Troxiglazone Lowers Islet Fat and Restores Beta Cell Function of Zucker Diabetic Fatty Rats*

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The thiazolidinedione compound troglitazone, which is used to treat non-insulin-dependent diabetes mellitus (NIDDM) in man, is also effective in the adipogenic NIDDM of Zucker diabetic fatty (ZDF) rats. To test the “lipotoxicity hypothesis,” which attributes the beta cell dysfunction of adipogenic NIDDM to an excessive accumulation of fat in the pancreatic islets, we sought to determine if troglitazone-mediated amelioration of beta cell function in islets of ZDF rats might be associated with a reduction in their elevated triglyceride (TG) content. Troglitazone (10 $\mu M$) in the culture medium reduced the TG content of ZDF rats by 52%; this was reflected by decreased esterification and increased oxidation of $[^3H]$palmitate. Glycerol-3-phosphate acyltransferase mRNA fell by 57% and acyl-CoA synthetase mRNA by 67% (brain isoform) and 38% (liver isoform), all consistent with the effects of troglitazone on TG metabolism. The 52% decrease in islet TG was accompanied by >30- and 2-fold improvements in glucose- and arginine-stimulated insulin secretion, respectively. We conclude that troglitazone exerts direct lipopenic activity in normal islets and in islets of obese prediabetic ZDF rats; in the latter, this correlated with improvement in beta cell function. The results are consistent with the lipotoxicity hypothesis for adipogenic diabetes.

The beta cell dysfunction and hyperglycemia of obese Zucker diabetic fatty (ZDF) rats have been attributed to the 15–17-fold increase in the fat content of their pancreatic islets, which coincides with onset of their overt diabetes (1–3). We have proposed that the overaccumulation of fat is the result of the mutation in the leptin receptor (OB-R) (4, 5) that blocks the normal triglyceride (TG)-lowering action of leptin on islets (6) and that this leads to so-called “lipotoxicity” (2, 7) through exaggerated production of nitric oxide (8). Indeed, overexpression of a normal leptin receptor in such islets lowers islet fat to normal levels in response to leptin and reduces nitric oxide production; it therefore appears that the OB-R mutation is the proximal cause of the fat overload and the resulting $\beta$-cytotoxicity.

If this is correct, any agent that lowers islet TG should improve beta cell function in islets of ZDF rats. The leptin resistance of ZDF rat islets is so severe that even the induction of marked hyperleptinemia by adenovirus-leptin gene transfer fails to lower their islet TG content (6). However, troglitazone, a member of the thiazolidinedione family of drugs, has a leptin-like TG-lowering effect on the fat-laden islets of ZDF rats as well as on normal islets (9). Since troglitazone is known to have an antidiabetic effect in obese ZDF rats (10–12) and in obese humans (13–15), this study was designed to determine if the therapeutic benefits of the drug might be related to a lipopenic effect on islets.

MATERIALS AND METHODS

Animals—Lean wild-type (+/+) male ZDF rats and obese homozygous (fa/fa) male ZDF rats were bred in our laboratory from ZDF/Dtfr-fa(F10) stock purchased from R. Peterson (University of Indiana School of Medicine, Indianapolis, IN). Their genotype was confirmed using the method of Phillips et al. (4).

Islet Isolation and Culture—Pancreatic islets were isolated from 7-week-old male ZDF rats according to the method of Naber et al. (16). Isolated islets were cultured in 60-mm Petri dishes at 37 °C in a humidified atmosphere of 5% CO$_2$ and 95% air as described previously (17). The culture medium consisted of RPMI 1640 medium supplemented with 8.0 mM glucose, 10% fetal bovine serum, 200 units/ml penicillin, 0.2 mg/ml streptomycin, and 2% bovine serum albumin (fraction V; Miles Inc., Kankakee, IL) and with 10 $\mu M$ troglitazone (Sankyo Co., Tokyo, Japan) or its vehicle (Me$_2$SO at a final concentration 0.01%).

