Thiazolidinediones (TZDs) are a new class of insulin-sensitizing drugs. To explore how and in which tissues they improve insulin action, we obtained fat and muscle biopsies from eight patients with type 2 diabetes before and 2 months after treatment with rosiglitazone (n = 5) or troglitazone (n = 3). TZD treatment was associated with a coordinated upregulation in the expression of genes and synthesis of proteins involved in fatty acid uptake, binding, \( \beta \)-oxidation and electron transport, and oxidative phosphorylation in subcutaneous fat but not in skeletal muscle. These changes were accompanied by a 13% increase in total body fat oxidation, a 20% decrease in plasma free fatty acid levels, and a 46% increase in insulin-stimulated glucose uptake. We conclude that TZDs induced a coordinated stimulation of fatty acid uptake, oxidation, and oxidative phosphorylation in fat of diabetic patients and thus may have corrected, at least partially, a recently recognized defect in patients with type 2 diabetes consisting of reduced expression of genes related to oxidative metabolism and mitochondrial function. *Diabetes* 54: 880–885, 2005

**RESEARCH DESIGN AND METHODS**

We studied eight patients with type 2 diabetes (Table 1). Two were treated with sulfonylureas, two with sulfonylureas and metformin, two received sulfonylureas, metformin, and NPH insulin (10–25 units at bedtime), and two received insulin twice daily. These medications were withheld at least 72 h 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.

All patients were admitted to the general clinical research center at Temple University Hospital the night before the study. At ~8:00 A.M. on the day of the study, a fat and muscle biopsy was obtained from the subcutaneous fat of the upper thigh and the lateral vastus muscle, respectively, and rates of fat and oxidative phosphorylation in subcutaneous adipose tissue were mobilized and excised. Through the same incision, biopsies (~1 inch) were made through the skin at the lateral aspect of the upper thigh (~15 cm above the patella) and ~200 mg of subcutaneous adipose tissue was mobilized and excised. Through the same incision, biopsies (~100 mg) were obtained from the lateral aspect of the vastus lateralis muscle. The excised fat and muscle samples were dropped immediately into isopentane, kept at its freezing point (~100°C) by liquid nitrogen, and stored at ~80°C until used for RNA extraction.

**Array hybridization.** The cDNA filter arrays were prepared by the Wistar Institute Genomics facility (available at www.wistar.upenn.edu/genomics and ref. 16). A 7.5 × 11.5-cm nylon filter (HA-04) carrying a total of 9,600 probes was used. Sequence-verified clones were purchased from Research Genetics (Huntsville, AL). Total RNA was isolated from the aqueous phase by isopropanol precipitation after homogenization in phenol-phenyldimethylcarboxylate
TABLE 1

Characteristics of study subjects

<table>
<thead>
<tr>
<th></th>
<th>Pre-TZD</th>
<th>Post-TZD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>5/3</td>
<td>5/3</td>
</tr>
<tr>
<td>Age (years)</td>
<td>53 ± 3</td>
<td>—</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>95.2 ± 6.2</td>
<td>95.5 ± 6.4</td>
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<tr>
<td>Body fat (kg)</td>
<td>35.9 ± 3.7</td>
<td>34.0 ± 4.0</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>32.7 ± 1.7</td>
<td>33.0 ± 1.8</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>8.4 ± 2.1</td>
<td>—</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.8 ± 0.5</td>
<td>8.7 ± 0.9</td>
</tr>
</tbody>
</table>

