

Pioglitazone Induces Mitochondrial Biogenesis in Human Subcutaneous Adipose Tissue In Vivo

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Thiazolidinediones such as pioglitazone improve insulin sensitivity in diabetic patients by several mechanisms, including increased uptake and metabolism of free fatty acids in adipose tissue. The purpose of the present study was to determine the effect of pioglitazone on mitochondrial biogenesis and expression of genes involved in fatty acid oxidation in subcutaneous fat. Patients with type 2 diabetes were randomly divided into two groups and treated with placebo or pioglitazone (45 mg/day) for 12 weeks. Mitochondrial DNA copy number and expression of genes involved in mitochondrial biogenesis were quantified by real-time PCR. Pioglitazone treatment significantly increased mitochondrial copy number and expression of factors involved in mitochondrial biogenesis, including peroxisome proliferator-activated receptor (PPAR)- γ coactivator-1 α and mitochondrial transcription factor A. Treatment with pioglitazone stimulated the expression of genes in the fatty acid oxidation pathway, including carnitine palmitoyltransferase-1, malonyl-CoA decarboxylase, and medium-chain acyl-CoA dehydrogenase. The expression of PPAR- α , a transcriptional regulator of genes encoding mitochondrial enzymes involved in fatty acid oxidation, was higher after pioglitazone treatment. Finally, the increased mitochondrial copy number and the higher expression of genes involved in fatty acid oxidation in human adipocytes may contribute to the hypolipidemic effects of pioglitazone. *Diabetes* 54:1392–1399, 2005

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ADRB3, adrenergic β -3 receptor; CPT-1, carnitine palmitoyltransferase 1; FPG, fasting plasma glucose; MCAD, medium-chain acyl-CoA dehydrogenase; MLYCD, malonyl-CoA decarboxylase; mtDNA, mitochondrial DNA; mtTFA, mitochondrial transcription factor A; NRF, nuclear respiratory factor; PGC-1 α , peroxisome proliferator-activated receptor coactivator-1 α ; PPAR, peroxisome proliferator-activated receptor; TZD, thiazolidinedione; UCP, uncoupling protein.

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Recent studies (1–4) report that mitochondrial metabolism in muscle and adipocytes is disturbed in patients with insulin resistance, type 2 diabetes, and even in subjects with positive family history for diabetes. This includes a reduction in the expression of peroxisome proliferator-activated receptor (PPAR) coactivator-1 α (PGC-1 α), a key regulator of mitochondrial biogenesis. Additionally, lower expression of PGC-1 α -responsive genes involved in oxidative phosphorylation was noted in morbid obesity and the pre-diabetic state in skeletal muscle (1,2,5). Little is known about the effect of thiazolidinediones (TZDs) on mitochondrial function. In Zucker diabetic rats, treatment with troglitazone restored impaired expression of PGC-1 α and nuclear respiratory factor (NRF)-1 in muscle (6). Exposure of 3T3-L1 adipocytes to rosiglitazone for 48 h increased the number of mitochondria per cell, and the mitochondria became more similar to brown adipocytes (7). It has also been shown that a PPAR- γ ligand is required for the transcription of the brown adipocyte uncoupling protein (UCP)-1 in white adipocytes cultured in vitro (8,9).

A number of transcription factors are involved in mitochondrial biogenesis, including PGC-1 α , mitochondrial transcription factor A (mtTFA), NRF-1 and -2, mitochondrial transcription factors B1 and B2, and estrogen-related receptor- α (5,10–12). PGC-1 α , originally described as a brown fat-specific coactivator (13), has been recently described as a nuclear factor critical for activation of genes required for mitochondrial biogenesis in cell culture and rodent skeletal muscle (13–15). Several pieces of evidence demonstrate that PGC-1 α is capable of interacting with and coactivating nuclear receptors, e.g., PPAR- γ (13), PPAR- α (16), and NRF-1 (14), indicating a broad spectrum of actions for PGC-1 α (rev. in 17). PGC-1 α may also modulate intracellular fatty acid transport and fatty acid β -oxidation (16). In muscle, mitochondrial number is increased in response to endurance exercise training and β -adrenergic receptor activation; both stimulate PGC-1 α expression, mitochondrial biogenesis, and lipid oxidation (18,19). Interestingly, ectopic expression of PGC-1 α in human white adipocytes induces the expression of brown adipocyte genes and promotes fatty acid oxidation in vitro (20).

