Effects of troglitazone on substrate storage and utilization in insulin-resistant rats

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Sreenan, Seamus, Sara Keck, Timothy Fuller, Brian Cockburn, and Charles F. Burant. Effects of troglitazone on substrate storage and utilization in insulin-resistant rats. Am. J. Physiol. 276 (Endocrinol. Metab. 39): E1119–E1129, 1999.—Elevated serum and tissue lipid stores are associated with skeletal muscle insulin resistance and diminished glucose-stimulated insulin secretion, the hallmarks of type 2 diabetes. We studied the effects of 6-wk treatment with the insulin sensitizer troglitazone on substrate storage and utilization in lean control and Zucker diabetic fatty (ZDF) rats. Troglitazone prevented development of diabetes and lowered serum triglycerides (TG) in ZDF rats. Soleus muscle glycogen and TG content were elevated twofold in untreated ZDF rats, and both were normalized by troglitazone to lean control levels (P < 0.05). Troglitazone also normalized insulin-stimulated glucose uptake as well as basal and insulin-stimulated glycogen synthesis, implying increased skeletal muscle glycogen turnover. The proportion of active pyruvate dehydrogenase (PDH) in soleus muscle was reduced in ZDF relative to lean control rat muscle (16 ± 2 vs. 21 ± 2%) but was restored by troglitazone treatment (30 ± 3%). Increased PDH activation was associated with a 70% increase in glucose oxidation. Muscle lipoprotein lipase activity was decreased by 35% in ZDF compared with lean control rats and was increased twofold by troglitazone. Palmitate oxidation and incorporation into TG were higher in ZDF relative to lean control rats but were unaffected by troglitazone treatment. Troglitazone decreased the incorporation of glucose into the acyl group of TG by 60% in ZDF rats. In summary, ZDF rats demonstrate increased skeletal muscle glycogen and TG stores, both of which were reduced by troglitazone treatment. Troglitazone appears to increase both glycogen and TG turnover in skeletal muscle. Normalization of PDH activity and decreased glucose incorporation into acyl TG may underlie the improvements in intracellular substrate utilization and energy stores, which lead to decreased serum TG and glucose.

insulin resistance; non-insulin-dependent diabetes mellitus; glucose metabolism; free fatty acid metabolism; thiazolidinedione

DECREASED SKELETAL MUSCLE glucose uptake and nonoxidative storage are hallmarks of the insulin resistance associated with high-fat feeding, obesity, and type 2 diabetes (28). Increased serum lipids as a result of increased consumption of dietary fat and obesity have been shown to lead to increased uptake of free fatty acids by skeletal muscle and accumulation of intramyocytic triglyceride (TG; Refs. 25, 35). Accumulation of skeletal muscle lipid leads to increased oxidation of free fatty acids (16) and increased cytoplasmic acyl-CoA levels (24), each of which is associated with decreased ability of the skeletal muscle to take up and store glucose. Indeed a primary pathological event in obesity may be the inability of insulin to suppress intramyocyte lipolysis, which leads to continued utilization of TG rather than glucose as a source of energy (9). In a number of studies, activation of the glucose-fatty acid cycle has been implicated as a pathological event in the generation of insulin resistance of skeletal muscle (3). More recent studies of fatty acid-induced insulin resistance suggest that activation of the glucose-fatty acid cycle is not a primary event in the defect in glucose uptake (10, 29).

High-fat feeding, obesity, and diabetes are also associated with accumulation of lipids in tissues other than skeletal muscle. Increased islet TG content has been demonstrated in Zucker fatty (fa/fa) rats (3), and it is believed that it may contribute to impairment of insulin secretion (13) in ZDF rats due to increased nitric oxide production (35). In the liver, fat feeding has also been associated with elevated TG stores associated with increased hepatic glucose production (24).

Troglitazone, a member of the thiazolidinedione group of insulin-sensitizing drugs, has been shown to improve insulin sensitivity in humans (17) and in animals (30). Although thiazolidinediones have profound effects on glucose utilization, they also are associated with improvement in lipid profiles (30, 33), and it has been suggested that the improvement in insulin action in skeletal muscle may be due to alterations in lipid metabolism (33). Although thiazolidinediones are associated with activation of adipose tissue lipoprotein lipase (LPL) and increased FFA uptake into the adipocyte (21), we have recently demonstrated that troglitazone-induced improvements in lipid profiles and insulin sensitivity can be independent of adipose tissue and suggest that the drug had direct actions in skeletal muscle (5). Understanding interactions between lipid and glucose metabolism in skeletal muscle should provide new insights into the mechanism of action of thiazolidinediones.

