Partial Resistance to Peroxisome Proliferator–Activated Receptor-α Agonists in ZDF Rats Is Associated With Defective Hepatic Mitochondrial Metabolism

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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 whether abnormal mitochondrial metabolism plays a role in the dysregulation of both hepatic fat and glucose metabolism during diabetes.

RESEARCH DESIGN AND METHODS—Mitochondrial fluxes were measured using $^{2}H/^{13}C$ tracers and nuclear magnetic resonance spectroscopy in ZDF rats during early and advanced diabetes. To determine whether defects in hepatic fat oxidation can be corrected by peroxisome proliferator–activated receptor (PPAR-)-α activation, rats were treated with WY14,643 for 3 weeks before tracer administration.

RESULTS—Hepatic mitochondrial fat oxidation in the diabetic liver was impaired twofold secondary to decreased ketogenesis, but tricarboxylic acid (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 overstimulated 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 codependence 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. ***Diabetes*** **57:2012–2021, 2008**

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The liver is a critical hub in systemic energy distribution. In the postprandial 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 to provide substrate for the synthesis of ketone bodies (ketogenesis). Because 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 codependent. 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 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 in animal models (13,14) with primary defects in hepatic fat oxidation. Based on these observations, it is 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 whether similar defects exist in liver and, if so, how they could coexist with the increased energetic requirements of elevated gluconeogenesis and lipogenesis found in the insulin-resistant liver.

The ZDF rat is a model of obesity, insulin resistance, and diabetes in which 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 mitochon-

drial fluxes of β-oxidation but induction of the mitochondrial fluxes of the tricarboxylic acid (TCA) cycle and pyruvate carboxylase, which tends to contribute to elevated rates of glucose production in diabetic rats. Treatment with a peroxisome proliferator–activated receptor (PPAR)-α agonist improved plasma nonesterified fatty acid (NEFA), ketones, and insulin levels but overstimulated TCA cycle flux, 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

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

Sprague-Dawley (~300 g), ZDF (Fa/fa) control (~300 g), and (*fa/fa*) diabetic ZDF (~450 g) rats were studied using a protocol approved by the University of Texas Southwestern Institutional Animal Care and Use Committee. ZDF rats were studied at ~12 and ~22 weeks of age. Five days before infusion, rats were anesthetized with an isoflourane/oxygen and a jugular vein catheter was surgically implanted (22). On day 5, rats were fasted for 24 h (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 μmol/100 g body wt i.p. injection 90 min before the infusion of isotope tracers, where applicable. Where noted, rats were infused with octanoate along with tracers at a rate of 30 μmol/min for 90 min to stimulate ketogenesis. WY14,643 (BIOMOL Research Laboratories, Plymouth Meeting, PA) was mixed with rat diet at 100 (low dose) or 300 (high dose) mg/kg diet and administered for 3 weeks before the study of 12-week-old rats.

Infusate preparation. On the morning of infusion, 28 mg [3,4-^{13}C]ethylacetoacetate was suspended in 4 ml deionized water and 80 μl of 4 M NaOH. This solution was stirred at 40°C for 75 min. The solution was neutralized with dilute HCl, and quantitative hydrolysis to [3,4-^{13}C]acetoacetate was confirmed by ^{1}H nuclear magnetic resonance (NMR) spectroscopy. To this solution, 27 mg [3,4-^{13}C]glucose and 21 mg [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-μ filter. This procedure gives ~20 mmol/l of each tracer, although the actual concentrations were assayed.

Tracer delivery. Rats received an intraperitoneal injection (20 μl/g rat) containing [U-^{13}C]propionate (5 mg/ml) dissolved in $^{2}H_2O$ (99%). A bolus of infusate (2.25 ml/h for 10 min) was administered, followed by continuous infusion at a rate of 0.5 ml/h for 90 min. 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-hydoxybutyrate 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° pulse and a 3-s repetition time. MAG was analyzed by ^{2}H and ^{13}C NMR as previously described (23,24). Peak areas (^{2}H and ^{13}C) were measured using the 1D NMR software ACD/Labs 9.0 (Advanced Chemistry Development, Toronto, ON, Canada).

