

Partial resistance to PPAR α agonists in Zucker diabetic fatty (ZDF) rats is associated with defective hepatic mitochondrial metabolism

Santhosh Satapati MS^{1,5}, TianTeng He MD¹, Takeshi Inagaki MD³, Matthew Potthoff PhD³, Matthew E. Merritt PhD¹, Victoria Esser MD², David J. Mangelsdorf PhD³, Steven A. Kliewer PhD^{3,4}, Jeffrey D. Browning PhD^{1,2} and Shawn C. Burgess PhD^{1,3,5*}

¹The Advanced Imaging Research Center, ²Department of Internal Medicine, ³Department of Pharmacology, and Howard Hughes Medical Institute ⁴Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, TX
⁵Department of Molecular and Cell Biology, University of Texas at Dallas, Richardson, TX

*Corresponding author:

Shawn C. Burgess, PhD
The University of Texas Southwestern Medical Center
The Advanced Imaging Research Center and
Department of Pharmacology
5801 Forest Park Road
Dallas, TX 75390-8568
E-mail: shawn.burgess@utsouthwestern.edu

Received for publication 18 February 2008 and accepted in revised form 04 May 2008.

Additional information for this article can be found in an online appendix at
<http://diabetes.diabetesjournals.org>

Objective: Fluxes through mitochondrial pathways are defective in insulin resistant skeletal muscle, but it is unclear whether similar mitochondrial defects play a role in the liver during insulin resistance and/or diabetes. The purpose of this study is to determine if abnormal mitochondrial metabolism might play a role in the dysregulation of both hepatic fat and glucose metabolism during diabetes.

Research Design and Methods: Mitochondrial fluxes were measured using $^2\text{H}/^{13}\text{C}$ tracers and nuclear magnetic resonance (NMR) spectroscopy in Zucker Diabetic Fatty (ZDF) rats during early and advanced diabetes. To determine whether defects in hepatic fat oxidation can be corrected by PPAR α activation, rats were treated with WY14,643 for 3-weeks prior to tracer administration.

Results: Hepatic mitochondrial fat oxidation in the diabetic liver was impaired 2-fold secondary to decreased ketogenesis, but TCA cycle activity and pyruvate carboxylase flux were normal in newly diabetic rats and elevated in older rats. Treatment of diabetic rats with a PPAR α agonist induced hepatic fat oxidation via ketogenesis and hepatic TCA cycle activity, but failed to lower fasting glycemia or endogenous glucose production. In fact, PPAR α agonism over-stimulated mitochondrial TCA cycle flux and induced pyruvate carboxylase flux and gluconeogenesis in lean rats.

Conclusions: The impairment of certain mitochondrial fluxes, but preservation or induction of others, suggests a complex defect in mitochondrial metabolism in the diabetic liver. These data indicate an important co-dependence between hepatic fat oxidation and gluconeogenesis in the normal and diabetic state and potentially explain the sometimes equivocal effect of PPAR α agonists on glycemia.

The liver is a critical hub in systemic energy distribution. In the post-prandial state the liver condenses dietary carbohydrate to glycogen or converts it to lipid for storage in peripheral adipose tissue. During fasting, the liver oxidizes fatty acids released by lipolysis to provide energy for the synthesis of glucose (gluconeogenesis) or provide substrate for the synthesis of ketone bodies (ketogenesis). Since both glucose and ketones are crucial for postabsorptive survival, their synthesis is tightly regulated by multiple mechanisms. However, during insulin resistance and diabetes these regulatory mechanisms fail, resulting in hepatic fat accumulation and uncontrolled glucose production. Understanding the precise metabolic perturbations that accompany these regulatory failures has important implications for the prevention and treatment of diabetes and fatty liver disease.

The relationship between hepatic fat metabolism and gluconeogenesis is complex and co-dependent. Gluconeogenesis and fatty acid oxidation share molecular mediators that coordinate enzyme expression in these pathways (1-4). Metabolically, hepatic glucose metabolism is linked to mitochondrial fat oxidation as evidenced by **1**) the dependence of gluconeogenesis on mitochondrial fat oxidation in the isolated liver (5; 6); **2**) the induction of hepatic insulin resistance during a short term a high fat diet (7); **3**) the stimulation of gluconeogenesis and reduction of glycogenolysis during acute lipid infusions (8-10); and **4**) impaired gluconeogenesis and hypoglycemia in humans (11; 12) and animal models (13; 14) with primary defects in hepatic fat oxidation. Based on these observations, it's reasonable to suspect that the abnormal lipid and glucose metabolism associated with insulin resistance and diabetes, might be related to defects in shared metabolic pathways, particularly those

in the mitochondria (15; 16). Mitochondrial "dysfunction" in the form of impaired energy generation (17-19) or incomplete fat oxidation (20) is associated with insulin resistant skeletal muscle but it remains unclear if similar defects exists in liver and, if so, how they could co-exist with the increased energetic requirements of elevated gluconeogenesis and lipogenesis found in the insulin resistant liver.

The Zucker diabetic fatty (ZDF) rat is a model of obesity, insulin resistance and diabetes where the regulation of both hepatic fat and glucose metabolism are substantially dysfunctional (21). We hypothesized that defects of hepatic fat and glucose metabolism are coupled via defects in mitochondrial fluxes. The data indicate impaired mitochondrial fluxes of β -oxidation, but induction of the mitochondrial fluxes of the TCA cycle and pyruvate carboxylase which tended to contribute to elevated rates of glucose production in diabetic rats. Treatment with a PPAR α agonist improved plasma NEFA, ketones and insulin levels but over-stimulated TCA cycle flux and did not normalize glucose homeostasis in diabetic rats, and even induced glucose production in lean rats. The data suggest that a defect in mitochondrial metabolism is a fundamental feature of this model of diabetes and that it cannot be fully corrected by PPAR α agonist treatment.

RESEARCH DESIGN AND METHODS

Materials

[3, 4-¹³C] glucose (98%) was purchased from Omicron Biochemicals (South Bend, IN). [3, 4-¹³C] ethylacetoacetate (98%), [1, 2-¹³C] sodium β -hydroxybutyrate (98%), were purchased from Isotec (St. Louis, MO). [U-¹³C] propionate and deuterium oxide (99%) were purchased from Cambridge Isotopes (Andover, MA). Other common chemicals were obtained from Sigma (St. Louis, MO).

Animal Protocol

Sprague-Dawley (~300g), ZDF (Fa/fa) control (~300g) and (fa/fa) diabetic ZDF (~450g) rats were studied using a protocol approved by the UT Southwestern Institutional Animal Care and Use Committee. ZDF rats were studied at ~12 weeks and ~22 weeks of age. Five days prior to infusion, rats were anesthetized with an isoflourane/oxygen and a jugular vein catheter was surgically implanted (22). On day five, rats were fasted for 24-hours (unless otherwise noted). An initial blood sample was collected from the tail vein to measure pre-experimental glucose and ketone concentrations. Etomoxir was given as a 0.5 $\mu\text{mol}/100\text{ g}$ body weight I.P injection 90 min prior to the infusion of isotope tracers where applicable. Where noted, rats were infused with octanoate along with tracers at a rate of 30 $\mu\text{moles}/\text{min}$ for 90 minutes to stimulate ketogenesis. WY14,643 (BIOMOL Research Laboratories Inc. Plymouth Meeting, PA) was mixed with chow at 100 (low dose) or 300 (high dose) mg/Kg chow and administered for 3-weeks prior to the study of 12-week old rats.

Infusate preparation

On the morning of infusion, 28 mg of [3,4- ^{13}C]ethylacetoacetate was suspended in 4 ml of D.I. water and 80 μl of 4M NaOH. This solution was stirred at 40 $^{\circ}\text{C}$ for 75 minutes. The solution was neutralized with dilute HCl and quantitative hydrolysis to [3,4- ^{13}C]acetoacetate was confirmed by ^1H NMR spectroscopy. To this solution, 27 mg of [3,4- ^{13}C]glucose and 21mg of [1,2- ^{13}C] β -hydroxybutyrate was added, and the volume was adjusted to 7.2 ml with saline before filtering through a 0.2 micron filter. This procedure gives ~20 mM of each tracer, though the actual concentrations were assayed.

