Effect of Thiazolidinediones on Glucose and Fatty Acid Metabolism in Patients With Type 2 Diabetes

Guenther Boden, Peter Cheung, Maria Mozzoli, and Susan K. Fried

The current study aimed to compare the effects of treatment (2 months) with thiazolidinediones (TZDs) and placebo on glucose and fat metabolism in patients with type 2 diabetes (T2DM) in a crossover design. Eight patients received placebo (2 months) followed by TZD (2 months). Two-stage (1.5 and 6.0 pmol/kg min) hyperinsulinemic-euglycemic clamps were performed in all 8 patients pre- and post-placebo and post-TZD (post-placebo = pre-TZD). We determined rates of glucose disappearance (G_{Rd}), glycolysis (GLS), glycogen synthesis (GS) (all with 3-^{3}H glucose), carbohydrate (CHO) oxidation (indirect calorimetry), endogenous glucose production (EGP), free fatty acid (FFA) release ($^2H_5$ glycerol), and oxidation (indirect calorimetry) and re-esterification, as well as plasma adiponectin and leptin concentrations, and fat cell size and number (determined in upper thigh biopsy samples). Placebo treatment had no effects on any of the measured parameters. TZD treatment improved insulin-stimulated glucose uptake (ISGU) from 17.1 to 26.4 μmol/kg min ($P < .01$) and insulin-stimulated GS from 4.8 to 13.4 μmol/kg min ($P < 0.03$), potentiated insulin-induced suppression of lipolysis from 4.3 to 2.3 μmol/kg min ($P < .03$) and FFA re-esterification from 1.9 to 1.0 μmol/kg min ($P < .02$), increased plasma adiponectin levels from 2.7 to 7.2 μg/mL ($P < .05$), and decreased plasma leptin levels from 30.8 to 23.4 ng/mL ($P < .02$). In addition, TZD tended to increase the number of small adipocytes (<50 μm) in subcutaneous adipose tissue. We conclude that TZDs have multiple actions and that many, but perhaps not all, can be accounted for by their action on adipose tissue.

THIAZOLIDINEDIONES (TZDs) are a new class of drugs that lower blood sugar primarily by improving insulin sensitivity.[1] The mechanism by which they do this remains incompletely understood. It is known, however, that TZDs are peroxisome proliferator–activated receptor γ (PPARγ) agonists and that the binding affinity between TZDs and PPARγ correlates well with their insulin-sensitizing activity.[2,3] It is, therefore, generally assumed that TZDs exert their action through PPARγ. The finding that PPARγ is expressed at highest concentrations in adipose tissue and at much lower concentration in liver and muscle,[4,5] suggests that the primary action of TZDs is on adipose tissue. In support of this notion, it has been shown that TZDs play an important role in adipocyte development and in expression of several genes involved in regulation of adipocyte metabolism.[2,6] Moreover, treatment with TZDs lowers plasma levels of free fatty acids (FFA).[7,8] Inasmuch as elevated plasma levels of FFA inhibit insulin-stimulated glucose uptake (ISGU),[9] it has been suggested that at least part of the insulin-sensitizing action of TZDs might be mediated by their action on FFA metabolism.[10,11] To investigate this possibility, we have compared the effects of treatment with 2 TZDs on glucose and FFA metabolism in patients with type 2 diabetes.

MATERIALS AND METHODS

Subjects

We studied 8 patients with type 2 diabetes mellitus (T2DM) (Table 1). Two were treated with sulfonylureas; 2 were treated with sulfonylureas and metformin; 2 received sulfonylureas, metformin, and neutral protein hagedorin (NPH) insulin (10 to 25 U at bedtime); and 2 received insulin twice daily. These medications were withheld at least 72 hours before hospital admission but were otherwise continued throughout the studies. Informed written consent was obtained from all subjects after explanation of the nature, purpose, and potential risks of the studies. The study protocol was approved by the Institutional Review Board of Temple University Hospital.