Extraction of Total RNA and Semiquantitation by Reverse Transcription-Polymerase Chain Reaction (PCR)—Total RNA was extracted by the TRIzol isolation method (Life Technologies, Inc.) from ~100 islets following 48 h in culture. RNA was treated with RNase-free DNase (Promega, Madison, WI), and first strand cdNA was generated from 2 $\mu g$ of RNA in a 20-$\mu$l volume using the oligo(dT) primer first strand cdNA synthesis kit (CLONTECH, Palo Alto, CA). One $\mu l$ of the reverse transcription reaction mixture was amplified with primers specific for rat acyl-CoA oxidase, carnitine palmitoyltransferase I, glycerol-3-phosphate acyltransferase, brain-type acyl-CoA synthetase, and liver-type acyl-CoA synthetase (Table I) in a total volume of 50 $\mu$l. Linearity of the PCR was tested by amplification of 200 ng of total RNA/reaction from 15 to 50 cycles. The linear range extended from 15 to 40 cycles. The samples were amplified for 30 cycles, each using the following parameters: 92 °C for 45 s, 55 °C for 1 min, and 72 °C for 1 min. $\beta$-Actin primers were used as a control. Results are expressed as the ratio of signal intensity for the target genes relative to that for $\beta$-actin. The PCR products were subjected to electrophoresis on 1.2% agarose gel followed by Southern blotting on a nylon membrane (8, 18). Radiolabeled probes (Table I) specific for each molecule were hybridized to the membrane and quantitated using the Molecular Image (Bio-Rad).

Oxidation and Esterification of $[^3H]$Palmitate by Pancreatic Islets—Rates of oxidation and esterification of $[^3H]$Palmitate by islets were determined as described (3, 6). Groups of 100–250 islets were incubated for 48 h with medium containing 9,10-$[^3H]$palmitate. The total palmitate concentration was 1 $\mu$mol/liter. Excess $[^3H]$Palmitate was removed by precipitating twice with an equal volume of 10% trichloroacetic acid.
**TABLE I**

**Sequences of PCR primers**

| Gene | Sense primer (5’ to 3’) | Antisense primer (5’ to 3’) | PCR products | Nucleotides | GeneBank™ accession no. |
|------|------------------------|-----------------------------|--------------|-------------|------------------------|
| ACO  | GCCCTGAGCTATGTTATAC    | AGGAAGCTTCCTAAGACATG       | 634          | 2891–3524   | J02572                 |
| CPT-I| TATGAGGAGGATGGTCCTCC   | CTCGGAGAGGATGGCTGGTTC      | 629          | 3094–3722   | L07736                 |
| GPAT | TGAATCAGCGAGGACGAGCTG  | AGAAGTTGCGGCACCTCTC        | 504          | 1827–2330   | M77003                 |
| B-ACS| ACCTCTGAGACGACAGCAGG   | CAAAGACTGGCACTCATGCG       | 380          | 741–1120    | D10041                 |
| L-ACS| TGATGACCAGCTCCACATCAG  | GCTGAGACTCGCTCAGAGCTG      | 514          | 1329–1842   | D00109                 |
| β-Actin| TGTAAACCACGTGGAGCATG    | GATCTGAGCTCATGTTGCGATG     | 764          | 1552–2991   | J00691                 |

*bp, base pairs; ACO, acyl-CoA oxidase; CPT-I, carnitine palmitoyltransferase I; GPAT, glycerol-3-phosphate acyltransferase; B-ACS and L-ACS, rat brain- and liver-type, long-chain acyl-CoA synthetases, respectively.*

**TABLE II**

**TG content (part A) and rates of oxidation and esterification of [3H]palmitate (part B) in islets from normal lean (+/+ and prediabetic obese (fa/fa) ZDF rats cultured with 1 mM palmitate for 48 h with or without troglitazone**

| Troglitazone | TG content | Oxidation | Esterification |
|--------------|------------|-----------|---------------|
| 0 µM         | 10 µM      | %         | %             |
| A. TG content| ng/islet   | ng/ng DNA | pmol/ng DNA/48 h | pmol/ng DNA/48 h |
| (+/+)        | 10.4 ± 0.9 | 6.4 ± 0.8 | 23.6 ± 0.21     | 1.48 ± 0.18       |
| (fa/fa)      | 58.7 ± 3.0b| 28.4 ± 3.0b| 3.86 ± 0.2b     | 1.74 ± 0.18       |
| B. [3H]Palmitate oxidation | 8.8 ± 1.1 | 13.7 ± 1.5 | 9.9 ± 1.5     | 13.2 ± 0.9         |
| [3H]Palmitate esterification | 3.9 ± 0.1 | 3.0 ± 0.2 | 5.3 ± 0.1     | 4.6 ± 0.2         |

*p < 0.01 versus 0 troglitazone.

b p < 0.01 versus lean (+/+ ) ZDF rats.*

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with 2% bovine serum albumin. The supernatants in a microcentrifuge tube were placed in a scintillation vial containing unlabeled water and incubated at 50 °C for 18 h. Palmitate oxidation was measured by measuring the tritiated water in the medium as described for usage of [3H]glucose (17). Esterification of [3H]palmitate by islets was determined by measuring [3H]palmitate uptake by islets as described (3). 