The aim of the presented study was to define whether treatment of obese diabetic patients with pioglitazone, a potent selective PPAR- γ ligand, affects 1) mitochondrial DNA (mtDNA) copy number, 2) expression of genes involved in mitochondrial biogenesis, and 3) expression of genes driving fatty acid oxidation in human subcutaneous

TABLE 1
Oligonucleotide sequences for primer/probe sets used Taqman analysis

Gene	Accession number	Forward primer	Probe	Reverse primer
ND 1		CCCTAAACCCCGCCACATCT	CCATCACCCCTTACATCACCCGCC	GAGCGATGCTGAGAGCTAAGGT
ND 4		CCATTCTCCTCTATCCCTCAAC	CCGACATCATTTACCGGGTTTCTCTTG	CACAATCTGATGTTTGGTTAAACTATATTT
LPL	NM_000237	CGAGTCGTCTTCTCCTGATGAT	ACATTCACCGAGGGTTC	TTCTGGATTCACATGCTTCGA
PGC-1 α	NM_013261	CACCAAAACCCACAGAGAACA	CGCAGTCACAAACAGTTCAAGCCAAAC	GGGTCAATTTGGTGACTCTCTGG
PGC-1 β	NM_133263	CAGCCACTGCGAAGGACTTGA	CTGAAACACCGCCCTTCTCTCACA	CGGATGCTGGCCGCTCTTG
mTFA	NM_003201	CCCAGATGCAAAAATACTAGCAACTAA	TCCAAAGCTGGGCAATTTCTCTTA	TCGCCOCTATAAAGCATCTTGA
Cyt C	NM_001916	TGGCCCTCCCATCTACAC	AGAGTTTGACGATGGCACCCCCAGCTA	ATCCCTGGCTATCTGGGACATG
NRF-1	NM_005011	CGTTGCCCAAGTGAATTTATTCTG	TTGTTCCACTCTCCATCAGGCCA	CCCTGTAACGTGGCCCAAT
PPAR- α	NM_005036	GCTTTGGCTTTACGGAATACCA	AGCCATCTGAGCCAGGACAGCCTTCTTAA	TGAAAAGCGTGTCCGTGATGA
MCAD	AF251043	TGCCCTTGAAAGGAAAACCTTT	TGTAGAGCACCAAGCAATATCATTTATG	GTCAACTTTCATTTGCCATTTTCAG
CPT-1	D87812	CCAGAGCAGCACCCCAAT	CATCTGCTACAGGCCCAAAAGCCACCT	CTGCAATCATGTAGGAACCTCCATAG
MLYCD	NM_012213	TGGCACTGCATGGTAGACATCTC	AGCATCTGAGCAATGCTGAAAGGAACATC	TGTTCTTCTCTCTGTCTGATGGA
UCP-1	NM_021833	ACGACACCGGTCCAGGAGTTTC	TCACCGCAGGGAAGAAAACAGCACCC	ACCAGCTAAAATCTTGCCTTCCCTAAAC
TTF-2	NM_006540	AGTGACCTCCCGTGCCTACGTT	AGGATCATTTAACCTGCTCGGACCCCAT	CAGGTTGCCCTCCCTCAGA
SRC-1	U40396	GCAGATGCCAGGAATGAAACA	TGCCCTGAGGAGATTAATGATTCCTC	TTGCAAGTAGAGGCCCTGTGTCT
ADRB3	NM_000025	CGCAGCCCAAGGCTTT	AGCCCCGTCCGAGCCGCTTG	CTTCTTTGTCCTTCAGGCCCTAAG
Cyclophilin B	XM_042251	GGAGATGGCAACAGGAGGAAA	CATCTACCGGTGAGCCGCTTCCCCCG	CGTAGTCTTCAGGTTTGAAGTTCTCA

ND, NADH dehydrogenase subunit 1; ND 4, NADH dehydrogenase subunit 4; LPL, lipoprotein lipase; Cyt C, cytochrome C; TTF-2, nuclear receptor coactivator-2; SRC-1, steroid receptor coactivator-1.

adipocytes in vivo. We hypothesized that pioglitazone might increase mitochondrial content, increase expression of PGC-1 α in diabetic patients, and alter the expression of genes involved in fatty acid β -oxidation. As a secondary aim, we measured the activity of the PPAR- α nuclear hormone receptor in these same samples by measuring the expression of PPAR- α and genes known to be transcribed in response to PPAR- α agonists.