Troglitazone has been shown to lower serum lipid levels and to prevent the onset of diabetes in Zucker diabetic fatty (ZDF) rats by 12 wk of age, in part by preserving β-cell function (34). If the ability of thiazolidinediones to improve skeletal muscle insulin sensitivity and β-cell function is associated with alterations in lipid metabolism, troglitazone treatment should be associated with changes in lipid stores in these tissues. To test this hypothesis, we treated ZDF rats with troglitazone from the age of 6 wk (before diabetes onset) to 12–14 wk (when untreated animals are frankly
The results demonstrate that troglitazone decreases skeletal muscle, liver, and islets TG, due, in part, to decreased de novo synthesis of TG from glucose. In addition, skeletal muscle glycogen stores are reduced despite increased insulin-stimulated glucose uptake and glycogen synthesis, which suggests increased glycogen turnover.

METHODS

Animals. Experiments were carried out in obese ZDF rats (ZDF/Gmi, fa/fa), lean litter mates (fa/+ or +/+), and Zucker fatty rats (fa/za). All experiments were approved by the Animal Care and Use Committee at the University of Chicago. Rats were housed at a constant 22°C with a fixed 12:12-h artificial light-dark cycle. Animals were fed a diet of either Purina 5008 chow or chow supplemented with 16.6 mg/g of troglitazone as previously described (34). The rats were housed 3–4 to a cage and were maintained on the diet from age 7 to 13 wk. In the experimental group described in Fig. 1, animals were weighed and food consumption was determined every other day. In these latter animals, serum glucose, TG, and free fatty acid levels were determined from blood obtained from the tail on a weekly basis after an overnight fast.

Experimental protocols. The studies were performed on ad libitum-fed animals except where indicated (4–6 animals/group). In the experimental group described in Figs. 2 and 3, one soleus muscle was removed from anesthetized rats (ketamine, Fort Dodge Animal Health, Ft. Dodge, IA; 90 mg/kg) and xylazine (Butler, Columbus, OH; 10 mg/kg) after a 15-h fast at 12 wk of age. After 1 wk, the animals were killed in the fed state when the other soleus was collected. Animals were weighed before the experiments, and blood for serum glucose was drawn from the tail. Anesthesia was performed with 480 mg/kg chloral hydrate (Sigma) intraperitoneally.

Serum chemistries. Serum glucose values were determined with a YSI serum glucose analyzer. Serum TG, cholesterol, and free fatty acids were determined with commercially available kits (TG/GB and cholesterol/HP, Boehringer Mannheim, Indianapolis, IN; nonesterified fatty acid C, Wako Chemicals, Richmond VA).

Measurement of tissue TG and glycogen content. Liver, soleus, or gastrocnemius muscle fragments (∼50–70 mg) were extracted as described previously (4, 5, 21). For determination of total TG content, tissue fragments were powdered under liquid nitrogen and extracted for 16 h at 4°C in 4 ml CHCl3-MeOH (2:1) after which 2 ml of 0.6% NaCl were added and the solution centrifuged at 2,000 g for 20 min (5, 21). The organic layer was removed and dried under a stream of argon. The resulting pellet was dissolved in PBS containing 1% Triton X-100, and the TG content was determined as described previously.

The glycogen content of ~50-mg fragments of liver or gastrocnemius was determined as previously described (4).

Isolation of islets of Langerhans. Islet isolation was accomplished as previously described (36). Isolated islets were incubated in RPMI 1640 (GIBCO) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 11.6 mM glucose, and incubated with 95% air-5% CO2 at 37°C in a humidified incubator for 2 h.

Determination of insulin release from perifused islets. Secretion of insulin from perfused islets was measured as previously described (5). Groups of 25 islets were perifused with modified Krebs-Ringer buffer (KRB) containing 5 mg/ml bovine serum albumin. After an equilibration period during which islets were perifused for 30 min at 37°C with KRB containing 2 mM glucose, sampling of the effluent perifusate was initiated at 1-min intervals. During the first 5 min, KRB containing 2 mM glucose was perifused after which the perifusate glucose concentration was increased to 20 mM containing 2 mM glucose, sampling of the effluent perifusate was initiated at 1-min intervals. During the first 5 min, KRB containing 2 mM glucose was perifused after which the perifusate glucose concentration was increased to 20 mM...
glucose. Effluent perifusate was collected for a further 15 min. The insulin concentration of the effluent perifusate was measured by radioimmunoassay as described (36) and expressed as microunits of insulin per milliliter per 25 islets. The mean insulin concentration of the effluent perifusate during perifusion with 2 mM glucose (basal insulin secretion) and during stimulation with 20 mM glucose was calculated and compared between groups.