Study Design

All patients were admitted to the General Clinical Research Center at Temple University Hospital the night before the study and underwent a 2-stage hyperinsulinemic clamp the following morning. Thereafter, they were discharged from the hospital. They were instructed to take 1 placebo tablet each day before breakfast; to continue their usual diet, exercise, and medication regimens; and to monitor glucose concentrations with a glucose monitor (twice weekly, 4 times per day). They returned to the hospital at biweekly intervals for inspection of the home glucose record. After 8 weeks, the patients were readmitted. At approximately 8 AM on the day of the study, a fat biopsy was obtained from the upper thigh followed by the 2-stage hyperinsulinemic clamp. After that, the patients were switched to TZD. After another 2 months, they were readmitted for another fat biopsy and hyperinsulinemic clamp. The initial 3 patients received troglitazone (600 mg/d), the other 5 patients received rosiglitazone (8 mg/d). The change was necessary because troglitazone was withdrawn from the US market after the study had begun.

Fat Biopsies

Under local anesthesia (1% lidocaine in a field block pattern) an incision (~1 inch) was made through the skin at the lateral aspect of the upper thigh and 100 to 200 mg of subcutaneous adipose tissue was mobilized and excised. One portion (50 mg) was placed into 5 mL of osmium fixation buffer (2% osmium in a 0.05 mol/L collidin buffer) and incubated for 5 days at 37°C. Another portion (~50 mg) was frozen at −15°C and was used for determination of lipid content (Folch extraction).

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Table 1. Study Subjects

	Pre-Placebo	Post-Placebo	Post-TZD
Gender (M/F)	5/3	5/3	5/3
Age (yr)	53 ± 3	—	—
Weight (kg)	94.7 ± 6.0	95.2 ± 6.2	95.5 ± 6.4
Body fat (kg)	34.1 ± 4.5	35.9 ± 3.7	34.0 ± 4.0
BMI (kg/m^2)	32.7 ± 1.7	32.7 ± 1.7	33.0 ± 1.8
Duration of diabetes (yr)	8.4 ± 2.1	—	—
HbA_{1c}	9.4 ± 0.8	8.8 ± 0.5	8.7 ± 0.9

Two-Stage Hyperinsulinemic Clamps

The studies began at approximately 8 AM after an overnight fast with the subjects reclining in bed. A short polyethylene catheter was inserted into an antecubital vein for infusions. Another catheter was placed into a contralateral forearm vein for blood sampling. This arm was wrapped with a heating blanket (70°C) to arterialize venous blood. Regular human insulin (Humulin R, Eli Lilly, Indianapolis, IN) was infused intravenously at a rate of 1.5 pmol/kg min (low-insulin clamp) for 2 hours starting at 0 minutes; after that, the rate of infusion was increased to 6 pmol/kg min (high-insulin clamp) for another 3 hours. During the low-insulin clamp, glucose concentrations decreased to approximately 7.5 mmol/L. During the high-insulin clamp, glucose decreased further to approximately 5.5 mmol/L and was then clamped at that concentration by a feedback controlled variable rate infusion of 20% glucose (Fig 1).

Measurements

Glucose turnover. Glucose turnover was determined with 3-^{3}H glucose. The tracer infusion was started 120 minutes before initiation of the clamp with a bolus adjusted to the degree of hyperglycemia (40 μCi × mmol/L glucose/5.5) followed by continuous infusion of 0.4 μCi/min. Glucose was isolated from blood for determination of 3-^{3}H glucose specific activity as described.[12] Changes in specific activity during hyperinsulinemia were avoided by adding 3-^{3}H glucose to the unlabeled glucose, which was infused at variable rates to maintain euglycemia.[13] Rates of total body glucose appearance (G_{Ra}) and disappearance (G_{Rd}) were calculated using Steele's equation for non–steady-state conditions.[14]

Glycolytic flux. Rates of glycolysis (GLS) were determined with 3-^{3}H glucose by dividing the whole-body $^3H_20$ production rate by the specific activity of its precursor, ie, plasma 3-^{3}H glucose.[15] To calculate the $^3H_20$ production rate, the time course of plasma $^3H_20$ concentration was plotted against time and the slope of the linear equation was estimated by the least square method.[15] The whole-body production rate was obtained by multiplying this value with the body water volume measured in each study subject assuming that total-body water occupies 73% of the fat-free mass.

Glycogen synthesis. Glycogen synthesis (GS) rates were obtained by subtracting rates of GLS from rates of G_{Rd}.

Endogenous glucose production. Endogenous glucose production (EGP) was determined by subtracting the rate of glucose infusion needed to maintain euglycemia (GIR) from the rate of G_{Ra}.

