The Effect of Pioglitazone on Peroxisome Proliferator-Activated Receptor-γ Target Genes Related to Lipid Storage In Vivo

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OBJECTIVE — Pioglitazone is a member of the thiazolidinediones (TZDs), insulin-sensitizing agents used to treat type 2 diabetes. The aim of this study was to define the effect of pioglitazone on the expression of genes related to carbohydrate and lipid metabolism in subcutaneous fat obtained from type 2 diabetic patients.

RESEARCH DESIGN AND METHODS — Forty-eight volunteers with type 2 diabetes were divided into two groups treated for 12 weeks with placebo or pioglitazone (30 mg/day). The expression of several genes was quantified by real-time RT-PCR.

RESULTS — Pioglitazone treatment increased the expression of genes involved in glycerol-3-phosphate synthesis. The mRNA expression of PEPCK-C and glycerol-3-phosphate dehydrogenase (GPDH) increased \((P < 0.01)\) in patients treated with pioglitazone. There was no difference in glycerol kinase (GyK) mRNA levels. The expression of genes that regulate fatty acid availability in adipocytes, including lipoprotein lipase (LPL) and acetyl-CoA synthetase (ACS), was higher \((P < 0.01)\) in pioglitazone-treated patients. Pioglitazone stimulated \((P < 0.0001)\) expression of c-Cbl-associated protein (CAP), whereas tumor necrosis factor-α, leptin, resistin, angioptatin like-4, and 11β-hydroxysteroid dehydrogenase type 1 \((11β \text{HSD} 1)\) were not affected by pioglitazone. The baseline peroxisome proliferator–activated receptor (PPAR)-γ1 mRNA was significantly correlated with mRNA for LPL, CAP, ACS, 11β HSD 1, GyK, fatty acid synthase, leptin, and GPDH, whereas PPAR-γ2 mRNA was correlated with CAP, PEPCK-C, leptin, and GPDH.

CONCLUSIONS — Treatment with pioglitazone increased body weight, and this is associated with upregulation of some, but not all, genes previously demonstrated as “TZD responsive” in subcutaneous fat. The results suggest that TZDs might increase body weight through the upregulation of genes facilitating adipocyte lipid storage in vivo.

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The peroxisome proliferator–activated receptor (PPAR)-γ is a member of the nuclear hormone receptor family of transcription factors and is highly expressed in human adipose tissue. This suggests that adipose tissue is the primary target for PPAR-γ agonists. PPAR-γ transcriptional activity is regulated by nutritional factors, cytokines, and cofactor assembly. Several investigators have identified a pivotal role for PPAR-γ in fat cell differentiation, lipid storage, vascular function, and energy metabolism \((2,3)\). PPAR-γ is alternately spliced to generate two isoforms: PPAR-γ1 and -γ2, but PPAR-γ1 is the major form existing in the adipose tissue \((4)\). PPAR-γ is activated by specific, naturally occurring ligands including polynsaturated fatty acids and prostaglandin J2 derivatives. However, there are a number of synthetic compounds that also activate this receptor. Thiazolinediones (TZDs) are a pharmacological insulin-sensitizing class of compounds that are high-affinity ligands for PPAR-γ and widely used for treatment of type 2 diabetes \((5,6)\).

