

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

Julia Tonelli,¹ Weijie Li,¹ Preeti Kishore,¹ Utpal B. Pajvani,² Elize Kwon,¹ Charles Weaver,¹ Philipp E. Scherer,^{1,2} and Meredith Hawkins¹

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/m²). 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 ([3-³H]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

From the ¹Department of Medicine, Diabetes Research and Training Center, Albert Einstein College of Medicine, Bronx, New York; and the ²Department of Cell Biology, Diabetes Research and Training Center, Albert Einstein College of Medicine, Bronx, New York.

Address correspondence and reprint requests to Meredith Hawkins, MD, Division of Endocrinology, Department of Medicine, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. E-mail: hawkins@aecom.yu.edu.

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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.

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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

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-main-

TABLE 1
Participant characteristics

<i>n</i>	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 sulfonylurea; I + M, metformin plus insulin; *Weight after 21 days of pioglitazone (P+) administration; †weight after 21 days of placebo (P-) administration.

taining 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.

“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

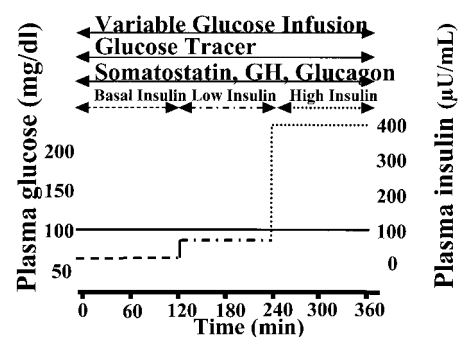


FIG. 1. Schematic depiction of “stepped clamp” protocol. Plasma glucose values were clamped at 90 mg/dl. Glucose fluxes were measured by infusing tritiated glucose. Somatostatin was infused to inhibit pancreatic hormone secretion with replacement of glucagon and growth hormone. Basal insulin infusion 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 μ g/h) infusions with replacement of glucoregulatory hormones (glucagon 1 ng \cdot kg⁻¹ \cdot min⁻¹; growth hormone 3 ng \cdot kg⁻¹ \cdot min⁻¹) to maintain fixed levels of these hormones throughout.

A primed-continuous infusion of high-performance liquid chromatography-purified [³H]glucose was initiated at $t = 0$ min. A bolus of 21.6 μ Ci was followed by continuous infusion of 0.15 μ Ci/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 \sim 90 mg/dl without the need for glucose infusion. Insulin infusion rates were then increased by 20 mU \cdot m⁻² \cdot 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 \cdot m⁻² \cdot 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 (\sim 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-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 \sim 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 \sim 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 [³H]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 μ l) 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 AGAAGGGAACCAAGAACCT, reverse GGAGTTTTCGTTGCTGT; CD36: forward AGATGCAGCCTCATTCCAC, reverse TGGGTTTTCAACTGGAGAGG; β -actin: forward 5'-ACTCTCCAGCCTTCCTCCT-3', reverse 5'-CGTCATACTCCTGCTTGTGA-3'; IL-6: forward CCTTCAAAGATGCTGAAA, reverse GTAGGGGTGTTATTGCAT; adiponectin: forward GGTGGGCTCTTACAGAACA, reverse TTCAAAGCATCACAGGACCA; TNF- α : forward TCCTTACAGACCCCTCAACC, reverse CAGGGATCAAGCTGTAGGC; TGF- β 1: forward AACCCGCTTCTCTCTCTCA, reverse CGCCCGGGTTATGCTGGTTGTA; leptin: forward ACAGCGAGAGGCAGAGAAAG, reverse AGGTGGTTGTGAGGATCTGC; fatty acid synthase (FAS): forward GTACACACCAAGGAATG, reverse GTGGGATGATGCTGATGATGG; muscle-specific isoform of acetyl-coenzyme A carboxylase (ACC2): forward CAAGAAGCAGGCAACATCA, reverse CAGATAAGACTCCAGGACA; fat-specific isoform of acetyl-coenzyme A carboxylase (ACC1): forward CACGCTCAAGTCACCAAGAA, reverse GCAAATGGGAGGCAATAAGA; carnitine palmitoyltransferase 1B (CPT1B); muscle isoform): forward TTCTTCCACATATCCAGCAA, reverse CACAGTGTGGG-GACGAAAG; stearoyl-CoA desaturase (SCD): forward TGGGAGTGTGTCTGTGCTGACT, reverse CTCAGGCCCTTTTCTACC. 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. ($P^- = 192 \pm 18$ mg/dl vs. $P^+ = 173 \pm 20$ 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 ($P^- = 1.63 \pm 0.22$ units/h vs. $P^+ = 1.55 \pm 0.36$ 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 \sim 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