Metabolic analysis. The ^{2}H and ^{13}C NMR spectra of MAG were used to measure glycogenolysis, gluconeogenesis from glycerol ($GNG_{glycerol}$), gluconeogenesis from phosphoenolpyruvate originating from the TCA cycle (GNG_{PEP}), and TCA cycle turnover (23,24) (see Supplemental Methods in the online appendix [available at http://dx.doi.org/10.2337/db08-0226]). Apparent ketone turnover was measured using a two-pool model of exchangeable acetoacetate (ACAC) and β-hydroxybutyrate (BHB) (25–27). Equations from reference 27 were adapted to NMR data (Supplemental Methods, online appendix).

Total ketone production is reported where:

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

Ketone production and hepatic TCA cycle flux were used to estimate an index of hepatic β-oxidation:

$$\beta\text{-}oxidation\ index\ (in\ 2\ carbon\ units) = TCA\ cycle\ flux + 2 \times ketone\ production$$

Gene expression analysis. Primers were designed using Primer Express software (Applied Biosystems, San Jose, CA) based on GenBank sequence data. Quantitative real-time PCR(10 μl) contained 25 ng cDNA, 150 nmol/l of each primer, and 5 μl SYBR Green PCR Master Mix (Applied Biosystems). 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 using cyclophilin as the internal control.

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

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

RESULTS

Simultaneous delivery of five stable isotope tracers and NMR analysis of plasma extracts provides insight into hepatic fat metabolism. Simultaneous administration of $^{2}H_2O$, [U-^{13}C]propionate, [3,4-^{13}C]glucose, [3,4-^{13}C]acetoacetate, and [1,2-^{13}C]BHB was used to measure gluconeogenesis and index hepatic fat oxidation by NMR isotopomer analysis of plasma glucose and ketones. Tracers of ketone turn over, the TCA cycle and gluconeogenesis have never been applied simultaneously; we therefore 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 (Supplemental Results and Supplemental Fig. 1 of the online appendix). Similarly, carbon-13 originating from [3,4-^{13}C]acetoacetate and [1,2-^{13}C]β-hydroxybutryrate 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 Fig. 1, online appendix).

Hepatic fat oxidation is impaired in the ZDF rat. As expected, fasting plasma glucose, NEFAs, insulin, and liver triglycerides were markedly elevated in diabetic rats (Table 1). Despite elevated NEFAs and liver triglycerides, fasting plasma ketone concentration was approximately fourfold lower in 12-week-old diabetic rats compared with lean littermates, suggesting a defect in hepatic fat oxidation. Ketone concentration doubled by 22 weeks in dia-

TABLE 1
Plasma metabolite and insulin concentrations in 12- and 22-week-old control (lean) and diabetic (ZDF) rats ($n = 7$)

	12 weeks		22 weeks	
	Lean	ZDF	Lean	ZDF
Glucose (mmol/l)	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 (μmol/l)	989 ± 53.2	212 ± 25.6*	919 ± 24.8	406 ± 67.6*†
Liver triglycerides (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*†

Data are means ± SE. *$P < 0.05$ between control and diabetic group. †$P < 0.05$ between young and old groups.

betic rats but remained markedly lower than in 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 (Fig. 1*A*). Consistent with low plasma ketone concentration, in vivo ketone turnover was fourfold lower in 12-week-old diabetic rats and twofold 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 ^{2}H 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 with lean controls (Fig. 1*B*). However, TCA cycle flux increased by 60% in the 22-week-old diabetic rats compared with their younger diabetic counterparts (Fig. 1*B*), consistent with the ketone data, suggesting an