RESEARCH DESIGN AND METHODS

Participants and sample collection. Two groups of subjects were included in this study. The first group had type 2 diabetes and was part of a clinical trial on the effects of pioglitazone on body composition (21). Briefly, it included 22 men and 26 women aged 54.7 ± 9.5 years, with a BMI 32.1 ± 5.1 kg/m². Diabetes was defined by a fasting plasma glucose (FPG) ≥ 126 mg/dl at entry or FPG > 115 mg/dl and a 2-h oral glucose tolerance test glucose ≥ 200 mg/dl or current use of either metformin or sulfonylureas. Baseline FPG at entry had to be ≤ 200 mg/dl. Insulin and triglyceride concentrations were 14.7 ± 9.4 mIU/ml and 213.9 ± 112.1 mg/dl, respectively. The second group consisted of nine men and three women without diabetes who had a mean age of 22.4 ± 3.2 years and mean BMI 28.9 ± 4.6 kg/m². All patients signed a consent form approved by the Pennington Biomedical Research Center ethical review board after risks and procedures had been explained.

The diabetic subjects were randomly divided into two groups of 24 patients each and treated with placebo or pioglitazone. Pioglitazone and matching placebos were prepared by Takeda Pharmaceuticals and were given as a single daily dose of 30 mg/day or placebo each morning for 12 weeks. The dose of pioglitazone was increased to 45 mg/day at week 8 if the FPG was > 100 mg/dl or the HbA_{1c} was $\geq 7.0\%$.

Treatment with pioglitazone after 12 weeks improved glucose control as evidenced by a decrease in HbA_{1c} ($-0.96 \pm 1.11\%$), a decrease in triglycerides (-54.18 ± 134.85 mg/dl), and an increase in body weight ($+1.68 \pm 2.4$ kg) (21). Subcutaneous adipose tissue biopsies were obtained from diabetic and nondiabetic subjects using a Bergstrom needle as previously described (22).

RNA and DNA extraction. Total RNA from 50 to 100 mg adipose tissue was isolated with Trizol reagent (Invitrogen, Carlsbad, CA) and quantified spectrophotometrically. The integrity of the RNA was confirmed by PAGE with Sybr green detection. DNA was extracted from the same tissue, after separation of protein and RNA with Trizol, by phenol-chloroform extraction and ethanol precipitation according to the manufacturer's procedure. The total amount of DNA recovered was determined by spectrophotometry.

Real-time RT-PCR for RNA. Real-time RT-PCR for tested genes was performed using a Taqman 100R \times n PCR Core Reagent Kit (Applied Biosystems, Roche, Branchburg, NJ) as described previously (24). Taqman probes and primers were designed using Primer Express Software version 2.0 (Applied Biosystems, Roche, Branchburg, NJ). The sequences of primers and probes and accession numbers for each gene are shown in Table 1. Real-time RT-PCR was carried out in an ABI PRISM 7700 sequence detector (Applied Biosystems, Branchburg, NJ) using the following parameters: one cycle of 48°C for 30 min, then 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All expression data were normalized by dividing the amount of target by amount of cyclophilin B used as internal control.

Real-time PCR for mitochondrial DNA. Relative amounts of nuclear DNA and mtDNA were determined by quantitative real-time PCR. The ratio of mtDNA to nuclear DNA reflects the tissue concentration of mitochondria per cell. For this purpose, a 120-nucleotide-long mtDNA fragment within the ND 1 gene was selected for quantification of mtDNA according to He et al. (23). The PCR product was cloned into the plasmid pCDNAII according to the manufacturer's protocol (Invitrogen, Carlsbad, CA) and verified by DNA sequencing. The concentration of DNA in the plasmid was estimated from the absorbance (260 nm) and calculated to give a stock of 2.5×10^{10} copy/ μ l. Plasmid standards of known copy number were used to generate a standard curve from which the copy number of the unknowns was determined. The quantification assay was performed in a total volume of 50 μ l containing 10 μ l DNA template, 5 μ l of 10 \times buffer A, 11 μ l of 25 mmol/l MgCl₂, 1 μ l of each dNTP, 0.5 μ l AMPERase UNG (uracil-N-glycosylase), 0.25 μ l AmpliTaq Gold DNA polymerase, and 15.25 μ l water. Amplification and detection were performed in an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Roche, Branchburg, NJ). The PCR was initiated with 2 min at 50°C (for the activation of AmpErase UNG), followed by 10 min at 95°C (for deactivation of AmpErase UNG and activation of AmpliTaq Gold). This was followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. To normalize results, a 120-bp region of nuclear DNA (lipoprotein lipase) was also amplified and cloned as

described above. The total amount of nuclear DNA was not affected by TZD. The PCR conditions were the same as for mtDNA.