Incubation media. Soleus muscles were incubated in a medium containing both glucose and sodium palmitate. Initially a solution of 10% fatty acid free bovine serum albumin (BSA, Sigma Fraction V) containing 5 mM sodium palmitate was prepared by ten 10-s sonications at 4°C in low calcium Krebs-Henseleit buffer (7). Subsequently, a portion of this solution was spiked with [3H]sodium palmitate (Amersham) and sonicated for another five cycles. Final incubation media were prepared by diluting the BSA-palmitate solution in Gey and Gey's balanced salt solution (4) (with calcium reduced to 0.5 mM), such that the final concentration of metabolizable substrates was 5 mM glucose and 0.5 mM sodium palmitate (incubation media). A portion of the incubation media contained either 0.1 mCi/ml [3H]palmitate or 0.5 µCi/ml [14C]glucose.

Substrate utilization in soleus muscle. After induction of anesthesia, the soleus muscle was removed from each hindlimb, and longitudinal strips of ~15–24 mg were preincubated for 30 min at 37°C in 24-well plates containing 1 ml of incubation media with or without 1 × 10⁻⁶ M insulin (20). The plates were gassed with 95% O₂-5% CO₂ mixture through a spigot attached to the center of the cover plate. At the end of the preincubation period, the muscles were transferred to identical media supplemented with [14C]glucose or [3H]palmitate and were incubated an additional 60 min. All substrate utilization data were corrected for muscle weight.

Glucose oxidation. After incubation of muscle strips in [14C]glucose, the muscle was removed and the 24-well plate fitted with a precut piece of Whatman 3MM paper that had been soaked in benzethonium hydroxide (Sigma) and overlaid with a sized rubber gasket (20). A 24-well plate top with holes drilled was clamped tightly, and 0.25 ml of 4 N H₂SO₄ was added to each well with a 25-g syringe needle introduced through the rubber gasket to release [14C]CO₂. The filter was dried, and the areas corresponding to the well were cut and placed into Scintiverse II after which 1 ml 4N H₂SO₄ was added. Background counts were determined by the release of radioactivity from incubation media that did not contain muscles.

Glucose incorporation into glycogen. Incorporation of glucose into glycogen in strips of skeletal muscle was measured as previously described (4).

Palmitate oxidation. The conversion of [3H]palmitate into H₂O was determined by incubation of muscle with [3H]palmitate as described (8). Excess palmitate was precipitated by the addition of 0.5 ml of 30% TCA and centrifugation. The acidified supernatant was extracted twice with CHCl₃-MeOH (2:1), and the aqueous phase was counted in scintillation fluid. Background counts were estimated by determining the counts present in media incubated in the absence of muscle.

Glucose or palmitate incorporation into neutral lipids. To estimate the incorporation of [14C]glucose or [3H]palmitate into extractable lipids, the muscles were quickly frozen in dry ice, crushed in a microfuge tube, and extracted overnight at 4°C in 1 ml of CHCl₃-MeOH (2:1). The unincorporated, labeled substrate was extracted twice by the addition of 0.5 ml of 0.6% NaCl, 100 mM Tris, pH 6.0. The extraction efficiency was estimated by the addition of [3H]trioleate (Dupont/New England Nuclear) to a muscle sample incu-
bated without label and was 86%. The presented data were correct for the extraction efficiency.

To determine the site (glycerol vs. acyl moiety) of glucose incorporated into neutral lipids, pooled CHCl₃ extracts from each treatment group were dried and resuspended in 200 µl of PBS with 1% Triton X-100 and hydrolyzed with 50 µl TG/GB lipase mix (Boehringer Mannheim). The solution was acidified and reextracted twice with CHCl₃, and the counts in the organic layer were used to estimate the amount of glucose incorporated into the acyl groups.

2-Deoxy-D-glucose uptake. Muscles were preincubated in the incubation media containing 0.5 mM sodium palmitate supplemented with 2 mM pyruvate with or without insulin (5). The muscles were then transferred to the same media supplemented with 2-deoxy-D-[3H]glucose (0.5 mM, 50 µCi/ml) and [14C]mannitol and incubated for 45 min at 37°C. The reaction was terminated by the addition of the 3.4 ml of Belfrage solution [CHCl₃-MeOH-n-heptane (1.25:1.4:1)] and 960 µl of 20 mM Na₂CO₃. After vigorous mixing, the aqueous phase was counted to determine [3H]oleic acid released from TG. A sample mixture without muscle was spiked with [14C]palmitate to estimate recovery of free fatty acid and release of [3H]oleic acid in the absence of muscle. The presented lipase activity data were corrected for background and recovery.