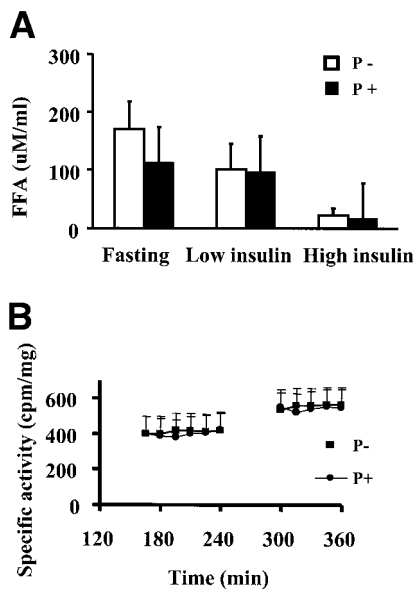


FIG. 2. A: Plasma FFA levels, fasting and during low and high insulin infusions, after 21 days of placebo (P-) or pioglitazone (P+) administration. B: Glucose specific activity (cpm/mg) during the “stepped clamp” shown after pioglitazone (P+) vs. placebo (P-).

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. 2A).

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. 2B).

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 \cdot kg $^{-1}$ \cdot min $^{-1}$ to 1.03 ± 0.11 mg \cdot kg $^{-1}$ \cdot min $^{-1}$ ($P = 0.0069$; Fig. 3A). 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 \cdot kg $^{-1}$ \cdot min $^{-1}$; $P = 0.064$; Fig. 3B). However, in response to pharmacological insulin concentrations (~ 400 μ U/ml, $t = 300-360$), there was a significant increase in GU from 9.46 ± 0.53 to 11.00 ± 0.74 mg \cdot kg $^{-1}$ \cdot 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 \cdot kg $^{-1}$ \cdot 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 μ g/ml vs. P+ = 16.51 ± 2.12 μ g/ml; $P = 0.002$; Table 2, Fig. 5A). 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 μ g/ml and P+ = 12.3 ± 1.8 μ g/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 $80.1 \pm 2.7\%$ of the circulating adiponectin pool in these poorly controlled type 2 diabetic participants, whereas the active HMW form composed only $19.9 \pm 2.7\%$. After pioglitazone therapy, the percentage of active HMW form rose to $38.4 \pm 3.2\%$, whereas the LMW form fell to $61.6 \pm 3.2\%$ of the total circulating pool (Fig. 5B). As shown in Fig. 3B, 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

Time	Placebo group		Pioglitazone group	
	180-240	300-360	180-240	300-360
Insulin (μ U/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 (μ mol/l)	83.53 ± 9.74	60.75 ± 6.17	91.22 ± 23.73	58.32 ± 11.23
Time	0	360	0	360
Adiponectin (μ g/ml)	6.1 ± 0.7	4.9 ± 0.7	$12.4 \pm 1.7^*$	12.3 ± 1.7
Leptin (ng/ml)	14.1 ± 5.6	13.8 ± 5.2	$11.7 \pm 4.9^*$	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	$216 \pm 49.9^*$	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 \pm SE. * $P \leq 0.05$ for placebo vs. pioglitazone.

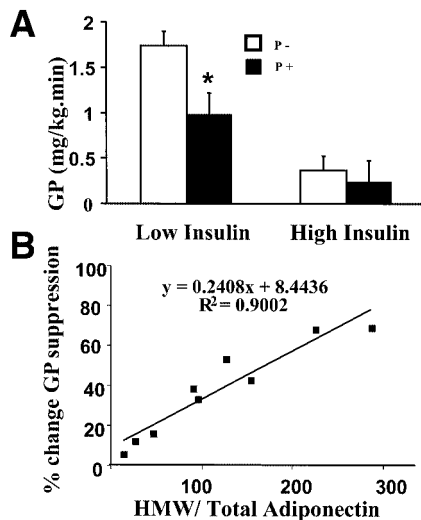


FIG. 3. A: Effect of pioglitazone (P+) vs. placebo (P-) on glucose production (GP) in $\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ in response to low and high plasma insulin concentrations. * $P < 0.05$. B: Correlation of change in HMW/total adiponectin ratio with percent suppression of EGP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

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 5C 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 ($P^- = 14 \pm 7$ ng/ml vs. $P^+ = 12 \pm 5$ ng/ml; $P = 0.029$) and TNF- α levels ($P^- = 257 \pm 51$ pg/ml vs. $P^+ = 217 \pm 50$ 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.