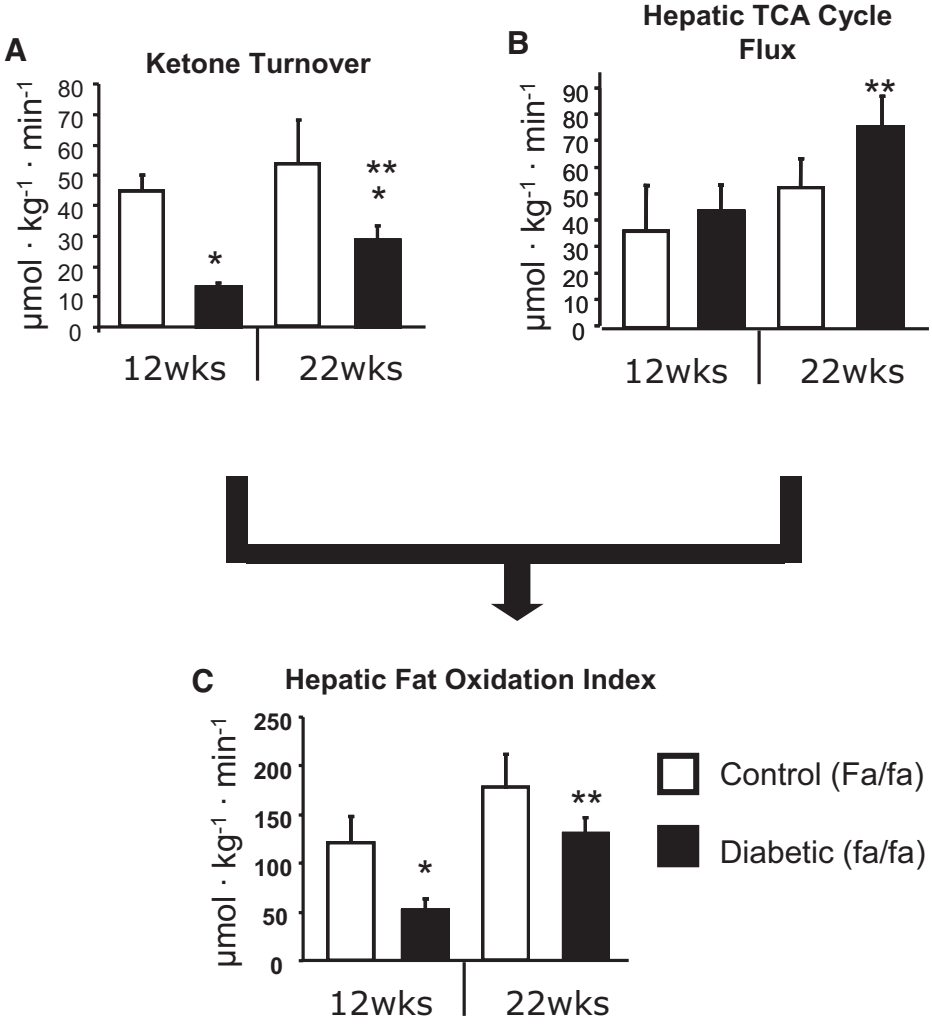

FIG. 1. In vivo fluxes associated with hepatic mitochondrial fat oxidation are defective in 24-h fasted 12-week-old and 22-week-old diabetic rats. *A*: Ketogenesis is impaired in both 12- and 22-week-old diabetic rats. Ketone turnover was measured by tracer dilution of [1,2-^{13}C]BHB and [3,4-^{13}C]ACAC. *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 ^{2}H 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 groups. **$P < 0.05$ between young and old diabetic groups.

