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## Synergistic Activation of Glucose-6-Phosphate Dehydrogenase and NAD(P)H oxidase by Src kinase Elevates Superoxide in Type 2 Diabetic, Zucker fa/fa, Rat Liver

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### Abstract

Glucose metabolism through glycolysis and hexosamine pathway has been shown to be altered in type 2 diabetes. However, its fate through the pentose phosphate pathway (PPP) is currently unclear. In this study, we determined whether the activity of glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting enzyme in the PPP, is modulated in the liver of Zucker obese fa/fa rats (9–11 weeks old). We found that G6PD expression and activity, NADPH levels and 6-phosphogluconate generation was significantly increased in liver of fa/fa rats. Inhibition of PI3 kinase and Src kinases decreased ( $P < 0.05$ ) G6PD activity in the fa/fa but not in the lean rat liver, suggesting that G6PD activity is regulated by PI3/Src kinase signaling pathways. G6PD-derived NADPH increased ( $P < 0.05$ ) superoxide anion levels by 70–90% in the fa/fa vs the lean rat liver, which was inhibited by NADPH oxidase inhibitor, gp91<sup>ds-tat</sup> (50  $\mu$ M), and G6PD inhibitors, 6-aminonicotinamide (1 mM) and dihydroepiandrosterone (100  $\mu$ M); therefore, indicating that elevated G6PD activity may be responsible for mediating superoxide generation. Interestingly, we also found a positive correlation between liver hypertrophy/increased G6PD activity ( $r^2 = 0.77$ ;  $P = 0.0009$ ) and liver hypertrophy/superoxide production ( $r^2 = 0.51$ ;  $P = 0.0091$ ) in fa/fa rats. Increased G6PD and NADPH oxidase expression and activity, in young hyper-glycemic and -insulinemic rats prior to the development of diabetes, appear to be contributing factors for the induction of oxidative stress. Since inhibition of G6PD activity decreases oxidative stress, we conclude that it behaves as a pro-oxidant in the fa/fa rat liver, in type 2 diabetes.

### Keywords

Diabetes; Src kinase; NADPH; NADPH oxidase; Oxidative stress; Superoxide anion; Hydrogen peroxide; Liver; Zucker obese fa/fa

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Type 2 diabetes affects approximately 170 million people around the world and these numbers are rapidly rising every year [1]. This metabolic syndrome is now widely recognized as a risk factor for liver disorders, obesity and cardiovascular diseases.

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Several studies have suggested that oxidative stress is elevated by chronic hyperglycemia in diabetic animal models as well as in diabetic patients [2–7]. Overproduction of superoxide anion ( $O_2^-$ ) by the mitochondria, polyol pathway, NAD(P)H oxidase, uncoupled nitric oxide synthase, and reduced endogenous antioxidant enzymes have been proposed to increase oxidative stress in hyperglycemia. Yet, the underlying mechanism that activates reactive oxygen species generation in hyper-glycemia or -insulinemia is far from clear. Brownlee and colleagues [8], have proposed that an increase in advanced glycation end products, activation of protein kinase C ( $\beta$  isoform), and activation of aldosereductase in diabetes, elevate  $O_2^-$  generation from mitochondrial respiratory chain. Furthermore, other studies have shown that in streptozotocin-treated (type 1 diabetes) rat model there is over-expression of NAD(P)H oxidase (Nox-2 and Nox-4) in the vascular system and kidneys with a concomitant elevation in  $O_2^-$  production [2,9].

In most tissue types, constitutively active NAD(P)H oxidase produces low levels of  $O_2^-$  [10]. Additionally, in pathophysiological conditions, activation of NADPH oxidase following stimulation by factors such as, angiotensin II, thrombin, and TNF $\alpha$  [10,11], leads to subsequent elevation in  $O_2^-$  generation. Other investigators and we have shown that NADPH derived from glucose-6-phosphate dehydrogenase (G6PD), a rate limiting enzyme in the pentose phosphate pathway (PPP) that produces a major portion of NADPH in the cell, fuels NADPH oxidase and  $O_2^-$  production in the failing heart [12,13], angiotensin-II and PKC agonist treated vasculature [14,15], and neutrophils in basal state [16] and during respiratory burst [17]. Since NADPH levels have been shown to be elevated in the endothelial cells of diabetic (type 1) BB rats [18], we postulated that an increase in G6PD-derived NADPH in type 2 diabetes could elevate NADPH oxidase-derived oxidative stress.

This study was, therefore, designed to investigate the implications of imbalance in glucose metabolism observed in type 2 diabetes, on oxidoreductases enzymes such as G6PD and NADPH oxidase in the liver of young Zucker fa/fa rats, an animal model of type 2 diabetes, resembling human diabetes. In particular we investigated whether; [i] the G6PD expression and activity is up-regulated, [ii] protein expression and activity of NAD(P)H oxidase is altered, and [iii] PI3Kinase and Src kinases-dependent signaling pathways and G6PD-derived NADPH evoke Nox-derived  $O_2^-$  in liver from young (9–11 weeks old animals) Zucker fa/fa rats as compared to the lean rats.

## Materials & Methods

NADPH, NADH, NADP $^+$ , NAD $^+$ , glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 6-phosphogluconolactone, 6-phosphogluconate dehydrogenase, bis(N-methylacridinium) nitrate (Lucigenin), antimycin, rotenone, dehydroepiandrosterone, 6-aminonicotinamide and other salts were purchased from Sigma (St. Louis, MO, USA). The stock solutions of 6-aminonicotinamide and dehydroepiandrosterone were made in dimethyl sulfoxide (DMSO; Sigma), and final 1:1000 dilutions in buffered physiological salt solution were used in the study. 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4-*d*] pyrimidine (PP2) and their controls were purchased from Calbiochem, Inc (Carlsberg, CA, USA).

## Animal model

The Institutional Animal Use Committee approved all protocols and surgical procedures, which were in accordance with National Institutes of Health and American Physiological Society guidelines. To examine our hypothesis, experiments were performed with adult male Zucker fa/fa rat, a prototype model of hyperglycemia and type 2 diabetes, purchased from Charles River laboratories, Wilmington, MA, USA. All rats were exposed to a 12:12-h light-dark cycle and allowed free access to standard rat food and water.

### Isolation of liver

Animals were anesthetized with Nembutal (10 mg/kg) and abdominal cavity was opened. The liver was cannulated through the portal vein and perfused with normal tyrode solution at 37°C for 5 minutes. The liver was excised and cut in small pieces. The minced liver was homogenized as described in individual protocols and used for biochemical analyses. In some experiments, the isolated liver was perfused with inhibitors of G6PD, NADPH oxidase, mitochondrial respiratory complex I and III, and PI3 kinase and Src kinases in normal tyrode solution for 30 minutes at 37°C and then used for biochemical analyses.

### Isolation of hepatocytes

Hepatic cells were isolated from liver by slight modification of previously described method [19]. Briefly, liver was washed with ice cold  $\text{Ca}^{2+}$ -free buffer, pH 7.6, consisting of NaCl (150 mM), KCl (2.8 mM), glucose (5.5 mM), and HEPES (25 mM) for 10 minutes. Next, liver was treated with same buffer containing  $\text{CaCl}_2$  (3.8 mM), collagenase type I (0.5 mg/ml) and soyabean trypsin inhibitor (10  $\mu\text{g/ml}$ ) at 37°C for 1 hr. The liver cells were dispersed by titration and centrifuged at  $1\times g$  for 5 minutes. Hepatocytes were separated from non-parenchymal cells by centrifugation in isotonic percoll (specific gravity=1.06) at  $50\times g$  for 5 minutes. Percoll was removed by two washes in buffer.

### Estimation of glucose and insulin levels

Blood glucose levels were determined by standard glucose strips using a glucometer (Abbot Laboratory, Chicago, IL, USA) and insulin levels were quantified by ELISA kit purchased from Cayman Chemical Co., Ann Arbor, MI, USA.

### Estimation of superoxide

Superoxide levels were determined by previously published protocols [12]; briefly tissue homogenates (10–15  $\mu\text{l}$ ) were incubated at 37°C in 20 mM MOPS buffer, pH 7.4 containing 250 mM sucrose for 10 minutes in absence or presence of drugs and  $\text{O}_2^-$  was detected by lucigenin (5  $\mu\text{M}$ ) chemiluminescence. Some experiments examined  $\text{O}_2^-$  production in the absence of the NADPH regenerating system to evaluate the role of NADPH and NADH.  $\text{O}_2^-$  in hepatocytes was detected in luminometer (Synergy 2, Biotek Instruments, MA, USA) instead of liquid scintillation counter described previously [12].

### Estimation of superoxide dismutase activity

SOD activity was assessed by measuring the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine in a convenient 96 well format using a kit from Cayman Chemical Co.

### Estimation of glutathione peroxidase and glutathione reductase activity

GSH peroxidase and reductase activity was measured using a kit from Cayman Chemical Co. The oxidation of NADPH to  $\text{NADP}^+$  was accompanied by a decrease in absorbance measured at 340 nm. The rate of decrease in the  $A_{340}$  was directly proportional to the peroxidase activity and the rate of decrease in the absorbance at 340 nm was directly proportional to the reductase activity in the sample.