Tracer delivery

Rats received an intraperitoneal injection (20 $\mu\text{L}/\text{g}$ rat) containing [U- ^{13}C]propionate (5 mg/ml) dissolved in $^2\text{H}_2\text{O}$ (99%). A bolus of infusate (2.25 ml/ hour for 10 minutes) was administered, followed by continuous infusion at a rate of 0.5 ml/hour for 90 mins. Rats were allowed unrestrained movement within their cage during the infusion period.

Sample preparation

After infusion, rats were anesthetized with isoflourane-oxygen gas and ~10 ml of blood was collected from the *vena cava*. A small portion (200 μl) was used for biochemical assays and the remainder was extracted with perchloric acid. Supernatant was passed through cation (H^+) resin and neutralized with LiOH. This solution was condensed to ~400 μl by incomplete lyophilization and 100 μl of D_2O was added before ^{13}C NMR analysis of acetoacetate and β -hydroxybutyrate multiplets. After analysis of ketones the glucose was converted to the 1,2-isopropylidene glucofuranose derivative (monoacetone glucose, MAG) (23; 24).

NMR analysis

Standard proton decoupled ^{13}C NMR spectra of plasma extracts were acquired on a 14T spectrometer equipped with a 5 mm broadband probe using a 45 $^{\circ}$ pulse and a 3s repetition time. MAG was analyzed by ^2H and ^{13}C NMR as previously described (23; 24). Peak areas (^2H and ^{13}C) were measured using the 1D NMR software *ACD/Labs 9.0* (Advanced Chemistry Development, Toronto, Ontario, Canada).

Metabolic Analysis

The ^2H and ^{13}C NMR spectra of MAG were used to measure glycogenolysis (GLY), gluconeogenesis from glycerol ($\text{GNG}_{\text{glycerol}}$), gluconeogenesis from phosphoenolpyruvate originating from the TCA cycle (GNG_{PEP}), and TCA cycle turnover (23; 24) (See **Supplemental Methods**). Apparent ketone turnover was measured using a two pool model of exchangeable acetoacetate and β -hydroxybutyrate (25-27). Equations in (27)

were adapted to NMR data (See **Supplemental Methods**).

Total ketone production is reported where:

$$\text{ketone production} = Ra_{ACAC} + Ra_{BHB}$$

Ketone production and hepatic TCA cycle flux were used to estimate an index of hepatic β -oxidation: **β -oxidation index** (in 2 carbon units) = TCA cycle flux + 2 x ketone production

Gene expression analysis

Primers were designed using Primer Express software (Applied Biosystems Inc., San Jose, CA) based on GenBank sequence data. Quantitative real-time PCR reactions (10 μ l) contained 25 ng of cDNA, 150 nM of each primer, and 5 μ l of SYBR Green PCR Master Mix (Applied Biosystems Inc., San Jose, CA). All reactions were performed in triplicate on an Applied Biosystems Prism 7900HT Sequence Detection System, and relative mRNA levels were calculated by the comparative threshold cycle method by using cyclophilin as the internal control.

Metabolite/hormone Measurements

Lipids were extracted from ~50 mg of liver using a standard methanol/chloroform extraction and triglyceride content of liver was measured using the L-type TGH triglyceride kit (Wako Chemicals Inc., Richmond, VA). Plasma-free fatty acids were measured using the NEFA kit (Wako Chemicals Inc., Richmond, VA). Glucose was assayed by standard enzyme coupled reactions. Total ketone concentration and β -hydroxybutyrate (BHB) were measured using a ketone kit (Wako Chemicals Inc., Richmond, VA) and acetoacetate (ACAC) levels were determined from the difference. Plasma insulin was measured by radioimmunoassay using the Rat Insulin RIA kit (Linco Research, Inc., St. Charles, MO). Plasma FGF-21 concentration was measured using an RIA kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA).

Statistics

Data are expressed as the mean and standard error. Differences between groups were analyzed for statistical significance using an unpaired Student's t-test where $P < 0.05$ was considered significant. Analysis of covariance (ANCOVA) was used to compare slopes between regression lines in Systat 12 (Systat Software, Inc., San Jose, CA). Correlations with $P < 0.05$ were considered significant.

RESULTS

Simultaneous delivery of 5 stable isotope tracers and NMR analysis of plasma extracts provides insight into hepatic fat metabolism.

Simultaneous administration of $^2\text{H}_2\text{O}$, [U - ^{13}C]propionate, [$3,4$ - ^{13}C]glucose, [$3,4$ - ^{13}C]acetoacetate and [$1,2$ - ^{13}C] β -hydroxybutyrate was used to measure gluconeogenesis and index hepatic fat oxidation by NMR isotopomer analysis of plasma glucose and ketones. Since tracers of ketone turnover, the TCA cycle and gluconeogenesis have never been applied simultaneously, we performed initial experiments to confirm that the techniques are compatible. [U - ^{13}C]propionate and [$3,4$ - ^{13}C]glucose generated ^{13}C multiplets in the NMR spectrum of plasma glucose, but did not significantly enrich plasma ketones, indicating that tracers of gluconeogenesis and the TCA cycle do not interfere with the analysis of plasma ketones (See **Supplemental Results and Supplemental Figure 1**). Similarly, Carbon-13 originating from [$3,4$ - ^{13}C]acetoacetate and [$1,2$ - ^{13}C] β -hydroxybutyrate did not enrich plasma glucose at low infusion rates, indicating that ketone tracers do not interfere with the determination of gluconeogenesis. In addition, we measured ketone turnover in a group of rats under various levels of hepatic fat oxidation to assess the responsiveness of the method. Data from fasted, fasted + etomoxir treated, fed, and fed + octanoate treated rats confirm that ketone turnover, as

measured by NMR, matches the expected effect of the interventions on hepatic fat oxidation (**Supplemental Results and Supplemental Figure 1**).

Hepatic fat oxidation is impaired in the ZDF rat.

As expected, fasting plasma glucose, non-esterified fatty acids (NEFA), insulin and liver triglycerides were markedly elevated in diabetic rats (**Table 1**). Despite elevated NEFA and liver triglycerides, fasting plasma ketone concentration was ~4-fold lower in 12-week old diabetic rats compared to lean littermates, suggesting a defect in hepatic fat oxidation. Ketone concentration doubled by 22-weeks in diabetic rats, but remained markedly lower than control rats.

Low plasma ketone levels in diabetic rats were investigated further using apparent ketone tracer turnover to estimate hepatic ketone production in diabetic rats (**Figure 1A**). Consistent with low plasma ketone concentration, *in vivo* ketone turnover was 4-fold lower in 12-week old diabetic rats and 2-fold lower in 22-week old diabetic rats. To investigate hepatic fat oxidation further, we measured terminal substrate oxidation in the hepatic TCA cycle by ^{13}C and ^2H NMR isotopomer analysis of plasma glucose (23; 24; 28). Despite dramatically impaired ketogenesis in diabetic rats, hepatic TCA cycle flux was normal in 12-week old diabetic rats compared to lean controls (**Figure 1B**). However, TCA cycle flux increased by 60% in the 22-week old diabetic rats compared to their younger diabetic counterpart (**Figure 1B**), consistent with the ketone data, suggesting an age (or loss of insulin) related increase in fat oxidation in diabetic animals.

The sum of hepatic TCA cycle flux and ketone turnover was used as an index of acetyl-CoA formation by *in vivo* hepatic β -oxidation. This data indicated a substantially lower rate of β -oxidation in newly diabetic rats compared to age-matched controls (**Figure 1C**). 22-week old diabetic rats were

not significantly different from control littermates due to increased ketogenesis and TCA cycle flux, but the relative contribution of these two pathways to β -oxidation remained abnormal. These data indicate that the early onset of fatty liver in these animals is partly due to impaired fasting hepatic fat oxidation despite increased peripheral lipolysis.

Sources of hepatic glucose production are abnormal in ZDF rats.

To determine the effect of impaired fat oxidation on gluconeogenesis, hepatic glucose production and its sources were measured in lean and diabetic rats. As we previously reported (22), elevated glucose production (**Figure 2A**) in newly diabetic ZDF rats (age 12-weeks) was affiliated with increased glycogenolysis (**Figure 2B**) and gluconeogenesis from glycerol ($\text{GNG}_{\text{glycerol}}$) (**Figure 2C**) but normal gluconeogenesis originating from substrates (i.e. lactate, pyruvate alanine) that pass through the TCA cycle (GNG_{PEP}) (**Figure 2D**). Flux through all of these pathways were exacerbated in older diabetic rats (age 22-weeks), including a 45% increase in GNG_{PEP} which was provoked by a 60% increase in anaplerotic flux (presumably via mitochondrial pyruvate carboxylase) in 22-week old ZDF rats compared to lean littermates (350 vs. 211 $\mu\text{mol}/\text{min}/\text{Kg}$, $P < 0.05$).