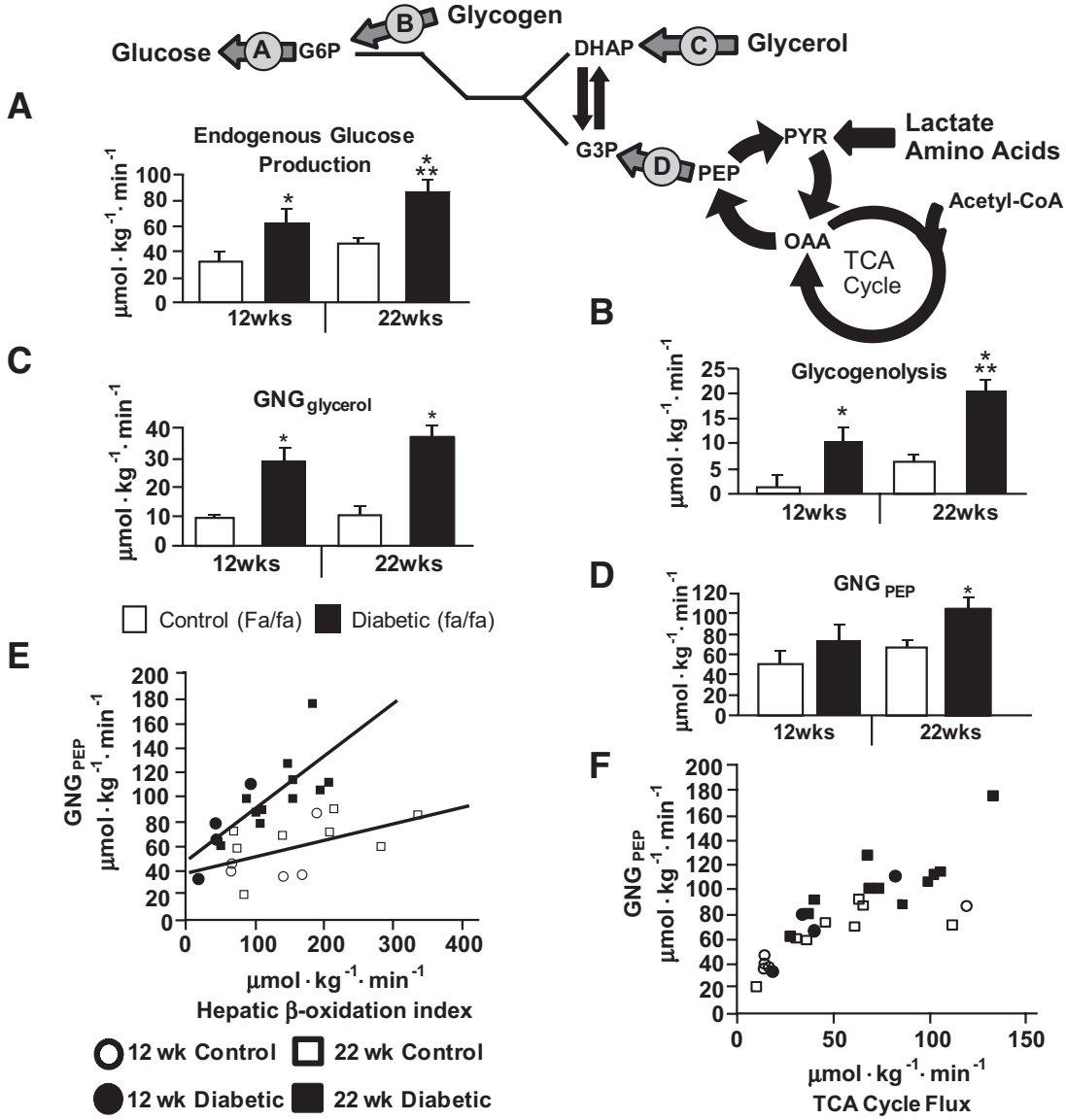

FIG. 2. Sources of endogenous glucose production are abnormal in 24-h–fasted 12- and 22-week-old diabetic rats. *A*: Endogenous glucose production is elevated in 12-week-old diabetic rats and further elevated in 22-week-old diabetic rats. Endogenous glucose production was measured by tracer dilution of [3,4-^{13}C]glucose (measured by ^{13}C NMR). Sources of endogenous glucose production were determined by ^{2}H incorporation in plasma glucose (measured by ^{2}H NMR) after administration of $^{2}H_2O$. Glycogenolysis (*B*) and gluconeogenesis from glycerol (*C*) are substantial sources of elevated glucose production in diabetic rats at both 12 and 22 weeks of age, whereas gluconeogenesis from phosphoenolpyruvate (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. *E*: Hepatic fat oxidation correlates with the rate of gluconeogenesis, but diabetic rats have a higher 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 (n = 4–11). **$P < 0.05$ between young and old diabetic groups. Data are represented as the mean and SE.

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 with age-matched controls (Fig. 1*C*). The 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 (Fig. 2*A*) in newly diabetic ZDF rats (age 12 weeks) was associated with increased glycogenolysis (Fig. 2*B*) and gluconeogenesis from glycerol ($GNG_{glycerol}$) (Fig. 2*C*) but with normal gluconeogenesis originating from substrates (i.e., lactate and pyruvate alanine) that pass through the TCA cycle (GNG_{PEP}) (Fig. 2*D*). Flux through all of these pathways was 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 (presum-