(Tri-Reagent Molecular Research Center, Cincinnati, OH). Total RNA samples were amplified (aRNA) using a modified T7 protocol for fat (available at http://cmgm.Stanford.edu/pbrown/protocols) and muscle (MessageAmp; Ambion, Austin, TX). Each sample (total 32) was hybridized to a single array, as materials were limited. The average relative error between arrays was <5% when they were hybridized in triplicate under our standard conditions (data not shown). All hybridization steps were carried out in a hybridization oven with constant rotation; prehybridization 1 was in 3 ml of MicroHyb (Research Genetics) and 1 µg/ml of salmon sperm DNA for 1 h at 42°C then removed. A total of 2.5 ml of MicroHyb with 1 µg/ml of denatured human Cot-1 DNA and 1 µg/ml of PolyD (Prehyb2) was added and filters incubated for 2 h at 42°C. The aRNA target was labeled with [33P] (2000-5000 Ci/mmol; Amersham Pharmacia Biotech) using reverse transcriptase. The denatured [33P] target (0.5 µg) was added to Prehyb 2, and filters were incubated at 42°C for 18 h. Filters were washed twice in 2× SSC/0.1% SDS at 60°C then all filters exposed to a phoshorimager screen for 6 and 14 days for fat and muscle, respectively. Screens were scanned at 50 µ resolution in a Storm PhosphorImager and visualized using ImageQuant (Molecular Dynamics).

Array analysis. Arrays were analyzed with ImaGene 5.1 (Biodiscovery, El Segundo, CA), and the median pixel value was calculated for each spot after subtraction of the local background. The calculated values were exported to Microsoft Excel and the values for each spot normalized by dividing the signal reference, which is the calculated median pixel value for all 9,000 background-subtracted spots on the array (normalized median density). The dynamic range of array signals was on average 7–10,000 (16). The detection limit for these conditions and arrays was calibrated by quantitative PCR. Standard curves were generated using known amounts of a purified gene-specific template. PCR values from the RNA were mapped to that standard curve. The lowest detectable signal that was considered reliable was a normalized median density of 100. All hybridization steps were carried out in a hybridization oven with constant rotation; prehybridization 1 was in 3 ml of MicroHyb (Research Genetics) and 1 µg/ml of salmon sperm DNA for 1 h at 42°C then removed. A total of 2.5 ml of MicroHyb with 1 µg/ml of denatured human Cot-1 DNA and 1 µg/ml of PolyD (Prehyb2) was added and filters incubated for 2 h at 42°C. The aRNA target was labeled with [33P] (2000-5000 Ci/mmol; Amersham Pharmacia Biotech) using reverse transcriptase. The denatured [33P] target (0.5 µg) was added to Prehyb 2, and filters were incubated at 42°C for 18 h. Filters were washed twice in 2× SSC/0.1% SDS, one time in 0.5× SSC/1% SDS, and one time in 0.1× SSC/0.5% SDS each for 30’ at 55°C. The fat filters received an additional wash of 0.1× SSC/0.1% SDS at 60°C then all filters exposed to a phoshorimager screen for 6 and 14 days for fat and muscle, respectively. Screens were scanned at 50 µ resolution in a Storm PhosphorImager and visualized using ImageQuant (Molecular Dynamics).

RESULTS

Adipose tissue

Effect of TZDs on gene expression. TZD treatment of the eight patients with type 2 diabetes (Table 1) was associated with a statistically significant twofold or greater increase in the expression (by microarray) of 107 gene sequences and a twofold or greater decrease in the expression of six gene sequences (online appendix [available at http://diabetes.diabetesjournals.org]). Of those that had increased, 95 included genes related to carbohydrate or protein metabolism, signal transduction, cell growth and development, transcription factors, nuclear proteins, and others (online appendix), and 12 gene sequences were functionally related to either fatty acid uptake and binding (fatty acid translocase/CD36, fatty acid binding proteins 4 and 6 and fatty acid CoA ligase) or β-oxidation (acyl-CoA dehydrogenase and acetyl-CoA acyltransferase) or mito-