Pyruvate dehydrogenase activity. A modification of the protocol of Mondon et al. (19) was used. Gastrocnemius muscle (40–60 mg) was minced and homogenized in 10 ml of 100 mM potassium phosphate, pH 7.0, 2 mM EDTA, and 5 mM 2-mercaptoethanol (buffer A) and centrifuged at 900 g for 5 min. The supernatant was filtered through glass wool and recentrifuged at 10,000 g for 15 min at 4°C, and the pellet was resuspended in 2 ml buffer A and recentrifuged. The pellet was resuspended in 2 ml of 30 mM tetraethylammonium, pH 7.0, 2 mM 1,4-dithiothreitol, and 5 µg/ml oligomycin. The protein content of the sample was determined by the Bradford method (Sigma). To determine the total pyruvate dehydrogenase (PDH) activity, 400 µl of the suspended mitocho-
RESULTS

In these studies, we determined the effect of troglitazone treatment on tissue energy stores and the utilization of carbohydrate and lipid in skeletal muscle in male lean control and ZDF rats. The rats were fed troglitazone as a food admixture from 7 wk of age, before the onset of diabetes, to 13–14 wk of age when, in the untreated ZDF rat, frank diabetes occurs (34).

Animal weight, food consumption, and serum TG. Figure 1 shows the changes in weight and food consumption in lean and ZDF rats over the duration of the study. The ZDF rats consumed more chow at all time points compared with the control animals (Fig. 1B). Troglitazone did not appear to affect the food consumption in lean rats. Troglitazone-treated ZDF rats consumed less chow but gained more weight after the first week of treatment (Fig. 1A). The weight gain may be due to the prevention of diabetes and glycosuria in the troglitazone-treated ZDF rats. Serum TG levels were significantly higher in the ZDF rats compared with the lean rats throughout the treatment period. Troglitazone treatment attenuated the rise in serum TG levels in the ZDF rats but did not restore them to the levels observed in lean animals (Fig. 1C). Although the serum glucose levels were similar at the beginning of the experiment (not shown), by 13 wk the untreated ZDF rats had significant hyperglycemia, which was prevented by the addition of troglitazone to the diet (Table 1). Liver weights were significantly higher in the ZDF animals than in lean rats. Troglitazone treatment did not affect liver weight in either group of animals (Table 1). Soleus and extensor digitorum longus weights were slightly lower in the ZDF rats compared with the lean animals (Table 1). Skeletal muscle weights were not changed by troglitazone treatment in either animal group (Table 1).

Tissue glycogen levels. The glycogen levels in the soleus muscle from ZDF rats at 13–14 wk of age were 50% higher in fasted rats and nearly twofold higher in fed rats compared with lean controls (Fig. 2, A and B). Troglitazone treatment caused a small, but nonsignificant, decrease in the glycogen of solei of lean animals in the fasted state but reduced the levels in the ZDF animals to levels similar to those seen in lean controls (Fig. 2, A and B) in both the fed and fasted states. The total glycogen levels were slightly higher in the fasted animals than fed animals in these experiments. This may be due to the effects of anesthesia or slight blood contamination in the solei surgically removed vs. those removed at time of death. We also examined the glycogen levels in gastrocnemius muscle in obese, non-diabetic, fa/fa rats and ZDF rats at 6 wk of age (prediabetic) and found elevations in hindlimb muscle glycogen levels in these animals as well (Table 2). As in the soleus of the ZDF rat, troglitazone treatment decreased hindlimb glycogen stores in the Zucker fatty rats (fa/fa; Table 2) as well as in the gastrocnemius of lean rats. The levels of glycogen in the liver of ad libitum-fed animals were not different between lean and ZDF rats (Fig. 2C), and troglitazone had no significant effect on hepatic glycogen stores.