### Estimation of triglycerides, free fatty acid, and liver function markers

The triglyceride, free fatty acid, alkaline phosphatase (ALP), alanine aminotransferase (ALT) and  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GT) levels were determined in the serum collected from Zucker lean and fa/fa rats using kits purchased from Teco Diagnostics, Anaheim, CA, USA.

## Western blot analysis

Homogenates were prepared from frozen tissues and total protein content was measured as described previously. Thirty-five micrograms of total protein was analyzed by SDS-PAGE. Western blot analyses was performed using rabbit polyclonal anti-G6PD (Sigma Chemical), mouse monoclonal anti-Nox-1, anti-Nox-2, and anti-p67<sup>phox</sup> (Transduction Laboratory, San Jose, CA, USA), goat polyclonal anti-Nox-4 and anti-p47<sup>phox</sup>, mouse monoclonal anti- $\alpha$ -tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Bands were visualized by autoradiography and quantified using densitometric analyses. Densitometric values for specific proteins were normalized with  $\alpha$ -tubulin and expressed as a ratio of the two proteins.

## Estimation of glucose-6-phosphate dehydrogenase activity

Glucose-6-phosphate dehydrogenase (G6PD) activity was measured in liver homogenates by following the reduction of NADP<sup>+</sup> to NADPH [14]. NADPH fluorescence was detected at 340 nm (Ex) and 460 nm (Em) using a Flx800 microplate fluorescence detector (BioTek Instruments, Winooski, Vermont, USA).

## Estimation of NAD(P)H levels

Frozen tissues were homogenized in an extraction medium containing NaOH (0.02 N) and cysteine (0.5 mmol/L) 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 and NADPH levels were estimated by determining NADPH fluorescence at 340 (Ex) and 460 (Em) using a previously described recycling method [20].

## Statistical analysis

Values are means  $\pm$ SEM. Comparisons between groups were made with Student's *t*-test or analysis of variance (ANOVA) with Scheffé's *post-hoc* test for multiple comparisons. Differences were considered significant at  $p < 0.05$ .

## Results

### Glucose and insulin levels are increased in young Zucker fa/fa rats

Nine to Eleven weeks old Zucker fa/fa rats (345 $\pm$ 6 g; n=6) were obese ( $P < 0.05$ ) as compared to lean rats (264 $\pm$ 15 g; n=6), and had high ( $P < 0.05$ ) non-fasting plasma glucose [127 $\pm$ 11 (lean) and 341 $\pm$ 12 (Zucker fa/fa) mg/dl] and insulin [0.67 $\pm$ 0.07 (lean) and 3.04 $\pm$ 0.22 (Zucker fa/fa) ng/ml] levels.

### Glucose-6-phosphate dehydrogenase activity is elevated in type 2 diabetes

Since glucose metabolism is altered in type 2 diabetes, it is possible that G6PD expression and/or activity may also be modulated. We performed Western blot analysis to determine whether G6PD expression was changed in liver of Zucker fa/fa rats. The results indicated that the expression of G6PD increased ( $P < 0.05$ ) by 117 % in liver (Fig 1 A top panel and B) and hepatocytes (Fig 1 A bottom panel) of Zucker fa/fa as compared to the lean rats. In addition, we found that the activity of G6PD was significantly higher in the liver and hepatocytes of Zucker fa/fa animals by 400 % (Fig 1 C) and 160 % ( $P < 0.05$ ), respectively, as compared to age-matched lean rat. Additionally, NADP<sup>+</sup>, substrate, -dependent enzyme activity curve was also significantly shifted to the left in hepatic tissue from Zucker fa/fa rats (Fig 1 D). Consistently, the NADPH (Fig 1 E) and 6-phospho-gluconate (Fig 1 F), products of G6PD, levels were significantly increased in the liver of Zucker fa/fa rats as compared to lean rats.

## Glucose-6-phosphate dehydrogenase activity is elevated by PI3 and Src kinases in type 2 diabetes

EGF associated tyrosine kinases-, PKC- and Src-dependent pathways have been shown to activate G6PD in renal cortical cells [21] and in the failing human hearts [22]. To elucidate how G6PD is activated in fa/fa, we studied the effects of kinases involved in the insulin signaling pathway such as, PI3 kinase [23] and Src family of protein tyrosine kinases [24], on G6PD activity. We found that there was an increase in the amount of total-Src (Fig 2 A & B) and phospho-Src<sub>416</sub> (Fig. 2 A & C) in fa/fa vs. lean rats. Next, we treated liver with (i) PI3 kinase inhibitor-LY294002 (active form; 10  $\mu$ M) and LY303511 (non-active form; 10  $\mu$ M); and (ii) Src kinase inhibitor-PP2 (active form; 10  $\mu$ M) and PP3 (non-active form; 10  $\mu$ M), respectively, to determine the role of PI3 kinase and Src kinase in activating G6PD. The liver was perfused with the inhibitors for 30 minutes as described earlier (See Methods) and then the activity of G6PD was determined in liver homogenates in the presence of LY294002/LY303511 and PP2/PP3. Both active form of inhibitors attenuated ( $P < 0.05$ ) G6PD activity in fa/fa, but not in lean liver homogenate (Fig 2 D & E). Total-Src was increased in isolated hepatocytes (Fig 2 A), and treatment with PI3 kinase and Src kinase inhibitors decreased ( $P < 0.05$ ) G6PD activity (Control:  $0.892 \pm 0.159$ ; LY294002:  $0.358 \pm 0.2893$ ; PP2:  $0.406 \pm 0.3802$  nmol/min/mg protein) in hepatocytes from fa/fa rats.

## Protein expression of NAD(P)H oxidase is altered in type 2 diabetic tissue

Considering the significant elevation of G6PD activity and subsequently NADPH levels, we performed experiments to determine whether expression of NADPH oxidase subunits were also altered in type 2 diabetes. We estimated expression of Nox-1, Nox-2, Nox-4, p47<sup>phox</sup> and p67<sup>phox</sup> in lean and fa/fa rat liver homogenates (Fig 3 A: top panel) and isolated hepatocyte lysate (Fig 3 A: bottom panel) by Western blot analysis. We were unable to detect Nox-2 (Fig 3 A; second panel), however, Nox-4 levels (Fig 3 A, third panel) were increased by approximately 2-fold ( $P < 0.05$ ; Fig 3 B) in the liver of fa/fa vs lean rats. Although, we observed a decreased expression of Nox-1 ( $P < 0.05$ , Fig 3 B), in fa/fa vs lean rats, increased levels of p47<sup>phox</sup> and p67<sup>phox</sup> (Fig 3 A, fourth and fifth panel, respectively) and significantly increased membrane-to-cytoplasmic ratio of p47<sup>phox</sup> (Fig 3 C), suggests an overall increase in the Nox-1/p47<sup>phox</sup> and p67<sup>phox</sup> complex, in the liver homogenates of fa/fa vs lean rat.

## Superoxide generated in type 2 diabetes is NADPH-dependent

Although studies have shown that O<sub>2</sub><sup>-</sup> is elevated in diabetes, the source and mechanism of O<sub>2</sub><sup>-</sup> generation are still unclear. Thus, to determine whether O<sub>2</sub><sup>-</sup> derived from NAD(P)H oxidase or mitochondrial respiratory chain is elevated in type 2 diabetes, we estimated O<sub>2</sub><sup>-</sup> levels in liver homogenates of lean and fa/fa rats. We found O<sub>2</sub><sup>-</sup> generation was elevated ( $P < 0.05$ ) in the liver (Fig 4 A) and in isolated hepatocytes (lean:  $7.85 \pm 2.69$  and fa/fa:  $13.34 \pm 3.93$  units/mg protein) of fa/fa as compared to lean rats. Concurrently, SOD activity was also significantly increased (Fig 4 B), suggesting oxidative stress was increased in fa/fa liver. Furthermore, to rule out non-specific effects, homogenates were pretreated with superoxide dismutase (peg-SOD; 300 U/ml) to scavenge superoxide produced in the liver tissue. As expected, peg-SOD treatment decreased O<sub>2</sub><sup>-</sup> levels by 60–80% ( $P < 0.05$ ) as compared to that of the untreated control. Consistently, by cytochrome reduction method, we found a 2-fold ( $P < 0.05$ ) elevation in O<sub>2</sub><sup>-</sup> generation in fa/fa ( $76.9 \pm 5.4$  nmol/mg protein) as compared to lean ( $32.5 \pm 8.1$  nmol/mg protein) rats. Interestingly, there was a decrease in activity of GSH-Px ( $P < 0.05$ ; Fig 5 A) and GSH reductase ( $P < 0.05$ ; Fig 5 B) in fa/fa liver.

Additionally, we found that mitochondrial respiratory chain blockers, antimycin (10  $\mu$ M) and rotenone (50  $\mu$ M), did not decrease O<sub>2</sub><sup>-</sup> production; while perfusion of the liver with an inhibitor of NADPH oxidase, gp91<sup>ds-tat</sup>, completely inhibited O<sub>2</sub><sup>-</sup> generation in both lean and fa/fa rat liver (Fig 6 A). Scrambled gp91<sup>ds-tat</sup> peptide (50  $\mu$ M), a negative control for

gp91<sup>ds-tat</sup>, did not decrease O<sub>2</sub><sup>-</sup> (981±120 Units/mg Protein). These experiments were performed in the presence of NADPH re-generating system.