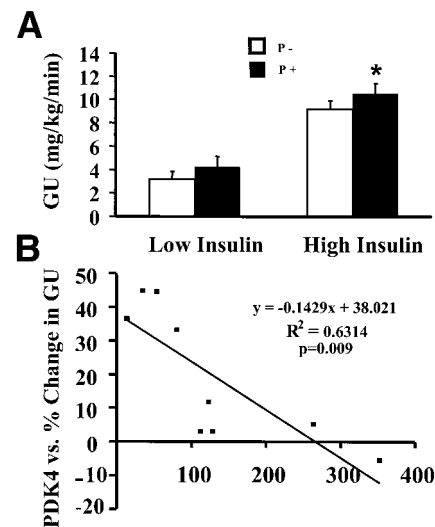


FIG. 4. A: Effect of pioglitazone (P+) vs. placebo (P-) on GU ($\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) in response to low and high plasma insulin concentrations. * $P < 0.05$. B: Correlation of change in PDK-4 gene expression in muscle of subjects after 21 days of pioglitazone administration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

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. 4B). 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., $40 \text{ mU} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$ (5). During a subsequent step in the same studies, there was no demonstrable difference in EGP with pioglitazone at insulin infusion rates of $160 \text{ mU} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$ (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 hypo adiponectinemia 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 $\leq 5\%$ 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).

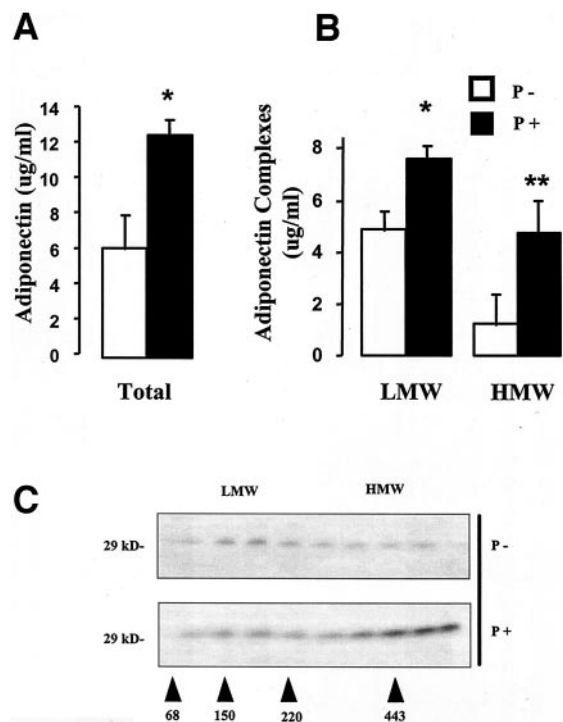


FIG. 5. A: Total adiponectin levels after 21 days of pioglitazone administration (P+) compared with placebo (P-). B: Circulating levels of LMW and HMW adiponectin complexes are compared in the P+ and P- groups. C: Representative pair of Western blot analyses demonstrating separation of adiponectin multimeric 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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REFERENCES

1. Vamecq J, Latruffe N: Medical significance of peroxisome proliferator-activated receptors. *Lancet* 354:141-148, 1999
2. Brun RP, Tontonoz P, Forman BM, Ellis R, Chen J, Evans RM, Spiegelman BM: Differential activation of adipogenesis by multiple PPAR isoforms. *Genes Dev* 10:974-984, 1996
3. Savage DB, Tan GD, Acerini CL, Jebb SA, Agostini M, Gurnell M, Williams RL, Umpleby AM, Thomas EL, Bell JD, Dixon AK, Dunne F, Boiani R, Cinti S, Vidal-Puig A, Karpe F, Chatterjee VK, O'Rahilly S: Human metabolic syndrome resulting from dominant-negative mutations in the nuclear receptor peroxisome proliferator-activated receptor-gamma. *Diabetes* 52: 910-917, 2003
4. Petersen KF, Krssak M, Inzucchi S, Cline GW, Dufour S, Shulman GI: Mechanism of troglitazone action in type 2 diabetes. *Diabetes* 49:827-831, 2000
5. Miyazaki Y, Mahankali A, Matsuda M, Mahankali S, Hardies J, Cusi K, Mandarino LJ, DeFronzo RA: Effect of pioglitazone on abdominal fat distribution and insulin sensitivity in type 2 diabetic patients. *J Clin Endocrinol Metab* 87:2784-2791, 2002

