# Mechanisms of Early Insulin-Sensitizing Effects of Thiazolidinediones in Type 2 Diabetes

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**Whereas thiazolidinediones (TZDs) are known to rapidly improve insulin action in animals, short durations of TZD therapy have never been studied in humans. Among the many known actions of TZDs, increased circulating levels of the high molecular weight (HMW) multimer of adiponectin may be an important insulin-sensitizing mechanism. We examined the effects of only 21 days of 45 mg of pioglitazone (P+) versus placebo (P−) in nine subjects with type 2 diabetes ($HbA_{1c}$, 10.9 ± 0.6%; BMI, 31.9 ± 1.5 kg/m$^2$). Total adiponectin levels increased by approximately twofold in P+ in association with increased adipose tissue gene expression. However, plasma free fatty acid and glucose levels were unchanged, and there were only minimal changes in other "adipokines." Glucose fluxes ([3-$^3$H]glucose infusion) were measured during 6-h euglycemic (5 mmol/l) "pancreatic clamp" studies (somatostatin/glucagon/growth hormone) with stepped insulin levels. Pioglitazone induced marked decreases in endogenous glucose production (P+ = 0.9 ± 0.1 vs. P− = 1.7 ± 0.3 mg · kg$^{-1}$ · min$^{-1}$; $P < 0.05$) at physiologic hyperinsulinemia (~50 μU/ml), which was highly correlated with an increased ratio of HMW adiponectin/total levels ($r^2$ = 0.90). Maximal insulin stimulation (~400 μU/ml) revealed pioglitazone-associated increases in glucose uptake (P+ = 10.5 ± 0.9 vs. P− = 8.9 ± 0.8 mg · kg$^{-1}$ · min$^{-1}$; $P < 0.05$), which did not correlate with HMW or total adiponectin levels. Thus, only 21 days of pioglitazone therapy improved insulin action in humans with type 2 diabetes. Increased abundance of the HMW adiponectin multimer may contribute to the hepatic insulin-sensitizing effects of these agents. *Diabetes* 53: 1621–1629, 2004**

Type 2 diabetes is becoming a global epidemic with grave health consequences. Insulin resistance is a characteristic feature of most patients with type 2 diabetes and therefore a principal target for intervention. The peroxisome proliferator–activated receptors (PPARs) are lipid-activated transcription factors that regulate the expression of genes that control lipid and glucose metabolism (1). The PPAR-γ isoform is most abundantly expressed in adipose tissue and immune cells and plays an important role in adipocyte differentiation and lipogenesis (2). The severe insulin resistance that characterizes individuals with dominant-negative PPAR-γ mutations (3) highlights the potential importance of these nuclear receptors in the treatment of diabetes. Activation of PPAR-γ by the thiazolidinedione (TZD) class of antidiabetic drugs improves glucose homeostasis and insulin action in type 2 diabetes (4–6) and may prevent the progression from impaired glucose tolerance to type 2 diabetes (7).

There are many potential mechanisms whereby TZDs improve insulin action in skeletal muscle (5,8) and liver (6,9). These include altered body composition (5,10), decreased intramyocellular and intrahepatocellular triglyceride content (6,11,12), reduction in circulating free fatty acid (FFA) levels (5,13), and decreased production and/or actions of circulating proinflammatory proteins (14,15). In addition, TZD treatment alters the expression of many metabolically important genes in adipose tissue, liver, and muscle, as demonstrated both in insulin resistant animal models (16,17) and in some cell culture systems (18,19). Among those genes whose expression is altered in muscle and liver are genes that regulate glucose metabolism and fatty acid handling (16). In adipose tissue, TZDs have been shown to alter expression of leptin, inflammatory molecules, and circulating proteins such as tumor necrosis factor α (TNF-α), which may be implicated in systemic insulin resistance. It remains to be determined whether altered expression of these key metabolic genes accompanies the insulin-sensitizing effects of TZDs in humans.

Of particular note, TZDs increase adipocyte gene expression and plasma levels of adiponectin (17,20,21), a circulating adipose-derived protein with favorable effects on insulin action (22). Adiponectin administration enhances hepatic insulin sensitivity, lowering glucose production in isolated hepatocytes (23) and in conscious mice (24). Adiponectin circulates predominantly as at least two

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ACC, acetyl-coenzyme A carboxylase; CPT, carnitine palmitoyltransferase; EGP, endogenous glucose production; ELISA, enzyme-linked immunosorbent assay; FAS, fatty acid synthase; FFA, free fatty acid; GU, glucose uptake; HMW, high molecular weight; IL-6, interleukin-6; LMW, low molecular weight; PDK-4, pyruvate dehydrogenase kinase-4; PPAR, peroxisome proliferator–activated receptor; SCD, stearoyl-CoA desaturase; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor α; TZD, thiazolidinedione.

discrete forms (25,26): a high–molecular weight (HMW) multimer that potently reduces glucose production in conscious mice and a more abundant low–molecular weight (LMW) trimer-dimer that seems to lack these insulin-sensitizing effects. Multimeric formation is critically dependent on the formation of disulfide bonds, and both HMW and LMW complexes seem to be formed within adipocytes without interconversion of these forms in the circulation (25,26). Recent studies have shown that the relative abundance of circulating HMW adiponectin is reduced by both hyperglycemia and hyperinsulinemia in normal mice (25). The potential relevance of these circulating complexes to insulin action in humans is highlighted by the recent observation that human adiponectin mutations that prevent the formation of HMW multimers were associated with increased risk of type 2 diabetes (26). It remains to be determined whether and how TZD administration might alter the relative abundance of these adiponectin multimers.