The co-dependence of gluconeogenesis and hepatic fat oxidation is altered in diabetic rats.

Hepatic fat oxidation induces glucose production by supplying energy rich co-factors (ATP and NADH) necessary for gluconeogenesis and by altering the intrahepatic concentration of allosteric effectors of gluconeogenic enzymes (acetyl-CoA and citrate) (5). To determine if this relationship is altered by impaired fat oxidation in the ZDF model we compared the hepatic fat oxidation index with the rate of gluconeogenesis (GNG_{PEP} ; $\text{PyT} \rightarrow \text{OAA} \rightarrow$

PEP → → Glucose) (**Figure 2E**). Increased hepatic β -oxidation was closely associated with increased gluconeogenesis in all animals (Control: $r=0.55$, $P=0.05$; Diabetic: $r=0.78$, $P=0.0006$). These data indicate that hepatic fat oxidation is an important component of in vivo gluconeogenic potential; however, gluconeogenesis in diabetic livers required less total fat oxidation than normal livers as evidenced by a higher slope for the correlation ($P < 0.0007$). To determine the nature of this efficiency we also compared TCA cycle flux with GNG_{PEP} and found a tight correlation between the two which did not differ between control and diabetic rats (**Figure 2F**). This data is similar to our previous description of TCA cycle flux as an important mediator of gluconeogenic control in isolated liver (6), and indicates that the diabetic liver responds to impaired fat oxidation by preferentially shunting acetyl-CoA away from ketogenesis and towards the TCA cycle to preserve energy production for gluconeogenesis.

PPAR α agonist treatment normalizes hepatic fat oxidation but not hepatic glucose production.

Since hepatic fat oxidation was markedly impaired in the ZDF liver, we administered WY14,643 for 3-weeks to young control and ZDF rats to determine if fasting hepatic fat oxidation could be corrected by PPAR α induction and, if so, how this intervention would affect gluconeogenesis. Plasma NEFA, ketones and insulin levels were significantly normalized by WY14,643 in a dose dependent manner, but hepatic triglyceride content was unresponsive (**Table 2**). Surprisingly, fasting plasma glucose concentration did not decrease in diabetic rats and increased in treated lean rats (**Table 2**). Ketogenesis normalized only at high doses (**Figure 3A**) and TCA cycle activity (**Figure 3B**) was driven to supra-normal levels by either dose. Thus, PPAR α agonism appeared to correct the hepatic fat oxidation index

(**Figure 3C**) in diabetic rats, but the manner in which the end-product (acetyl-CoA) was further metabolized by hepatic mitochondria (TCA cycle oxidation vs. ketogenesis) remained dysfunctional.

Despite improved aspects of insulin resistance, (fasting insulin, NEFA) endogenous glucose production was not improved by PPAR α agonist treatment. Glycogenolysis was slightly reduced in treated diabetic rats, but gluconeogenesis was slightly increased (**Figure 4A-D**), resulting in unabated hyperglycemia. A similar induction of gluconeogenesis was observed in control rats which led to increased glucose production at the high dose of WY14,643, consistent with increased plasma glucose concentration during treatment (**Table 2**). Interestingly, pyruvate carboxylase flux and pyruvate cycling through either the malic or pyruvate kinase enzymes was induced 2-fold by WY14,643 in diabetic rats, but to a much lesser degree in control rats (**Figure 4E-F**). These data suggest that unabated fasting glucose can occur in rodents treated with PPAR α agonists (29-31) due, in part, to stimulation of hepatic gluconeogenesis via induction of TCA cycle flux (6).

Expression of enzymes in hepatic fat oxidation are slightly impaired in diabetic rats, but are induced by PPAR α agonist treatment.

We measured the expression of hepatic enzymes associated with mitochondrial, peroxisomal and microsomal fat oxidation by QPCR to investigate the molecular basis of attenuated hepatic β -oxidation in ZDF rats (**Table 3**). Of the mRNA we measured, only CPT-1a (mitochondrial fat transporter) and Cyp-4a (microsomal ω -oxidation) were significantly decreased, while CD36/FAT (cellular fat transporter) was overexpressed 5-fold. Treatment with WY14,643 dramatically stimulated the expression of nearly all measured FAO genes (**Table 3**), in agreement with the measures of ketogenesis and TCA

cycle activity after treatment. Surprisingly, despite increased hepatic fat oxidation and unaffected gluconeogenesis, PGC-1 α expression was decreased by 2-3-fold in WY14,643 treated lean and diabetic rats.

Diabetic rats are resistant to the normal ketogenic effects of FGF-21.

FGF-21 is an endocrine hormone produced by the liver that mediates the pleiotropic actions of PPAR α by stimulating lipolysis and ketogenesis (32; 33). To determine whether impaired hepatic fat oxidation in ZDF rats might be associated with defects in FGF-21, we measured both hepatic FGF-21 expression (**Figure 5A**) and plasma FGF-21 protein (**Figure 5B**). FGF-21 plasma protein was consistently, but not dramatically elevated in diabetic rats. However, FGF-21 expression was elevated 6-fold in the liver of newly diabetic rats. These findings are somewhat paradoxical since FGF-21 induces hepatic ketogenesis (32) and has been found to have substantial anti-diabetogenic effects (34). Expression of FGF-21 was induced by WY14,643 in control, but not diabetic, livers. Conversely, WY14,643 increased plasma FGF-21 protein in both control and diabetic rats. These data suggest that FGF-21 maintains its downstream responsiveness to PPAR α in these diabetic rats, but that their livers may be resistant to the normal induction of fat oxidation by FGF-21.

DISCUSSION

Insulin resistance and diabetes have profound effects on hepatic carbohydrate and lipid metabolism. *In vivo* hepatic fat oxidation was severely impaired in the fasted 12-week old ZDF rat, consistent with the previous reports of increased *de novo* lipogenesis in the fed state (35; 36). By 22-weeks, hepatic fat oxidation index in the ZDF rat was no longer impaired, but remained dysfunctional with regard to the distribution between ketogenesis (2-fold lower than normal) and TCA cycle oxidation (2-fold higher than normal)

suggesting a reorganization of mitochondrial fat oxidation with the onset of insulinopenia. Hepatic glucose production in diabetic rats was also remarkably age dependent. Together with previous work, the current data indicate that elevated fasting glucose production in the ZDF rat occurs initially as a consequence of increased glycogen breakdown (28), followed shortly by increased conversion of glycerol to glucose (22) and then, in later stages of the phenotype, due to increased gluconeogenesis from substrates like lactate and amino acids (**Figure 4**). However, other studies have revealed less remarkable changes in the sources of glucose production (37), perhaps due to differences in fasting times or methodological approaches.

Abnormal mitochondrial metabolism is a key feature of insulin resistant skeletal muscle (17-20) and has also been implicated in human insulin resistant liver (15; 16). However, there are few *in vivo* data regarding mitochondrial metabolism in insulin resistance liver. Here, FAO gene expressions, including PGC-1 α were not robustly altered, indicating that defects in hepatic mitochondrial fat oxidation may be metabolically mediated. Impaired fat oxidation in hepatocytes of non-diabetic Zucker fatty rats (38) and ZDF rats (39) has been attributed to increased levels of malonyl-CoA (39) and inhibition of CPT-1 mediated transport of LCFA into mitochondria (40). Liver mitochondria from non-diabetic Zucker fatty rats may (40) or may not (41) have a primary defect in oxidative capacity. In humans, abnormal mitochondrial respiratory chain activity is associated with NAFLD (16). In any case, defects in hepatic energy generation seem inconsistent with the increased energy requirements of excessive hepatic gluconeogenesis and lipogenesis that is associated with hepatic insulin resistance and diabetes.