6. Bajaj M, Suraamornkul S, Pratipanawatr T, Hardies LJ, Pratipanawatr W, Glass L, Cersosimo E, Miyazaki Y, DeFronzo RA: Pioglitazone reduces hepatic fat content and augments splanchnic glucose uptake in patients with type 2 diabetes. *Diabetes* 52:1364–1370, 2003
7. Buchanan TA, Xiang AH, Peters RK, Kjos SL, Marroquin A, Goico J, Ochoa C, Tan S, Berkowitz K, Hodis HN, Azen SP: Preservation of pancreatic β -cell function and prevention of type 2 diabetes by pharmacological treatment of insulin resistance in high-risk Hispanic women. *Diabetes* 51:2796–2803, 2002
8. Maggs DG, Buchanan TA, Burant CF, Cline G, Gumbiner B, Hsueh WA, Inzucchi S, Kelly D, Nolan J, Olefsky J, Polonsky KS, Silver D, Valiquett TR, Shulman GI: Metabolic effects of troglitazone monotherapy in type 2 diabetes mellitus: a randomized, double-blind, placebo-controlled trial. *Ann Intern Med* 128:176–185, 1998
9. Ye JM, Frangioudakis G, Iglesias MA, Furler SM, Ellis B, Dzamko N, Cooney GJ, Kraegen EW: Prior thiazolidinedione treatment preserves insulin sensitivity in normal rats during acute fatty acid elevation: role of the liver. *Endocrinology* 143:4527–4535, 2002
10. Nakamura T, Funahashi T, Yamashita S, Nishida M, Nishida Y, Takahashi M, Hotta K, Kuriyama H, Kihara S, Ohuchi N, Nishimura T, Kishino BI, Ishikawa K, Kawamoto T, Tokunaga K, Nakagawa C, Mineo I, Watanabe F, Tarui S, Matsuzawa Y: Thiazolidinedione derivative improves fat distribution and multiple risk factors in subjects with visceral fat accumulation—double-blind placebo-controlled trial. *Diabetes Res Clin Pract* 54:181–190, 2001
11. Ye JM, Iglesias MA, Watson DG, Ellis B, Wood L, Jensen PB, Sorensen RV, Larsen PJ, Cooney GJ, Wassermann K, Kraegen EW: PPAR α / γ agonist eliminates fatty liver and enhances insulin action in fat-fed rats in the absence of hepatomegaly. *Am J Physiol Endocrinol Metab* 284:E531–E540, 2003
12. Mayerson AB, Hundal R, Dufour S, Lebon V, Befroy D, Cline GW, Enocksson S, Inzucchi SE, Shulman GI, Petersen KF: The effects of rosiglitazone on insulin sensitivity, lipolysis, and hepatic and skeletal muscle triglyceride content in patients with type 2 diabetes. *Diabetes* 51:797–802, 2002
13. Tordjman J, Chauvet G, Quette J, Beale EG, Forest C, Antoine B: Thiazolidinediones block fatty acid release by inducing glyceroneogenesis in fat cells. *J Biol Chem* 278:18785–18790, 2003
14. Alleva DG, Johnson EB, Lio FM, Boehme SA, Conlon PJ, Crowe PD: Regulation of murine macrophage proinflammatory and anti-inflammatory cytokines by ligands for peroxisome proliferator-activated receptor- γ : counter-regulatory activity by IFN- γ . *J Leukoc Biol* 71:677–685, 2002
15. Sigrist S, Bedoucha M, Boelsterli UA: Down-regulation by troglitazone of hepatic tumor necrosis factor- α and interleukin-6 mRNA expression in a murine model of non-insulin-dependent diabetes. *Biochem Pharmacol* 60:67–75, 2000
16. Way JM, Harrington WW, Brown KK, Gottschalk WK, Sundseth SS, Mansfield TA, Ramachandran RK, Willson TM, Klier SA: Comprehensive messenger ribonucleic acid profiling reveals that peroxisome proliferator-activated receptor γ activation has coordinate effects on gene expression in multiple insulin-sensitive tissues. *Endocrinology* 142:1269–1277, 2001
17. Albrechtsen T, Frederiksen KS, Holmes WE, Boel E, Taylor K, Fleckner J: Novel genes regulated by the insulin sensitizers rosiglitazone during adipocyte differentiation. *Diabetes* 51:1042–1051, 2002