Carbohydrate and fat oxidation. Carbohydrate (CHO) and fat oxidation rates were determined by indirect calorimetry with a metabolic measurement cart (Deltatrac II, Sensormedics, Yorba Linda, CA) before and at 30-minute intervals during the clamps as described previously.[16] Rates of protein oxidation were estimated from urinary nitrogen excretion after correction for changes in urea nitrogen pool size.[17] Rates of protein were used to determine the nonprotein respiratory quotient (nPRQ). Rates of CHO and FFA oxidation were determined with tables of Lusk, which are based on a nPRQ of 0.707 for 100% fat oxidation and 1.00 for 100% carbohydrate oxidation.

Glycerol turnover. [$^2H_5$] glycerol (98 atom percent deuterium, Tracer Technologies, Somerville, MA) dissolved in normal saline was infused from −90 until 300 minutes starting with a priming dose of 1.6 μmol/kg followed by a continuous infusion of 0.11 $\mu mol \cdot kg^{-1} \cdot min^{-1}$. Blood for determination of [$^2H_5$] glycerol enrichment was collected at 30- to 60-minute intervals from before the start (−90 minutes) until the end (300 minutes) of the clamp. Plasma was immediately separated at 4°C and stored at −20°C until analyzed. The trimethylsilyl derivative of glycerol was prepared as described previously.[18] [$^2H_5$] glycerol enrichment was determined by gas chromatography–mass spectrometry (Hewlett Packard, Palo Alto, CA, 5989 MS, 5890 GC) with the use of electron impact ionization and monitoring of ions at m/e 205 and 208.

Calculation. Because [$^2H_5$] glycerol enrichments were stable before (−30 to 0 minutes) and at the end of the clamp, the R_a of glycerol was calculated according to the steady-state equation of Steele corrected for the amount of exogenously infused stable isotope[19]: Glycerol $R_a = (IE_{inf}/IE_{pla} - 1) \times F$, where R_a is the rate of appearance of glycerol ($\mu mol \cdot kg^{-1} \cdot min^{-1}$), IE_{inf} is the isotope enrichment of the infusate (APE), IE_{pla} is the isotope enrichment of the plasma at isotopic equilibrium and F is the isotope rate of infusion ($\mu mol \cdot kg^{-1} \cdot min^{-1}$).

Glycerol $R_a \times 3$ was assumed to reflect rates of whole-body lipolysis.

FFA re-esterification. The difference between the rate of lipolysis (glycerol $R_a \times 3$) and the rate of FA oxidation provides an index of the

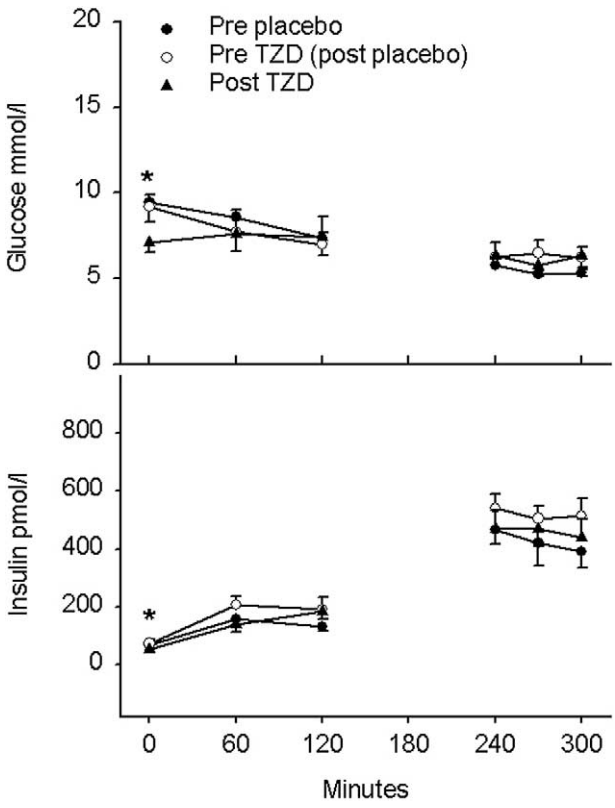

Fig 1. Plasma glucose and serum insulin concentrations before (0 min), and during low-insulin (1.5 pmol/kg min) (0-120 min) and high-insulin (6 pmol/kg min) (240-300 min) infusions in 8 patients with T2DM pre- and post-placebo (pre-TZD) and post-TZD therapy. Values are mean ± SE. *$P < .01$ pre- *v* post-TZD.

rate of total FFA re-esterification because FFA re-esterification is ultimately the fate of all nonoxidative FFAs.[18] Thus, FFA re-esterification = glycerol $R_a \times 3$ − FFA oxidation