Humans (11; 12) and animal models (13; 14) with primary defects in hepatic fat

oxidation become hypoglycemic, yet the ZDF rat has elevated fasting glucose production despite impaired fat oxidation. This is possible because elevated glucose production in the ZDF liver comes largely from the non-energy demanding pathways of glycogen breakdown and conversion of glycerol to glucose (GNG_{glycerol}) (**Figure 4**). The former process is essentially energy neutral while the latter process contributes to net energy production by way of NADH generated in the α -glycerophosphate dehydrogenase step. Additionally, although mitochondrial metabolism is dysfunctional in the diabetic liver, only total β -oxidation and ketogenesis are impaired; the mitochondrial pathways of pyruvate carboxylase, α -glycerophosphate dehydrogenase and the TCA cycle are, in fact, elevated. The inappropriate segregation of β -oxidation products towards oxidation is reminiscent, but seemingly opposite to mitochondrial metabolism in insulin resistant skeletal muscle where fatty acid overload induces fat oxidation but results in the build-up of acyl-carnitine/CoA intermediates (20) due to impaired TCA cycle flux (18).

A reasonable response to impaired hepatic fat oxidation is to correct the condition by pharmacological intervention. While PPAR α agonists (i.e. WY14,643 and fibrate drugs) stimulate fat oxidation and improve insulin resistance, they do not always improve glycemia and/or endogenous glucose production in diabetic rodent models (29-31) or humans (42). Here, WY14,643 stimulated hepatic β -oxidation in diabetic rats by over-induction of TCA cycle flux even at a relatively low dose (1/3 the typical rodent dose) and also ketogenesis at a higher dose (typical rodent dose). Concurrently, hepatic pyruvate carboxylase flux was stimulated by WY14,643 treatment and although much of the effect was dissipated by an induction of pyruvate cycling, GNG tended to be increased rather than decreased (**Figure 4**). Moreover, hepatic gluconeogenesis was increased in lean

control animals treated with WY14,643, reinforcing the indication that induction of hepatic fat oxidation stimulates hepatic glucose production. These data do not diminish the utility of PPAR α agonist drugs, which are commonly used to treat hyperlipidemia, but highlight an unanticipated effect on liver metabolism that may go unnoticed because improved insulin sensitivity can metabolically supersede the adverse effect of stimulated gluconeogenesis on glycemia. This may be particularly true in humans where hepatic PPAR α expression is less abundant than in rodents (43).

It is unclear whether paradoxically increased FGF-21 expression in the hypoketotic liver of ZDF rats and other diabetic rodents (44) is due to a PPAR α related defect or some other form of resistance to the paracrine effects of FGF-21. However, increased lipolysis and circulating NEFA in these animals suggests that FGF-21's endocrine effects on adipose tissue (32) remain intact. Further studies are required to determine if overproduction of FGF-21 by the liver is a diabetogenic feature meant to compensate for impaired fat oxidation, and whether this also contributes to hyperlipidemia by exacerbating the lipolytic state of insulin resistant adipose.

Methodological considerations and limitations

Measurements of ketogenesis by ketone tracer dilution may be vulnerable to overestimation via extrahepatic exchange processes (45), termed pseudoketogenesis (46). This was demonstrated in hepatectomized dogs given a bolus of ketone tracers and the PDH activator trichloroacetate (47). However, others showed that steady state infusion of low enrichments of ketone tracers matched the "gold standard" of hepatic ketone A/V difference in both fasted normal and diabetic dogs (25; 26; 48). We cannot rule out the possibility that the method overestimate ketogenesis in the rat, but we consider it

unlikely that the approach would underestimate ketone turnover in diabetic rats compared to controls. Most importantly, the data correctly predict changes in hepatic fat metabolism after interventions (i.e. fasting, feeding, etomoxir treatment, octanoate infusion, **see supplemental data**).

With regard to impaired hepatic fat oxidation in the ZDF, it is unclear whether this finding is a general feature of obesity and insulin resistance or a defect specific to the absence of a functioning leptin signaling pathway (49). Thus the hepatic fluxes should also be studied in non-leptin based rodent models to understand more clearly the role of these defects in the insulin resistant liver. Moreover, the approaches used here are completely translatable to human subjects and will be valuable tools for probing fluxes in the liver during metabolic pathophysiologies and/or drug therapies.

CONCLUSIONS

These data reveal abnormal mitochondrial metabolism in the ZDF liver leading to inefficient fat oxidation, a process known to interfere with insulin signaling in muscle (50); but induction of other mitochondrial pathways (TCA cycle flux and pyruvate carboxylase) reveal a complex defect in mitochondrial metabolism in the liver during

diabetes. PPAR α agonist treatment lowered insulin and NEFA levels and improved mitochondrial ketogenesis and total fat oxidation in diabetic rats, but also induced the mitochondrial fluxes of pyruvate carboxylase and TCA cycle flux and the stimulation of gluconeogenesis. Future studies on other models of insulin resistance and in human subjects will help to determine if defects in hepatic mitochondrial metabolism are a universal feature of insulin resistance.

ACKNOWLEDGMENTS

D.J.M. is an investigator of the Howard Hughes Medical Institute. SCB is the recipient of an American Diabetes Association Junior Faculty Award 1-50-JF-05. JDB is the recipient of an NIH training grant K23DK074396. Support for this work was provided by the Robert A Welch Foundation (SAK and DJM), NIH DK078184 (SCB), RL1GM084436 (SAK and DJM), the Howard Hughes Medical Institute (DJM), RL1DK081187 (SCB and JB), and Cores within RR02584, DK076269 (Courtesy of Craig R. Malloy). Excellent technical assistance was provided by Zheng Yan, Charles Storey, Angela Milde and Kristen Wertz. Portions of this work have been reported in abstract form to the ADA Scientific Sessions.

References:

1. Lin J, Handschin C, Spiegelman BM: Metabolic control through the PGC-1 family of transcription coactivators. *Cell Metabolism* 1:361-370, 2005
2. Koo S-H, Flechner L, Qi L, Zhang X, Scretton RA, Jeffries S, Hedrick S, Xu W, Boussouar F, Brindle P, Takemori H, Montminy M: The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism. 437:1109-1111, 2005
3. Zhang W, Patil S, Chauhan B, Guo S, Powell DR, Le J, Klotsas A, Matika R, Xiao X, Franks R, Heidenreich KA, Sajjan MP, Farese RV, Stolz DB, Tso P, Koo S-H, Montminy M, Unterman TG: FoxO1 Regulates Multiple Metabolic Pathways in the Liver *J. Biol. Chem.* 281:10105-10117, 2006
4. Wolfrum C, Asilmaz E, Luca E, Friedman JM, Stoffel M: Foxa2 regulates lipid metabolism and ketogenesis in the liver during fasting and in diabetes. 432:1027-1032, 2004
5. Williamson JR, Kreisberg RA, Felts PW: Mechanism for the Stimulation of Gluconeogenesis by Fatty Acids in Perfused Rat Liver. *Proceedings of the National Academy of Sciences of the United States of America* 56:247-254, 1966
6. Burgess SC, He T, Yan Z, Lindner J, Sherry AD, Malloy CR, Browning JD, Magnuson MA: Cytosolic Phosphoenolpyruvate Carboxykinase Does Not Solely Control the Rate of Hepatic Gluconeogenesis in the Intact Mouse Liver. *Cell Metabolism* 5:313-320, 2007
7. Samuel VT, Liu Z-X, Qu X, Elder BD, Bilz S, Befroy D, Romanelli AJ, Shulman GI: Mechanism of Hepatic Insulin Resistance in Non-alcoholic Fatty Liver Disease. *J. Biol. Chem.* 279:32345-32353, 2004
8. Chu CA, Sherck SM, Igawa K, Sindelar DK, Neal DW, Emshwiller M, Cherrington AD: Effects of free fatty acids on hepatic glycogenolysis and gluconeogenesis in conscious dogs. *Am J Physiol Endocrinol Metab* 282:E402-411, 2002
9. Lam TKT, Carpentier A, Lewis GF, van de Werve G, Fantus IG, Giacca A: Mechanisms of the free fatty acid-induced increase in hepatic glucose production. *Am J Physiol Endocrinol Metab* 284:E863-873, 2003
10. Roden M, Stingl H, Chandramouli V, Schumann W, Hofer A, Landau B, Nowotny P, Waldhausl W, Shulman G: Effects of free fatty acid elevation on postabsorptive endogenous glucose production and gluconeogenesis in humans. *Diabetes* 49:701-707, 2000
11. Sovik O: Inborn errors of amino acid and fatty acid metabolism with hypoglycemia as a major clinical manifestation. *Acta Paediatrica Scandinavica* 78:161-170, 1989
12. Stanley CA: New genetic defects in mitochondrial fatty acid oxidation and carnitine deficiency. *Advances in pediatrics* 34:59-88, 1987
13. Kurtz DM, Rinaldo P, Rhead WJ, Tian L, Millington DS, Vockley J, Hamm DA, Brix AE, Lindsey JR, Pinkert CA, O'Brien WE, Wood PA: Targeted disruption of mouse long-chain acyl-CoA dehydrogenase gene reveals crucial roles for fatty acid oxidation. *PNAS* 95:15592-15597, 1998
14. Ibdah JA, Paul H, Zhao Y, Binford S, Salleng K, Cline M, Matern D, Bennett MJ, Rinaldo P, Strauss AW: Lack of mitochondrial trifunctional protein in mice causes neonatal hypoglycemia and sudden death. *J. Clin. Invest.* 107:1403-1409, 2001
15. Petersen KF, Shulman GI: Etiology of Insulin Resistance. *The American Journal of Medicine* 119:S10-S16, 2006