The antidiabetic effect of TZDs is well established, although the precise mechanism of action is unknown. Chronic treatment with PPAR-γ activators \((e.g., \text{pioglitazone, rosiglitazone})\) improves glucose homeostasis by increasing insulin sensitivity in various animal models of obesity and diabetes as well as in humans \((7,8)\). However, several studies have observed a modest weight gain in diabetic subjects treated with TZDs \((9)\). Diabetic patients treated with TZDs have increased subcutaneous adipose tissue and decreased or unchanged visceral adipose tissue \((10)\). One mechanistic hypothesis used to explain the insulin-sensitizing effect of TZDs includes the differentiation of mesenchymal and preadipocytic precursors in adipose tissue \((i.e., \text{adipose tissue remodeling})\), exemplified by an increase in the number of small adipocytes and a shift in lipids from large adipocytes into new small more insulin-sensitive adipocytes \((11)\). Small adipocytes may be more efficient at storing lipids, and body weight could increase as a result. This might also reduce the production of harmful adipokines such as tumor necrosis factor-α or resistin. Alternate mechanisms, including a reduction in circulating free fatty acids, have been proposed as a link between TZDs and improved insulin sensitivity. A reduction in free fatty acids is observed early after PPAR-γ acti-
viation and occurs before plasma glucose and triglycerides fall (12).

In humans, triglyceride storage occurs mainly by coupling existing fatty acids to glycerol-3-phosphate; de novo fatty acid synthesis makes also a significant contribution (13). The activation of PPAR-γ-responsive genes could increase body fat by increasing the genes that facilitate either or both of these processes. As genes that produce enzymes involved directly or indirectly in the formation of glycerol-3-phosphate, we selected the genes for glycerol kinase (GyK), glycerol-3-phosphate dehydrogenase (GPDH), PEPCK-C, and c-Cbl–associated protein (CAP). As examples of genes involved in the supply of fatty acids, we selected lipoprotein lipase (LPL), fatty acid synthase (FAS), and acetyl-CoA synthetase (ACS). We hypothesize that the activation of PPAR-γ-responsive genes in one or more of these pathways will account for the carbon skeletons that make the extra triglyceride for storage in fat cells.

Thus, the aim of this study was to relate the expression of PPAR-γ–responsive genes in adipose tissue to the expression of PPAR-γ and to define the effect of pioglitazone to regulate these same genes in patients with type 2 diabetes.

**RESEARCH DESIGN AND METHODS** — Forty-eight men and women with type 2 diabetes were enrolled in a clinical trial to determine the effect of pioglitazone on body composition (22 men and 26 women, mean age 54.7 ± 9.5 years, BMI 32.1 ± 5.1 kg/m²). Diabetes was defined by a fasting plasma glucose ≥126 mg/dl at entry or fasting plasma glucose >115 mg/dl and a 2-h oral glucose tolerance test glucose >200 mg/dl and a 2-h oral glucose tolerance test glucose ≥200 mg/dl or current use of either metformin or sulfonylureas. Fasting plasma glucose at entry had to be ≤115 mg/dl and a 2-h oral glucose tolerance test glucose ≤200 mg/dl or the HbA1c was ≥7.0%.

Subcutaneous adipose tissue was obtained by Bergstrom needle biopsies of subcutaneous adipose tissue 6–10 cm lateral to the umbilicus at baseline and after 12 weeks of treatment following an overnight fast as previously described (14). Fat samples were cleaned of visible connective tissue and blood vessels under a bright light, immediately frozen in liquid nitrogen, and then stored at −70°C until assays.

**RNA extraction and real-time RT-PCR**

Total RNA from 50–100 mg of adipose tissue was isolated with Trizol reagent (Invitrogen, Carlsbad, CA) as previously described (14), quantified spectrophotometrically, and the integrity of the RNA confirmed by PAGE with Sybr green detection.