Fat cell size. Mean fat cell size was determined as described by Hirsch and Gallian,[20] with minor modifications. After fixation in osmium for 5 days at 37°C, fat cells were filtered through a 250-μm nylon mesh, captured on a 25-μm mesh, and washed thoroughly with 0.9% saline. Fixed fat cells were resuspended in 10% glycerol, aliquots were placed on a slide, and the diameter of 500 cells were measured on a calibrated ocular micrometer (in 5-μm divisions). Fixed fat cells were differentiated from occasional debris by their rounded edges. The mean fat cell diameter and mean fat cell weight (μg of lipid per cell) were calculated as described previous.[22] Histograms of fat cell size distribution for each subject were examined for individual shifts in each size class.

Analytical Procedures

Plasma glucose was measured with a glucose analyzer (YSI, Yellow Springs, OH). Insulin was determined in serum after protein precipitation with polyethylene glycol (PEG) by radioimmunoassay with a specific antibody that crossreacts only minimally (<0.2%) with proinsulin (Linco, St Charles, MO). Adiponectin and leptin were determined by radioimmunoassay with kits from (Linco). Total plasma FFA was determined enzymatically in plasma containing EDTA and the lipoprotein lipase inhibitor paroxam (0.25 mg/mL blood, Sigma, St Louis, MO) with a kit from Wako (Richmond, VA).

Statistical Analysis

All data are expressed as the mean ± SE. Statistical significance was assessed using analysis of variance (ANOVA) with repeated measures and paired Student's 2-tailed *t* test when applicable. No significant differences were found comparing glucose and lipid turnover, EGP, adiponectin, and leptin data obtained from all 8 patients with those obtained from the 5 patients on rosiglitazone. Also, there were no significant differences in values for plasma glucose, FFA, G_{Rd}, GS, GLS, CHO, and FFA oxidation, lipolysis, and FFA re-esterification comparing troglitazone- with rosiglitazone-treated patients.

RESULTS

Glucose and Insulin

Basal plasma glucose concentrations were the same before and after placebo (10.1 ± 0.6 *v* 9.5 ± 0.9 mmol/L), but basal glucose concentrations were significantly lower after than before TZD (9.5 ± 0.9 *v* 7.3 ± 0.7 mmol/l, $P < .01$) (Fig 1).

At the end of the low-insulin clamp (at 120 minutes), plasma glucose levels were 7.0 ± 0.4, 6.8 ± 0.6 and 7.4 ± 1.3 mmol/L, respectively (not significant [NS]) pre- and post-placebo and post-TZD.

During the last hour of the high-insulin clamp (240 to 300 minutes), plasma glucose concentrations were 5.9 ± 0.8, 6.3 ± 0.7 and 6.2 ± 0.6 mmol/L, respectively (NS).

Basal serum insulin concentrations were similar pre- and post-placebo but were lower post-TZD than pre-TZD (75 ± 15 *v* 52 ± 9 pmol/L, $P < .05$). At the end of the low-insulin clamp, insulin levels were 158 ± 19, 219 ± 37 and 182 ± 24 pmol/L, respectively (NS) (Fig 1).

During the last hour of the high-insulin clamp, insulin levels were 419 ± 51, 520 ± 46, and 460 ± 60 pmol/L, respectively (NS).