16. Pérez-Carreras M, Hoyo PD, Martín MA, Rubio JC, Martín A, Castellano G, Colina F, Arenas J, Solis-Herruzo JA: Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. *Hepatology* 38:999-1007, 2003
17. Kelley DE, He J, Menshikova EV, Ritov VB: Dysfunction of Mitochondria in Human Skeletal Muscle in Type 2 Diabetes *Diabetes* 51:2944-2950, 2002
18. Petersen KF, Dufour S, Befroy D, Garcia R, Shulman GI: Impaired Mitochondrial Activity in the Insulin-Resistant Offspring of Patients with Type 2 Diabetes. *N Engl J Med* 350:664-671, 2004
19. Sparks LM, Xie H, Koza RA, Mynatt R, Hulver MW, Bray GA, Smith SR: A High-Fat Diet Coordinately Downregulates Genes Required for Mitochondrial Oxidative Phosphorylation in Skeletal Muscle. *Diabetes* 54:1926-1933, 2005
20. Koves TR, Ussher JR, Noland RC, Slentz D, Mosedale M, Ilkayeva O, Bain J, Stevens R, Dyck JR, Newgard CB, Lopaschuk GD, Muoio DM: Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell Metab* 7:45-56, 2008
21. Clark JB, Palmer CJ, Shaw WN: The diabetic Zucker fatty rat. *Proc Soc Exp Biol Med* 173:68-75, 1983
22. Jin ES, Burgess SC, Merritt ME, Sherry AD, Malloy CR: Differing mechanisms of hepatic glucose overproduction in triiodothyronine-treated rats vs. Zucker diabetic fatty rats by NMR analysis of plasma glucose. *Am J Physiol Endocrinol Metab* 288:E654-662, 2005
23. Burgess SC, Jeffrey FMH, Storey C, Milde A, Hausler N, Merritt ME, Mulder H, Holm C, Sherry AD, Malloy CR: Effect of murine strain on metabolic pathways of glucose production after brief or prolonged fasting. *Am J Physiol Endocrinol Metab* 289:E53-61, 2005
24. Jin ES, Jones JG, Merritt ME, Burgess SC, Malloy CR, Sherry AD: Glucose production, gluconeogenesis, and hepatic tricarboxylic acid cycle fluxes measured by nuclear magnetic resonance analysis of a single glucose derivative. *Analytical Biochemistry* 327:149-155, 2004
25. Miles JM, Schwenk WF, McClean KL, Haymond MW: A dual-isotope technique for determination of in vivo ketone body kinetics. *American journal of physiology* 251:E185-191, 1986
26. Bailey JW, Haymond MW, Miles JM: Validation of two-pool model for in vivo ketone body kinetics. *Am J Physiol* 258:E850-855, 1990
27. Bougneres PF, Ferre P: Study of ketone body kinetics in children by a combined perfusion of carbon-13 and deuterium (²H₃) tracers. *American Journal of Physiology* 253:E496-E502, 1987
28. Jin ES, Park B-H, Sherry AD, Malloy CR: Role of Excess Glycogenolysis in Fasting Hyperglycemia Among Pre-Diabetic and Diabetic Zucker (fa/fa) Rats. *Diabetes* 56:777-785, 2007
29. Bergeron R, Yao J, Woods JW, Zycband EI, Liu C, Li Z, Adams A, Berger JP, Zhang BB, Moller DE, Doebber TW: Peroxisome proliferator-activated receptor (PPAR)-alpha agonism prevents the onset of type 2 diabetes in Zucker diabetic fatty rats: A comparison with PPAR gamma agonism. *Endocrinology* 147:4252-4262, 2006
30. Kim H, Haluzik M, Asghar Z, Yau D, Joseph JW, Fernandez AM, Reitman ML, Yakar S, Stannard B, Heron-Milhavet L, Wheeler MB, LeRoith D: Peroxisome proliferator-activated receptor-alpha agonist treatment in a transgenic model of type 2 diabetes reverses the lipotoxic state and improves glucose homeostasis. *Diabetes* 52:1770-1778, 2003
31. Chou CJ, Haluzik M, Gregory C, Dietz KR, Vinson C, Gavrilova O, Reitman ML: WY14,643, a peroxisome proliferator-activated receptor alpha (PPARalpha) agonist, improves

- hepatic and muscle steatosis and reverses insulin resistance in lipoatrophic A-ZIP/F-1 mice. *J Biol Chem* 277:24484-24489, 2002
32. Inagaki T, Dutchak P, Zhao G, Ding X, Gautron L, Parameswara V, Li Y, Goetz R, Mohammadi M, Esser V, Elmquist JK, Gerard RD, Burgess SC, Hammer RE, Mangelsdorf DJ, Kliewer SA: Endocrine Regulation of the Fasting Response by PPAR α -Mediated Induction of Fibroblast Growth Factor 21. *Cell Metab* 5:415-425, 2007
33. Badman MK, Pissios P, Kennedy AR, Koukos G, Flier JS, Maratos-Flier E: Hepatic Fibroblast Growth Factor 21 Is Regulated by PPAR α and Is a Key Mediator of Hepatic Lipid Metabolism in Ketotic States. *Cell Metabolism* 5:426-437, 2007
34. Kharitononkov A, Shiyanova TL, Koester A, Ford AM, Micanovic R, Galbreath EJ, Sandusky GE, Hammond LJ, Moyers JS, Owens RA, Gromada J, Brozinick JT, Hawkins ED, Wroblewski VJ, Li D-S, Mehrbod F, Jaskunas SR, Shanafelt AB: FGF-21 as a novel metabolic regulator. *J. Clin. Invest.* 115:1627-1635, 2005
35. Kakuma T, Lee Y, Higa M, Wang Z-w, Pan W, Shimomura I, Unger RH: Leptin, troglitazone, and the expression of sterol regulatory element binding proteins in liver and pancreatic islets. *Proceedings of the National Academy of Sciences* 97:8536-8541, 2000
36. Lee WNP, Bassilian S, Lim S, Boros LG: Loss of regulation of lipogenesis in the Zucker diabetic (ZDF) rat. *Am J Physiol Endocrinol Metab* 279:E425-432, 2000
37. Fujimoto Y, Torres TP, Donahue EP, Shiota M: Glucose Toxicity Is Responsible for the Development of Impaired Regulation of Endogenous Glucose Production and Hepatic Glucokinase in Zucker Diabetic Fatty Rats. *Diabetes* 55:2479-2490, 2006
38. Triscari J, Greenwood MR, Sullivan AC: Oxidation and ketogenesis in hepatocytes of lean and obese Zucker rats. *Metabolism: clinical and experimental* 31:223-228, 1982
39. Yu X, McCorkle S, Wang M, Lee Y, Li J, Saha AK, Unger RH, Ruderman NB: Leptinomimetic effects of the AMP kinase activator AICAR in leptin-resistant rats: prevention of diabetes and ectopic lipid deposition. *Diabetologia* 47:2012-2021, 2004
40. Clouet P, Henninger C, Bezaud J: Study of some factors controlling fatty acid oxidation in liver mitochondria of obese Zucker rats. *Biochem J* 239:103-108, 1986
41. Brady LJ, Hoppel CL: Hepatic mitochondrial function in lean and obese Zucker rats. *Am J Physiol Endocrinol Metab* 245:E239-245, 1983
42. Keech A, Simes RJ, Barter P, Best J, Scott R, Taskinen MR, Forder P, Pillai A, Davis T, Glasziou P, Drury P, Kesaniemi YA, Sullivan D, Hunt D, Colman P, d'Emden M, Whiting M, Ehnholm C, Laakso M: Effects of long-term fenofibrate therapy on cardiovascular events in 9795 people with type 2 diabetes mellitus (the FIELD study): randomised controlled trial. *Lancet* 366:1849-1861, 2005
43. Palmer CNA, Hsu MH, Griffin KJ, Raucy JL, Johnson EF: Peroxisome proliferator activated receptor- α expression in human liver. *Molecular Pharmacology* 53:14-22, 1998
44. Lundasen T, Hunt MC, Nilsson L-M, Sanyal S, Angelin B, Alexson SEH, Rudling M: PPAR α is a key regulator of hepatic FGF21. *Biochemical and Biophysical Research Communications* 360:437-440, 2007
45. Landau BR: A potential pitfall in the use of isotopes to measure ketone body production. *Metabolism* 35:94, 1986
46. Fink G, Desrochers S, Des Rosiers C, Garneau M, David F, Daloze T, Landau BR, Brunengraber H: Pseudoketogenesis in the perfused rat heart. *J. Biol. Chem.* 263:18036-18042, 1988