Citrate synthase activity. Citrate synthase activity was measured according to the method described previously (24). Briefly, 50 mg tissue was placed in extraction buffer (10:1 vol/vol) containing 50 mmol/l Tris/HCl, 1 mmol/l MgCl₂, 100 mmol/l KCl, 250 mmol/l sucrose, and 30 mmol/l 2-mercaptoethanol. The suspension was homogenized by polytron and centrifuged twice (9,000g, 15 min, 4°C). The pellet and lipid were carefully removed. For the measure, the activity the assay buffer containing 0.11 mmol/l 5-5'-dithio-bis (2-nitrobenzoic acid), 0.25 mmol/l acetyl-CoA, and 0.28 mmol/l oxaloacetate was used. The reaction was initiated by adding of 30 μl of enzyme extract. Changes in absorbance at 412 nm in 25°C were measured during 10 min using kinetic software (Microplate Manager; Bio-Rad Laboratories). The results are presented as changes in activity in the two treatment groups (μmol · min⁻¹ · g tissue⁻¹).

Statistical analysis. All clinical data were entered into the Pennington Biomedical Research Center database, extracted and combined with a gene expression database, and analyzed using JMP version 5.0.1a (SAS, Cary, NC). Correlations were performed in a pairwise fashion using the Pearson product-moment statistic. Two-way ANOVA was performed using time and treatment as factors to assess changes in gene expression within subject during the study. The effect of metformin and sulfonylureas on gene expression was tested using variance-covariance analysis (SAS version 8). All values are presented in figures and tables as sample (raw) means ± SE. Significant differences were assumed for $P < 0.05$.

RESULTS

Characteristics of the study populations. The patients treated with pioglitazone and those treated with placebo were well matched at baseline. The nondiabetic subjects were markedly younger and leaner than the diabetic subjects.

Expression of PGC-1α and mtDNA copy number in type 2 diabetes. mtDNA copy number was significantly higher in healthy patients compared with diabetic nontreated subjects ($P < 0.05$; Fig. 1A). Mitochondrial number did not change in the placebo group but significantly increased after pioglitazone treatment ($P < 0.01$; Fig. 1A). There was no significant difference between nondiabetic control subjects and diabetic patients after treatment with pioglitazone.

PGC-1α mRNA was significantly higher in nondiabetic control subjects compared with diabetic nontreated subjects ($P < 0.05$; Fig. 1B). The placebo-treated subjects showed no significant changes for any of the genes that were measured. Treatment with pioglitazone increased the mRNA for PGC-1α in diabetic patients ($P < 0.01$; Fig. 1B).

Citrate synthase activity, a marker of mitochondrial aerobic capacity, increased in pioglitazone-treated patients (8.19 ± 0.9 vs. 6.07 ± 0.3 μmol · min⁻¹ · g tissue⁻¹; NS; change in activity: 2.12 ± 0.77 vs. -0.09 ± 0.48 μmol · min⁻¹ · g⁻¹ tissue; $P < 0.05$; Fig. 1C). Patients in the placebo group showed no differences in the citrate synthase activity (6.94 ± 0.49 vs. 7.03 ± 0.86 μmol · min⁻¹ · g tissue⁻¹; data not shown). A modest positive relationship was observed between mtDNA copy number and citrate synthase activity ($R = 0.52$; $P < 0.05$; data not shown).