Tissue TG levels. Skeletal muscle TG levels were approximately twofold higher in the ZDF rats than in

Table 1. Characteristics of study animals

<table>
<thead>
<tr>
<th>Rat</th>
<th>Body Weight, g</th>
<th>Plasma Glucose, mg/dl</th>
<th>Liver Weight, g</th>
<th>Soleus Weight, g</th>
<th>EDL Weight, g</th>
<th>Epididymal Fat Pad Weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean</td>
<td>293 ± 5</td>
<td>127 ± 2</td>
<td>10.8 ± 0.6</td>
<td>123.5 ± 2.8</td>
<td>157.2 ± 3.8</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>Lean + Tro</td>
<td>304 ± 9</td>
<td>119 ± 5</td>
<td>8.3 ± 1.2</td>
<td>121.5 ± 5.8</td>
<td>150.0 ± 7.4</td>
<td>3.3 ± 0.5</td>
</tr>
<tr>
<td>ZDF</td>
<td>388 ± 7*</td>
<td>407 ± 25*</td>
<td>24.6 ± 1.2*</td>
<td>113.4 ± 3.8*</td>
<td>134.8 ± 0.9*</td>
<td>13.1 ± 1.5*</td>
</tr>
<tr>
<td>ZDF + Tro</td>
<td>414 ± 6*†</td>
<td>136 ± 5*†</td>
<td>22.1 ± 0.9*</td>
<td>113.9 ± 3.7*</td>
<td>139.5 ± 2.5*</td>
<td>14.6 ± 2.3*</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 4–6/group. Tro, troglitazone; ZDF, Zucker diabetic fatty. *P < 0.05 vs. lean group. †P < 0.05 vs. ZDF group.

Table 2. Gastrocnemius glycogen and triglyceride content

<table>
<thead>
<tr>
<th>Animal (n)</th>
<th>Glycogen, nmol glucose/mg</th>
<th>Triglyceride, ng/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean (4)</td>
<td>9.9 ± 1.7</td>
<td>3.3 ± 0.4</td>
</tr>
<tr>
<td>Lean + Tro (3)</td>
<td>6.2 ± 0.6*</td>
<td>2.2 ± 0.4*</td>
</tr>
<tr>
<td>ZDF (5)</td>
<td>13.3 ± 4.4*</td>
<td>6.7 ± 1.1*</td>
</tr>
<tr>
<td>ZDF + Tro (6)</td>
<td>10.3 ± 2.4</td>
<td>2.6 ± 0.6†</td>
</tr>
<tr>
<td>fa/fa (4)</td>
<td>10.8 ± 1.5</td>
<td>5.2 ± 1.0*</td>
</tr>
<tr>
<td>fa/fa + Tro (4)</td>
<td>8.1 ± 1.4</td>
<td>2.1 ± 0.3‡</td>
</tr>
<tr>
<td>Prediabetic ZDF (5)</td>
<td>12.1 ± 4.1*</td>
<td>4.9 ± 0.8</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P < 0.05 vs. lean group. †P < 0.05 vs. non-troglitazone-treated group.
lean animals in both the fed and fasted states (Fig. 3, A and B). Troglitazone treatment lowered soleus TG content in both lean and ZDF rats, decreasing the latter to near control levels (Fig. 3, A and B). The gastrocnemius TG levels in fa/fa and prediabetic ZDF rats were also elevated and were also reduced by troglitazone (Table 2).

As previously reported (12), tissue TG levels were significantly elevated in the liver of ZDF rats compared with lean controls (Fig. 3C). Troglitazone treatment had no effect on liver TG in lean rats but dramatically lowered the levels in the ZDF (Fig. 3C).

In islets from control rats, the levels of TG were below the level of detection in our assay (average of ~200 islets measured). TG in the islets obtained from ZDF rats was readily measurable and was decreased ~85% after treatment with troglitazone (Fig. 3D).

Insulin secretion by perifused islets. Untreated ZDF rat islets exhibited marked basal hypersecretion of insulin (mean insulin secreted during perfusion of KRB with 2 mM glucose of 53.2 ± 7 µU·ml⁻¹·25 islets⁻¹·2 min vs. 2.3 ± 1.1 µU·ml⁻¹·25 islets⁻¹·2 min in lean control islets, P < 0.05) and were poorly responsive to glucose (Fig. 4). An increase in the glucose concentration in the perfusion system, from 2 to 20 mM glucose, led to a transient 20% increase in insulin secretion in ZDF-derived islets compared with a 20-fold increase in secretion in control islets. Troglitazone treatment reduced insulin secretion at 2 mM glucose to 20.5 ± 8 µU·ml⁻¹·25 islets⁻¹·2 min and resulted in a 3.5-fold response to stimulation with 20 mM glucose. The drug had no effect on insulin secretion from control lean islets.