In general, O<sub>2</sub><sup>-</sup> generation by NAD(P)H oxidase requires either NADPH or NADH; however, which of these reducing potential is required for O<sub>2</sub><sup>-</sup> production in type 2 diabetes is unknown. Thus, to investigate whether NADPH or NADH was responsible to potentiate O<sub>2</sub><sup>-</sup> generation in fa/fa liver tissues, we estimated O<sub>2</sub><sup>-</sup> levels in presence of either NADPH (100 μM) or NADH (100 μM). The results suggested that O<sub>2</sub><sup>-</sup> production was significantly augmented by NADPH, but not by NADH, therefore, demonstrating that NADPH is primarily utilized by NAD(P)H oxidase for O<sub>2</sub><sup>-</sup> generation in the liver of fa/fa rats (Fig 6 B).

### **NADPH oxidase and glucose-6-phosphate dehydrogenase synergistically increases superoxide production in diabetic tissues**

We have recently suggested that G6PD-derived NADPH fuels O<sub>2</sub><sup>-</sup> production [12,14,22] and have demonstrated (Fig 6 B) that NADPH oxidase requires NADPH for activity. These observations taken together raised the question of whether NADPH oxidase and G6PD function in a coordinated manner to maintain elevated O<sub>2</sub><sup>-</sup> in fa/fa rats. To test this hypothesis we first perfused the liver and incubated tissue homogenates in the absence and presence of PI3K and Src kinase inhibitors, since this signaling cascade is found to activate G6PD. We found that both types of inhibitors decreased ( $P<0.05$ ) the O<sub>2</sub><sup>-</sup> generation by ~50–60% in fa/fa rat liver (Fig 7 A) and abolished the O<sub>2</sub><sup>-</sup> generation (under detectable limits) in hepatocytes. Next, we incubated the tissues with competitive and non-competitive inhibitors of G6PD, 6-aminonicotinamide (5 mM) and dihydroepiandrosterone (100 μM), respectively. The results indicated that O<sub>2</sub><sup>-</sup> levels were decreased ( $P<0.05$ ) by ~67–78% by both 6-aminonicotinamide and dihydroepiandrosterone (Fig 7 B).

### **A positive correlation exists between liver hypertrophy and increased G6PD activity/superoxide production in Zucker fa/fa rats**

We found activity of γGT [1.17±0.15 (fa/fa) and 7.03±2.55 (lean) U/L], alkaline phosphatase [40.32±1.02 (fa/fa) and 142.40±28.40 (lean) U/L], and ALT [54.42±7.29 (fa/fa) and 263.00 ±100.70 (lean) U/L] in blood serum was significantly elevated in fa/fa vs lean rats. Furthermore, we also found that liver weight (Fig 8 A) and liver weight-to-body weight ratio (Fig 8 B) was significantly increased in fa/fa as compared to lean rats. We extracted DNA and estimated protein-to-DNA ratio (Fig 8 C) with a previously described method [25], and PCNA expression by Western blot analysis (Fig 8 D). Interestingly, we found that PCNA expression was down-regulated and protein-to-DNA ratio was elevated ( $P<0.05$ ) in the liver of fa/fa as compared to lean rats suggesting decreased cell proliferation and a strong possibility of liver hypertrophy in fa/fa rats. Additionally, we found a positive correlation between G6PD activity (Fig 8 E) and O<sub>2</sub><sup>-</sup> production with liver weight by regression analysis (Fig 8 F).

## **Discussion**

Type 2 diabetes is now widely recognized as a risk factor for liver dysfunction, obesity, cardiovascular diseases, and organ failure. This study was, therefore, undertaken to elucidate possible molecule(s)/mechanism(s) that may be responsible/involved for triggering liver dysfunction, in type 2 diabetes.

Glucose metabolism is considerably altered in various organs in type 2 diabetes. The fate of glucose through the PPP has not been well characterized and there is conflicting evidence about the activity of G6PD, the first/rate limiting enzyme in the PPP, in diabetes. For example, some studies suggest that G6PD activity is inhibited and NADPH is decreased in islets cells [26], Leydig cells [27], kidneys [28], lens [29], heart [30] and the liver [31] of type 1 diabetic rats

(induced by streptozotocin- and alloxan-treatment); and in the liver [32], mononuclear leukocytes [33] and erythrocytes [34] of chronic diabetic patients. In contrast, recent studies have shown that G6PD is over-expressed in the adipocytes of obese (including *db/db*, *ob/ob*, and diet-induced obesity) mice, adipocytes and stromal-vascular cells of diabetic *db/db* mice, and adipocytes cultured, *in vitro*, under high glucose conditions [35,36]. In lieu of these contrasting observations, we determined the expression and the activity of G6PD in liver and isolated hepatocytes of Zucker lean and *fa/fa* rats. Our data indicated that G6PD protein levels and activity was much higher in the liver of *fa/fa* vs lean rats, which was consistent with previous findings [37], and in addition we found that G6PD expression and activity was significantly higher in the isolated hepatocytes of *fa/fa* vs lean rats. Next, we proceeded to identify the pathways that could be responsible for increasing the expression and/or activity of G6PD in the *fa/fa* rat liver. Interestingly, we found significantly higher amounts of activated Src kinase (determined by phospho-Src<sub>416</sub>) in the *fa/fa* but not lean liver. Concomitantly, G6PD activity was significantly elevated in the *fa/fa* vs lean liver and hepatocytes by 4.4-fold and 1.6-fold, respectively. Inhibition of either PI3Kinase or Src kinase using LY294002 or PP2 inhibitors, respectively, significantly inhibited G6PD activity nearly to the levels observed in the lean liver and hepatocytes. However, no inhibitory effect of PI3kinase and Src kinase on G6PD activity was observed in the lean liver. Moreover, G6PD activity was completely abolished (Fig. 2 C & D) by alkaline phosphatase (50 U). This data provides an insight to the possibility that in *fa/fa* liver, G6PD activation may involve phosphorylation of G6PD *via* a signaling cascade that includes PI3Kinase and/or Src kinases. This notion is supported by the previous independent observations that induction of G6PD expression by insulin, in hepatocytes, requires PI3 kinase [23] and that EGF associated tyrosine kinases activate G6PD in renal cortical cells [21]. Hence it is possible that activation of G6PD by Src kinase probably occurs *via* phosphorylation of key tyrosine residues, located (as determined by scansite.mit.edu program) at its C-terminal, viz., Y279 (GRGGYFDEFG) and Y537 (YEGTYKWVN). Taken together, these findings indicate that PI3 kinase/Src kinase-dependent pathways could mediate an increase in G6PD expression as well as activity and this presumably shunts glucose through the PPP even though glucose uptake is impaired under hyperinsulinemic and/or hyperglycemic conditions prior to the development of diabetes in the *fa/fa* rats.

The major biological significance of G6PD is to generate NADPH, the cell's most abundant reducing coenzyme. NADPH is an important cofactor for many enzymatic reactions in the cell, for example, fatty acid synthesis/oxidation, nitric oxide production and generation of GSH by glutathione reductase. We and others, have reported that G6PD-derived NADPH fuels NADPH oxidase-derived O<sub>2</sub><sup>-</sup> production [14,15]. Studies have suggested that increased oxidative stress is responsible for multi-organ dysfunction and damage in streptozotocin- and alloxane-treated rats. However, our understanding about the mechanism(s) that increase(s) reactive oxygen species in type 2 diabetes is still unclear. This led us to examine G6PD-derived NADPH levels and its effect on oxidative stress. We found that inhibition of G6PD by 6-aminonicotinamide and dihydroepiandrosterone (G6PD inhibitors) significantly decreased O<sub>2</sub><sup>-</sup> generation in *fa/fa* as well as lean rat liver. These findings taken together with the observations in figure 6B, suggest that NADPH, but not NADH, is consumed by constitutively active and by activated oxidases to produce O<sub>2</sub><sup>-</sup> in lean and *fa/fa* liver and that G6PD-derived NADPH is an important co-factor in regulating NADPH oxidase activity and generating sub-maximal level of O<sub>2</sub><sup>-</sup> in *fa/fa* rat liver. Interestingly, we also found that inhibition of PI3 kinase or Src kinase significantly decreased O<sub>2</sub><sup>-</sup> production in liver of *fa/fa*, but not in lean, rats; thereby indicating that activation of G6PD and NADPH oxidase by Src kinase was responsible to increase O<sub>2</sub><sup>-</sup> generation in *fa/fa* rat liver.