18. Malerod L, Sporstol M, Juvet LK, Mousavi A, Gjoen T, Berg T: Hepatic scavenger receptor class B, type I is stimulated by peroxisome proliferator-activated receptor γ and hepatocyte nuclear factor 4 α . *Biochem Biophys Res Commun* 305:557–565, 2003
19. Cha BS, Ciaraldi TP, Carter L, Nikoulina SE, Mudaliar S, Mukherjee R, Paterniti JR Jr, Henry RR: Peroxisome proliferator-activated receptor (PPAR) γ and retinoid X receptor (RXR) agonists have complementary effects on glucose and lipid metabolism in human skeletal muscle. *Diabetologia* 44:444–452, 2001
20. Yu JG, Javorschi S, Hevener AL, Kruszynska YT, Norman RA, Sinha M, Olefsky JM: The effect of thiazolidinediones on plasma adiponectin levels in normal, obese, and type 2 diabetic subjects. *Diabetes* 51:2968–2974, 2002
21. Iwaki M, Matsuda M, Maeda N, Funahashi T, Matsuzawa Y, Makishim M, Shimomura I: Induction of adiponectin, a fat-derived antidiabetic and antiatherogenic factor, by nuclear receptors. *Diabetes* 52:1655–1663, 2003
22. Maeda N, Shimomura I, Kishida K, Nishizawa H, Matsuda M, Nagaretani H, Furuyama N, Kondo H, Takahashi M, Arita Y, Komuro R, Ouchi N, Kihara S, Tochino Y, Okutomi K, Horie M, Takeda S, Aoyama T, Funahashi T, Matsuzawa Y: Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nat Med* 8:731–737, 2002
23. Berg AH, Combs TP, Du X, Brownlee M, Scherer PE: The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. *Nat Med* 7:947–953, 2001
24. Combs TP, Berg AH, Obici S, Scherer PE, Rossetti L: Endogenous glucose production is inhibited by the adipose-derived protein Acrp30. *J Clin Invest* 108:1875–1881, 2001
25. Pajvani UB, Du X, Combs TP, Berg AH, Rajala MW, Schulthess T, Engel J, Brownlee M, Scherer PE: Structure-function studies of the adipocyte-secreted hormone Acrp30/adiponectin: implications for metabolic regulation and bioactivity. *J Biol Chem* 278:9073–9085, 2003
26. Waki H, Yamauchi T, Kamon J, Ito Y, Uchida S, Kita S, Hara K, Hada Y, Vasseur F, Froguel P, Kimura S, Nagai R, Kadowaki T: Impaired multimerization of human adiponectin mutants associated with diabetes: molecular structure and multimer formation of adiponectin. *J Biol Chem* 278:43052–43063, 2003
27. Kim JK, Fillmore JJ, Gavrilova O, Chao L, Higashimori T, Choi H, Kim H, Yu C, Chen Y, Qu X, Haluzik M, Reitman ML, Shulman GI: Differential effects of rosiglitazone on skeletal muscle and liver insulin resistance in A-ZIP/F-1 fatless mice. *Diabetes* 52:1311–1318, 2003
28. Jiang H, Dallas-Yang Q, Li Z, Szalkowski D, Liu F, Shen X, Wu M, Zhou G, Doebber T, Berger J, Moller DE, Zhang BB: Potentiation of insulin signaling in tissues of Zucker obese rats after acute and long-term treatment with PPAR γ agonists. *Diabetes* 51:2412–2419, 2002
29. Ikeda T, Fujiyama K: The effect of pioglitazone on glucose metabolism and insulin uptake in the perfused liver and hindquarter of high-fructose-fed rats. *Metabolism* 47:1152–1155, 1998
30. Sugiyama Y, Shimura Y, Ikeda H: Effects of pioglitazone on hepatic and peripheral insulin resistance in Wistar fatty rats. *Arzneimittelforschung* 40:436–440, 1990
31. Miyazaki Y, Mahankali A, Matsuda M, Glass L, Mahankali S, Ferrannini E, Cusi K, Mandarino LJ, DeFronzo RA: Improved glycemic control and enhanced insulin sensitivity in type 2 diabetic subjects treated with pioglitazone. *Diabetes Care* 24:710–719, 2001