Real-time RT-PCRs were performed in a 50-μl final volume using a Taqman 100Rxn PCR Core Reagent Kit (Applied Biosystems, Roche, Branchburg, NJ). Ten microliters of RNA was mixed with 40 μl of Taqman Universal Master Mix (5 μl of 10X buffer A; 11 μl of 25 mMol/l MgCl₂; 1 μl of each dNTP; 0.25 μl of reverse transcriptase; 0.25 μl of RNase inhibitor; 0.25 μl of AmpliTaq Gold DNA polymerase; and 15.25 μl of water). The concentration of the PCR primers was 300–450 nmol/l and the fluorogenic probes 100–200 nmol/l (depending on the gene). Taqman probes (complementary to the genes of interest, labeled with FAM [6-FAM, 6-carboxyfluorescein] dye) and primers were designed using Primer Express Software, version 2.0 (Applied Biosystems). The sequences of primers and probes and accession numbers for each gene are listed in Table 1. Real-time RT-PCR was carried out in duplicate for each sample in optical 96-well reaction plates in an ABI PRISM 7700 sequence detector (Applied Biosystems) using the following parameters: one cycle of 48°C for 30 min, then 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All expression data were normalized by dividing the amount of target by the amount of cyclophilin used as internal control. The specificity of the PCR product for each tested gene was confirmed by gel electrophoresis.

**Statistical analysis**

All clinical data were entered into the Pennington Biomedical Research Center database, extracted, and combined with a gene expression database and analyzed using JMP version 5.0.1a (SAS, Cary, NC). Correlations were performed in a pairwise fashion using the Pearson product-moment statistic. Two-way ANOVA was performed using time and treatment as factors to assess changes in gene expression within subject during the study. All values are presented in figures and tables as sample (raw) means ± SE. Significant differences were assumed for P ≤ 0.05.

**RESULTS**

**Subjects**

As previously reported, the body weight of patients treated with pioglitazone increased by 1.68 ± 2.4 and 3.68 ± 3.1 kg at 12 and 24 weeks, respectively (P < 0.001). The body weight of the placebo-treated patients decreased slightly at each time point (−0.71 ± 2.19 and −0.70 ± 3.3 kg at 12 and 24 weeks, respectively) (15). Body fat measured by dual-energy X-ray absorptiometry rose 3.55 ± 2.52 kg over the 24 weeks and accounted for almost all of the increase in body weight in the pioglitazone-treated patients. Body fat in this group increased in the arms, legs, and abdomen but not in the visceral fat compartment (15).

**Genes involved in glycerol-3-phosphate synthesis**

Expression for three of the four genes in this group was increased by treatment with pioglitazone (Fig. 1A–D). The mRNA level of PEPCK-C was increased significantly to 1.07 ± 0.10 arbitrary units in the pioglitazone-treated group compared with 0.59 ± 0.09 in the placebo-treated group (P < 0.01) (Fig. 1A). The mRNA
for GPDH was $1.46 \pm 0.07$ in the TZD-treated group, which was significantly higher than the $1.13 \pm 0.07$ seen in the placebo-treated group ($P < 0.01$) (Fig. 1B). In contrast, there was no difference between the two treatment groups in the mRNA for GyK ($0.39 \pm 0.03$ vs. $0.37 \pm 0.03$ in the TZD- vs. placebo-treated group) (Fig. 1C). The expression of CAP, a signaling protein involved in insulin-stimulated glucose transport, was markedly stimulated by pioglitazone ($4.31 \pm 0.29$ vs. $2.64 \pm 0.28$; $P < 0.0001$) (Fig. 1D).

**Genes involved in fatty acid metabolism, synthesis, or storage**

The mRNA expression for LPL was $1.14 \pm 0.07$ in the pioglitazone-treated patients and was significantly higher than the $0.74 \pm 0.06$ arbitrary units in the placebo-treated group ($P < 0.001$) (Fig. 1E). The mRNA for ACS was also significantly higher in the pioglitazone-treated patients than in the placebo-treated control subjects ($1.63 \pm 0.12$ vs. $0.94 \pm 0.11$; $P < 0.001$) (Fig. 1F). FAS mRNA expression tended to increase in the pioglitazone-treated patients ($5.42 \pm 0.54$ vs. $3.67 \pm 0.51$; $P = 0.09$) (Fig. 1G).