Over-expression of G6PD in the 3T3-L1 adipocytes, cultured in high glucose, *in vitro*, has been shown to increase oxidative stress by inducing the expression of Nox-2, p22<sup>phox</sup>, and p40<sup>phox</sup> subunits [36]. We found that Nox-4, p67<sup>phox</sup> and p47<sup>phox</sup> were significantly over-

expressed in the fa/fa vs lean rat liver and hepatocytes, while we were unable to detect Nox-2 in both the rat livers. Increased levels of Nox-4 observed in this study and a significant increase in membrane-to-cytoplasmic ratio of p47<sup>phox</sup>, which may probably increase complex formation of Nox-1 with p67<sup>phox</sup> and p47<sup>phox</sup>, in turn maybe responsible for the elevation of O<sub>2</sub><sup>-</sup> production in fa/fa rats. Furthermore, NADPH oxidase inhibitor, gp91<sup>ds-tat</sup>, that inactivates Nox-2 as well as Nox-1 by binding to p47<sup>phox</sup> binding sites [38], significantly attenuated O<sub>2</sub><sup>-</sup> generation in the lean and fa/fa liver. However, no such effect was observed by the mitochondrial respiratory chain inhibitors; antimycin or rotenone. These results suggest that majority of O<sub>2</sub><sup>-</sup> was generated by NADPH oxidase but not by the mitochondria. Interestingly, inactivation of anti-oxidant systems [39]; including GSH-Px activity (see figure 5), presumably led to the poor removal of reactive oxygen species, which could be one of the probable causes for elevating the oxidative stress observed in the hepatic tissue prior to the on-set of diabetes. Taken together, these observations provide novel evidence that elevation in G6PD activity in the liver of fa/fa rats, functions to sustain and compensate for increased NADPH consumption by activated NADPH oxidases and that G6PD-derived NADPH fuels NADPH oxidases to over-produce O<sub>2</sub><sup>-</sup>, in hyper-glycemic and -insulinemic liver. Alternatively, increased reactive oxygen species could upregulate G6PD expression and increase intracellular NADPH, which may potentially be consumed by the activated pro-oxidant pathways. Additionally, G6PD-derived NADPH could promote GSH reductase activity and reduce oxidized GSH levels. However, in the event of a decrease in GSH peroxidase and reductase activity (figure 5), and a reduction in total GSH in liver [40], it is unlikely that NADPH may be involved in regulating the GSH redox cycling. In light of our observations, we propose that an increased G6PD-derived-NADPH, functions as a pro-oxidant and increases oxidative stress (O<sub>2</sub><sup>-</sup>), instead of preventing oxidative stress, in the liver tissue in pathological conditions such as type 2 diabetes.

Studies have suggested that elevated NADPH oxidase-derived O<sub>2</sub><sup>-</sup> found in the upper abdomen in the Zucker diabetic fatty rats [41], in the aorta and the kidney in streptozotocin-treated rats [2,9], and in the diabetic human internal mammary artery and saphenous vein [3], could play a role in impairing organ function. Consistently, others have reported that over-expression of NADPH oxidase subunits ( $\alpha$ -subunit of neutrophilic NADPH oxidase and p47<sup>phox</sup> [42,43] in hepatic tissue plays a predominant role in the pathogenesis of early alcohol-induced hepatitis [44]; in the activation of mitogens in Kupfer cells by peroxisome proliferators [45]; in the NF- $\kappa$ B mediated production of mitogens that causes hepatocellular proliferation [46]; and in mediating the actions of Ang II on hepatic stellate cells and in liver fibrogenesis [47]. However, the precise signaling pathways involved in activating NADPH oxidases and NADPH oxidase-derived oxidative stress that cause liver dysfunction in diabetic animals are still elusive. The results presented in this study have elucidated a possible mechanism involving with two key players, G6PD and NADPH oxidase, which seem to be activated simultaneously in hepatic tissue and hepatocytes in a tyrosine kinase-dependent manner, prior to the on-set of diabetes (i.e. in 9–10 weeks old Zucker fa/fa rats), and operate in synergy to up-regulate O<sub>2</sub><sup>-</sup>, perhaps contributing to the development of hyperglycemia/diabetes-associated hepatic complications.

We found an increase in liver weight in fa/fa as compared to lean rats, consistent with other studies [48,49]. Our results show that expression of PCNA is down-regulated and the protein-to-DNA ratio is increased in fa/fa rats, thus suggesting that liver hypertrophy and not hyperplasia occurs in obese, hyper-insulinemic and -glycemic rats. Interestingly, we also found a positive correlation between liver weight and increased [1] G6PD activity and [2] O<sub>2</sub><sup>-</sup> levels, indicating that concomitant activation of NADPH oxidase and G6PD, by hyperglycemia and/or hyperinsulinemia, could be a signal to either trigger or sustain hepatic cell growth in the congenital model of obesity and type 2 diabetes. In this regards, there is suggestive evidence that reactive oxygen species may play a role in the initiation of liver regeneration/growth, because of increased electron transport, resulting from the high metabolic load imposed on hepatocytes after partial hepatectomy of rat and mouse liver [50,51]. Alternatively, elevated



oxidants in conjunction with upregulation of G6PD activity could increase fatty acid synthesis and play a potential role in evoking either liver dysfunction or damage (determined by increase in the serum  $\gamma$ GT, alkaline phosphatase and ALT levels) in fa/fa rats.

As summarized in the schematic model (figure 9), we have demonstrated that in liver of fa/fa rats G6PD expression and activity is increased by PI3 kinase- and Src kinase-dependent pathways; increase in NADPH oxidase-derived  $O_2^-$  generation is dependent on G6PD activity; and GSH-Px as well as GSH reductase activity is decreased. Interestingly, we have found that elevated G6PD activity and  $O_2^-$  levels have a positive correlation with hypertrophy of the liver; therefore, suggesting a plausible role for reactive oxygen species in triggering either liver dysfunction or growth. More importantly, suppression of G6PD activity by 6-aminonicotinamide and dihydroepiandrosterone decreased oxidative stress. Although it is unclear whether the increase in G6PD activity and  $O_2^-$  levels is a effect of hyper-insulinemia or -glycemia and further work is required to determine the consequences of their up-regulation on liver function, our results nevertheless imply that G6PD might be a novel target for therapeutic intervention to reduce oxidative stress, ameliorate organ dysfunction, and improve liver physiology in type 2 diabetes.

## Acknowledgments

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## Abbreviation

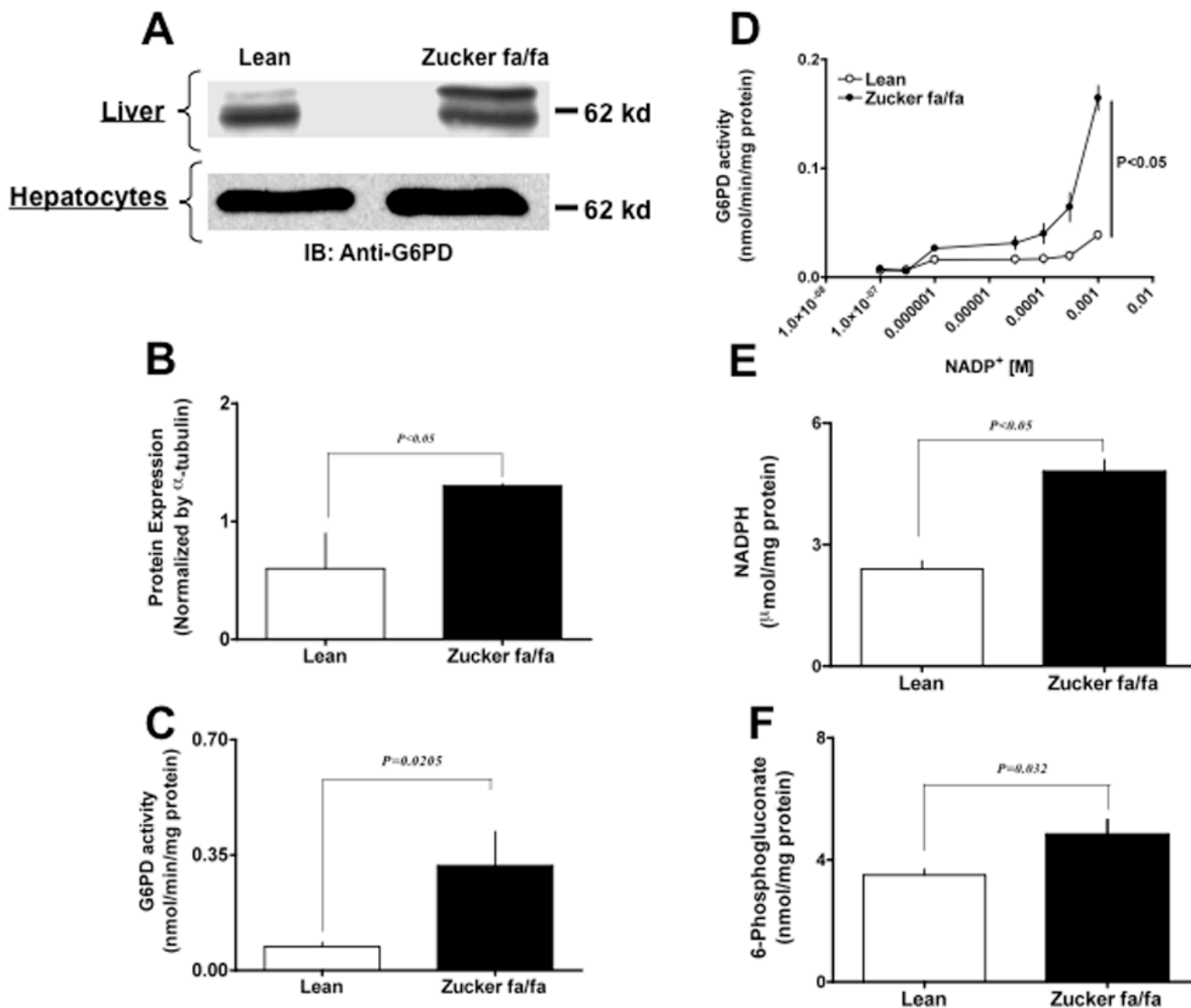
<b>ALP</b>	alkaline phosphatase
<b>ALT</b>	alanine aminotransferase
<b>DMSO</b>	dimethyl sulfoxide
<b>G6PD</b>	glucose-6-phosphate dehydrogenase
<b><math>\gamma</math>-GT</b>	$\gamma$ -glutamyl transpeptidase
<b>Nox-1</b>	Nox-2 and Nox-4, NAD(P)H oxidase
<b><math>O_2^-</math></b>	superoxide anion
<b>PPP</b>	pentose phosphate pathway
<b>TNF<math>\alpha</math></b>	Tumor necrotic factor $\alpha$