Although it is known that the effects of TZDs on insulin action (27,28) and gene expression (16) occur rapidly in rodents, no human studies have examined the effects of TZDs for <8 weeks. Some rodent studies suggest that the rapid improvement in hepatic insulin action may precede that in skeletal muscle (29,30). Indeed, it has been proposed that any improvement in muscle insulin action observed with short-term TZD treatment in rodents may be secondary to decreased FFA levels, whereas TZD-induced enhancement in hepatic insulin sensitivity is independent of FFA lowering (9).

On the basis of the established findings discussed above, we hypothesized that *1*) TZDs have an impact on insulin action in humans far more rapidly than previously identified, with earlier improvement in hepatic than in peripheral insulin action, and *2*) relative increases in HMW adiponectin multimers may correlate more strongly with improved suppression of endogenous glucose production (EGP) than total adiponectin levels. Indeed, it remains to be determined whether many of the effects of TZDs discussed above are pertinent to their insulin-sensitizing effects in humans. We proposed that administering TZDs to individuals with type 2 diabetes over a short time course would avoid the confounding effects of improved plasma glucose levels, which have been demonstrated after 4 weeks or more of TZD therapy (31). Thus, these studies examined the effects of only 3 weeks of pioglitazone treatment in humans with type 2 diabetes on hepatic and peripheral insulin action and circulating adipose-derived proteins, including adiponectin multimers. We also examined effects of TZDs on gene expression in adipose tissue and muscle in concert with these measures of in vivo physiology, selecting those genes that were demonstrated to be most potently regulated by TZDs in rodent models (16).

## RESEARCH DESIGN AND METHODS

Nine individuals with type 2 diabetes and no concurrent illnesses were studied (Table 1). Before individuals' enrollment in the study, the purpose, nature, risks, and benefits of the study were explained and voluntary, informed, written consents were obtained. All individuals with a history of hypertension, microvascular complications, heart disease, cerebrovascular disease, seizures, bleeding disorders, muscle disease, or smoking were excluded from the studies.

Each participant was instructed to follow his or her usual weight-maintaining diet plan, maintaining consistent dietary composition (confirmed by food records) for 3 days before each study. Given the potential interactions of fish oils with PPARs, participants were instructed to limit intake of fish products for 21 days before the study. In addition, they were instructed not to change their level of activity while participating in the studies but to refrain from vigorous activity for 3 days before each study. The participants discontinued sulfonylureas 3 days and long-acting insulin 24 h before the clamp study. Before each study, participants were given a 21-day supply of unmarked capsules that contained either pioglitazone (45 mg) or placebo. Each participant received the experimental agents in random order, and both the participant and the investigators were masked as to treatment assignment. During the time they were taking the experimental capsules, the participants also continued their usual diabetes medication. Each individual participated in a pair of studies, a placebo study and a pioglitazone study, for 21 days. Before each clamp study, blood samples were collected for liver function tests. There was a washout period of at least 3 weeks between studies.

TABLE 1
Participant characteristics

| | |
|---|---|
| *n* | 9 |
| Age (years) | 47 ± 2.8 |
| Sex (male/female) | 7/2 |
| BMI | 33.5 ± 2.1 |
| $HbA_{1c}$ (%) | 10.3 ± 0.6 |
| Weight P+ (kg)* | 96.5 ± 4.8 |
| Weight P− (kg)† | 95.8 ± 4.7 |
| Waist-to-hip ratio | 1.0 ± 0.01 |
| Medication | 2 I, 1 M, 5 M + S, 1 M + I |

Data are means ± SE unless otherwise indicated. I, insulin alone; M, metformin alone; M + S, metformin plus sufonylurea; I + M, metformin plus insulin; *Weight after 21 days of pioglitazone (P+) administration; †weight after 21 days of placebo (P−) administration.

**"Stepped" hyperinsulinemic clamp studies.** All participants were admitted to the General Clinical Research Center 1 day before the study. Intravenous access was established for overnight control of plasma glucose with intravenous insulin. For minimizing the effects of insulin on the end points of interest, insulin infusions were begun at 3:00 A.M. and were adjusted on the basis of hourly plasma glucose measurements to gradually attain euglycemia. Participants fasted overnight but took their capsules on the morning of the study. At 7:30 A.M., an additional intravenous cannula was inserted in a dorsal vein of the opposite arm for blood sampling. For obtaining arterialized venous blood samples, this hand was maintained at 65°C in a thermoregulated plexiglas box.

To examine the effects of 21 days of pioglitazone on both hepatic and peripheral insulin action, we used 6-h "stepped" hyperinsulinemic clamp studies (Fig. 1). Individualized basal insulin requirements were established during the first 2 h of each study. Glucose fluxes were then measured with labeled isotopes at two distinct levels of hyperinsulinemia: high physiologic levels (plasma insulin levels ~50 μU/ml), designed to suppress EGP, and pharmacologic levels (~400 μU/ml) to stimulate maximal insulin-mediated

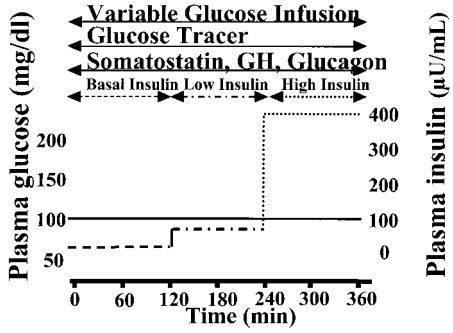


**FIG. 1. Schematic depiction of "stepped clamp" protocol. Plasma glucose values were clamped at 90 mg/dl. Glucose fluxes were measured by infusing tritiated glucose. Somatostatin was infused to inhibit pancreatic hormone secretion with replacement of glucagon and growth hormone. Basal insulin infusion rates (—) were established for the first 2 h, and then the rates were increased by 20 mU · $m^{-2}$ · $min^{-1}$ to reproduce physiologic hyperinsulinemia (-.-.-). During the final 2 h of the clamp, the insulin infusion rate was increased by 150 mU · $m^{-2}$ · $min^{-1}$ above basal ( . . . ).**

glucose uptake (GU). All experiments consisted of 360-min euglycemic (90 mg/dl) insulin/somatostatin (250 μg/h) infusions with replacement of glucoregulatory hormones (glucagon 1 ng · $kg^{-1}$ · $min^{-1}$; growth hormone 3 ng · $kg^{-1}$ · $min^{-1}$) to maintain fixed levels of these hormones throughout.

