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 2H/13C tracers and nuclear 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

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 non-esterified 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

[3,4-13C]glucose (98%) was purchased from Omicron Biochemicals (South Bend, IN). [3,4-13C]ethanolacetate (98%) and [1,2-13C]sodium β-hydroxybutyrate (98%) were purchased from Isotec (St. Louis, MO). [1,2-13C]propanol and deuterium oxide (98%) 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 isoflurane/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. Etoxomix was given as a 0.5 mg/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) ppm to activate the PPAR-γ receptor (23,24). Where noted, rats were infused with etomoxir as a 0.5 mg/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) ppm to activate the PPAR-γ receptor (23,24). 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) ppm to activate the PPAR-γ receptor (23,24).

Metabolite/hormone measurements. Lipids were extracted from ~50 mg liver using a standard methanol/chloroform extraction, and triglyceride content of liver was measured with the type TG/TH triglyceride kit (Wako Chemicals, Richmond, VA). Plasma free fatty acids were measured using a 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 tail vein of the experimental group. Where noted, rats were infused with etomoxir as a 0.5 mg/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) ppm to activate the PPAR-γ receptor (23,24). 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) ppm to activate the PPAR-γ receptor (23,24).

RESULTS

Simultaneous delivery of five stable isotope tracers and NMR analysis of plasma extracts provides insight into hepatic fat metabolism. Simultaneous administration of [2H2O, [U-13C]propionate, [3,4,13C]glucose, [3,4,13C]acetoacetate, and [1,2-13C]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; therefore, performed initial experiments to confirm that the techniques are compatible. [U-13C]propionate and [3,4,13C]glucose generated 13C 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 [3,4,13C]acetoacetate and [1,2-13C]β-hydroxybutyrylrate 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-

Total ketone production is reported where:

\[
\text{keto} \text{ne} \text{production} = R_{a,hac} + R_{a,\text{min}}
\]

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

\[
\text{β-oxidation index (in } 2 \text{ carbon units)} = \text{TCA cycle flux} \times 2 \times \text{keto} \text{ne} \text{production}
\]

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 7900HT Sequence Detection System, and relative mRNA levels were calculated by the comparative threshold cycle method using cyclophilin as the internal control.

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.
Betetic 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. 1A). 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. 1B). However, TCA cycle flux increased by 60% in the 22-week-old diabetic rats compared with their younger diabetic counterparts (Fig. 1B), consistent with the ketone data, suggesting an

| TABLE 1 | Plasma metabolite and insulin concentrations in 12- and 22-week-old control (lean) and diabetic (ZDF) rats ($n = 7$) |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
|                                |       Lean       | ZDF                            |       Lean       | ZDF                            |       Lean       | ZDF                            |
| Glucose (mmol/l)               | 4.4 ± 0.79    | 10.2 ± 0.73*                   | 6.9 ± 0.35      | 17.7 ± 1.92†                  | 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†                  | 0.88 ± 0.08     | 2.45 ± 0.26†                  |
| Total ketones (µmol/l)         | 989 ± 53.2    | 212 ± 25.6*                    | 919 ± 24.8      | 406 ± 67.6†                   | 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*                    | 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†                   | 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.

![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 [1,2-13C]BHB and [3,4-13C]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.](image-url)
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 \(\beta\)-oxidation. This data indicated a substantially lower rate of \(\beta\)-oxidation in newly diabetic rats compared with age-matched controls (Fig. 1C). 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 \(\beta\)-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. 2A) in newly diabetic ZDF rats (age 12 weeks) was associated with increased glycogenolysis (Fig. 2B) and gluconeogenesis from glycerol (GNGglycerol) (Fig. 2C) but with normal gluconeogenesis originating from substrates (i.e., lactate and pyruvate alanine) that pass through the TCA cycle (GNGPEP) (Fig. 2D). Flux through all of these pathways was exacerbated in older diabetic rats (age 22 weeks), including a 45% increase in GNGPEP, which was provoked by a 60% increase in anaplerotic flux (presum-
ably via mitochondrial pyruvate carboxylase) in 22-week-old ZDF rats compared with lean littermates (350 vs. 211 \(\mu\)mol · min\(^{-1}\) · 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 was slightly increased (Fig. 4).---oxidation induces glucose production by supplying ener-

### PPAR-\(\alpha\) 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-\(\alpha\) agonist 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. 3A), and TCA cycle activity (Fig. 3B) was driven to supra-normal levels by either dose. Thus, PPAR-\(\alpha\) agonism appeared to correct the hepatic fat oxidation index (Fig. 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.