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>CD36</td>
<td>N39161 Forward 5’-AGGTCAACCTATTTGGTCAAGC-3’ Reverse 5’-AGATCATTTCTATCAGGCCAAGGA-3’</td>
</tr>
<tr>
<td>FABP4</td>
<td>N92901 Forward 5’-TTGACGAGGAATGAGTCAA-3’ Reverse 5’-TCTTCTAAAACCTGCTGGAAGT-3’</td>
</tr>
<tr>
<td>FACL3</td>
<td>AA424965 Forward 5’-GTGTCACAATGGGGTACT-3’ Reverse 5’-CACAGCTTCTCCCAAG-3’</td>
</tr>
<tr>
<td>ACAT1</td>
<td>AB71165 Forward 5’-AGGGAAAGGTCTCAAT-3’ Reverse 5’-GTCATAAGCAACAACTCCTG-3’</td>
</tr>
<tr>
<td>ACADM</td>
<td>N70794 Forward 5’-TTGTGCAACAGGTCTTGGAGAT-3’ Reverse 5’-GTTGTTTCAGGGGCTAATAAAGTCT-3’</td>
</tr>
<tr>
<td>CYS</td>
<td>R52654 Forward 5’-GTCACACCGTTGAAAAG-3’ Reverse 5’-AGATAAAGTATTTAGTCTGCC-3’</td>
</tr>
<tr>
<td>NDUF2</td>
<td>BA94779 Forward 5’-AACCAGCAGACCTTACG-3’ Reverse 5’-CTCACAGCATAGCCGTGTC-3’</td>
</tr>
<tr>
<td>ATP5I</td>
<td>AA504465 Forward 5’-TGCTGATTTCCGTCAGGC-3’ Reverse 5’-GGGAAAATGTTTCTGCTG-3’</td>
</tr>
</tbody>
</table>

Western blot. Fat tissues were homogenized in T-Per tissue protein extraction reagent and the hald protease inhibitor cocktail for 60 s (Pierce, Rockford, IL). The soluble proteins were collected after centrifugation at 10,000g for 15 min at 4°C. Protein concentrations were determined with Coomassie plus protein assay reagent using BSA as standard.
Mitochondrial electron transport and oxidative phosphorylation (cytochrome c, NADH dehydrogenase and ATPase) (Table 3). Individual changes in the expression of eight of those gene sequences are shown in Fig. 1 (upper panels).

Real time RT-PCR confirmed the differential induction of these eight gene sequences (Fig. 1, lower panels). There were no consistent differences between troglitazone- and rosiglitazone-induced changes (Fig. 1). The results of both were therefore combined for statistical evaluation (Table 3).

### Effect on FAT/CD36, ACADM, and cytochrome C protein.
Protein mass was determined with immunoblots in fat biopsies for three proteins for which antibodies were commercially available. FAT/CD36 mass increased 3.8-fold in four patients (2.2-, 4.1-, 7.7-, and 1.2-fold, respectively; two treated with troglitazone and two with rosiglitazone) after TZD treatment ($P < 0.03$) (Fig. 2). ACADM mass increased 2.0-fold ($P < 0.05$, Fig. 2) in fat from three patients (1.7-, 1.4-, and 3.0-fold, respectively; one treated with troglitazone and two with rosiglitazone). Cytochrome C (HCS) mass increased 5.7-fold in fat from two patients (8.4- and 3.0-fold, respectively) treated with rosiglitazone.

### Skeletal muscle
#### Effects of TZDs on gene expression.
None of the gene sequences whose expression was increased in fat was significantly induced in skeletal muscle. Expression of FAT/CD36, in fact, was significantly reduced (Table 3).

#### Plasma FFA, FOX, and glucose uptake.
As reported previously, TZD treatment in these patients resulted in a 20% lowering of basal plasma FFA levels ($P < 0.02$), a 13% increase in basal total body FOX ($P < 0.05$), and a 46%...
increase in insulin-stimulated glucose uptake ($P < 0.01$) (Table 4) (14).