A primed-continuous infusion of high-performance liquid chromatography–purified [3-$^3$H]glucose was initiated at $t = 0$ min. A bolus of 21.6 μCi was followed by continuous infusion of 0.15 μCi/min for the entire study in all participants (for total duration of 6 h) to quantify glucose turnover, specifically rates of peripheral glucose disposal and hepatic glucose production. For reducing interstudy variability, individualized basal insulin replacement rates were established from 0 to 120 min (B) by means of variable rates of insulin infusion (Novolin Regular; Novo-Nordisk, Princeton, NJ; prepared in albumin-containing saline) to keep plasma glucose levels at ~90 mg/dl without the need for glucose infusion. Insulin infusion rates were then increased by 20 mU · $m^{-2}$ · $min^{-1}$ above these basal rates to reproduce physiologic hyperinsulinemia (B + 20) from 120 to 240 min. Infusion rates were then increased further, to 150 mU · $m^{-2}$ · $min^{-1}$ above the basal rate (B + 150), for the final 2 h of the studies.

Plasma lipids (LDL, HDL, total cholesterol, and triglycerides) were measured at $t = 0$. Plasma glucose levels were measured every 5–10 min in duplicate using a Beckman glucose analyzer (Fullerton, CA; glucose oxidase method) and were maintained at euglycemic concentrations (~90 mg/dl) by a variable infusion of 20% dextrose for the entire study. From $t = 0$ to $t = 360$ min, blood samples were obtained at hourly intervals for determinations of plasma glucose, insulin, C-peptide, total FFAs, glycerol, lactate, adiponectin, leptin, interleukin-6 (IL-6), transforming growth factor β (TGF-β), and TNF-α. Additional samples for 3-$^3$H-glucose determinations were also obtained every 15 min.

All infusions were stopped at $t = 360$ min. The participants were given a standard meal, and plasma glucose levels were monitored for the next 60 min. Dextrose infusion was continued for ~25 min after the study to avoid hypoglycemia. The participants were discharged after 1 h, provided that their condition remained stable.

**Fat and muscle biopsies.** At $t = 345$ min of the clamp studies, fat and muscle biopsies were obtained. Fat biopsies were obtained from the periumbilical region. A small 0.25-cm cutaneous incision was performed under local anesthesia (Lidocaine 1%), and 1–2 g of adipose tissue was obtained by aspiration (32). A skeletal muscle biopsy of ~50 mg was obtained with a spring-loaded biopsy needle (Bard Instruments) in the mid-thigh region 15 cm above the knee after administration of local anesthesia. Biopsy specimens were homogenized immediately in TRIzol reagent (Invitrogen Technologies) at the bedside to inhibit any RNAase activity and subsequently stored at −80°C.

**Analytical procedures**

***Plasma hormones and substrates.*** Plasma insulin and C-peptide were measured by radioimmunoassay, plasma FFA levels were measured by an acyl-CoA oxidase–based colorimetric kit (Wako, Osaka, Japan), glycerol was measured by colorimetric enzymatic methods, and plasma lactate was measured by fluorometric enzyme techniques, as previously described (33).

***Glucose turnover.*** Plasma [3-$^3$H]glucose and tritiated water specific activity were measured as previously described (34). Rates of glucose appearance and GU (or glucose disappearance) were calculated using Steele's steady-state equation (35). Rates of EGP were calculated as previously described (34). Data for glucose turnover, plasma hormones, and substrate concentrations represent the mean values during the final 60 min of the physiological hyperinsulinemia period ($t = 180$–240 min) and the final 60 min of the pharmacological hyperinsulinemia period ($t = 300$–360 min).

***Circulating cytokines and acute-phase reactants.*** Plasma leptin concentrations were measured by immunoassay (ELISA kit; Linco Research, St. Charles, MO). TNF-α and IL-6 levels were measured by sandwich enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, CA). Plasma levels of PAI-1 and TGF-β were also measured by ELISA (Trinity Biotech, Ireland, and Amersham Biosciences, Piscataway, NJ, respectively).

***Adiponectin measurements.*** Total plasma adiponectin was quantified by Human Adiponectin RIA kit (Linco Research). HMW and LMW adiponectin multimers were measured following separation by velocity sedimentation/gel filtration chromatography as described by Pajvani et al. (25). Briefly, 5–20% sucrose gradients in 10 mmol/l HEPES (pH 8) and 125 mmol/l NaCl were poured stepwise (5, 10, 15, and 20%) in 2-ml thin-walled ultracentrifuge tubes (BD Biosciences) and allowed to equilibrate overnight at 4°C. After layering of the sample on top (diluted 1:10 with 10 mmol/l HEPES [pH 8] and 125 mmol/l NaCl in the case of serum), the gradients were spun at 55,000 rpm for 4 h at 4°C in a TLS55 rotor in a Sorvall TL-100 table-top ultracentrifuge. Gradient fractions (150 μl) were retrieved sequentially from the top of the gradient and analyzed either by quantitative Western blot analysis or by scintillation counting in the case of iodinated protein. Alternatively, pooled serum or recombinant protein was loaded on two Superose 6 gel filtration columns arranged in tandem, and 0.5-ml fractions were collected (0.2 ml/min flow rate for 4 h in PBS) and analyzed by SDS-PAGE.