## References

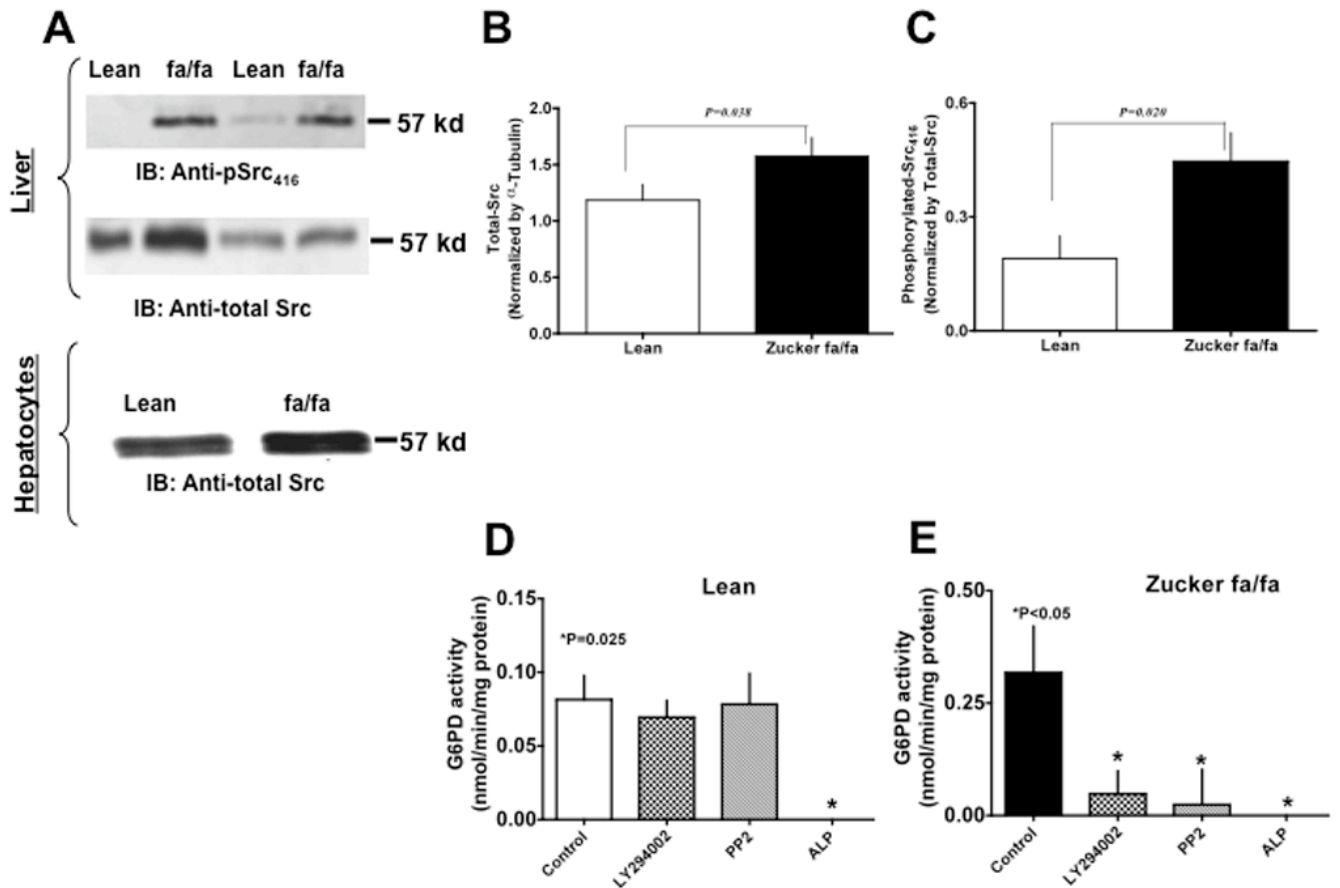
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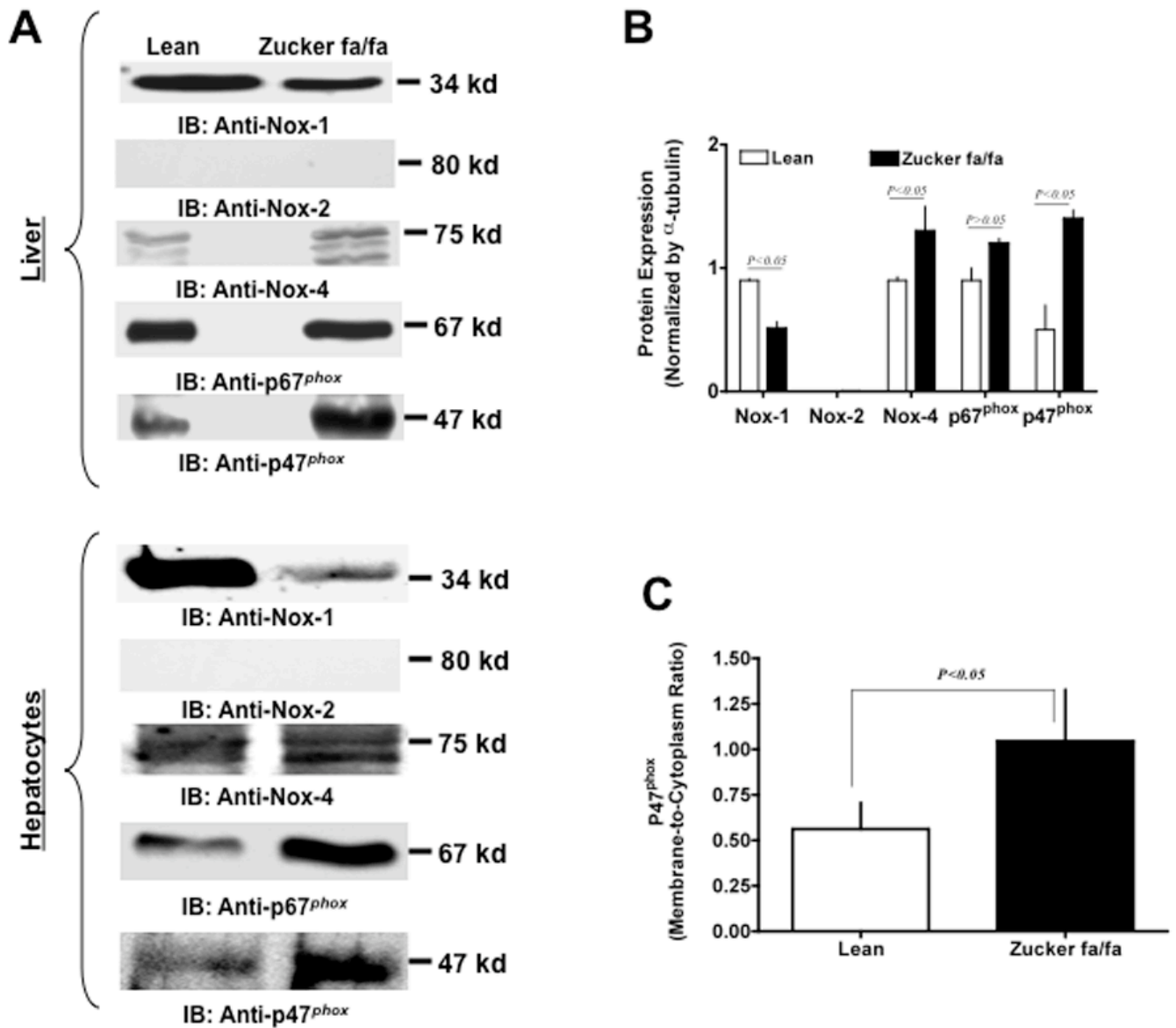