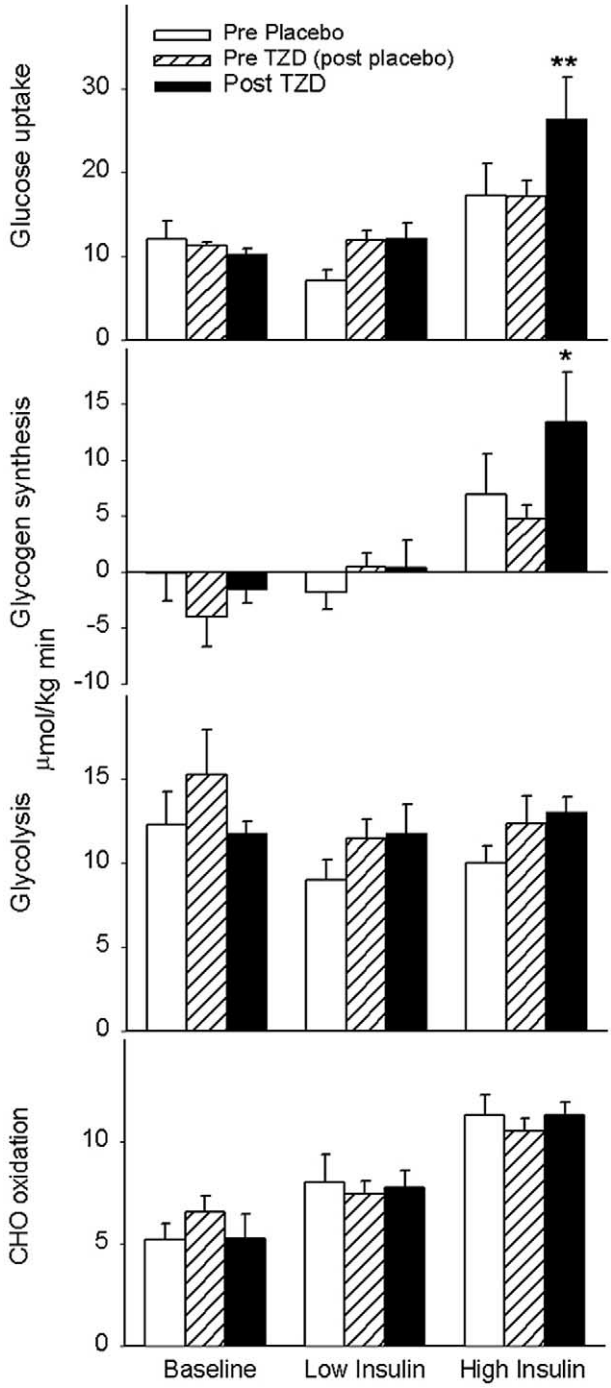

Fig 2. Rates of glucose uptake (G_{Rd}), glycogen synthesis (GS), glycolysis (GLS), and CHO oxidation (COX) before (0 min) and during low- and high-insulin infusions in 8 patients with T2DM pre- and post-placebo and post-TZD treatment. *$P < .03$; **$P < .01$ pre- *v* post-TZD.

G_{Rd}, GS, GLS, and CHO Oxidation

Effect of placebo. Placebo therapy (pre- *v* post-placebo) had no effects on any of the measured parameters during low- or high-insulin clamps (Fig 2).

Effects of TZDs. TZDs had no significant effects on basal rates nor on low-insulin–stimulated rates of G_{Rd}, GS, GLS, or CHO oxidation.

High-insulin–stimulated G_{Rd} was higher after TZD than after placebo (26.4 ± 5.0 *v* 17.1 ± 1.9 μmol/kg min, $P < .01$). Similarly, high-insulin–stimulated GS was higher after TZD than after placebo (13.4 ± 4.5 *v* 4.8 ± 1.3 μmol/kg min, $P < .03$). TZDs had no effect on GLS or on insulin-stimulated CHO oxidation (Fig 2).

Lipolysis, FFA Oxidation, Re-esterification, and Plasma Levels

Effect of Placebo. Placebo treatment (pre- *v* post-placebo) had no significant effects on lipolysis, FFA oxidation, re-esterification, or plasma levels during low or high-insulin infusions (Fig 3).

Effect of TZDs. Basal rates of lipolysis (7.7 *v* 6.8 μmol/kg min), FFA re-esterification (5.0 *v* 4.7 μmol/kg min), FFA oxidation (2.9 *v* 3.3 μmol/kg min), and plasma FFA levels (700 *v* 595 μmol/L) tended to be lower in response to TZDs than to placebo, but only the difference in FFA oxidation reached statistical significance ($P < .05$) (Fig 3).

Low insulin sharply reduced rates of lipolysis, re-esterification, and plasma FFA levels. TZDs compared to placebo potentiated the insulin effects on lipolysis (3.4 *v* 4.2 μmol/kg min, $P < .05$).

High insulin did not significantly depress rates of lipolysis and FFA re-esterification beyond the levels reached with low insulin. TZD compared to placebo potentiated the insulin effect on lipolysis (4.3 *v* 2.3 μmol/kg min, $P < .03$), FFA re-esterification (1.9 *v* 1.0 μmol/kg min, $P < .02$), and plasma FFA levels (207 *v* 98 μmol/L, $P < .02$). High-insulin infusion suppressed fat oxidation more than low insulin ($P < .001$), but TZDs had no further potentiating effects on this action.