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

	Untreated		Low dose		High dose	
	Lean	ZDF	Lean WY14,643	ZDF	Lean WY14,643	ZDF WY14,643
Glucose (mmol/l)	4.4 ± 0.79	10.2 ± 0.73*	6.9 ± 0.10†	9.2 ± 0.30*	9.8 ± 0.88†	10.4 ± 0.24
NEFAs (mEq/l)	0.67 ± 0.03	1.81 ± 0.55*	0.31 ± 0.05†	0.64 ± 0.08*†	0.33 ± 0.04†	0.86 ± 0.20*†
Total ketones (μmol/l)	989 ± 53.2	212 ± 25.6*	790 ± 39.5	482 ± 76.5*†	886 ± 90.9	1200 ± 106†
Liver triglycerides (mg/g tissue)	3.8 ± 0.22	15 ± 5.5*	3.7 ± 0.58	17 ± 4.3*	4.8 ± 0.22	13 ± 1.6*
Insulin (ng/ml)	0.22 ± 0.04	2.6 ± 0.87*	0.13 ± 0.02†	1.2 ± 0.40*†	0.33 ± 0.10	0.65 ± 0.07*†

Data are means ± SE. *$P < 0.05$ between control and diabetic group. †$P < 0.05$ between treated and untreated groups.

ably via mitochondrial pyruvate carboxylase) in 22-week-old ZDF rats compared with lean littermates (350 vs. 211 $\mu mol \cdot min^{-1} \cdot kg^{-1}$, $P < 0.05$).

The codependence of gluconeogenesis and hepatic fat oxidation is altered in diabetic rats. Hepatic fat oxidation induces glucose production by supplying energy-rich cofactors (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 whether 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}; Pyr → OAA → PEP → → glucose, where OAA is oxaloacetate and PEP is phosphoenolpyruvate) (Fig. 2*E*). 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 in 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 that did not differ between control and diabetic rats (Fig. 2*F*). These data are reminiscent of our previous description of TCA cycle flux as an important mediator of glucongeogenic 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 toward the TCA cycle to preserve energy production for gluconeogenesis.

PPAR-α agonist treatment normalizes hepatic fat oxidation but not hepatic glucose production. Because 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 whether fasting hepatic fat oxidation could be corrected by PPAR-α induction and, if so, how this intervention would affect gluconeogenesis. Plasma NEFAs, 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 (Fig. 3*A*), and TCA cycle activity (Fig. 3*B*) was driven to supra-normal levels by either dose. Thus, PPAR-α agonism appeared to correct the hepatic fat oxidation index (Fig. 3*C*) 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 and NEFAs), endogenous glucose production was not improved by PPAR-α agonist treatment. Glycogenolysis was slightly reduced in treated diabetic rats, but gluconeogenesis was slightly increased (Fig. 4*A*–*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 twofold by WY14,643 in diabetic rats but to a much lesser degree in control rats (Fig. 4*E* and *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 is slightly impaired in diabetic rats but is induced by PPAR-α agonist treatment. We measured the expression of hepatic enzymes associated with mitochondrial, peroxisomal, and microsomal fat oxidation by quantitative PCR to investigate the molecular basis of attenuated hepatic β-oxidation in ZDF rats (Table 3). Of the mRNA measured, only carnitine palmitoyltransferase (CPT)-1a (mitochondrial fat transporter) and Cyp-4a (microsomal ω-oxidation) were significantly decreased, while CD36/FAT (cellular fat transporter) was overexpressed fivefold. Treatment with WY14,643 dramatically stimulated the expression of nearly all measured FAO (fatty acid oxidation) 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, PPAR-γ coactivator (PGC)-1α expression was decreased by two- to threefold in WY14,643-treated lean and diabetic rats.

Diabetic rats are resistant to the normal ketogenic effects of fibroblast growth factor-21. Fibroblast growth factor (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 (Fig. 5*A*) and plasma FGF-21 protein (Fig. 5*B*). FGF-21 plasma protein was consistently, but not dramatically, elevated in diabetic rats. However, FGF-21 expression was elevated sixfold in the liver of newly diabetic rats. These findings are some-