**Figure 1. Glucose-6-phosphate dehydrogenase activity is elevated in type 2 diabetic model Zucker lean and fa/fa rat liver homogenates (Panel A top) and hepatocyte lysates (Panel A bottom) were analyzed on 9% SDS-PAGE. Western blot analysis was performed using rabbit polyclonal anti-G6PD antibody (A). All input lanes contain 35  $\mu$ g of total protein content. Panel (A) represents one blot of six such independent experiments. (B) The graph represents G6PD protein expression in lean (n=6) and ZDF (n=6) rat liver as estimated by densitometric analysis (total: upper and lower band) and normalized with  $\alpha$ -tubulin values. The graph represents G6PD activity (C), effect of NADP<sup>+</sup>-dependent on activity (D), NADPH levels (E) and 6-phospho-gluconate levels (F) from lean (n=6) and Zucker fa/fa (n=6) rat liver, respectively. Statistical analysis for (B–F) was performed using Student's t-test and  $P < 0.05$  (lean versus Zucker fa/fa) was considered statistically significant.**

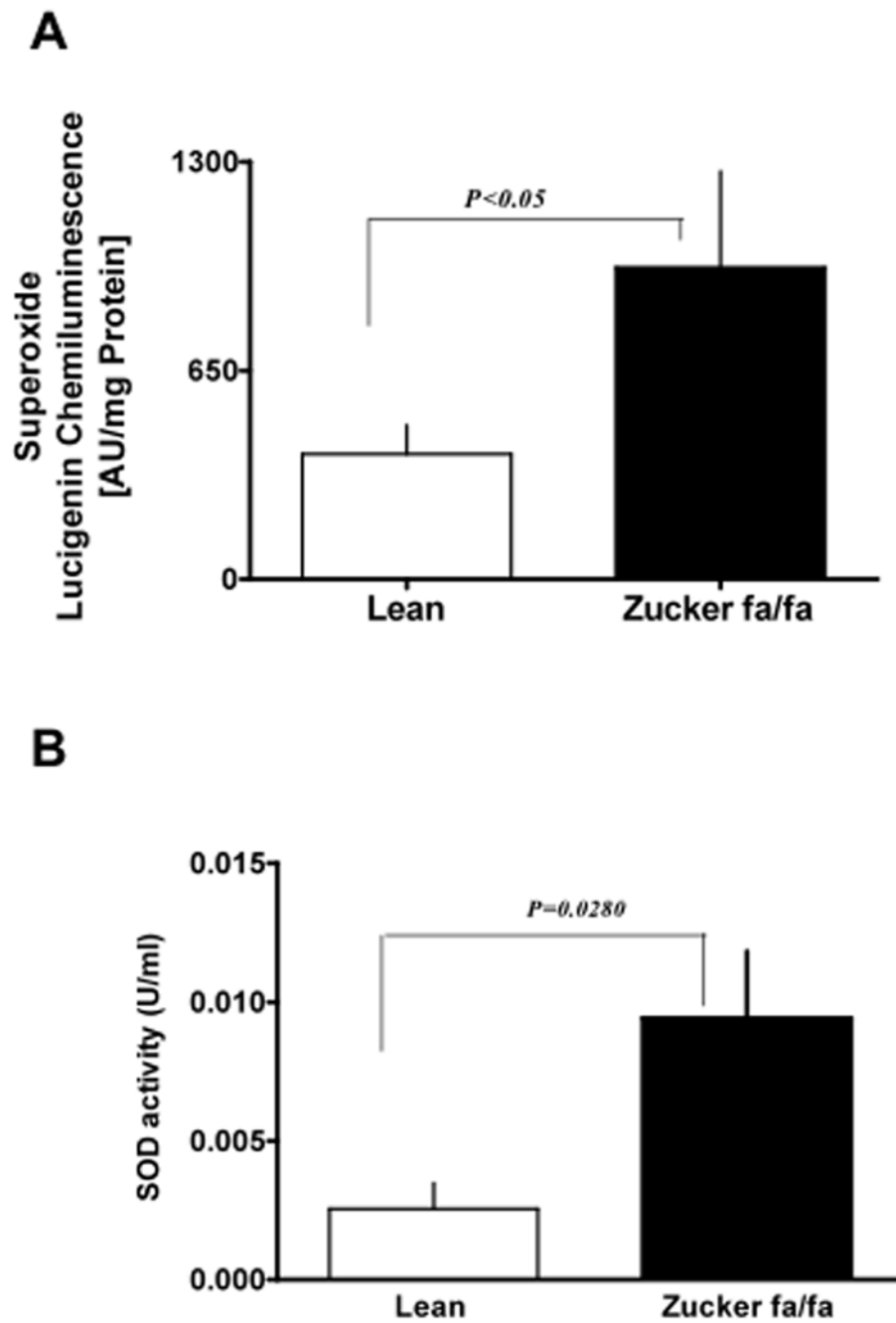


**Figure 2. Glucose-6-phosphate dehydrogenase is activated by Src kinase in the liver of Zucker fa/fa rats**

(A) Zucker lean (lanes 1 & 3) and fa/fa (lanes 2 & 4) rat liver homogenates (Panel A top) and hepatocyte lysates (Panel A bottom) were analyzed on 9% SDS-PAGE. Western blot analysis was performed using rabbit polyclonal anti-pSrc (first panel) and anti-total Src antibody (second panel). All input lanes contain 35  $\mu$ g of total protein content. Panel (A) represents one blot of six such independent experiments. (B) & (C) Graphs represent total-Src (B) and phosphorylated-Src<sub>416</sub> (C) in lean (n=6) and ZDF (n=6) rat liver as estimated by densitometric analysis and normalized with total-Src or  $\alpha$ -tubulin values. (D) & (E) Lean (D) and Zucker fa/fa (E) liver tissues were incubated at 37°C for 10 minutes without and with PI3 kinase inhibitor, LY294002 (10  $\mu$ M), Src kinase inhibitor, PP2 (10  $\mu$ M), and alkaline phosphatase (200 U/ml), respectively. Graphs represent glucose-6-phosphate dehydrogenase activity in Lean (D) and Zucker fa/fa (E) liver homogenates, respectively. Statistical analysis was performed using Student's t-test and *P* < 0.05 (lean versus Zucker fa/fa) was considered statistically significant.



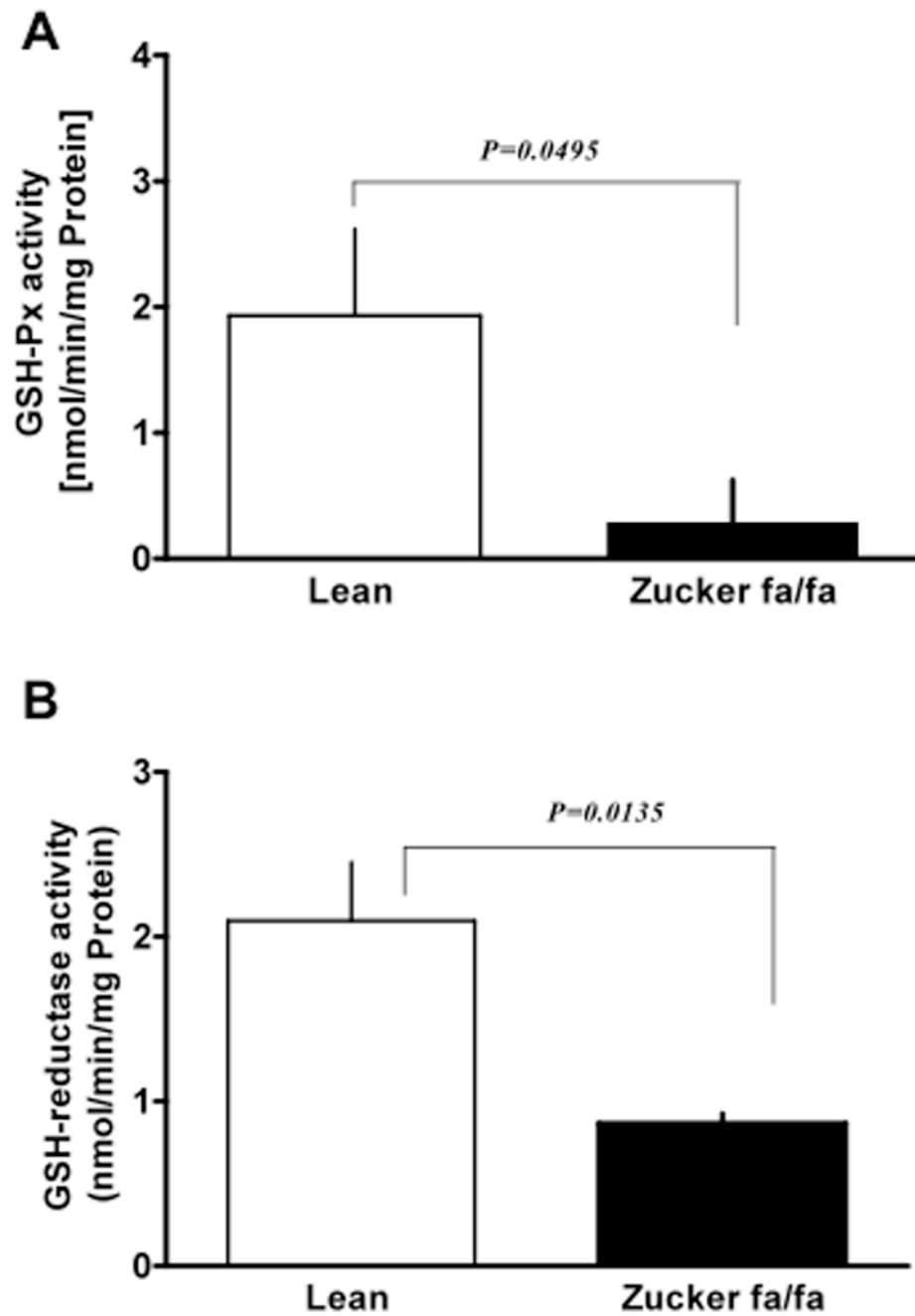
**Figure 3. Protein expression of NAD(P)H oxidase is altered in Zucker fa/fa rat liver tissue**  
 Lean and Zucker fa/fa rat liver homogenates (Panel A top) and hepatocyte lysates (Panel A bottom) were analyzed on 9% SDS-PAGE. Western blot analysis was performed using mouse monoclonal anti-Nox1 and -Nox2 (A; first & second panels), goat polyclonal anti-Nox4 (A; third panel), mouse monoclonal anti-p67<sup>phox</sup> (A; fourth panel) and goat polyclonal anti-p47<sup>phox</sup> (A; bottom panel) antibodies, respectively. All lanes contain 35  $\mu$ g of total protein content. Panel (A) represents one blot of six such independent experiments. Panel B: Graph represents NADPH oxidase subunit expression in lean (n=6) and Zucker fa/fa (n=6) rat liver as estimated by densitometric analysis and normalized with  $\alpha$ -tubulin values. Panel C: Lean and Zucker fa/fa rat liver tissue was fractionated by ultracentrifugation at 100,000 $\times$ g to separate the membrane and cytoplasmic fractions. Graph represents levels of p47<sup>phox</sup> found in membrane as compared to cytoplasmic fraction in Zucker fa/fa rat liver. Statistical analysis was performed using Student's t-test and  $P < 0.05$  (lean versus ZDF) was considered statistically significant.