EGP

There was no placebo effect on EGP nor was there a TZD effect on basal EGP (10.9 ± 1.1 *v* 9.9 ± 1.0 μmol/kg min, NS). The low-insulin infusion suppressed EGP by approximately 60% (from 10.9 to 4.5 μmol/kg min), while the high-insulin infusion suppressed EGP by approximately 80% (from 10.9 to 2 μmol/kg min), demonstrating the exquisite sensitivity of EGP to even small increases in serum insulin concentration. TZDs, however, did not potentiate these actions of insulin.

Adiponectin and Leptin

The was no placebo effect on plasma adiponectin levels, which were severely depressed compared to levels in 10 non-diabetic non-obese controls who were not part of this study (2.7 ± 0.4 *v* 18.2 ± 3.1 μg/mL). TZD therapy increased basal adiponectin levels approximately 2.5-fold (from 2.7 ± 0.4 to 7.2 ± 2.1 μg/mL, $P < .05$). Neither low- or high-insulin infusions affected plasma adiponectin levels.

Basal leptin levels were significantly lower after than before TZD treatment (23.4 ± 7.6 *v* 30.8 ± 10.9 ng/mL, $P < .02$), whereas placebo treatment had no effect (Fig 4).

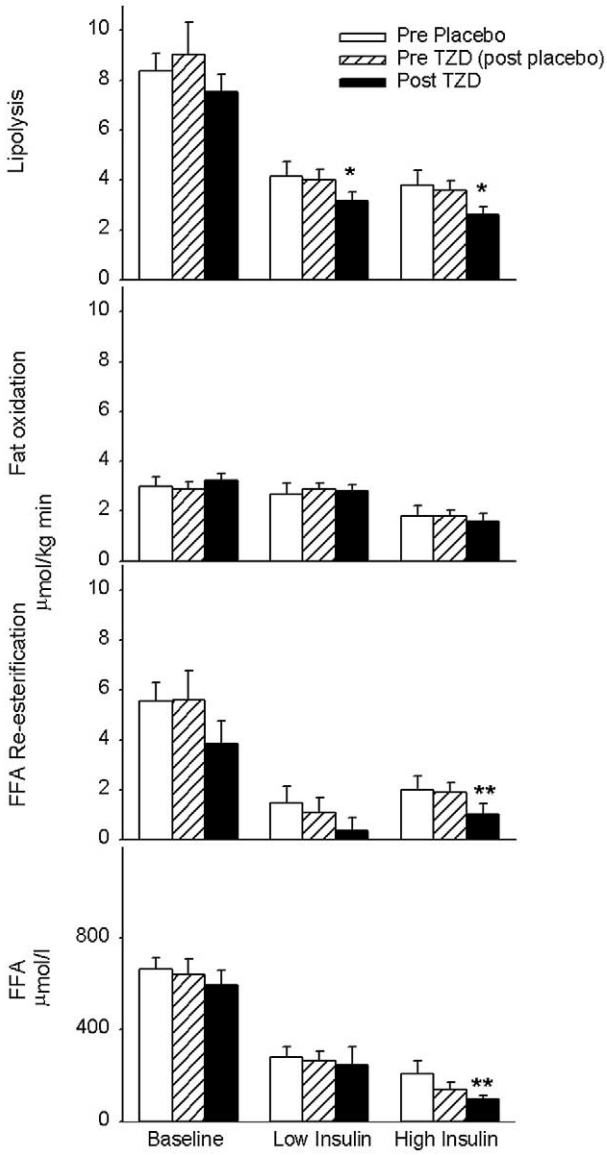

Fig 3. Rates of lipolysis, fat oxidation (FOX), FFA re-esterification, and plasma FFA levels before and during low- and high-insulin infusions in 8 patients with T2DM pre- and post-placebo (pre-TZD) and post-TZD. *$P < .05$; **$P < .02$ comparing pre- and post-TZD.

Fat Cell Size

The average fat cell size was not changed after TZD treatment (0.65 ± 0.06 *v* 0.59 ± 0.07 μg of lipid per cell). However, there seemed to be more smaller fat cells (<50 μm or 0.25 μg lipid per cell) after than before TZD treatment, although the difference was not statistically significant (Fig 5).