Effect of pioglitazone on gene expression. Pioglitazone increased the gene expression of several factors involved in mitochondrial biogenesis (Fig. 2A–C). mtTFA, a downstream target of PGC-1α, increased in pioglitazone-treated patients ($P < 0.05$; Fig. 2A). The mRNA for cytochrome C tended to increase in the pioglitazone-treated group (Fig. 2B). Unexpectedly, the mRNA for NRF-1 was not affected by pioglitazone and did not differ between the two groups (Fig. 2C). The expression of

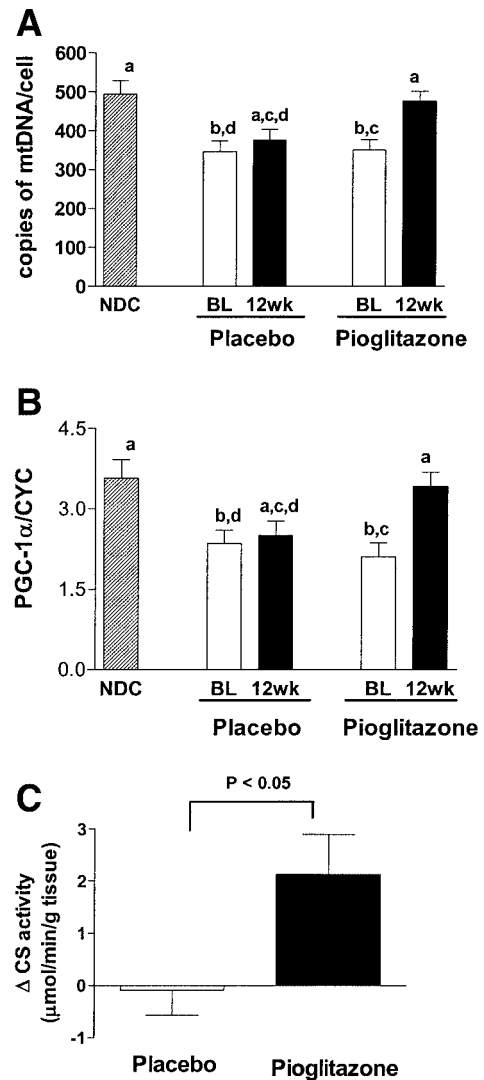


FIG. 1. Mitochondrial copy number (A) and PGC-1α expression (B) in young healthy nondiabetic patients (NDC) and in patients with type 2 diabetes treated with placebo or pioglitazone during 12 weeks. C: Changes in citrate synthase activity in two groups of patients: placebo and pioglitazone. Data are shown as means ± SE. Significant differences ($P < 0.05$) are indicated by different letters (a and b) and not significant between the same letters.

UCP-1, a marker of brown adipose tissue, increased in pioglitazone-treated subjects ($P < 0.05$; Fig. 2H). In contrast, there was no difference between the two groups in the mRNA level for adrenergic β-3 receptor (ADRB3; Fig. 2I). Pioglitazone increased the expression of genes required for fatty acid oxidation (Fig. 2D–G), including PPAR-α ($P < 0.05$; Fig. 2D), medium-chain acyl-CoA dehydrogenase (MCAD; $P < 0.0001$; Fig. 2E), carnitine palmitoyltransferase I (CPT-1; $P < 0.05$; Fig. 2F), and malonyl-CoA decarboxylase (MLYCD; $P < 0.001$; Fig. 2G). The expression of PGC-1β, a homolog of PGC-1α implicated in the regulation of fatty acid oxidation (25), tended to be higher after pioglitazone treatment, although the difference was not statistically significant (0.82 ± 0.05 vs. 0.69 ± 0.05 ; NS; data not shown). The expression of two members of the p160 coregulator family, steroid receptor coactivator-1 and nuclear receptor coactivator-2, both involved in energy homeostasis in white and brown adi-