32. Mauriege P, Imbeault P, Langin D, Lacaille M, Almeras N, Tremblay A, Despre JP: Regional and gender variations in adipose tissue lipolysis in response to weight loss. *J Lipid Res* 40:1559–1571, 1999
33. Hawkins M, Gabriely I, Wozniak R, Reddy K, Rossetti L, Shamon H: Glycemic control determines hepatic and peripheral glucose effectiveness in type 2 diabetic subjects. *Diabetes* 51:2179–2189, 2002
34. Hawkins M, Gabriely I, Wozniak R, Vilcu C, Shamon H, Rossetti L: Fructose improves the ability of hyperglycemia per se to regulate glucose production in type 2 diabetes. *Diabetes* 51:606–614, 2002
35. Steele R: Influences of glucose loading and of injected insulin on hepatic glucose output. *Ann N Y Acad Sci* 82:420–430, 1959
36. Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB, Kadowaki T: Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* 8:1288–1295, 2002
37. Inzucchi SE, Maggs DG, Spollett GR, Page SL, Rife FS, Walton V, Shulman GI: Efficacy and metabolic effects of metformin and troglitazone in type II diabetes mellitus. *N Engl J Med* 338:867–872, 1998
38. Boden G, Cheung P, Mozzoli M, Fried SK: Effect of thiazolidinediones on glucose and fatty acid metabolism in patients with type 2 diabetes. *Metabolism* 52:753–759, 2003
39. Kondo H, Shimomura I, Matsukawa Y, Kumada M, Takahashi M, Matsuda M, Ouchi N, Kihara S, Kawamoto T, Sumitsuji S, Funahashi T, Matsuzawa Y: Association of adiponectin mutation with type 2 diabetes: a candidate gene for the insulin resistance syndrome. *Diabetes* 51:2325–2328, 2002
40. Stefan N, Vozarova B, Funahashi T, Matsuzawa Y, Weyer C, Lindsay RS, Youngren JF, Havel PJ, Pratley RE, Bogardus C, Tataranni PA: Plasma adiponectin concentration is associated with skeletal muscle insulin receptor tyrosine phosphorylation, and low plasma concentration precedes a decrease in whole-body insulin sensitivity in humans. *Diabetes* 51:1884–1888, 2002
41. Best JD, Kahn SE, Ader M, Watanabe RM, Ni TC, Bergman RN: Role of glucose effectiveness in the determination of glucose tolerance. *Diabetes Care* 19:1018–1030, 1996
42. Hawkins M, Tonelli J, Kishore P, Stein D, Ragucci E, Gitig A, Reddy K: Contribution of elevated free fatty acid levels to the lack of glucose effectiveness in type 2 diabetes. *Diabetes* 52:2748–2758, 2003
43. Van Cauter E, Polonsky KS, Scheen AJ: Roles of circadian rhythmicity and sleep in human glucose regulation. *Endocr Rev* 18:716–738, 1997
44. Majer M, Popov KM, Harris RA, Bogardus C, Prochazka M: Insulin downregulates pyruvate dehydrogenase kinase (PDK) mRNA: potential mechanism contributing to increased lipid oxidation in insulin-resistant subjects. *Mol Genet Metab* 65:181–186, 1998

45. Holness MJ, Kraus A, Harris RA, Sugden MC: Targeted upregulation of pyruvate dehydrogenase kinase (PDK)-4 in slow-twitch skeletal muscle underlies the stable modification of the regulatory characteristics of PDK induced by high-fat feeding. *Diabetes* 49:775–781, 2000
46. Hevener AL, Reichart D, Janez A, Olefsky J: Thiazolidinedione treatment prevents free fatty acid-induced insulin resistance in male Wistar rats. *Diabetes* 50:2316–2322, 2001
47. Rosa G, Di Rocco P, Manco M, Greco AV, Castagneto M, Vidal H, Mingrone G: Reduced PDK4 expression associates with increased insulin sensitivity in postobese patients. *Obes Res* 11:176–182, 2003