**DISCUSSION**

To explore where and how TZDs improve insulin sensitivity, we examined gene expression in subcutaneous fat and skeletal muscle biopsies of eight patients with type 2 diabetes. The key findings were that 2 months of treatment with TZDs was associated with a coordinated twofold or greater increased expression in fat but not in skeletal muscle of a number of genes coding for proteins related to uptake, binding, β-oxidation, electron transport, and oxidative phosphorylation of fatty acids. Specifically, we found significantly increased expression of FAT/CD36, fatty acid binding proteins 4 and 6 (proteins involved in high-affinity, saturable, fatty acid transport and utilization [17]) of fatty acid CoA ligase (a protein that makes fatty acid transport unidirectional by converting fatty acids, which have crossed the cell membrane, into acyl-CoA derivatives that prevent their efflux out of cells) (18), ACADM and acetyl-CoA acetyltransferase (the first and the last of the 4 β-oxidation enzymes), HCS, the β-subcomplex of NADH-dehydrogenase, and the F0 and F1 complexes of ATPase (sequences that are part of the electron transport chain and of oxidative phosphorylation). TZD induction of these gene sequences was confirmed by real-time RT-PCR (Fig. 1). These results confirm, in human fat, previous findings by others in white adipose tissue of rodents that have shown that TZDs induced expression of FAT/CD36, fatty acid binding protein, fatty acid CoA ligase, and ACADM (12,18,19). The current study is, as far as we know, the first to demonstrate that TZDs induced expression of gene sequences that are part of the electron transport chain and of oxidative phosphorylation and, more importantly, that they promote a coordinated up-regulation of all these functionally related genes in human adipose tissue.

TZDs had no significant effects in skeletal muscle on expression of any of these gene sequences. This suggested that these PPARγ agonists had little if any direct action on fatty acid uptake and oxidation in skeletal muscle and is in line with the observation that PPARγ is expressed very little in muscle (4,5). Nevertheless, direct effects of TZDs on skeletal muscle have been reported (20) and cannot be completely ruled out with this study because it is possible that even smaller changes than those we considered significant in the expression of several functionally related genes can have biological effects (21,22) and because of the relatively short period of treatment (2 months) and the fact that most patients were on other antidiabetic medications.

The TZD-mediated induction of expression of genes related to FFA uptake, β-oxidation, and electron transport (FAT/CD36, ACADM, and HCS) in fat was accompanied by increases of comparable magnitude of the respective proteins.

It is currently believed that in adipose tissue, PPARγ is critically important for adipocyte differentiation and fat storage, whereas in the liver, PPARα plays an important role in fatty acid oxidation (23). Our results suggest that this concept should be expanded to include TZD stimulation of FOX in adipose tissue. How TZDs stimulated FOX, however, is not clear. It is possible that TZDs induced

![FIG. 2. FAT/CD36 and acyl-CoA dehydrogenase (ACADM) proteins in subcutaneous fat from patients with type 2 diabetes before and after 2 months of treatment with TZDs. A: Individual FAT/CD4 immunoblots of four patients. B: Densitometric ratios of FAT to CD36 protein normalized to arbitrary units by assigning the value 1 to the 36-protein ratio before treatment. C: Individual ACADM immunoblots of three patients. D: Densitometric ratios of ACADM protein normalized to arbitrary units by assigning the value 1 to the ACADM ratio before treatment. E: Individual cytochrome C (HCS) immunoblots of two patients. F: densitometric ratios of HCS protein normalized with β-actin. Shown are means ± SE ($n = 3$).](Image)

| Table 4: Effects of TZDs on basal plasma FFA levels, rates of FOX, and insulin-stimulated rates of glucose disappearance |
|-------------------|-----------------|----------------|
| TZD               | Pre             | Post            | P       |
| Plasma FFA ($\mu$mol/l) | 721 ± 51         | 574 ± 57         | <0.02   |
| FOX ($\mu$mol·kg$^{-1}$·min$^{-1}$) | 2.87 ± 0.27  | 3.25 ± 0.28   | <0.05   |
| $R_d$ ($\mu$mol·kg$^{-1}$·min$^{-1}$) | 17.1 ± 1.9 | 26.4 ± 5.0   | <0.01   |

Data are means ± SE ($n = 8$). $R_d$ (rate of glucose disappearance) at the end of a 4-h hyperinsulinemic-euglycemic clamp. FOX was determined, and hyperinsulinemic clamps were performed as described (14,15). From Boden et al. (14).
expression of all these FFA uptake and oxidation-related genes simultaneously. It appears more likely, however, that the primary event was induction of the transport-related genes, particularly FAT/CD36 (24,25), and that the increased fatty acid flux then enhanced expression of the oxidation-related genes. In support of this notion, Ibrahim et al. (25) have shown that muscle targeted overexpression of FAT/CD36 produced a large (five- to sixfold) increase in FOX. This indicated not only a tight coupling between FFA uptake and oxidation but also that FFA uptake was rate limiting (25).