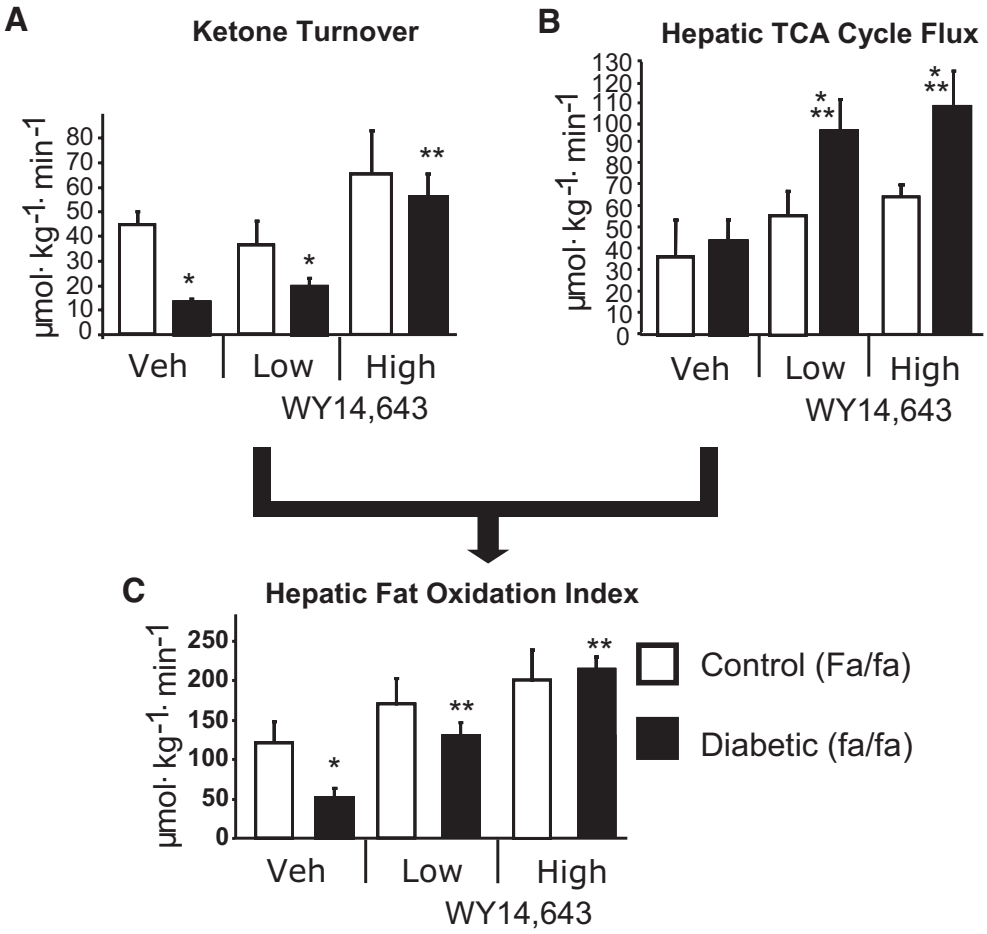

FIG. 3. In vivo hepatic fat oxidation is stimulated by WY14,643 in the diabetic liver and to a lesser extent in the normal liver. Twenty-four-hour–fasted 12-week-old rats fed a normal diet (Veh) or diet containing WY14,643 at either 100 mg/Kg diet (Low) or 300 mg/Kg diet (High). *A*: Ketone turnover measured by tracer dilution of [1,2-^{13}C]BHB and [3,4-^{13}C]ACAC was stimulated at high, but not low, doses of WY14,643. *B*: Hepatic TCA cycle flux was measured by ^{13}C and ^{2}H 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 vs. control group. ***P* < 0.05 vs. untreated control group (*n* = 4–11) .

what paradoxical, since FGF-21 induces hepatic ketogenesis (32) and has been found to have substantial antidiabetogenic 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 (twofold lower than normal) and TCA cycle oxidation (twofold 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, it occurs due to increased gluconeogenesis from substrates like lactate and amino acids (Fig. 2). 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 been implicated in human insulin-resistant liver (15,16). However, there are few in vivo data regarding mitochondrial metabolism in insulin-resistant liver. Here, FAO gene expressions, including PGC-1α were not robustly altered, indicating that defects in hepatic mitochondrial fat oxidation may be metabolically mediated. Impaired fat oxidation in hepatocytes of nondiabetic 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 long-chain fatty acids into mitochondria (40). Liver mitochondria from nondiabetic 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 nonalcoholic fatty liver