DISCUSSION

In this placebo controlled study, we confirmed the finding by others that TZDs potentiated ISGU.[1,22] Extending these previous reports, the current study showed for the first time that TZDs also potentiated insulin-stimulated GS. In fact, all of the

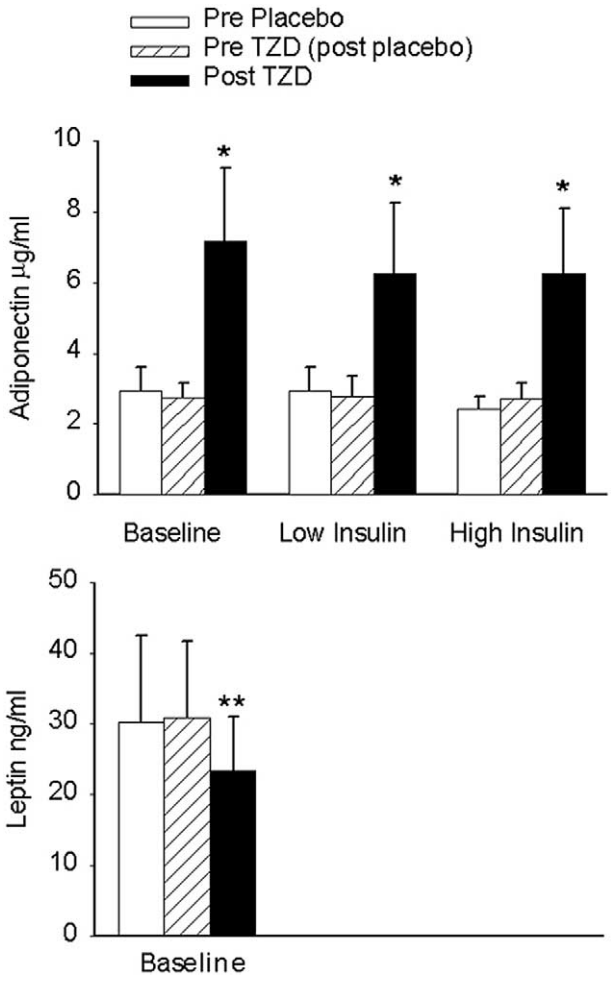

Fig 4. Plasma adiponectin levels before and during low- and high-insulin infusions in 8 patients with T2DM pre- and post-placebo (pre-TZD) and post-TZD. Plasma leptin levels were determined only during basal conditions. *$P < .05$; **$P < .02$ pre- *v* post-TZD.

TZD-enhanced glucose uptake went into GS. There is evidence that insulin increases GS primarily by stimulating glucose uptake[23,24] and that TZDs improve insulin signaling.[25] We, therefore, interpret our findings to suggest that TZDs increased ISGU and GS by improving insulin signaling through the insulin receptor, insulin receptor substrate-1/phosphoinositide 3 (IRS-1/PI3) kinase pathway (which is common to insulin action on both glucose uptake and GS).[26]

Since most (~80%) of ISGU occurs in muscle,[27] it follows that TZDs should also improve insulin action in muscle. It remains uncertain, however, whether this occurs by direct action on muscle or indirectly via action on adipose tissue. The current study provided evidence pointing to adipose tissue as a major target for TZDs. Noteworthy, TZDs potentiated insulin-induced suppression of lipolysis, plasma FFA levels and FFA re-esterification. These effects were only found in TZD-treated but not in placebo-treated patients and, in contrast to the effects on ISGU and GS, were also seen during the low-insulin infusions. This is consistent with greater insulin sensitivity of adipose tissue than muscle.[28] Two reports have recently appeared which failed to detect significant TZD effects on insulin suppression of glycerol turnover; however, in one of these studies, troglitazone potentiated insulin inhibition of palmitate release,[29] while in the other study, rosiglitazone potentiated the insulin suppression of glycerol release from subcutaneous fat (determined by microdialysis).[30] A third study[31] reported a small, nonsignificant decrease in palmitate turnover in response to high-insulin infusions (40 and 160 mU/m^2 min). However, because of intracellular fatty acid re-esterification, use of palmitate release frequently underestimates lipolysis, which could have contributed to the negative results. Thus, taken together, the available literature suggests that TZDs potentiate insulin action on FFA release from adipocytes.