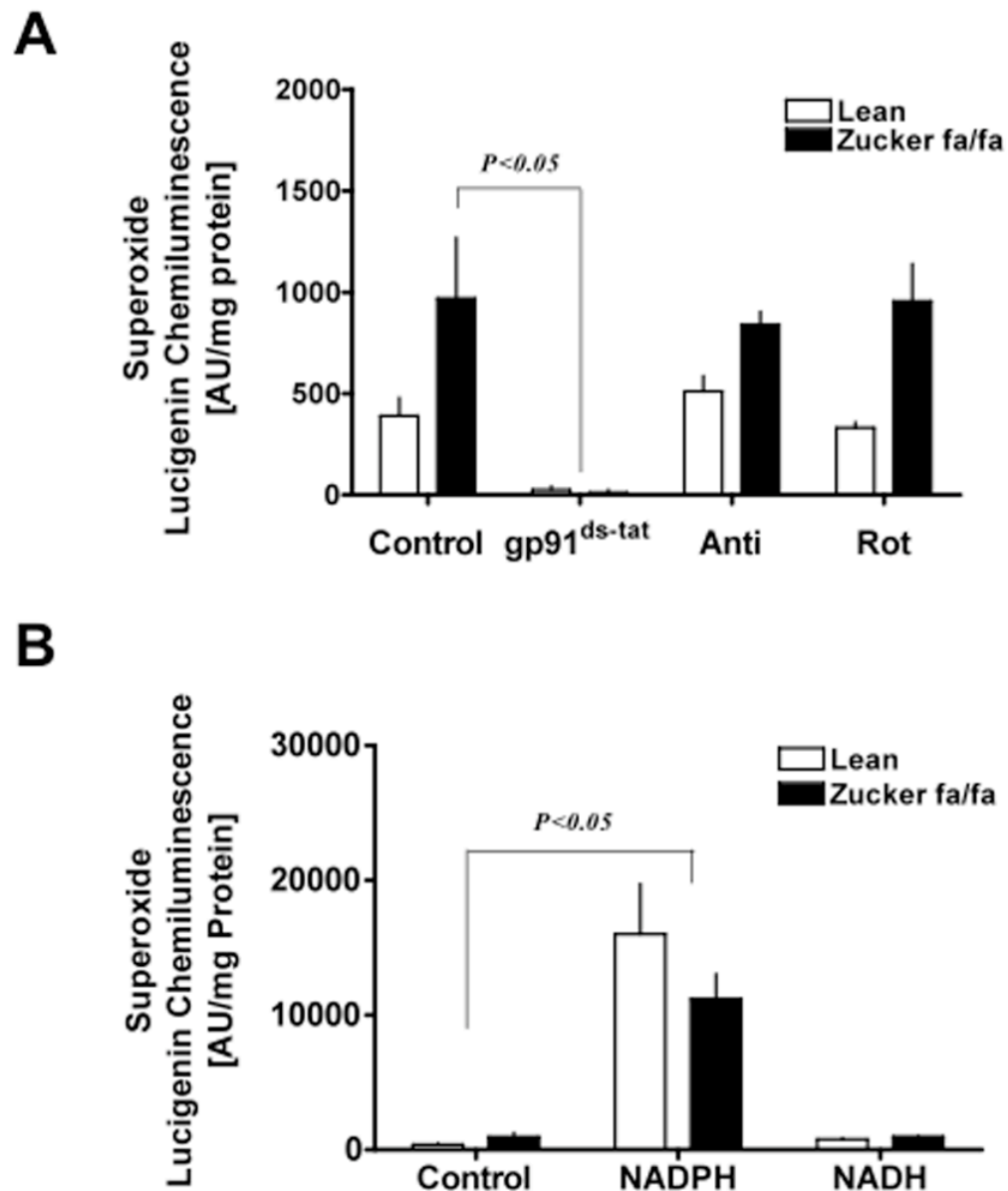
**Figure 4. Superoxide generation is elevated in the liver of Zucker fa/fa rats**

Liver homogenates obtained from lean (n=6) and Zucker fa/fa (n=6) rat were used to detect  $O_2^-$  production by lucigenin (5  $\mu$ M) chemiluminescence (A); Superoxide dismutase activity (SOD; B). Statistical analysis was performed using Student's t-test and  $P < 0.05$  (lean versus Zucker fa/fa) was considered statistically significant.



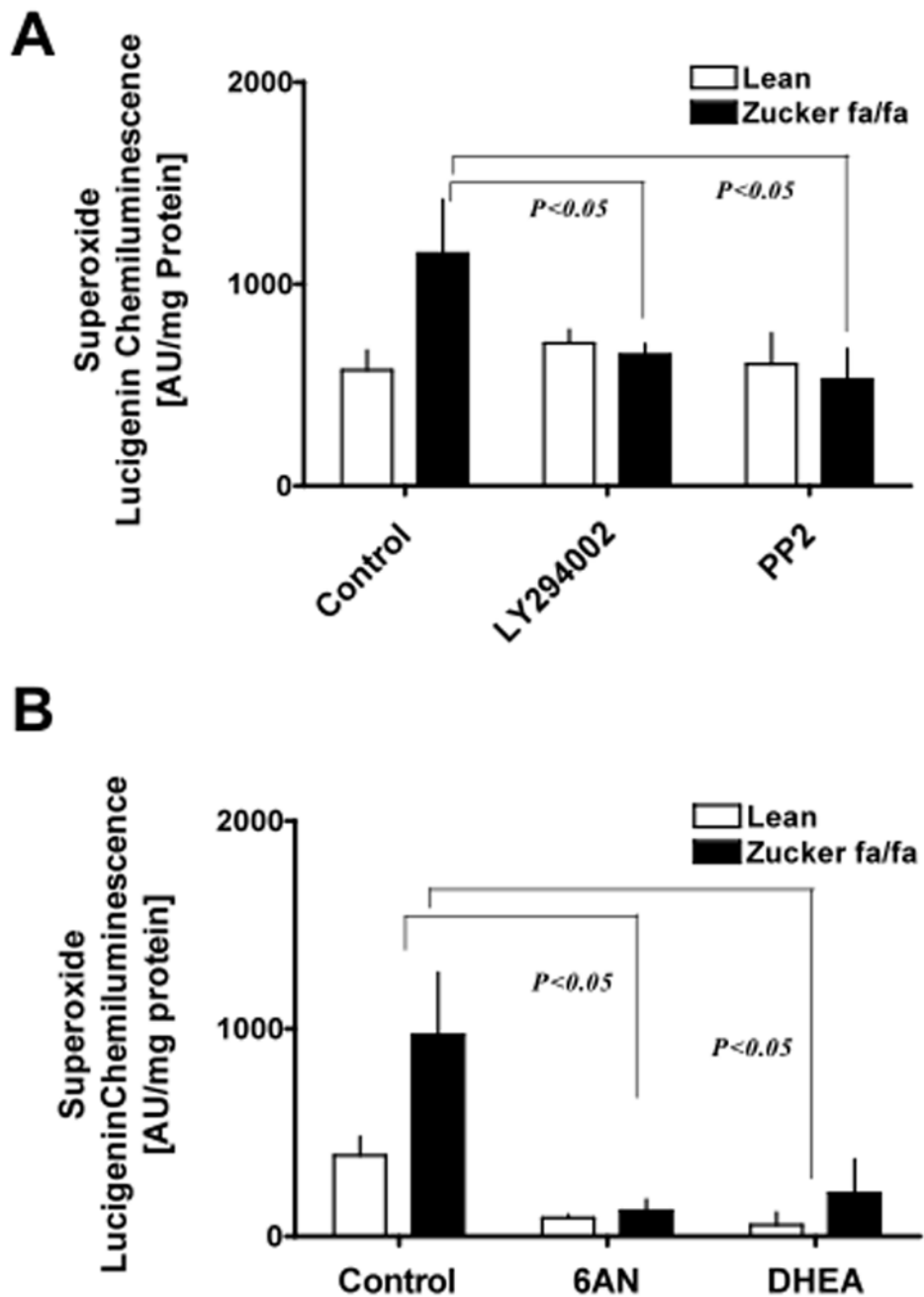


**Figure 5. Glutathione peroxidase and reductase activity is decreased in the liver of Zucker fa/fa rats**  
Liver homogenates obtained from lean (n=6) and Zucker fa/fa (n=6) rat were used to estimate glutathione peroxidase (GSH-Px; A) and glutathione reductase (GSH reductase; B) activities.



