) rats. C: effects of 6-AN or gp91\(^{ds\text{-tat}}\) \( \text{O}_2^- \) production in aortic rings from Zucker lean (n = 6) and fa/fa (n = 6) rats. D: phenylephrine-induced contraction curves in lean (n = 6) and fa/fa (n = 6) rat aortic rings. E and F: relaxation evoked by acetylcholine (E) or nitroglycerine (F) in aortic rings precontracted with phenylephrine. In some cases, Zucker fa/fa aortic rings were pretreated with polyethylene glycidyl-SOD (peg-SOD; 300 U/ml) or gp91\(^{ds\text{-tat}}\) (50 \( \mu \)M) before treatment with acetylcholine. Statistical analysis was performed using Student’s t-test or ANOVA with Scheffé’s post hoc test. P < 0.05 was considered significant; *P < 0.05 vs. Zucker fa/fa.
O$_2^-$ in Zucker lean and fa/fa rats. It is evident from these findings that Nox and mitochondria are two major sources of O$_2^-$ in the hearts of both healthy and diseased animals. Even though it is unclear whether any effect of Nox-derived ROS on mitochondrial ROS generation or vice versa, we speculate that O$_2^-$ production by Nox and mitochondria could be interlinked. This suggests that incomplete reduction of O$_2^-$ to water by mitochondria, activation of Nox, and inactivation of antioxidant systems (33) all contribute to increasing oxidative stress in the fa/fa heart before the onset of diabetes.

Increases in Nox-derived O$_2^-$ in the upper abdomen in Zucker rats (50), in the aortas and kidneys of streptozotocin-treated rats (13, 22), and in the internal mammary artery and saphenous vein of diabetic humans (20) may all be involved in impairing organ function. Consistent with that idea, we found that endothelium-dependent relaxation was completely abolished in the fa/fa aorta but was restored by treatment with peg-SOD and gp91ds-tat. Although reports from other laboratories have suggested that endothelium-dependent relaxation of cremaster muscle arteries (14), mesenteric and cerebral arteries (43, 49), and aorta (39) is impaired in obese Zucker (15–17 wk old) rats, the precise mechanism causing the endothelial dysfunction and cardiovascular complications.

It is well known that coronary arterial- and subendocardial endothelium-derived NO regulates myocardial mitochondrial respiration, substrate utilization, and contractility (7, 47, 52). Consequently, the depressed endothelial function seen in the fa/fa rat could affect myocardial O$_2^-$ generation, metabolism, and function. In that regard, it has already been shown that endothelium-derived NO release is downregulated in heart failure, which alters myocardial substrate utilization, eventually leading to cardiac dysfunction (46). In particular, myocardial glucose uptake and oxidation are increased (12, 40), although glycolysis and the mitochondrial capacity to oxidize carbohydrates appear reduced (29, 45). It is, therefore, reasonable to speculate that, as a result of endothelial dysfunction, glucose is shunted through the PPP in the failing heart. Previous studies have shown that glucose is metabolized via the PPP in both healthy and diseased hearts (16, 18, 44, 57, 58). In the present study, we have shown that endothelial dysfunction and upregulated G6PD activity, in turn, leads to increased Nox-catalyzed O$_2^-$ production in the hearts and aortas of obese, hyperglycemic, hyperinsulinemic, and hyperlipidemic rats. Chronic hyperglycemia and hyperlipidemia evokes cytotoxicity in the heart and has adverse outcome in hospitalized heart failure patients (10). Since LV diameters during diastole and systole appear to be slightly but not significantly increased in 9- to 11-wk-old Zucker fa/fa rats, our data suggest that the activation of G6PD and elevation in Nox-derived O$_2^-$ are a prelude to LV dilation and loss of cardiac contractile function, which have been reported in Zucker fa/fa rats by the time they reach 20 wk of age (56). Other studies have demonstrated that an increase in G6PD-derived NADPH fuels Nox enhanced O$_2^-$ production/oxidative stress in failing hearts from dogs and humans (16, 18) and that augmented G6PD expression and activity evokes dilated cardiomyopathy in R120GCryAB mutant mice (44). In light of these observations, we postulate that increased metabolism of glucose through the activated G6PD and PPP augmented Nox-derived ROS generation in both the heart and vasculature underlie the myocardial and vascular dysfunction seen in type 2 diabetes.

In summary, the findings of the present investigation suggest that in a model of severe hyperlipidemia and hyperglycemia, Nox-derived O$_2^-$ generation in the myocardium is fueled by elevated levels of G6PD-derived NADPH. Thus G6PD may be a useful therapeutic target for treating the cardiovascular disease associated with type 2 diabetes, if second-generation drugs specifically reducing the activity of G6PD to near normal levels are developed.

Limitations. In this study, measurement of ROS, antioxidants, and G6PD were made in heart tissue, and the results may be confounded with changes in cell types other than cardiomyocytes (i.e., fibroblasts, endothelial cells, smooth muscle cells) since their relative contribution cannot be segregated. In addition, as we have observed endothelial- and vascular dysfunction, the direct cardiac effects versus the vascular effects of diabetes cannot be separated. We speculate that some of the cardiac dysfunction may be tied to the adverse vascular effects, therefore rendering the cardiac effects secondary to the vascular dysfunction and, hence, not idiopathic.

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