**Perifusion of Cultured Islets—Groups of 50–100 islets were collected under a stereoscopic microscope, washed twice with Krebs-Ringer bicarbonate/Hepes buffer (pH 7.4, 3 mM glucose), and loaded into a 13-mm chamber containing an 8-mm nylon membrane filter (Millipore Corp., Bedford, MA). Islets were perifused with buffer containing 3 or 23 mM glucose or 20 mM arginine at a flow rate of 0.8 ml/min for 15 min each (18). Effluent fractions were collected at 2-min intervals and stored at −20 °C until insulin assay. Immunoactive insulin was determined by radioimmunoassay using charcoal separation.

**Statistical Analyses—**All results are expressed as mean ± S.E. Statistical significance was evaluated using Student’s t test or one-way analysis of variance followed by Bonferroni’s multiple comparison test.

**RESULTS**

**Effect of Troglitazone on Islet Fat Content—**To establish the leptin-like TG-lowering action of troglitazone, we cultured normal islets from wild-type lean (+/+ ) ZDF rats and fat-laden islets from obese prediabetic (fa/fa) ZDF rats with 10 µM troglitazone. At the end of 48 h, we measured the islet TG content. Without treatment, the TG content of fa/fa islets was significantly above that of normal islets, even when normalized for the differences in islet size (p < 0.01) (Table II, part A). With treatment, there were 39 and 52% declines in TG content in fat-laden fa/fa islets (Table II, part A). Although the TG content of the troglitazone-treated fa/fa islets was still almost 3 times that of untreated normal islets, when corrected for differences in islet size, there was no difference from normal islets.

**Effect of Troglitazone on Expression of Enzymes of Fatty Acid Oxidation and Esterification—**We have previously observed that leptin induces in normal islets the expression of two critical enzymes of fatty acid oxidation, acyl-CoA oxidase and carnitine palmitoyltransferase I, and lowers the expression of glycerol-3-phosphate acyltransferase, an enzyme of fatty acid esterification (18). Troglitazone, by contrast, did not up-regulate either the carnitine palmitoyltransferase I or acyl-CoA oxidase/β-actin mRNA ratio in islets from lean or obese rats (Figs. 1 and 2). It did, however, significantly reduce the mRNA ratios for glycerol-3-phosphate acyltransferase and both the brain and liver isoforms of acyl-CoA synthetase in both groups (Figs. 1 and 2). In islets from obese rats, the ratios declined by 57, 67, and 38%, respectively.

Direct measurement of [3H]palmitate oxidation in islets of obese ZDF rats cultured for 48 h in 10 µM troglitazone revealed a 33% increase. Esterification of [3H]palmitate was decreased by 13% (Table II, part B).

**Effect of Troglitazone on Insulin Secretion—**If the impaired beta cell function of the obese fa/fa rats were secondary to the high TG content, the 52% troglitazone-mediated reduction in fat content per islet from −6 times normal to 2.7 times normal (Table II, part A) should be associated with at least some
Functional improvement. Fig. 3 indicates the effect of troglitazone on basal, glucose-stimulated, and arginine-stimulated insulin secretion by the islets of obese ZDF rats perifused at the end of the culture period. Basal insulin secretion was reduced by 25% in the islets of obese prediabetic ZDF rats after 48 h of culture with troglitazone, whereas the increments in glucose- and arginine-stimulated insulin secretion were increased 3- and 5-fold, respectively, above control values (p < 0.01). These troglitazone-induced changes in insulin secretion resemble the hyperinsulinemic patterns of compensated islets of obese rats reported previously (19).

Discussion

Earlier studies have demonstrated that acute and subacute exposure of islets to exogenously produced elevations of free fatty acid may alter beta cell function (1–3, 20–22). In this report, we have examined the effects of troglitazone on beta cell function, islet fat content, and metabolism of islets chronically exposed in vivo to the endogenously elevated free fatty acid levels of obesity (1–3). Previously, we reported a close relationship between the accumulation of islet fat and the appearance of the beta cell abnormalities and hyperglycemia (1–3); we had further observed that all changes in islets could be prevented by caloric restriction, a measure that reduces islet fat content (23). Here we have studied the in vitro effects of troglitazone, a drug shown to be effective in the treatment of non-insulin-dependent diabetes mellitus in humans (13–15) and in ZDF rats (10–12), to determine if its effectiveness, like that of caloric restriction, might be associated with a reduction in islet TG content.