**Expression of other “TZD responsive” genes**

The mRNA expression levels for tumor necrosis factor-$\alpha$, leptin, and resistin, three adipocytokines, were not affected by treatment with pioglitazone and did not differ between the two groups (data not shown). Furthermore, no changes were observed between these two groups in angiopoietin like-4 (ANGPTL-4, also known as PGAR) and 11-$\beta$-hydroxysteroid dehydrogenase type 1 (11$\beta$HSD 1) mRNA.

**Correlations with the expression of PPAR-$\gamma 1$/PPAR-$\gamma 2$**

The baseline PPAR-$\gamma 1$ mRNA was significantly correlated with mRNA for LPL (Fig. 2A), CAP (Fig. 2B), ACS (Fig. 2C), 11$\beta$HSD 1 (Fig. 2D), GyK (Fig. 2E), FAS ($r = 0.33$; $P < 0.05$; data not shown), leptin ($r = 0.32$; $P < 0.05$), and GPDH ($r = 0.29$; $P < 0.05$). The expression of PPAR-$\gamma 2$ was significantly correlated with LPL ($r = 0.70$; $P < 0.001$; data not shown), CAP ($r = 0.48$; $P < 0.001$), PEPCK-C ($r = 0.39$; $P < 0.01$), leptin ($r = 0.44$; $P = 0.01$), and GPDH ($r =$
Figure 1—Effect of the 12-week treatment with pioglitazone or placebo on PEPCK-C (A), GPDH (B), GyK (C), CAP (D), LPL (E), ACS (F), FAS (G), and ANGPTL-4 (H).
The expression of mRNA for FAS was highly correlated with ACS (Fig. 2G) and GPDH (Fig. 2H). Additionally, the mRNA for ACS was correlated with GPDH (Fig. 2I).

Treatment with pioglitazone did not change the mRNA levels of the two tested isoforms of PPAR-γ (PPAR-γ1 or PPAR-γ2) in adipose tissue. The PPAR-γ protein expression was determined by a Western blotting with an antibody that recognizes a region common to PPAR-γ1 and PPAR-γ2 isoforms and did not show any major differences in PPAR-γ protein content before versus after TZD treatment (data not shown).

There was no significant correlation between the change in the expression of the lipogenic genes and the change in body weight or body fat after TZD treatment. The expression of PPAR-γ1 was inversely related to the fasting insulin value (P < 0.01). However, the slopes were not different across sexes. Interestingly, men had higher insulin values for any given level of PPAR-γ1 gene expression (P < 0.05).

**CONCLUSIONS** — The antidiabetic TZDs have revolutionized the pharmacotherapy of diabetes. TZDs as ligands for the PPAR-γ nuclear hormone receptors exert pleuripotent effects on many cell types (6,16). The aim of this study was to define the effect of TZDs to alter transcription of lipogenic and lipid storage genes in adipose tissue in vivo. There are several notable findings from our study of gene expression in adipose tissue from diabetic subjects treated with pioglitazone. First, we measured the expression of these same genes before and after 12 weeks’ treatment with TZD or placebo. We measured gene expression at this time point to be certain that subjects were in “steady state” and because several weeks are necessary to obtain the full antidiabetic effect of the TZDs. The expression of genes involved in glycerol-3-phosphate synthesis was increased after pioglitazone treatment. For example, PEPCK-C, an enzyme critical for glyceroneogenesis from pyruvate or lactate (17,18) increased by 84% in TZD-treated subjects. A recent report shows that glyceroneogenesis is the main metabolic pathway supplying glycerol for triacylglycerol synthesis in fasted humans (19). The higher mRNA level was also observed for GPDH after TZD treatment. In contrast, GyK, which has been proposed as an important enzyme for re-esterification of glycerol in adipocytes and the reduction in circulating free fatty acids in rodents and in vitro experiments (20), was unchanged by TZD treatment in the present study. This suggests that TZD up-regulation of GyK is a transient effect or, alternately, may not be so important in human adipose tissue. Our observation is in accordance with a recent study showing that GyK is not induced by rosiglitazone in human adipocytes in vitro (21). The changes in PEPCK-C and GPDH gene