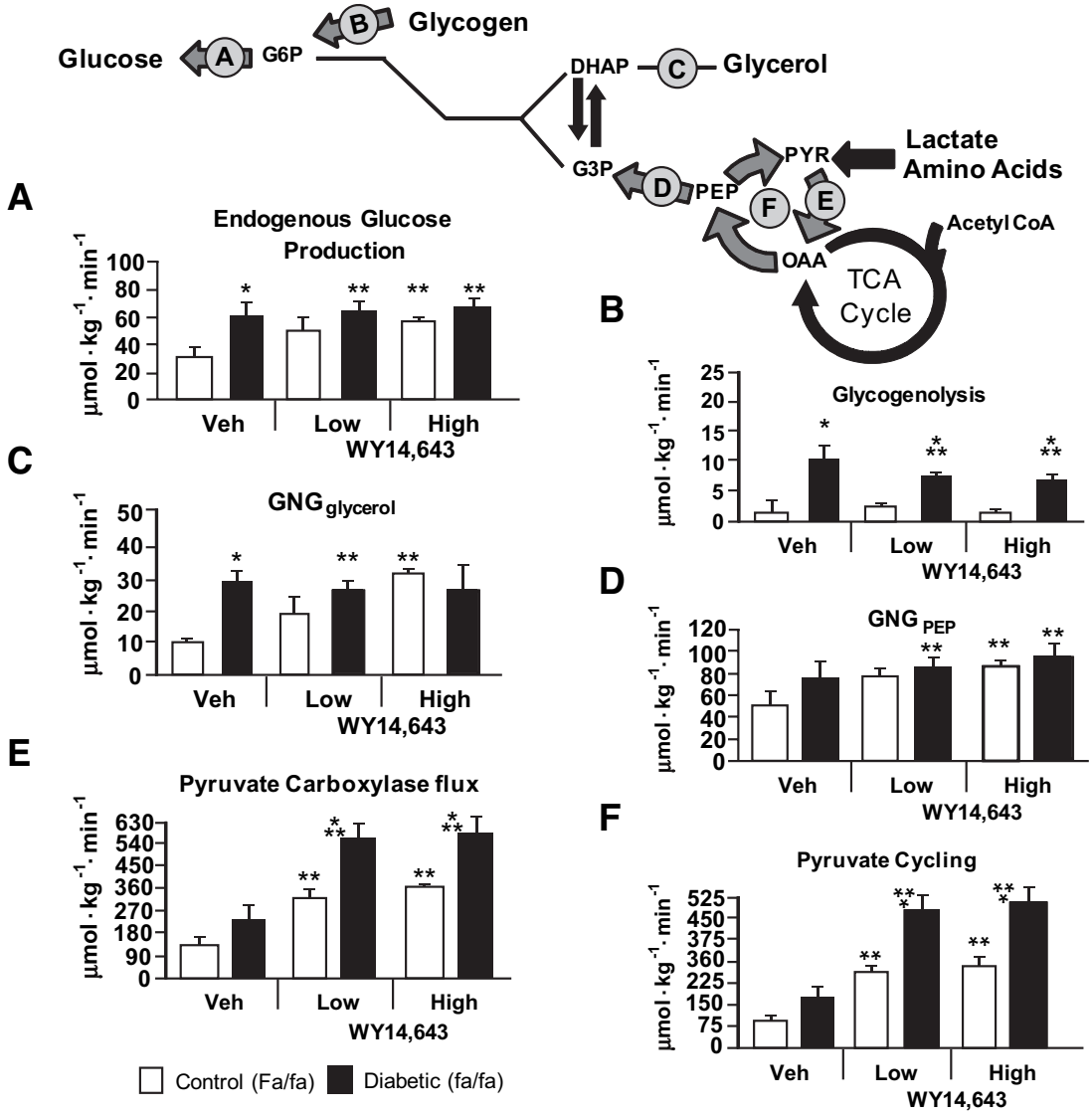

FIG. 4. Sources of in vivo glucose production are not corrected in 24-h– fasted 12-week-old diabetic rats treated with WY14,643. *A*: Endogenous glucose production is not corrected in diabetic rats and is stimulated in control rats by WY14,643 treatment. Sources of endogenous glucose production were determined by ^{2}H incorporation in plasma glucose (measured by ^{2}H NMR) after administration of ^2H_2O. *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. *$P < 0.05$ vs. control group. **$P < 0.05$ vs. untreated control group ($n = 4$–11).