Fig. 1. Representative Southern blot of reverse transcription-PCR products for acyl-CoA oxidase, carnitine palmitoyltransferase I, glycerol-3-phosphate acyltransferase, brain- and liver-type acyl-CoA synthetases, and β-actin in islets isolated from lean (+/+) and obese (fa/fa) ZDF rats and cultured for 48 h with or without troglitazone. ACO, acyl-CoA oxidase; CPT-I, carnitine palmitoyltransferase I; GPAT, glycerol-3-phosphate acyltransferase; B-ACS, brain-type acyl-CoA synthetase; L-ACS, liver-type acyl-CoA synthetase.

Fig. 2. Effect of troglitazone on mRNA expression of acyl-CoA oxidase, carnitine palmitoyltransferase I, glycerol-3-phosphate acyltransferase, and brain- and liver-type acyl-CoA synthetases in islets isolated from lean (+/+) and obese (fa/fa) ZDF rats and treated for 48 h with or without troglitazone. Levels of mRNA expression are expressed as the ratio of signal intensity for each mRNA relative to that for β-actin. Values are the mean ± S.E. of three experiments. * and **, p < 0.05 and p < 0.01 versus 0 troglitazone, respectively; †, p < 0.05 versus lean (+/+) ZDF rats; ††, p < 0.01 versus lean (+/+) ZDF rats. ACO, acyl-CoA oxidase; CPT-I, carnitine palmitoyltransferase I; GPAT, glycerol-3-phosphate acyltransferase; B-ACS, brain-type acyl-CoA synthetase; L-ACS, liver-type acyl-CoA synthetase.

Fig. 3. A, effect of troglitazone on insulin secretion in islets isolated from obese (fa/fa) (left panel) and lean (+/+) (right panel) ZDF rats cultured for 48 h with or without troglitazone. The islets were perfused with 3 mm glucose (unshaded zones) or with 23 mm glucose or 20 mm arginine (shaded zones) for 15 min. B, change in insulin production above the base-line level (3 mm glucose) during glucose or arginine stimulation. Values are the mean ± S.E. of three to four experiments. *, p < 0.01 versus 0 troglitazone. IRI, immunoreactive insulin.
We have found that troglitazone has a potent lipopinic effect on both the normal islets and fat-laden islets of obese ZDF rats with the OB-R mutation. In addition to bypassing the leptin receptor, the mechanism by which troglitazone induces lipopinia must differ somewhat from leptin-induced lipopinia in normal islets (6, 18) since, in contrast to leptin, there was no increase in mRNA of genes encoding two enzymes of fatty acid oxidation, acyl-CoA oxidase and carnitine palmitoyltransferase I. Nevertheless, oxidation of [3H]palmitate was increased by oxidation, acyl-CoA oxidase and carnitine palmitoyltransferase I, which is profoundly reduced by leptin (18), and of the brain and liver acyl-CoA synthetase isoforms was significantly decreased by troglitazone, as it is by leptin (6). Thus, troglitazone appears to exert a direct TG-lowering effect on islet cells. The 52% reduction in TG content still left the islets of the obese rats with a fat content 2.7 times that of untreated islets of lean rats (28.4 versus 10.4 ng/islet), but normal when corrected for the difference in islet size (Table II). The beta cell response to glucose rose >30-fold (Fig. 3B), whereas the arginine response doubled. This pattern of increased insulin secretion (Fig. 3A) resembles that of islets from fully compensated obese prediabtetic ZDF rats (17, 19). Thus, troglitazone produced a pattern of insulin secretion capable of compensating for insulin resistance. TG reduction in +/+ islets with a normal TG content and normal beta cell function did not alter beta cell function (Fig. 3).

While these correlative findings do not establish a cause-effect relationship between excessive islet fat content and beta cell dysfunction, they support the lipotoxicity hypothesis by providing yet another association between the two abnormalities. A possible causal link between fatty acids and β-cytotoxicity has been provided by the recent demonstration that fat-laden ZDF islets have a greatly increased nitric oxide response to long-chain free fatty acids (8, 9). Given the well established β-cytotoxic effects of excess nitric oxide (24–26), it is possible that the ameliorating effect on beta cell function of the reduction in islet fat content may be a consequence of decreased nitric oxide generation. The ability to manipulate pharmacologically the dynamics of intracellular lipid homeostasis in islets may herald a new strategy for designing drugs that prevent the diabetes of obesity.

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