***Quantitative real-time RT-PCR.*** From the biopsy samples obtained as described above, total RNA was extracted with TRIzol. cDNA was made using Superscript First Strand Synthesis System for RT-PCR (Invitrogen Technologies). Gene expression was then studied by quantitative, real-time RT-PCR using the specific protocol for the LightCycler instrument (Roche Diagnostics, Indianapolis, IN). SYBRGreen 1 dye (Roche Diagnostics) was used for fluorescent detection of double-stranded DNA. Standard curves were generated for β-actin and the genes of interest using plasmids to calculate mRNA copy number. Melting curves were used to determine the specificity of the gene products, which were subsequently confirmed by running the PCR products on agarose gels. All of the reactions were performed at least three times. Primers used were as follows: pyruvate dehydrogenase kinase-4 (PDK-4): forward AGAAGGGAACCAAAGAACCT, reverse GGAGTTTTCGTTG CTGT; CD36: forward AGATGCAGCCTCATTTCCAC, reverse TGGGTTTTCA ACTGGAGAGG; β-actin: forward 5′-ACTCTTCCAGCCTTCCTTCCT-3′, reverse 5′-CGTCATACTCCTGCTTGCTGA-3′; IL-6: forward CCTTCCAAAGATGGCTG AAA, reverse GTAGGGGTGGTTATTGCAT; adiponectin: forward GGTGGG CTCCTTACAGAACA, reverse TTCAAAGCATCACAGGACCA; TNF-α: forward TCCTTCAGACACCCTCAACC, reverse CAGGGATCAAAGCTGTAGGC; TGF-β1: forward AACCGGCCTTTCCTGCTTCTCA, reverse CGCCCGGGTTATGCTGGTT-GTA; leptin: forward ACAGCGAGAGGCAGAGAAAG, reverse AGGTGGTTGTGA GGATCTGC; fatty acid synthase (FAS): forward GTACACACCCAAGGCAATG, reverse GTGGGATGATGCTGATGATGG; muscle-specific isoform of acetyl-coenzyme A carboxylase (ACC2): forward CAAGAAGCAGGCAAACATCA, reverse CAGATAAGACTCCAGGGACA; fat-specific isoform of acetyl-coenzyme A carboxylase (ACC1): forward CACGCTCAAGTCACCAAGAA, reverse GCAAAT GGGAGGCAATAAGA; carnitine palmitoyltransferase 1B (CPT1B; muscle isoform): forward TTCTTCCACATATCCAGCAA, reverse CACAGTGTGGG-GACGAAAG; stearoyl-CoA desaturase (SCD): forward TGGGAGTGTGTCTG TGCTGACT, reverse CTCAGGCCCCTTTTTCTACC. Reaction conditions were as follows: 40 cycles, denaturation at 95°C for 2 s, annealing at 59°C for 5 s, and elongation at 74°C for 12 s. Results are expressed as fold change by determining the ratio of copy number of the gene of interest in a given individual after pioglitazone versus placebo treatment, corrected for relative expression of β-actin and glyceraldehyde-3-phosphate dehydrogenase in the same pair of samples.

**Statistical analysis.** Statistical analysis of the data over time was performed using SPSS statistical software version 11.5 (SPSS, Chicago, IL). The random effect considered in this mixed model is the error measurement of individual participants, and the within-individual fixed effect is the difference between groups. For averaged data, paired $t$ tests were used for comparisons of pioglitazone versus placebo studies. Because all correlations were performed using data from all nine participants, the significance of linear correlations was determined with a two-tailed analysis, with 7 (= $n - 2$) degrees of freedom, yielding critical $r^2$ values of 0.444, 0.637, and 0.806 for $P$ values of 0.05, 0.01, and 0.001, respectively.

## RESULTS

**Effect of pioglitazone on metabolic parameters.** Plasma glucose levels did not differ between the two experimental conditions before the insulin infusion was started at 3:00 A.M. (P− = 192 ± 18 mg/dl vs. P+ = 173 ± 20 mg/dl; $P = 0.49$). Liver enzymes remained unchanged in all participants after 21 days of pioglitazone administration. The rate of overnight insulin infusion required to maintain plasma glucose in the target range of 90–120 mg/dl was similar after both pioglitazone and placebo (P− = 1.63 ± 0.22 units/h vs. P+ = 1.55 ± 0.36 units/h; $P = 0.86$). Indeed, the rates of overnight insulin infusion likely reflected the need to overcome the effects of hyperglycemia on insulin action and thus did not differ with pioglitazone.

After an overnight fast and insulin infusion for ~4 h, plasma insulin concentrations were similar in all participants. On the morning of the clamp studies (after overnight insulin), plasma glucose levels were not different between the two groups and averaged 119 ± 6 mg/dl for P− and 108 ± 14 mg/dl for P+ ($P = 0.52$). There was no

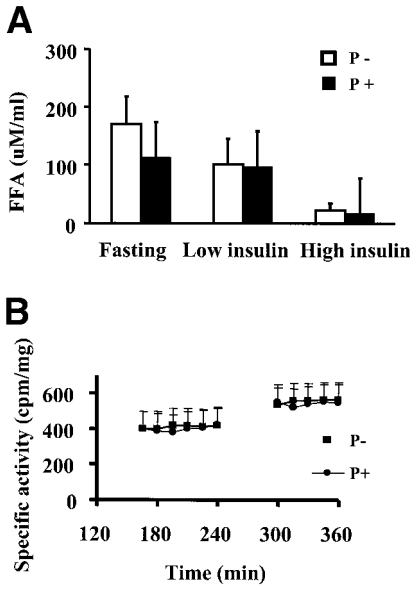


**FIG. 2.** *A*: **Plasma FFA levels, fasting and during low and high insulin infusions, after 21 days of placebo (P-) or pioglitazone (P+) administration.** *B*: **Glucose specific activity (cpm/mg) during the "stepped clamp" shown after pioglitazone (P+) vs. placebo (P-).**

change in triglyceride ($P = 0.644$), HDL ($P = 0.475$), LDL ($P = 0.915$), or total cholesterol ($P = 0.380$) levels with P+ versus P−, and fasting plasma FFA levels did not differ significantly (Fig. 2*A*).