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**Figure 2** — Correlations between mRNAs at baseline for selected genes involved in lipid synthesis and storage in subcutaneous fat.
expression are consistent with the observed increase in insulin-stimulated fatty acid storage in rodent adipose tissue cultured in vitro (22–24) and the decrease in hepatic fat content (25,26) seen with TZD treatment. In other words, higher uptake and storage of fatty acids in adipose tissue might prevent the delivery of fatty acids to liver and skeletal muscle (27,28), and our results demonstrate for the first time that PEPCK-C may be an important factor driving this process in human adipocytes in vivo. The expression of CAP, an important component of the alternate insulin-signaling pathway required for GLUT-4 translocation and glucose uptake recently described (29,30), was higher after pioglitazone treatment, as observed in our study, in 3T3-L1 adipocytes, and in a rodent model in vivo (31,32). The action of TZDs to increase CAP might improve adipocyte insulin sensitivity and provide the glucose (9) necessary for the production of pyruvate/glycerol, which is finally converted into triglycerides (Fig. 3).

Next, we investigated the relationship between PPAR-γ mRNA and expression of our genes of interest. The expression of several genes was highly correlated with PPAR-γ1 and/or PPAR-γ2 expression before TZD treatment. This observation can be interpreted in at least two ways. First, the expression of PPAR-γ1/PPAR-γ2 might directly lead to increased PPAR-γ protein and gene expression of CAP, LPL, ACS, GyK, 11β HSD 1, FAS, and GPDH. An alternate explanation is that a common transcription factor drives both PPAR-γ1/PPAR-γ2 and these same genes.

**Figure 3**—Schematic representation of the main biochemical pathways of triglyceride synthesis/lipid storage in adipose tissue. Briefly, treatment with pioglitazone increased mRNA expression for PEPCK-C and GPDH from the glyceroneogenesis pathway and FAS from the lipid synthesis pathway. GS, glucogen synthase.
These strong relationships of mRNA for certain genes (LPL, CAP, ACS) with PPAR-γ mRNA suggest that the regulation of PPAR-γ mRNA is an important control point in vivo. Several other genes that have been demonstrated to be “TZD responsive” in vitro were not correlated with PPAR-γ mRNA at baseline in our study. Specifically, ANGPTL-4 and PECK-C were not correlated to PPAR-γ1 mRNA, although it was previously reported that each can be upregulated by agonists for PPAR-γ (23,35).

Interestingly, the expression of FAS, GPDH, and ACS, three genes critical for fatty acid storage as triglycerides in adipose tissue, were highly correlated with each other and with PPAR-γ. This suggests an important coordinate transcriptional control system for these lipogenic genes. One possible pathway includes activation of PPAR-γ by pioglitazone, which contributes to the increased glucose uptake by fat cells (higher CAP, GLUT-4, and signaling molecules in the insulin-signaling pathway [our results, 38,39]) that are finally incorporated into triglycerides (Fig. 3).

PPAR-γ has been suggested as an important determinant of body weight based on studies in individuals with activating or inhibitory mutations of PPAR (40,41). We showed that neither PPAR-γ1 nor PPAR-γ2 mRNA was altered by TZD treatment, and our results are in agreement with others (9), suggesting that TZD effects possibly occur through activation of PPAR-γ transcription and not through upregulation of the receptor expression (2).

In conclusion, 3 months of treatment with pioglitazone increased body weight, and this may be associated with upregulation of some, but not all, genes previously demonstrated as “TZD responsive” in abdominal subcutaneous adipose tissue. Combined with previous studies showing an increase in insulin-stimulated fatty acid uptake in adipose tissue and the weight gain commonly seen with TZDs in the clinical setting, these results suggest that TZDs might increase body weight through the upregulation of genes of adipocyte lipid storage pathways in vivo, including PECK-C, GPDH, and ACS.

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References