47. Des Rosiers C, Montgomery JA, Garneau M, David F, Mamer OA, Daloze P, Toffolo G, Cobelli C, Landau BR, Brunengraber H: Pseudoketogenesis in hepatectomized dogs. *Am J Physiol Endocrinol Metab* 258:E519-528, 1990
48. Keller U, Cherrington A, Liljenquist J: Ketone body turnover and net hepatic ketone production in fasted and diabetic dogs. *Am J Physiol Endocrinol Metab* 235:E238-247, 1978
49. Unger RH: Minireview: Weapons of Lean Body Mass Destruction: The Role of Ectopic Lipids in the Metabolic Syndrome. *Endocrinology* 144:5159-5165, 2003
50. Shulman GI: Cellular mechanisms of insulin resistance. *J. Clin. Invest.* 106:171-176, 2000

Table 1. Plasma metabolite and insulin concentrations in 12 week old and 22 week old control and diabetic rats (n = 7).

	Lean 12 weeks	ZDF 12 weeks	Lean 22 weeks	ZDF 22 weeks
Glucose (mM)	4.4 ± 0.79	10.2 ± 0.73*	6.9 ± 0.35	17.7 ± 1.92*‡
NEFA (mEQ/L)	0.67 ± 0.03	1.81 ± 0.55*	0.88 ± 0.08	2.45 ± 0.26*‡
Total Ketones (uM)	989 ± 53.2	212 ± 25.6*	919 ± 24.8	406 ± 67.6*‡
Liver TG (mg/g tissue)	3.8 ± 0.22	15 ± 5.5*	3.3 ± 0.27	11 ± 0.10*
Insulin (ng/ml)	0.22 ± 0.04	2.6 ± 0.87*	0.28 ± 0.02	1.0 ± 0.15*‡

* p < 0.05 between control and diabetic group. ‡ p < 0.05 between young and old groups.

Table 2. Plasma metabolite and insulin concentrations in 12 week old control and diabetic rats treated with a low or high dose of the PPAR α agonist WY14,643 (n = 4-7).

	Lean (Untreated)	ZDF (Untreated)	Lean WY14,643 (Low dose)	ZDF WY14,643 (Low dose)	Lean WY14,643 (High dose)	ZDF WY14,643 (High dose)
Glucose (mM)	4.4 \pm 0.79	10.2 \pm 0.73*	6.9 \pm 0.10#	9.2 \pm 0.30*	9.8 \pm 0.88#	10.4 \pm 0.24
NEFA (mEQ/L)	0.67 \pm 0.03	1.81 \pm 0.55*	0.31 \pm 0.05#	0.64 \pm 0.08*#	0.33 \pm 0.04#	0.86 \pm 0.20*#
Total Ketones (μ M)	989 \pm 53.2	212 \pm 25.6*	790 \pm 39.5	482 \pm 76.5*#	886 \pm 90.9	1200 \pm 106#
Liver TG (mg/g tissue)	3.8 \pm 0.22	15 \pm 5.5*	3.7 \pm 0.58	17 \pm 4.3*	4.8 \pm 0.22	13 \pm 1.6*
Insulin (ng/ml)	0.22 \pm 0.04	2.6 \pm 0.87*	0.13 \pm 0.02#	1.2 \pm 0.40*#	0.33 \pm 0.10	0.65 \pm 0.07*#

* P < 0.05 between control and diabetic group. # P < 0.05 between treated and untreated groups.

Table 3. mRNA levels in 12 week old and 22 week old control and diabetic rats and 12 week old rats treated with WY14,643 measured by QPCR (n = 3).

	Lean 12 weeks	ZDF 12 weeks	Lean 22 weeks	ZDF 22 weeks	Lean WY14,643 (Low dose)	ZDF WY14,643 (Low dose)	Lean WY14,643 (High dose)	ZDF WY14,643 (High dose)
MCAD	1 ± 0.16	0.84 ± 0.12	1.0 ± 0.13	1.1 ± 0.12	0.97 ± 0.34	0.79 ± 0.04	2.3 ± 0.50#	1.8 ± 0.40#
CPT1a	1 ± 0.14	0.49 ± 0.09*	0.75 ± 0.18	0.67 ± 0.09	0.74 ± 0.27	0.38 ± 0.05*	0.67 ± 0.15	0.60 ± 0.10
HMGCS2	1 ± 0.12	1.0 ± 0.10	0.82 ± 0.03	0.92 ± 0.05	1.0 ± 0.30	1.5 ± 0.11#	1.2 ± 0.20#	1.4 ± 0.40
PDK4	1 ± 0.30	1.3 ± 0.53	1.3 ± 0.14	2.3 ± 0.56* ‡	1.3 ± 0.39	3.9 ± 1.7*#	22 ± 2.5#	9.8 ± 0.50*#
CD36/FAT	1 ± 0.20	5.6 ± 1.1*	1.7 ± 0.15‡	3.9 ± 0.22*‡	5.2 ± 1.2#	2.2 ± 0.20*#	9.3 ± 0.81#	5.7 ± 0.45*
Cyp4a	1 ± 0.16	0.41 ± 0.21*	0.95 ± 0.24	0.37 ± 0.20*‡	3.7 ± 2.0#	1.5 ± 0.31*#	4.9 ± 0.50#	3.2 ± 0.92*#
ACOX1	1 ± 0.15	0.80 ± 0.06	0.88 ± 0.7	1.20 ± 0.04‡	4.2 ± 2.3#	2.4 ± 0.20*#	9.5 ± 1.0#	8.8 ± 1.5#
PGC1α	1 ± 0.06	1.0 ± 0.27	0.66 ± 0.09‡	0.85 ± 0.19	0.55 ± .06#	0.34 ± 0.06*#	0.44 ± 0.15#	0.42 ± 0.20#*
PPAR α	1 ± 0.40	0.77 ± 0.22	0.94 ± 0.26	0.95 ± 0.05	0.77 ± 0.23	0.61 ± 0.20	1.1 ± 0.20	1.0 ± 0.15

* p < 0.05 between control and diabetic groups. ‡ p < 0.05 between young and old groups. # p < 0.05 between treated and untreated groups.