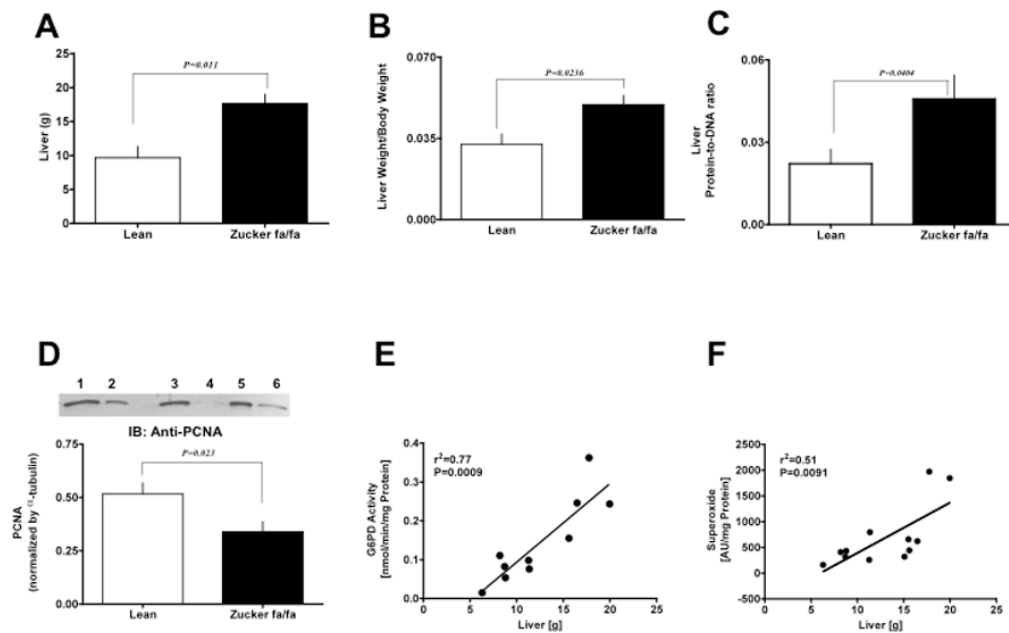
**Figure 6. Superoxide generated in liver is NADPH-dependent**

Liver homogenates obtained from lean (n=6) and Zucker fa/fa (n=6) animals were treated with: (A) either NADPH oxidase inhibitor, gp91<sup>ds-tat</sup> (50  $\mu$ M), mitochondrial respiratory chain inhibitors, antimycin (10  $\mu$ M) and rotenone (50  $\mu$ M), and superoxide levels were determined by lucigenin (5  $\mu$ M) chemiluminescence. (B) NADPH (100  $\mu$ M) or NADH (100  $\mu$ M) for 10 minutes at 37°C, and superoxide levels were determined as described above. These experiments were performed in the absence of NADPH regenerating system. Statistical analysis was performed using Student's t-test and  $P < 0.05$  was considered statistically significant.



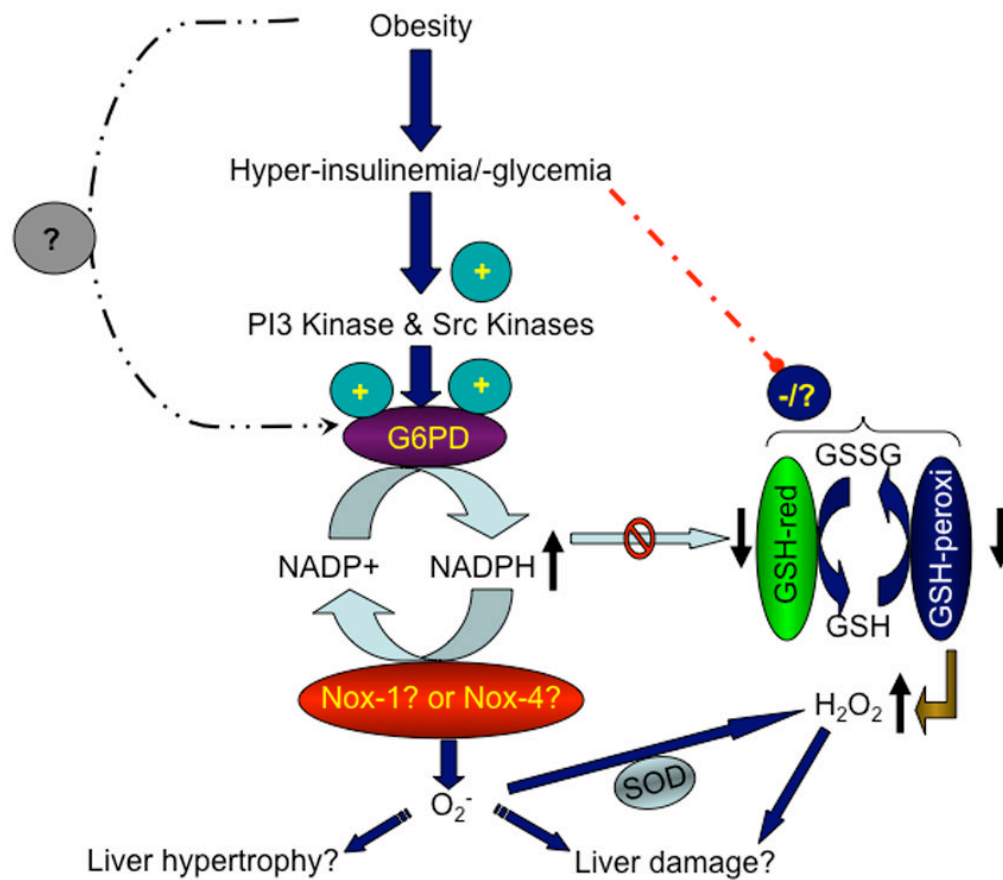
**Figure 7. NADPH oxidase and glucose-6-phosphate dehydrogenase synergistically increases superoxide production in diabetic tissues**

Liver tissues obtained from lean (n=6) and ZDF (n=6) animals were incubated (A) with PI3 kinase inhibitor, LY294002 (10  $\mu$ M), and Src kinase inhibitor, PP2 (10  $\mu$ M), for 10 minutes at 37°C and (B) either with 6-aminonicotinamide (5 mM) or dehydroepiandrosterone (DHEA; 100  $\mu$ M), inhibitors of glucose-6-phosphate dehydrogenase, for 10 minutes at 37°C in the presence of NADPH regenerating system and superoxide levels were determined by lucigenin (5  $\mu$ M) chemiluminescence. Statistical analysis was performed using Student's t-test and  $P < 0.05$  (lean versus ZDF) was considered statistically significant.



#### Figure 8. Liver hypertrophy occurs in young Zucker fa/fa rats

Liver weight (A) and liver-to-body weight (B) was also determined in these rats. Graph represents the liver protein-to-DNA ratio in lean and Zucker fa/fa rats (C). The expression of PCNA in lean (lanes 1, 3, and 5) and Zucker fa/fa (lanes 2, 4, and 6) liver was determined by western blot analysis using mouse monoclonal anti-PCNA antibody (D). Correlation between liver weight and G6PD activity (E) or superoxide levels (F) was determined by regression analysis. Statistical analysis was performed using Student's t-test and  $P < 0.05$  was considered statistically significant.



**Figure 9. A schematic illustration of G6PD and NADPH oxidase activation in Zucker fa/fa rats** In the fa/fa rats, a congenital model of obesity and type 2 diabetes, changes in adipose tissue hormones and elevation in either insulin or glucose increases expression and activity of glucose-6-phosphate dehydrogenase (G6PD) and NADPH oxidases (Nox-1 and Nox-4). Conversely, glutathione reductase (GSH reductase) activity is decreased. This leads to an increase in superoxide ( $O_2^-$ ) levels, which consequently either evokes liver growth or liver dysfunction.