TABLE 3
mRNA levels in 12- and 22-week-old control (lean) and diabetic (ZDF) rats and 12-week-old rats treated with WY14,643 measured by quantitative PCR ($n = 3$)

	12 weeks		22 weeks		Low dose		High dose	
	Lean	ZDF	Lean	ZDF	Lean WY14,643	ZDF WY14,643	Lean WY14,643	ZDF WY14,643
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*
PGC-1α	1 ± 0.06	1.0 ± 0.27	0.66 ± 0.09‡	0.85 ± 0.19	0.55 ± 0.06*	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

Data are means ± SE. *$P < 0.05$ between treated and untreated groups. †$P < 0.05$ between control and diabetic groups. ‡$P < 0.05$ between young and old groups. ACOX, acyl-CoA oxidase; HMGCS, hydroxymethylglutaryl-CoA synthase; MCAD, medium-chain acyl-CoA dehydrogenase; PDK, pyruvate dehydrogenase kinase.

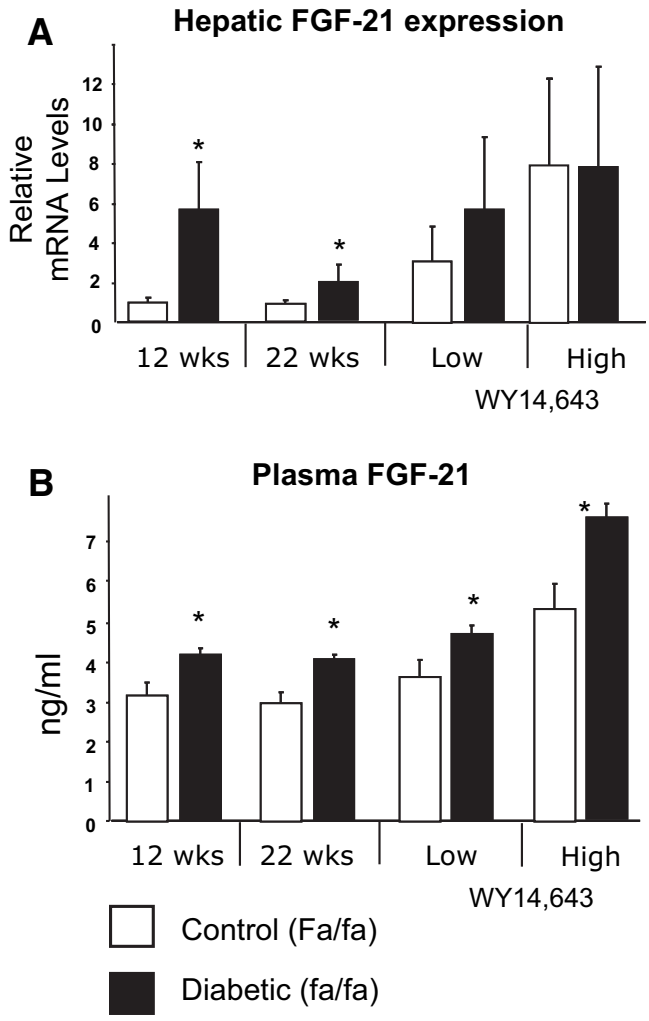

FIG. 5. Diabetic rats are resistant to the normal induction of hepatic fat oxidation by FGF-21. *A*: Hepatic FGF-21 expression measured by quantitative PCR is substantially elevated in diabetic rats. WY14,643 induced hepatic FGF-21 expression sevenfold in control rats but only 20% in diabetic rats. *B*: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

disease (16). In any case, defects in hepatic energy generation seem inconsistent with the increased energy requirements of excessive hepatic gluconeogenesis and lipogenesis 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 nonenergy demanding pathways of glycogen breakdown and conversion of glycerol to glucose ($GNG_{glycerol}$) (Fig. 2). 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 toward 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 acylcarnitine/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 overinduction of TCA cycle flux, even at a relatively low dose (one-third 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 (Fig. 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 rather 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 NEFAs in these animals suggests that FGF-21's endocrine effects on adipose tissue (32) remain intact. Further studies are required to determine whether 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 pyruvate dehydrogenase 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 overestimated ketogenesis in the rat, but we consider it unlikely that the approach would underestimate ketone turnover in diabetic rats compared with controls. Most importantly, the data correctly predict changes in hepatic fat metabolism after interventions (i.e., fasting, feeding, etomoxir treatment, and octanoate infusion; see supplemental data, online appendix).

With regard to impaired hepatic fat oxidation in the ZDF rat, 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 rat 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) reveals 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 whether defects in hepatic mitochondrial metabolism are a universal feature of insulin resistance.

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