Glucose metabolism in isolated soleus. Troglitazone treatment of ZDF rats restored the ability of insulin to stimulate transport of 2-deoxy-d-[³H]glucose in soleus strips isolated from fed rats. The increase was to the levels seen in lean control solei (Fig. 5A).

To examine the effect of troglitazone on substrate utilization, solei were incubated in media containing 5 mM glucose and 0.5 mM palmitate supplemented with either [¹⁴C]glucose or [¹⁴C]palmitate. Glycogen synthesis, as measured by glucose incorporation into glyco- gen, was significantly increased in the presence of insulin in the solei from lean rats and was not affected by troglitazone supplementation of their diet (Fig. 5B). In contrast, glycogen synthesis in the solei of untreated ZDF rats was not stimulated by insulin. Both basal and insulin-stimulated glycogen syntheses were increased in ZDF rat soleus after troglitazone treatment, with a significant demonstrable response to insulin treatment (Fig. 5B).

Glucose oxidation, as determined by the trapping of [¹⁴C]CO₂ generated during incubation of solei, was not increased by insulin treatment in any of the animal groups tested (Fig. 5C). Compared with lean animals, there was a slight but significant elevation in glucose oxidation in the ZDF rat soleus. This was slightly increased by treatment with troglitazone (Fig. 5C). When the partitioning of glucose utilization was determined by dividing the amount of glucose stored as glycogen in an individual animal by the amount of glucose oxidized, troglitazone significantly increased the ratio of glucose stored to oxidized in response to insulin in ZDF rats (Fig. 5D). This suggests that troglitazone promotes the storage relatively more than the oxidation of glucose in skeletal muscle.

Palmitate metabolism in isolated soleus. The metabolism of palmitate was investigated under conditions identical to those described in Glucose metabolism in isolated soleus for glucose utilization with paired soleus strips from the same animals. In lean rats, storage of palmitate as determined by the incorporation into neutral lipids (largely TG, Ref. 9) was not stimulated by insulin. Both basal and insulin-stimulated palmitate oxidation were increased in ZDF rats (Fig. 5D). This suggests that troglitazone promotes the storage relatively more than the oxidation of glucose in skeletal muscle.
were extracted from the individual muscle groups, and the amount of label associated with the acyl component was determined. The results showed that in the control animals, with or without troglitazone treatment, >90% of the incorporated label was associated with the glycerol backbone (Table 3). In contrast, in the soleus of ZDF rats, the proportion of label associated with the acyl group was nearly doubled. The glucose incorporation into the acyl component of TG in the ZDF solei was decreased to near control levels with troglitazone treatment (Table 3). Insulin had no apparent effect on the distribution of label in these experiments. Because samples were pooled, no statistical evaluation was performed.

LPL activity. The improvement in serum TG after troglitazone treatment may be due to increased delivery and metabolism of TG by skeletal muscle (5) as well as adipose tissue (14). In contrast, in the soleus of ZDF rats, the proportion of label associated with the acyl group was nearly doubled. The glucose incorporation into the acyl component of TG in the ZDF solei was decreased to near control levels with troglitazone treatment (Table 3). Insulin had no apparent effect on the distribution of label in these experiments. Because samples were pooled, no statistical evaluation was performed.

Skeletal muscle substrate utilization. We examined the activation state of PDH in the hindlimb muscle of untreated rats and in rats treated with troglitazone. Total PDH activity in the crude mitochondrial fraction from hindlimb skeletal muscle measured in the absence of phosphatase inhibition was not different on a per milligram basis in any of the animal groups (Fig. 8, insets). However, the PDH activity fraction in both fed and fasted rats was significantly lower in the ZDF rats than in the lean animals (Fig. 8). Whereas troglitazone treatment had no significant effect on the PDH activity fraction in the lean rats, there was a normalization of PDH activity proportion in the ZDF animals treated with troglitazone (Fig. 8).