**Effect of pioglitazone on insulin action.** There was no difference in basal insulin requirements (established in the initial step of the study, $t = 0$–120) between P+ and P−, and plasma insulin levels did not differ between P+ and P− during the three-step insulin clamp studies (Table 2). Plasma glucose levels were similar in both P+ and P− during the basal insulin step ($t = 0$–120, 111 ± 21 mg/dl), as well as during the physiological hyperinsulinemic step ($t = 120$–240, 96 ± 18 mg/dl) and the pharmacological hyperinsulinemic step ($t = 240$–360, 89 ± 18 mg/dl). C-peptide levels were suppressed by overnight insulin infusion and by somatostatin infusion throughout the length of the study. Plasma lactate concentrations were unchanged throughout the duration of the studies, and there were no differences in plasma glycerol levels (Table 2). Glucose specific activity was constant after tracer equilibration during the last hour of the physiological insulin ($t = 180$–240 min) and high insulin ($t = 300$–360 min) "steps," during which time glucose fluxes were measured. Specific activity did not differ between P+ and P− for either interval (Fig. 2*B*).

Pioglitazone administration for 21 days markedly enhanced the ability of insulin to suppress EGP compared with placebo in response to low plasma insulin concentrations ($t = 180$–240 min), with a decrement in glucose production from 1.73 ± 0.22 mg · kg$^{-1}$ · min$^{-1}$ to 1.03 ± 0.11 mg · kg$^{-1}$ · min$^{-1}$ ($P = 0.0069$; Fig. 3*A*). A trend toward improved total body GU in P+ versus P− in response to low plasma insulin values did not reach significance (3.27 ± 0.33 vs. 4.51 ± 0.69 mg · kg$^{-1}$ · min$^{-1}$; $P = 0.064$; Fig. 3*B*). However, in response to pharmacological insulin concentrations (~400 μU/ml, $t = 300$–360), there was a significant increase in GU from 9.46 ± 0.53 to 11.00 ± 0.74 mg · kg$^{-1}$ · min$^{-1}$ (P− vs. P+, respectively; $P = 0.042$). By contrast, EGP was comparably and nearly completely suppressed in response to high insulin concentrations in both P− and P+ (P− 0.43 ± 0.15 vs. P+ 0.30 ± 0.24 mg · kg$^{-1}$ · min$^{-1}$; $P = 0.31$).

**Effect of pioglitazone on circulating adiponectin.** There was a twofold increase in total fasting adiponectin levels after 21 days of pioglitazone (P− = 8.21 ± 1 μg/ml vs. P+ = 16.51 ± 2.12 μg/ml; $P = 0.002$; Table 2, Fig. 5*A*). There was no significant effect of insulin on plasma adiponectin levels during the "stepped insulin" clamp studies in either P+ or P− ($t = 360$ min: P− = 5.0 ± 0.8 μg/ml and P+ = 12.3 ± 1.8 μg/ml; $P = 0.302$ and 0.952 vs. respective fasting values). Despite the considerable rise in total circulating adiponectin levels, there were no significant correlations between the percentage increase in adiponectin levels with pioglitazone and either the percentage decrease in EGP ($r^2 = 0.018$) or the percentage increase in GU ($r^2 = 0.436$). Thus, we also examined the relative abundance of adiponectin multimers as a potentially more physiologically relevant marker of biologically active adiponectin (25). Under baseline (placebo) conditions, the LMW form composed 80.1 ± 2.7% of the circulating adiponectin pool in these poorly controlled type 2 diabetic participants, whereas the active HMW form composed only 19.9 ± 2.7%. After pioglitazone therapy, the percentage of active HMW form rose to 38.4 ± 3.2%, whereas the LMW form fell to 61.6 ± 3.2% of the total circulating pool (Fig. 5*B*). As shown in Fig. 3*B*, there was a strong correlation ($r^2 = 0.90$, $P < 0.001$) between the percentage increase in HMW/(HMW + LMW) adiponectin

TABLE 2
Plasma hormone, substrate, and "adipokine" levels after 21 days of placebo or pioglitazone therapy

| | Placebo group | | Pioglitazone group | |
|---|---|---|---|---|
| Time | 180–240 | 300–360 | 180–240 | 300–360 |
| Insulin (μU/ml) | 60.39 ± 7.32 | 395.7 ± 32.74 | 25.58 ± 6.99 | 389.3 ± 35.18 |
| C-peptide (nmol/ml) | 0.10 ± 0.01 | 0.06 ± 0.01 | 0.12 ± 0.01 | 0.06 ± 0.01 |
| Lactate (mmol/l) | 1.26 ± 0.10 | 1.40 ± 0.10 | 1.11 ± 0.08 | 1.49 ± 0.14 |
| Glycerol (μmol/l) | 83.53 ± 9.74 | 60.75 ± 6.17 | 91.22 ± 23.73 | 58.32 ± 11.23 |
| Time | 0 | 360 | 0 | 360 |
| Adiponectin (μg/ml) | 6.1 ± 0.7 | 4.9 ± 0.7 | 12.4 ± 1.7* | 12.3 ± 1.7 |
| Leptin (ng/ml) | 14.1 ± 5.6 | 13.8 ± 5.2 | 11.7 ± 4.9* | 11.9 ± 4.9 |
| IL-6 (pg/ml) | 62.3 ± 15.1 | 47.0 ± 10.1 | 53.4 ± 8.1 | 67.1 ± 14.3 |
| TNF-α (pg/ml) | 256.9 ± 51.4 | 189.2 ± 35.4 | 216 ± 49.9* | 227.9 ± 49.9 |
| TGF-β (pg/ml) | 0.30 ± 0.02 | 0.15 ± 0.01 | 0.27 ± 0.03 | 0.16 ± 0.01 |

Data are means ± SE. *$P \leq 0.05$ for placebo vs. pioglitazone.