The demonstrated TZD-induced induction of genes involved in fatty acid uptake and oxidation in adipose tissue raises several questions. First, to what extent could it account for the observed increase in total body FOX? Second, to what extent could it account for the decrease in plasma FFA? And third, to what extent could it account for the improvement in insulin sensitivity in these patients (14) (Table 4)? With respect to FOX, TZDs have been shown to not affect FFA uptake and oxidation by the liver (19). The current study showed that they also have little or no effect on muscle. This leaves fat, where a 2- to 2.5-fold increase in fat oxidation (i.e., an increase similar to the observed change in gene expression) could have been sufficient to explain the observed 13% increase in total body FOX. This is based on the following consideration. In vitro O2 consumption by adipose tissue from obese adults has been determined to be 0.39 ml·kg−1·min−1 (26). Thus, O2 consumption by fat in our patients can be estimated to have been ~14 ml O2/min (0.39 ml O2·kg−1·min−1 × 35.87 kg) or ~5% of their total O2 consumption, which was 273 ml/min. These in vitro results, however, might not accurately reflect O2 in vivo adipocyte O2 consumption.

With respect to plasma FFA, our results suggested that it was the TZD-mediated increase in adipose tissue FOX that was responsible for the observed 20% decrease in basal plasma FFA levels (14). The reason for this is that there is usually a close and positive correlation between plasma FFA levels and FOX. Therefore, had the decrease in plasma FFA been the primary TZD effect, FOX should have decreased. In addition, TZDs could have lowered FFAs by increased FFA esterification via increased glyceroenogenesis (by induction of PEPCK) (27).

The results of the current and other studies suggest that TZDs improve insulin sensitivity directly and by several mechanisms. First, there is strong evidence that TZDs increase insulin sensitivity by lowering plasma FFAs (7,12). These FFA-induced changes in insulin sensitivity in muscle are now recognized to be associated with changes in intramyocellular diacylglycerol content and protein kinase C activity, which can cause insulin resistance by serine phosphorylation of the insulin receptor substrate-1, resulting in interruption of insulin signaling (28). Further, by selectively stimulating FFA uptake and FOX in fat, TZDs can decrease FFA uptake in muscle (29), which would increase insulin sensitivity (30).

FFA-mediated changes in insulin sensitivity are dose dependent and proportional (31). It is therefore not likely that the observed ~40% increase in insulin-stimulated glucose uptake (Table 4) can be fully explained by the 20% decrease in plasma FFA levels. This suggested that other factors contributed to the TZD effect on insulin sensitivity. Such factors may include the TZD-mediated increase in the expression and protein concentration of adiponectin that occurred in these patients (14). Adiponectin is a protein that is exclusively produced by adipocytes and that has been shown to increase insulin sensitivity (32–36). TZDs have also been shown to increase the number of small adipocytes that are more insulin sensitive than large adipocytes (14,37). Moreover, TZDs have been shown to shift the balance of adipose tissue away from visceral to subcutaneous fat (38,39), which may be important because visceral fat seems to cause more insulin resistance than subcutaneous fat. Lastly, TZDs activate AMP kinase and lower malonyl-CoA, which increases FOX (40).

In summary, we have shown that 2 months of treatment with TZDs induced a coordinated upregulation of transcripts of proteins involved in fatty acid uptake, binding, β-oxidation, electron transport, and oxidative phosphorylation in adipose tissue of patients with type 2 diabetes. These changes were associated with an increase in total body FOX, a decrease in plasma FFA levels, and an increase in insulin-stimulated glucose uptake (insulin sensitivity). We conclude that the TZD-induced expression of fat uptake and oxidation genes and of FOX in human adipose tissue contributed to the TZD-mediated decrease in plasma FFA levels and the increased insulin sensitivity. In addition, microarray and RT-PCR data suggested that TZDs improved expression of multiple genes in fat related to oxidative metabolism and mitochondrial function, which has recently been reported to be defective in the skeletal muscle of patients with type 2 diabetes (22,41–43).

ACKNOWLEDGMENTS
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REFERENCES