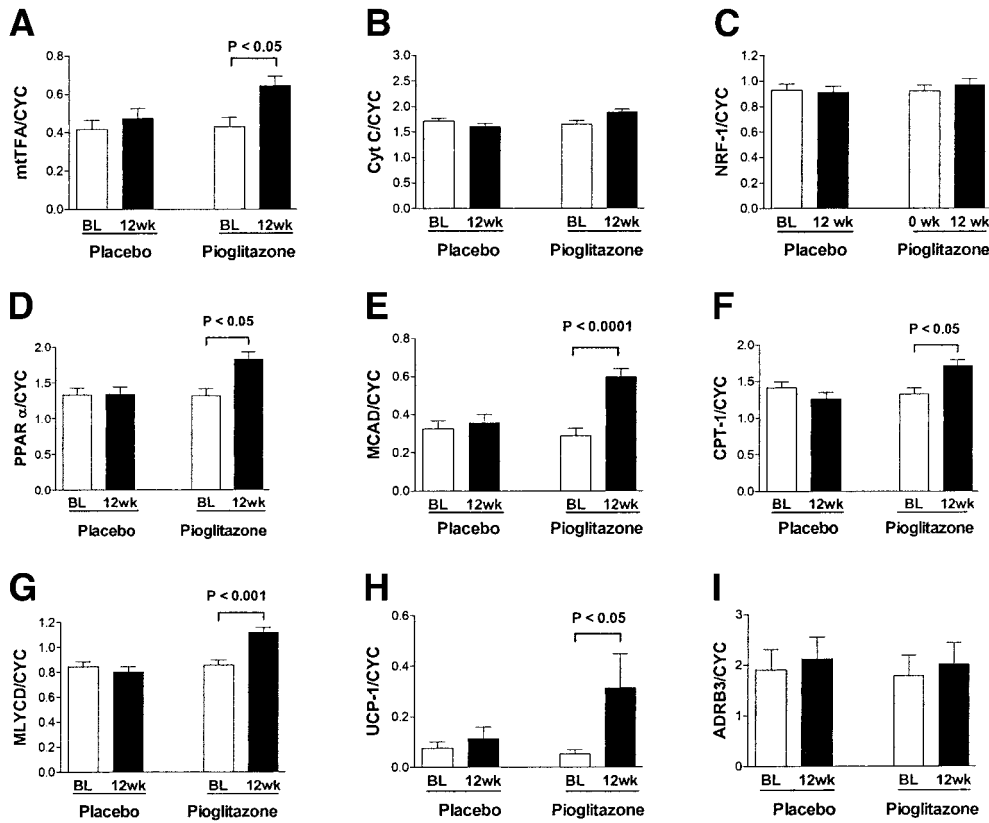


FIG. 2. Effect of the 12-week treatment with placebo or pioglitazone on expression of mtTFA (A), cytochrome C (Cyt C) (B), NRF-1 (C), PPAR- α (D), MCAD (E), CPT-1 (F), MLYCD (G), UCP-1 (H), and ADRB3 (I). Data are shown as means \pm SE.

pose tissue by formation of complexes with PGC-1 α (26) did not change after pioglitazone treatment (data not shown).

Because many of the subjects were treated with sulfonylureas and/or metformin, we tested the effect of these treatments on gene expression at baseline by ANOVA.

There were no significant differences in gene expression at baseline in patients treated with metformin or sulfonylurea as compared with patients not taking these drugs at study entry.

The mRNA for PGC-1 α was highly correlated with mRNA for several genes in the mitochondrial biogenesis