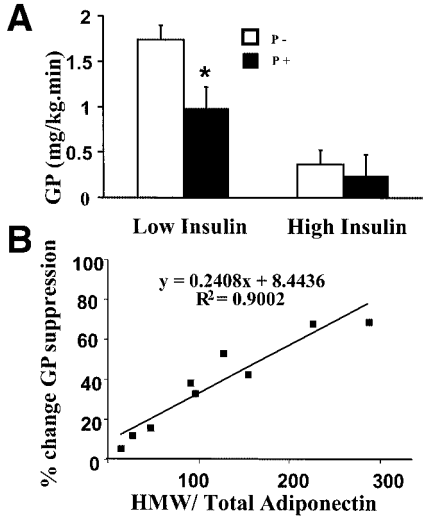


FIG. 3. *A*: Effect of pioglitazone (P+) vs. placebo (P-) on glucose production (GP) in mg · kg$^{-1}$ · min$^{-1}$) in response to low and high plasma insulin concentrations.*$P < 0.05$. *B*: Correlation of change in HMW/ total adiponectin ratio with percent suppression of EGP after 21 days of pioglitazone administration.

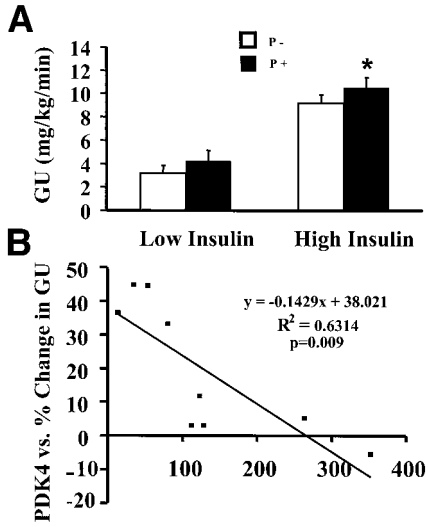


FIG. 4. *A*: Effect of pioglitazone (P+) vs. placebo (P-) on GU (mg · kg$^{-1}$ · min$^{-1}$) in response to low and high plasma insulin concentrations.*$P < 0.05$. *B*: Correlation of change in PDK-4 gene expression in muscle of subjects after 21 days of pioglitazone administration with percent change in whole-body GU. Relative PDK expression is the absolute copy numbers in P+ studies corrected for GAPDH.

ratio and the percentage change in EGP (pioglitazone versus placebo), suggesting that the HMW form of adiponectin may play an important role in the insulin-sensitizing effect of TZDs on EGP. By contrast, there was a complete lack of correlation between induction of HMW/(HMW + LMW) adiponectin ratio and the relative percentage change in GU ($r^2$ = 0.060). Figure 5*C* shows representative Western blots from one participant's plasma samples after pioglitazone versus placebo, demonstrating increased enrichment of the HMW after pioglitazone.

**Effect of pioglitazone on circulating levels of other "adipokines."** Pioglitazone for 21 days induced modest decreases in plasma leptin (P− = 14 ± 7 ng/ml vs. P+ = 12 ± 5 ng/ml; $P = 0.029$) and TNF- α levels (P− = 257 ± 51 pg/ml vs. P+ = 217 ± 50 pg/ml, $P = 0.023$). However, we did not observe any change in circulating IL-6 levels (62 ± 15 pg/ml for P− vs. 53 ± 8 pg/ml for P+; $P = 0.39$) or TGF-β values (0.304 ± 0.026 pg/ml for P− vs. 0.278 ± 0.03 pg/ml for P+; $P = 0.50$; Table 2).

**Effect of pioglitazone on adipose and muscle tissue gene expression.** The relative levels of expression of several genes from subcutaneous adipose tissue and skeletal muscle biopsies performed at the end of the stepped insulin clamp studies after 21 days of placebo and pioglitazone administration were analyzed by real-time RT-PCR. mRNA copy numbers were calculated using plasmid standard curves, and fold changes in gene expression were corrected for both β-actin and glyceraldehyde-3-phosphate dehydrogenase expression, then averaged (Table 3). Adiponectin mRNA copy number was increased by 1.72-fold after pioglitazone administration, whereas there was a 47% decrease in resistin and a modest (28%) decrease in the expression of leptin with pioglitazone. There was a trend toward increased expression of the fat-specific isoform of ACC, consistent with lipogenic effects of TZDs shown in rodents (16); however, this was not significant. This duration of pioglitazone did not affect gene expression of TNF-α, TGF-β, IL-6, glycerol kinase, or FAS in adipose tissue.

PDK-4 gene expression in skeletal muscle showed a heterogeneous response to pioglitazone. Although overall PDK-4 gene expression did not change, there was a significant negative correlation ($r^2$ = 0.6314, $P = 0.01$) between the change in expression levels of muscle PDK-4 mRNA and the percentage change in GU (P+ versus P−; Fig. 4*B*). Indeed, all participants in whom insulin-stimulated GU improved with pioglitazone had suppressed muscle PDK-4 expression, whereas those participants in whom muscle PDK-4 expression was unchanged or increased demonstrated no improvement in GU. By contrast, there was a significant increase in muscle expression of the lipogenic enzyme SCD, consistent with rodent models (16). There were no significant changes in CD36, ACC2, CPT-1B, or uncoupling protein-3 gene expression or correlations between their relative levels of gene expression and improved insulin action. FAS was expressed at very low levels in muscle, but its expression did not seem to change with pioglitazone. The lack of effects of pioglitazone on expression of ACC, FAS, and CPT-1 in skeletal muscle is consistent with the findings of Way et al. (16), suggesting that most effects of PPAR-γ stimulation and/or adiponectin on skeletal muscle fatty acid metabolism are likely to be mediated through changes in enzyme activity rather than gene expression (36).