**DISCUSSION**

The increased energy intake in the ZDF rat, due to the leptin receptor mutation, is manifested primarily as increased adiposity, which occurs early in the life of the animal (27). The increase in adipose TG tissue stores in the ZDF rat is paralleled by an increase in the
amount of TG associated with liver, skeletal muscle, and pancreatic islets (Fig. 3). The increased TG in skeletal muscle is apparently not due to the diabetic state per se because elevated levels of muscle TG are also seen in the Zucker fa/fa rats and in the prediabetic ZDF rat (Table 2; Ref. 12). In the liver, TG are elevated in prediabetic ZDF and non-diabetic Zucker fatty rats (12), and there appears to be an even greater accumulation in the frankly diabetic ZDF rat (Fig. 3), likely due to increased glucose delivery to the liver and augmented TG synthesis (1). Troglitazone treatment of both lean and ZDF rats resulted in decreased content of TG in skeletal muscle and a marked decrease in liver TG content in ZDF rats (Fig. 3). Because troglitazone increased the body weight and fat pad weight of the ZDF rat (Table 1), we believe that the decrease in muscle-associated TG seen in the ZDF rat is due to a decrease in intramyocyte TG rather than intermyocyte adipose tissue-associated TG. However, definitive proof awaits more direct measures of these different TG pools.

Table 3. Incorporation of [14C]glucose into acyl component of triglyceride

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Cpm Total</th>
<th>Cpm in Acyl</th>
<th>Percentage in Acyl Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean – Ins</td>
<td>33,453</td>
<td>2,984</td>
<td>8.9</td>
</tr>
<tr>
<td>Lean + Ins</td>
<td>23,239</td>
<td>1,893</td>
<td>8.1</td>
</tr>
<tr>
<td>Lean + Tro – Ins</td>
<td>26,370</td>
<td>1,674</td>
<td>6.3</td>
</tr>
<tr>
<td>Lean + Tro + Ins</td>
<td>35,832</td>
<td>3,471</td>
<td>9.7</td>
</tr>
<tr>
<td>ZDF</td>
<td>36,721</td>
<td>5,798</td>
<td>15.8</td>
</tr>
<tr>
<td>ZDF + Ins</td>
<td>19,076</td>
<td>3,234</td>
<td>17.0</td>
</tr>
<tr>
<td>ZDF + Tro – Ins</td>
<td>34,927</td>
<td>3,583</td>
<td>10.3</td>
</tr>
<tr>
<td>ZDF + Tro + Ins</td>
<td>22,098</td>
<td>2,623</td>
<td>11.9</td>
</tr>
</tbody>
</table>

Equal volumes of extracted neutral lipids from each muscle group (n = 4) were pooled and treated with lipase acidified and extracted with CHCl₃-methanol (2:1), and organic layer was counted for 14C. Cpm, counts/min; Ins, insulin.

A number of studies have demonstrated a relationship between increased TG stores, insulin resistance (25, 35), and deterioration in insulin secretion from the pancreas (13, 38). Parallel to a decrease in secretory function of the isolated ZDF islets, we observed an elevated TG content, although the multiples of increase over controls could not be determined because the TG concentration found in the islets from lean animals was below the limit of detection in our assay (Fig. 3).
animals fed a high-fat diet, manipulation of TG stores of the β-cell has been associated with improvements in insulin sensitivity and in β-cell function (31). In this study, we found reduced basal secretion and greater glucose responsiveness in ZDF islets from troglitazone-treated rats compared with untreated ZDF islets (Fig. 4). The improved β-cell function may be due, in part, to the improvement in peripheral insulin sensitivity and also to the lowering of islet TG content. The in vivo effects of troglitazone on islet cell function that we have observed in this study are identical to the in vitro effects of troglitazone demonstrated by Shimabukuro et al. (31).

In the present study we also found, in the ZDF rat skeletal muscle, an increased capacity to incorporate palmitate into TG as well as an increase in de novo TG synthesis. The results suggest that, as in the isolated islet (32), activation of synthetic pathways underlies the increased accumulation of TG. However, we have been unable to detect increases in skeletal muscle acyl-CoA synthase mRNA levels (Burant, unpublished observation) similar to those seen in isolated islets (32). Although the TG levels decreased in skeletal muscle after troglitazone treatment, we observed no significant change in palmitate incorporation into TG or changes in palmitate oxidation (Fig. 6). These results suggest that these metabolic changes are due to the increased substrate delivery to muscle (secondary to hyperphagia) or to deficiency in leptin action, which is not ameliorated by troglitazone. In incubated skeletal muscle, leptin treatment has been shown to increase the oxidation relative to storage of palmitate (20). This may partially explain the relative increase in storage seen in the ZDF rat muscle because they are deficient in leptin action.