### Expression of enzymes in hepatic fat oxidation is slightly impaired in diabetic rats but is induced by PPAR-\(\alpha\) 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 \(\beta\)-oxidation in ZDF rats (Table 3). Of the mRNA measured, only carnitine palmitoyltransferase (CPT)-1a (mitochondrial fat transporter) and Cyp-4a (microsomal \(\omega\)-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-\(\gamma\) coactivator (PGC)-1\(\alpha\) 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-\(\alpha\) 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 plasma FGF-21 protein (Fig. 5A) and plasma FGF-21 mRNA (Fig. 5B). FGF-21 protein was consistent, 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-
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). 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 disease.
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 $^2$H₂O. 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       |
| MCAD      | 1.0 ± 0.16 | 0.84 ± 0.12 | 1.0 ± 0.13 | 1.1 ± 0.12 |
| CPT1a     | 1.0 ± 0.14 | 0.49 ± 0.09† | 0.75 ± 0.18 | 0.67 ± 0.09 |
| HMGCS2    | 1.0 ± 0.12 | 1.0 ± 0.10 | 0.82 ± 0.03 | 0.92 ± 0.05 |
| PDK4      | 1.0 ± 0.30 | 1.3 ± 0.53 | 1.3 ± 0.14 | 2.3 ± 0.56‡ |
| CD36/FAT  | 1.0 ± 0.20 | 5.6 ± 1.1* | 1.3 ± 0.14 | 5.6 ± 1.1* |
| Cyp4a     | 1.0 ± 0.16 | 0.41 ± 0.21‡ | 0.95 ± 0.24 | 0.37 ± 0.20‡ |
| ACOX1     | 1.0 ± 0.15 | 0.80 ± 0.06 | 0.88 ± 0.14 | 1.20 ± 0.04‡ |
| PGC-1α    | 1.0 ± 0.06 | 1.0 ± 0.27 | 0.66 ± 0.09‡ | 0.85 ± 0.19 |
| PPAR-α    | 1.0 ± 0.40 | 0.77 ± 0.22 | 0.94 ± 0.26 | 0.95 ± 0.05 |

Data are means ± SE. *P < 0.05 vs. control group. †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.
diabetic liver, only total mitochondrial metabolism is dysfunctional in the energy production by way of NADH generated in the energy neutral, while the latter process contributes to net breakdown and conversion of glycerol to glucose (GNG_glycerol) (Fig. 2). The former process is essentially 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 acetyl-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 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 agonists, 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 pathophysiology 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 oxidation, a process known to interfere with insulin signaling, permits 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 in conscious dogs.

**Acknowledgments**

S.C.B. is the recipient of an American Diabetes Association Junior Faculty Award (1-50-JF-05). J.D.B. is the recipient of a National Institutes of Health (NIH) training grant (K23DK074396). Support for this work was provided by the Robert A. Welch Foundation (to S.A.K. and D.J.M.), NIH Grant DK078184 (to S.C.B.), grant R01GM084436 (to S.A.K. and D.J.M.), the Howard Hughes Medical Institute (to D.J.M.), grant R01DK081187 (to S.C.B. and J.B.), and cores within grant RR02584 DK076269 (Courtesy of Craig R. Malloy).

Portions of this work have been reported in abstract form at the 67th and 68th annual meetings of the American Diabetes Association, Chicago, Illinois, 22–26 June 2007, and San Francisco, California, 6–10 June 2008, respectively.