## DISCUSSION

These studies established for the first time that TZD therapy for only 21 days can significantly improve insulin action in humans with type 2 diabetes, while being of insufficient duration for confounding effects on plasma glucose and FFA levels. Consistent with previous rodent studies, all participants demonstrated a marked early hepatic response to TZDs with improved suppression of EGP by insulin, but the effects of pioglitazone on peripheral insulin action were more variable. The improved suppression of EGP with pioglitazone was strongly correlated with the relative increase in the HMW form of adiponectin.

TABLE 3
Changes in gene expression after 21 days of pioglitazone therapy

| | Fold change | 95% CI |
|---|---|---|
| Skeletal muscle P+ vs. P− | | |
| ACC2 | 1.34 | 0.38–2.29 |
| CD36 | 1.33 | 0.99–1.67 |
| CPT1B | 1.23 | 0.92–1.54 |
| UCP-3 | 0.97 | 0.80–1.13 |
| SCD | 5.54* | 2.82–8.27 |
| Adipose tissue P+ vs. P− | | |
| Adiponectin | 1.72* | 1.18–2.21 |
| ACC1 | 4.25 | 0.44–8.05 |
| FAS | 1.32 | 0.94–1.72 |
| Glycerol kinase | 0.93 | 0.70–1.16 |
| IL-6 | 1.14 | 0.68–1.60 |
| Leptin | 0.84 | 0.22–1.07 |
| Resistin | 0.53* | 0.19–0.86 |
| TGF-β | 1.37 | 0.37–1.75 |
| TNF-α | 0.83 | 0.66–1.01 |

Fold change in gene expression of uncoupling protein-3 (UCP-3), carnitine palmitoyltransferase 1B (CPT-1B), fatty acid synthase (FAS), acetyl CoA carboxylase 2 (ACC2), stearoyl CoA desaturase (SCD), and CD36 in mRNA obtained from pairs of skeletal muscle were analyzed by RT-PCR. Fold change in gene expression of adiponectin, leptin, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), transforming growth factor β (TGF-β), glycerol kinase, resistin, acetyl-CoA carboxylase-1 (ACC1), and fatty acid synthase (FAS) in mRNA obtained from pairs of subcutaneous (SC) adipose tissue biopsies performed after 345 min of the stepped insulin clamp were also analyzed by RT-PCR. *$P < 0.05$. P+, pioglitazone; P−, placebo.

In the current studies, sophisticated "stepped" insulin clamp methods were used to compare hepatic and peripheral insulin action under optimal conditions and to individualize basal insulin needs, because our preliminary data suggested considerable intra-individual variability in basal insulin needs on separate study days. A review of the literature highlights the importance of insulin infusion rates in revealing effects of TZDs on hepatic insulin action. Of note, the effects of 16 weeks of pioglitazone on EGP were apparent only under clamp conditions with similar insulin rates infused during the physiological insulin interval in our studies, i.e., 40 mU · $m^{-2}$ · $min^{-1}$ (5). During a subsequent step in the same studies, there was no demonstrable difference in EGP with pioglitazone at insulin infusion rates of 160 mU · $m^{-2}$ · $min^{-1}$ (5). Of note, effects of TZDs on EGP were not apparent during supraphysiologic hyperinsulinemia in the current studies or during other clamp studies using high insulin infusion rates (37,38). It is likely that higher insulin levels, by markedly suppressing EGP, obscure the effects of TZD therapy on EGP (~50% suppression in the presence of physiologic insulin levels in these studies).

There was a striking correlation between pioglitazone-induced improvements in suppression of EGP and relative increases in HMW adiponectin levels. This was consistent with the potent insulin-sensitizing effects of HMW adiponectin multimers on EGP in conscious mice and the lack of suppression of EGP with infusion of the LMW form alone (24 and unpublished observations). In addition, the HMW multimer was the only form of adiponectin that activated AMPK in hepatocytes (26), the putative mechanism whereby adiponectin suppresses hepatic glucose production (36). Recently, several mutations known to be associated with both hypoadiponectinemia and type 2 diabetes were demonstrated to have mutations in the adiponectin gene that do not permit the formation of HMW multimers (26). In particular, of the nine individuals in a Japanese population (1.3% of that population) identified to have an I164T mutation in the adiponectin gene and markedly reduced HMW adiponectin levels, seven had type 2 diabetes, and the remaining two had impaired glucose tolerance (39). By contrast, individuals with mutations in the adiponectin gene that did not affect multimer formation seemed to have a normal phenotype (26). Together, these observations suggest that measuring the relative quantity of HMW multimers may be of greater biological significance than total adiponectin levels. The current studies are the first to demonstrate TZD-induced selective increases in the HMW multimer and to suggest that this might contribute importantly to the insulin-sensitizing effects of these agents on EGP.

In contrast with the more uniform EGP response, four of the participants exhibited ≤5% improvement in insulin-mediated stimulation of GU, and the latter was not correlated with either the change in total adiponectin levels or the increase in relative abundance of HMW adiponectin. This is consistent with the observation that infusing adiponectin in nondiabetic mice enhanced hepatic but not peripheral insulin action (24). By contrast, total adiponectin levels were positively correlated with both whole-body insulin-stimulated glucose disposal and the fold increase in skeletal muscle insulin receptor phosphorylation in a large group of mainly nondiabetic Pima Indians (40).