One of the more salient findings in this study is the increase in the proportion of glucose incorporated into TG by skeletal muscle of the ZDF rat. Not only is there an increase in the amount incorporated into total TG, but also, the amount incorporated into the acyl group increased approximately twofold. The increased conversion of glucose into TG was normalized by troglitazone treatment. A shunting of glucose to de novo synthesis of TG explains the increased accumulation of TG seen in the skeletal muscle of the ZDF rat. Because the metabolic route would be via acetyl-CoA to malonyl-CoA, the increase in malonyl-CoA could result in decreased carnitine palmitoyltransferase I activity (18), which would result in increased cytosolic long chain fatty acyl-CoA levels. Increases in fatty acyl-CoA levels have been implicated in defects in insulin signaling in high-fat feeding (23) and may play a role in peripheral insulin resistance (6).

In humans, an elevated skeletal muscle TG content is also associated with a decrease in insulin-stimulated
glucose uptake and metabolism (25, 26). Whereas troglitazone is very effective at increasing insulin action in human skeletal muscle (17), to our knowledge its effects on the TG content in skeletal muscle have not been determined. Given the data presented in this report, we predict that troglitazone will have similar effects.

In addition to increased muscle-associated TG content, we also have demonstrated increased stores of glycogen in the muscle of the diabetic ZDF and the prediabetic ZDF rats and in the nondiabetic Zucker fatty rat muscles (Table 2). Previous studies have shown a correlation between muscle glycogen content and insulin sensitivity. Thus exercise- or epinephrine-induced lowering of skeletal muscle glycogen content enhances insulin sensitivity (11), whereas increasing glycogen by fasting-refeeding protocols (11) or by muscle denervation (4) results in attenuated insulin action. Whether this is indirect because of the elevated TG or LC-acyl-CoA levels (39) or the purported direct glyco- gen feedback (2) is not clear. Because troglitazone decreased the steady-state glycogen levels in soleus from ZDF rats despite increased glycogen synthesis rates, there must be an increase in glycogenolysis, which leads to the increased glycogen turnover rate. Whether the stimulation of glycogen turnover is part of the actions of troglitazone in humans is also not clear. Although it is clear that insulin resistance in skeletal muscle is associated with decreased glucose incorporation into glycogen (28, 29, 32), there is no compelling evidence for elevated glycogen in the skeletal muscle of patients with type 2 diabetes (37).

An increase in lipid oxidation results in decreased oxidation of glucose both in vivo and in vitro (4, 32). This may be due to inhibition of PDH and increased citrate levels, as suggested by Randle (reviewed in Ref. 3), or due to a defect at the stimulation of glucose uptake or phosphorylation (10, 29). In either case, an increased utilization of fats as an energy substrate leads to a decrease in the utilization of glucose that is indicative of the glucose-fatty acid cycle (3). In the present study, whereas the total PDH activity in mitochondria from ZDF rats in the fed or fasted state was not changed, the PDH active form was 33% lower than in lean control rats and was increased to near control levels with troglitazone treatment (Fig. 7). Thus, even in the face of increased oxidation of lipids, troglitazone was able to increase the PDH active fraction in the skeletal muscle of these animals. Further investigations are needed to determine whether this is due to alterations in the enzyme itself or to changes in PDH kinases or phosphatases that control the activation state of the enzyme.

In summary, the diabetic ZDF rat has a profound insulin resistance, which is associated with increased skeletal muscle energy stores in the form of both glycogen and TG. Troglitazone treatment reverses the defects in glucose metabolism but only partially reverses the changes in TG metabolism. The metabolic changes induced by troglitazone treatment may be enhanced or attenuated by the persistent hyperphagia of the ZDF rat, and the actions of thiazolidinediones in other animal models of insulin resistance, such as high-fat feeding, may more closely reflect their actions in humans.

The main findings in the current study are, first, that troglitazone increases the turnover of skeletal muscle energy stores, profoundly reducing the glycogen and TG levels within the myocyte. The apparent dichotomy of increased capacity of skeletal muscle to transport glucose and synthesize glycogen after troglitazone treatment and decreased glycogen stores will need to be resolved. Detailed examination of the glycogen synthesis and glycogenolysis pathways will provide insights into the actions of the thiazolidinediones on skeletal muscle. Second, the ability of troglitazone to decrease de novo synthesis of TG within the skeletal muscle may be the primary way in which TG stores are normalized. Finally, insulin resistance in skeletal muscle of the ZDF rat is characterized by impaired glucose uptake and storage, decreased LPL activity and PDH activation, and increased oxidative capacity toward free fatty acids. Troglitazone treatment reverses these defects along with decreasing intracellular energy stores. These findings might suggest that it is not "lipotoxicity" per se that results in insulin resistance of skeletal muscle; rather, insulin resistance develops as a way for the muscle to defend against over increasing total energy stores.

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