Enhanced insulin-induced inhibition of lipolysis should contribute to a decrease in plasma FFA levels. Indeed, several studies have shown that treatment with TZDs was associated with a decrease in plasma FFAs.[7,8,22,30,31] In the current study, the decrease in basal plasma FFA levels from 700 to 595 μmol/L was not statistically significant, possibly because of the relatively small number of study participants and also because the length of TZD treatment (2 months) was shorter than that in other studies. Lowering of plasma FFA has been demonstrated to improve ISGU.[32] Newer data have suggested that the mechanism by which FFAs produce insulin resistance involves intramyocellular re-esterification of FFAs and accumulation of long-chain acyl coenzyme A and diacylglycerol and activation of protein kinase C.[33,34] The results of this study showing a decrease in FFA release and re-esterification are therefore compatible with the hypothesis that TZDs improve insulin action by lowering plasma FFA turnover.

TZD treatment was also associated with an approximately 2.5-fold increase in plasma adiponectin and a 30% decrease in plasma leptin levels. Both adiponectin and leptin are produced and released from fully differentiated adipocytes.[35] TZDs have been shown to increase expression of adiponectin and to raise plasma adiponectin levels.[36-38] In two animal models of T2DM, characterized by obesity and insulin resistance (db/db and KKA

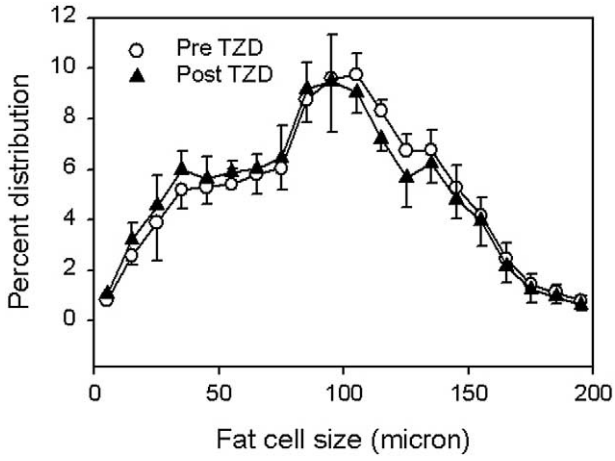

Fig 5. Distribution of fat cells according to size (in μm) pre- and post-TZD treatment in 8 patients with T2DM. Shown are smoothed averages.

mice), infusion of adiponectin, which restored the initially depressed levels, lowered blood glucose and plasma FFA levels and intramyocellular triglyceride (IMCL-TG) and increased fat oxidation (FOX).[36] These data suggested that adiponectin acted on skeletal muscle where it increased influx and oxidation of FFA resulting in a decrease of IMCL-TG.[38] The latter has been shown to correlate closely with ISGU.[39] In another study in rats, adiponectin inhibited hepatic glucose production.[40] Thus, it is possible that the rise in plasma adiponectin levels in our TZD-treated patients may have contributed to their improved insulin action on glucose uptake.[41]

PPARγ agonists including TZDs have been shown to transcriptionally inhibit leptin expression.[42,43] It is difficult to know, however, whether the decrease in plasma leptin levels in our study contributed to the improved ISGU. On one hand, several studies found that leptin inhibited insulin action in liver and fat cells,[44,45] while others found that leptin decreased FFA transport into cells and improved insulin sensitivity.[46,47]

TZDs are known to promote adipocyte differentiation[6] and both troglitazone and rosiglitazone have been found to increase the number of smaller more insulin sensitive fat cells in white adipose tissue of obese Zucker rats.[48,49] In the current study, we did observed a trend towards smaller fat cells. However, there was considerable interindividual variability, which could have been due in part to the fact that once formed, small, insulin-sensitive adipocytes can rapidly fill, resulting in no change in mean fat cell size at a given time point.

In addition to these effects of TZDs on adipose tissue other effects have been reported, including the stimulation of FAD/CD36[50] and the redistribution of fat from the subcutaneous to the visceral depots,[48] all of which may have contributed to the increase in ISGU. Lastly, there is evidence to suggest that TZDs may have direct actions on skeletal muscle.[51,52]

Summary

We have treated 8 patients with T2DM with placebo (for 2 months) and with TZDs (for 2 months). Placebo treatment had no effect on any of the measured parameters. TZDs (troglitazone and rosiglitazone) improved ISGU and insulin-stimulated GS, suppressed lipolysis and FFA re-esterification, increased plasma adiponectin levels, and decreased plasma leptin levels. In addition, it tended to increase the number of small adipocytes. We conclude that TZDs have many actions and that much (but perhaps not all) of the improvement in ISGU can be accounted for by TZD actions on adipose tissue.

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