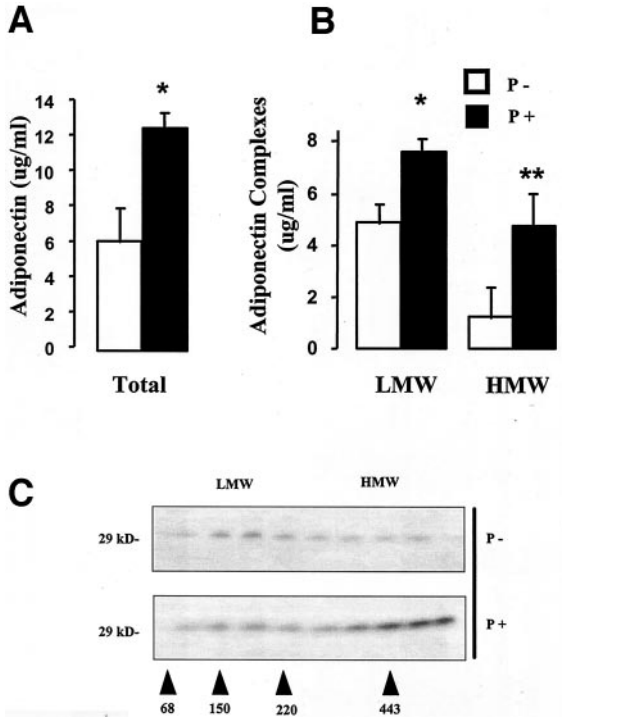


**FIG. 5. *A*: Total adiponectin levels after 21 days of pioglitazone administration (P+) compared with placebo (P-). *B*: Circulating levels of LMW and HMW adiponectin complexes are compared in the P+ and P- groups. *C*: Representative pair of Western blot analyses demonstrating separation of adiponectin multimeric complexes. Patient serum samples were analyzed before and after pioglitazone treatment for 21 days. Adiponectin complex formation was assessed by velocity sedimentation. Fractions were collected and analyzed by quantitative Western blot analysis.**

However, the current study specifically examines the effects of short-term TZD therapy in a small and relatively homogeneous group of obese type 2 diabetic subjects and is not powered to examine baseline interindividual differences in insulin action. Of note, there was a significant correlation between lipid content and insulin action in liver and particularly muscle with adiponectin levels in fat-fed rats (11). This is consistent with important chronic effects of adiponectin on muscle triglyceride content and ultimately on insulin action and may explain the lack of correlation of increased adiponectin with peripheral insulin action after TZD treatment for this short duration.

It is noteworthy that the hepatic insulin-sensitizing effects of these agents were of such magnitude at a time point that preceded many of the known effects of TZDs. The lack of significant reductions in baseline plasma glucose levels despite the marked improvement in hepatic insulin action may suggest that concomitant increases in peripheral GU are required for TZDs to affect glucose levels. In addition, suppression of EGP by other stimuli, e.g., glucose, may still be unaltered. Indeed, hepatic "glucose effectiveness" may be at least as important as hepatic insulin action in the regulation of EGP (41), and correction of this defect in type 2 diabetes has been demonstrated with reduction in FFA levels (42). Furthermore, because baseline plasma glucose levels were measured at 3:00 A.M. before initiating insulin infusion, insulin levels were relatively low, and thus improved hepatic insulin action would have been less relevant. In addition, at 3:00 A.M., such counterregulatory hormones as cortisol and growth hormone would be exerting important effects on blood glucose levels (43), and pioglitazone should not affect secretion of those hormones.

Whereas altered levels of FFAs or other "adipokines" may contribute to the insulin-sensitizing effects of TZDs over longer durations, these effects were also not significant after 21 days of pioglitazone despite the improved insulin sensitivity. Indeed, we did not observe any significant changes in FFA levels under either fasting or insulin clamp conditions. However, it is important to note that the effects of TZDs on FFA levels in previous studies were both dose and duration dependent (8). Indeed, the degree of suppression of FFA levels after 16 weeks of maximal doses of pioglitazone was only modest, whereas the effects on insulin action were considerably greater (5). Furthermore, fasting FFA levels were not correlated with improved plasma glucose levels either fasting or 2 h after an oral glucose tolerance test.

By contrast, there was a significant correlation between pioglitazone-induced decreases in muscle PDK-4 gene expression and increased insulin-stimulated GU. Indeed, PDK-4 gene expression was downregulated only in those who demonstrated improved peripheral insulin action. Because PDK-4 is predominantly regulated by gene transcription, its gene expression has been shown to be an appropriate measure of PDK-4 protein levels and activity (44). Although more study is clearly necessary to establish such an association, this potentially intriguing finding is consistent with suggestions that lowering skeletal muscle PDK-4 might improve insulin action. Indeed, increased PDK-4 gene expression in skeletal muscle is associated with insulin resistance in humans and rodents (44,45), and TZDs normalized these gene expression patterns in association with improved insulin action in animal models and in vitro (16,46). Indeed, suppression of PDK-4 gene expression after treatment with PPAR-γ ligands in insulin-resistant rats immediately preceded improvements in insulin action (16). In obese humans, a significant decrease of skeletal muscle PDK-4 mRNA after bariatric surgery was correlated with both increased GU and decreased intramyocytic triglyceride content (47).

These are the first studies to examine the effects of short-term in vivo administration of TZDs in humans on adiponectin multimers and on gene expression in muscle and fat and to correlate these measures with rigorous quantification of peripheral and hepatic insulin action. Indeed, these studies demonstrated for the first time in humans that pioglitazone induces *1*) rapid and marked effects on hepatic insulin action after only 3 weeks with more heterogeneous effects on peripheral insulin action and *2*) marked increases in the HMW adiponectin multimer, which correlated strikingly with the improved hepatic insulin action. These effects preceded significant changes in plasma glucose, FFA, or adipokine levels. The striking correlation of increased HMW adiponectin with enhanced suppression of EGP but not with increased insulin-stimulated GU suggests different insulin-sensitizing mechanisms of pioglitazone on liver and muscle. These findings could have important implications for the therapeutic use of TZDs in humans with type 2 diabetes and for better understanding their mechanisms of action.

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