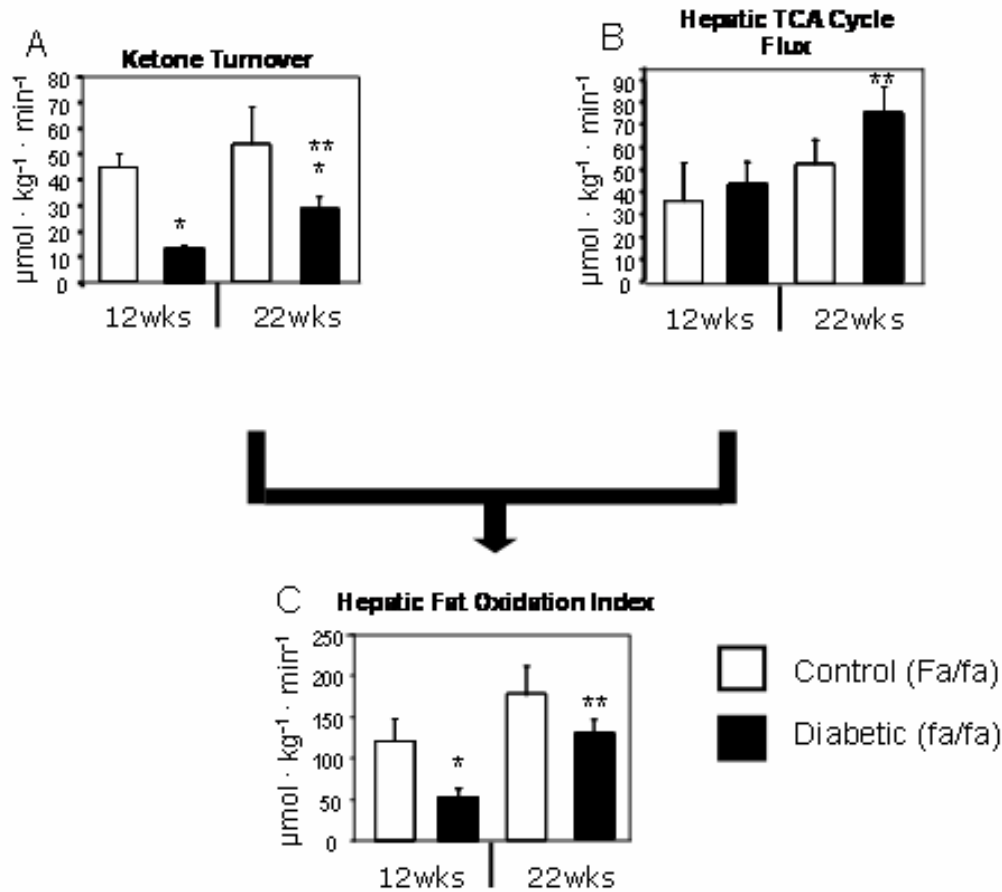


Figure 1

Figure 1. In vivo fluxes associated with hepatic mitochondrial fat oxidation are defective in, 24-hour fasted, 12 week old and 22 week old diabetic rats. **(A)** Ketogenesis is impaired in both 12 and 22 week old diabetic rats. Ketone turnover was measured by tracer dilution of $[1,2-^{13}\text{C}]\text{BHB}$ and $[3,4-^{13}\text{C}]\text{ACAC}$. **(B)** In vivo hepatic TCA cycle flux is normal in 12 week old diabetic rats, but is abnormally high in the more severe 22 week old diabetic rats. TCA cycle flux was measured by ^{13}C and ^2H NMR isotopomer analysis of plasma glucose. **(C)** In vivo hepatic fat oxidation index is impaired in 12 week old diabetic rats but not 22 week old rats. Hepatic fat oxidation index was calculated by adding A and B in 2-carbon units ($n = 4-11$). Data are represented as the mean and SE. * $p < 0.05$ between control and diabetic group. ** $p < 0.05$ between young and old diabetic groups.

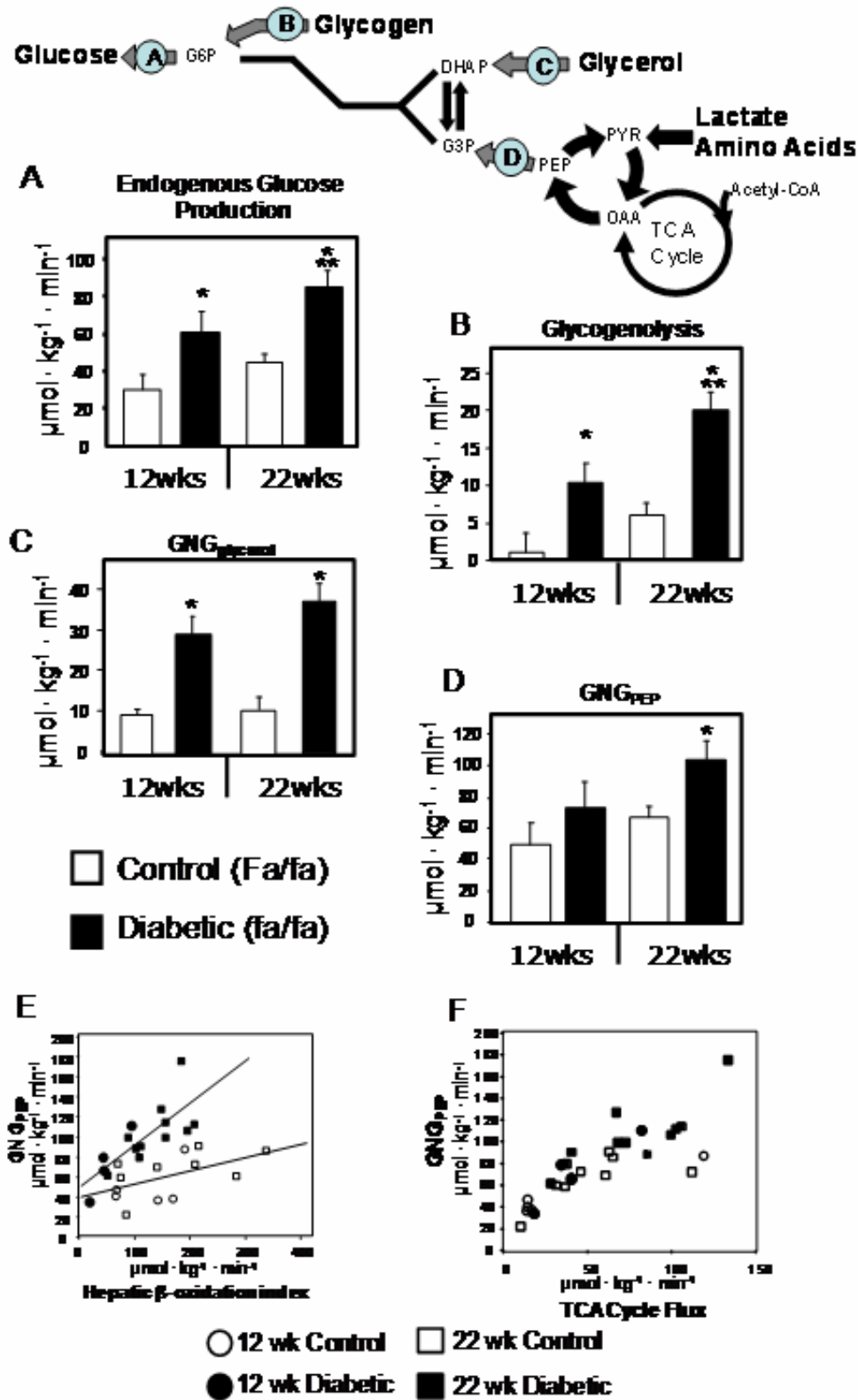


Figure 2

Figure 2. Sources of endogenous glucose production are abnormal in 24-hour fasted 12 week old and 22 week old diabetic rats. **(A)** Endogenous glucose production is elevated in 12-week old diabetic rats and further elevated in 22-week old diabetic rats. Endogenous glucose production was measured by tracer dilution of [3,4-¹³C]glucose (measured by ¹³C NMR). Sources of endogenous glucose production were determined by ²H incorporation in plasma glucose (measured by ²H NMR) after administration of ²H₂O. Glycogenolysis **(B)** and Gluconeogenesis from glycerol **(C)** are substantially sources of elevated glucose production in diabetic rats at both 12 and 22 weeks of age; whereas gluconeogenesis from PEP, derived from lactate, pyruvate or amino acids **(D)** is not different between control and diabetic rats at 12 weeks of age, but is elevated in the more severe 22 week old diabetic rats (n = 4-11). ** p < 0.05 between young and old diabetic groups. Data are represented as the mean and SE. **(E)** Hepatic fat oxidation correlates with the rate of gluconeogenesis, but diabetic rats have a lower slope for this correlation, indicating that GNG_{PEP} is supported with a lower than normal rate of fat oxidation. **(F)** TCA cycle oxidation correlates with GNG_{PEP} similarly in control and diabetic animals.