**Excellent technical assistance was provided by Zheng Yan, Charles Storey, Angela Milde, and Kristen Wertz.**

**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. Sato S, Flechner L, Qi L, Zhang X, Scriver 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. *Nature* 437:1109–1111, 2005
3. Zhang W, Patil S, Chauhan B, Guo S, Powell DR, Le J, Klotsas A, Matika R, Zhang W, Patil S, Chauhan B, Guo S, Powell DR, Le J, Klotsas A, Matika R, Zhang BB, Moller DE, Doebber TW: Peroxisome proliferator-activated receptor (PPAR-α) agonism prevents the onset of type 2 diabetes in a transgenic model of type 2 diabetes reverses the lipotoxic state and improves hepatic insulin resistance. *Proc Natl Acad Sci USA* 95:15592–15597, 1998
4. Bdhala JA, Paul H, Zhao Y, Binford S, Saulleng 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
5. Petersen RF, Shulman GI: Etiology of insulin resistance. *Am J Med* 110(S1–S16, 2006
6. 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
7. Kelley DE, He J, Moshinskova EV, Rittov VB: Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes* 51:2944–2956, 2002
8. Petersen KP, Davour 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
9. 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* 54:1026–1033, 2005
10. Kovacs TR, Ussher JR, Noland RC, Szentz D, Mosedale M, Ilkayeva O, Bain J, Stevens R, Dyck JR, Newgard CB, Lopaschuk GD, Muonio DM: Mitochondrial overoxidation and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell Metab* 7:45–56, 2008
11. Clark JB, Palmer CJ, Shaw WN: The diabetic Zucker fatty rat. *Proc Soc Exp Biol Med* 173:68–75, 1983
12. 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:E564–E565, 2005
13. Burgess SC, Jeffrey PMH, Stoye M, 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–E61, 2005
14. 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. *Anal Biochem* 327:140–155, 2004
15. Miles JM, Schwenk WF, McClean KL, Haymond MW: A dual-isotope technique for determination of in vivo ketone body kinetics. *Am J Physiology 251:E185–E191, 1986
16. Bailey JW, Haymond MW, Miles JM: Validation of two-pool model for in vivo ketone body kinetics. *Am J Physiol 258:E380–E385, 1990
17. Bougueres P, Ferre P: Study of ketone body kinetics in children by a combined perfusion of carbon-13 and deuterium (2H3) tracers. *Am J Physiol 253:E496–E502, 1987
18. Jin ES, Park BH, Sherry AD, Malloy CR: Role of excess glycosylation in fasting hyperglycemia among pre-diabetic and diabetic Zucker (fa/fa) rats. *Diabetes* 56:777–785, 2007
19. 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-)α agonism prevents the onset of type 2 diabetes in Zucker diabetic fatty rats: a comparison with PPAR-γ gamma agonism. *Endocrinology* 147:4525–4532, 2006
20. Kim H, Huluzik M, Asghar Z, Yao 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 lowers glucose and improves glucose homeostasis. *Diabetes* 52:1770–1778, 2003
21. Chou CJ, Huluzik M, Gregory C, Dietz KR, Vinson C, Gavrilova O, Reitman ML: *WY14,643, a peroxisome proliferator-activated receptor alpha (PPAR-α) 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. Imagaki T, Dutchak P, Zhao G, Ding X, Gautron L, Parameswara V, Li Y, Goetz R, Mohammadi M, Esder V, Elmquist JK, Gerhard RD, Burgess SC, Hammer RE, Mangelsdorf DJ, Kiewer SA: Endocrine regulation of the fasting response by PPAR-alpha-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. Kharitonenkov A, Shiyanova TL, Koester A, Ford AM, Micanovic R, Galbreath EJ, Sandusky GE, Hammond LJ, Moyer J, Owens RA, Gromada J, Brozinick JT, Hawkins ED, Wroblewski VJ, Li D-S, Mehrbod F, Jaskunas SR, Shaafelt 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. *Proc Nat Acad Sci* 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–E432, 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. *Metab Clin Exp* 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, Bezard 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:E529–E524, 1983

42. Keech A, Simes RJ, Barter P, Best J, Scott R, Taskinen MR, Forder P, Pillai A, Davis T, Glassziou 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-alpha expression in human liver. *Mol Pharmacol* 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 FGF-21. *Biochem Biophys Res Comm* 360:437–440, 2007

45. Landau BR: A potential pitfall in the use of isotopes to measure ketone body production (Letter). *Metabolism* 35:94–95, 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–E528, 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–E247, 1978

49. Unger RH: Minireview: Weapons of lean body mass destruction: the role of eortopic 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