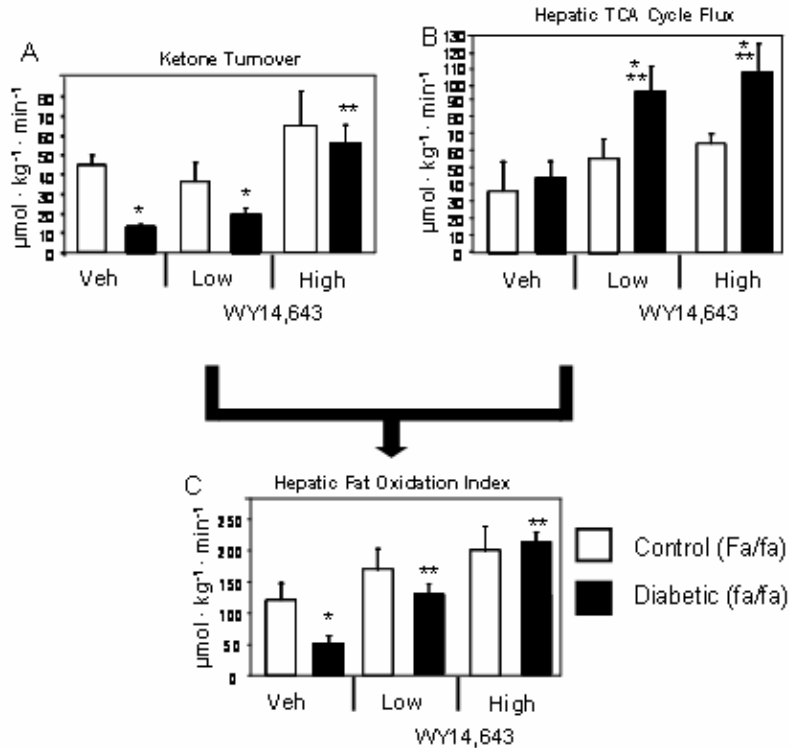


Figure 3

Figure 3. In vivo hepatic fat oxidation is stimulated by WY14,643 in the diabetic liver and to a lesser extent in the normal liver. 24-hour fasted 12 week old rats fed a normal chow (Veh) or chow containing WY14,643 at either 100 mg/Kg chow (Low) or 300 mg/Kg chow (High). **(A)** Ketone turnover measured by tracer dilution of $[1,2-^{13}\text{C}]\text{BHB}$ and $[3,4-^{13}\text{C}]\text{ACAC}$ was stimulated at high, but not low doses of WY14,643. **(B)** Hepatic TCA cycle flux was measured by ^{13}C and ^2H NMR isotopomer analysis of plasma glucose is over stimulated by both low and high doses of WY14,643. **(C)** Hepatic fat oxidation index calculated by adding A and B in 2-carbon units is corrected by low and high doses of WY14,643. All data are represented as the mean and SE. * $p < 0.05$ versus control group. ** $p < 0.05$ versus untreated control group ($n = 4-11$).

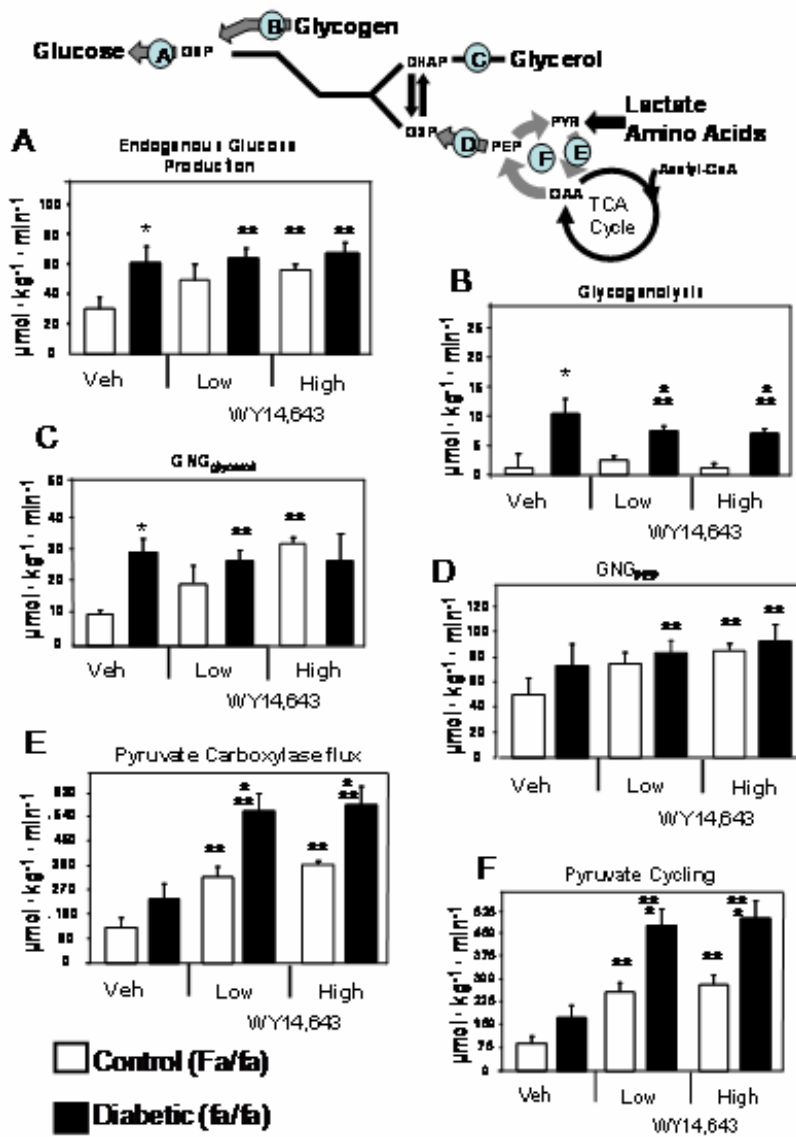


Figure 4

Figure 4. Sources of *in vivo* glucose production are not corrected in, 24-hour fasted, 12 week old diabetic rats treated with WY14,643. (A) Endogenous glucose production is not corrected in diabetic rats and is stimulated in control rats by WY14,643 treatment. Sources of endogenous glucose production were determined by ^2H incorporation in plasma glucose (measured by ^2H NMR) after administration of $^2\text{H}_2\text{O}$. (B) Abnormal hepatic glycogenolysis is not effected by WY14,643 treatment. (C) and (D) Gluconeogenesis is not corrected in diabetic rats and is stimulated in control rats. All data are represented as the mean and SE. * $p < 0.05$ versus control group. ** $p < 0.05$ versus untreated control group (n = 4-11).

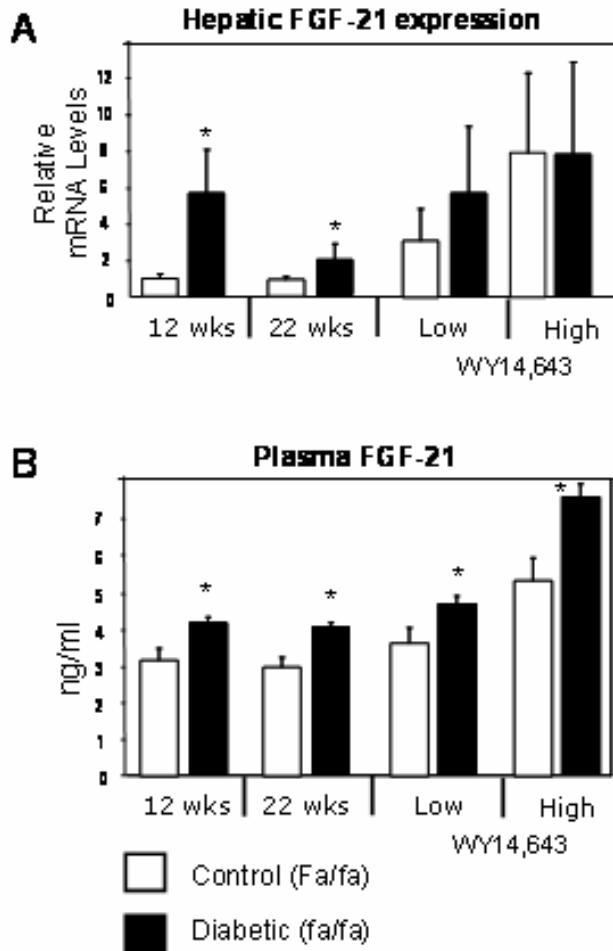


Figure 5

Figure 5. Diabetic rats are resistant to the normal induction of hepatic fat oxidation by FGF-21. **(A)** Hepatic FGF-21 expression measured by QPCR is substantially elevated in diabetic rats. WY14,643 induced hepatic FGF-21 expression 7-fold in control rats but only 20% in diabetic rats. **(B)** Plasma FGF-21 protein concentration is consistently increased in diabetic rats. All data is represented as the mean and SE. * $p < 0.05$ between control and diabetic group ($n=3$).