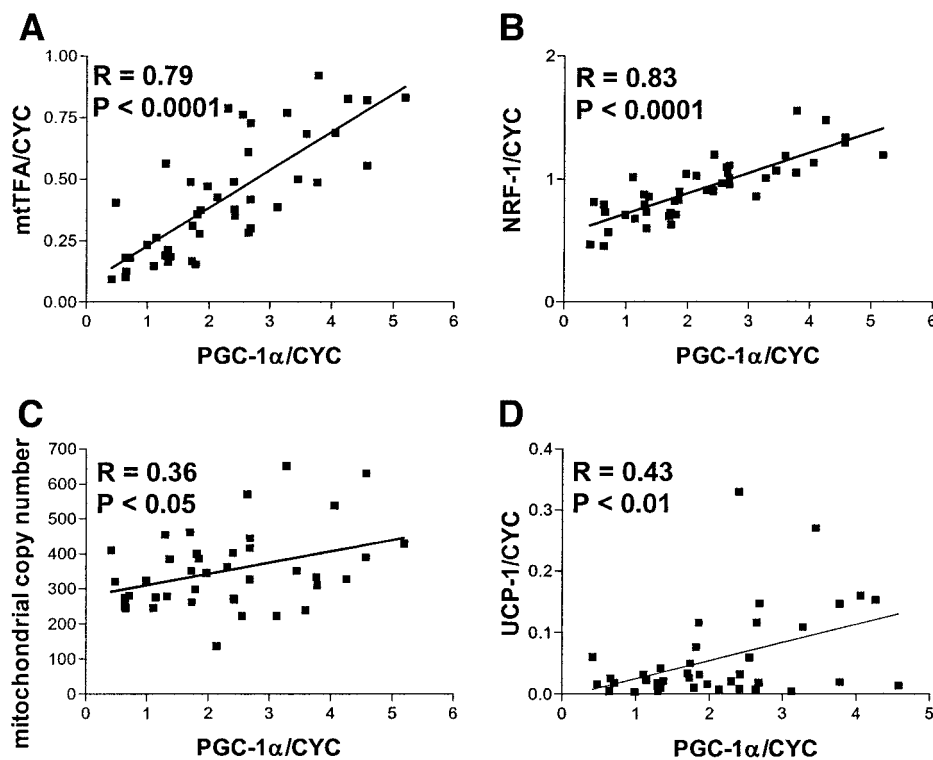


FIG. 3. Correlations between mRNAs at baseline for PGC-1 α and mtTFA (A), NRF-1 (B), mitochondrial copy number (C), or UCP-1 (D) in human subcutaneous fat.

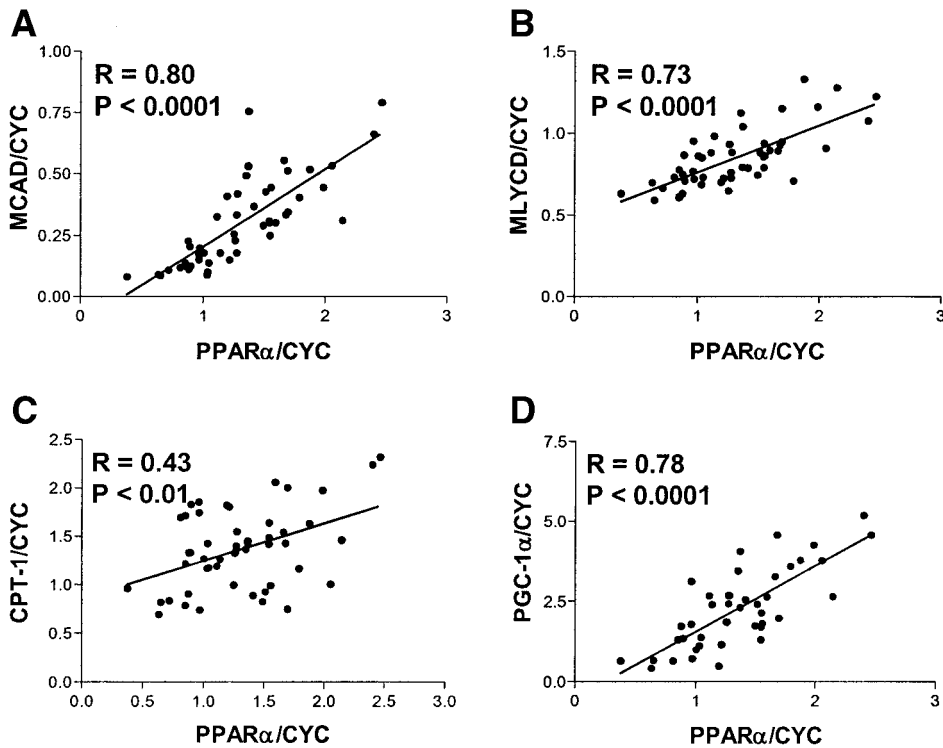


FIG. 4. Correlations between mRNAs at baseline for PPAR- α and downstream targets of PPAR- α : MCAD (A), MLYCD (B), CPT-1 (C), or PGC-1 α (D) in human subcutaneous fat.

pathway, including mtTFA (Fig. 3A) and NRF-1 (Fig. 3B). Statistically significant but much smaller correlations were observed between mRNA for PGC-1 α and mitochondrial copy number and UCP-1 (Fig. 3C and D). No relationship was found between PGC-1 α and ADRB3 or cytochrome C (data not shown). Interestingly, PGC-1 α expression was highly correlated with the mRNA for two members of p160 family coregulators: nuclear receptor coactivator-2 ($R = 0.84$; $P < 0.0001$; data not shown) and steroid receptor coactivator-1 ($R = 0.77$; $P < 0.0001$; data not shown).

The baseline expression of mRNA for PPAR- α was significantly correlated with several PPAR- α targets: MCAD (Fig. 4A), MLYCD (Fig. 4B), and CPT-1 (Fig. 4C). Additionally, a strong correlation was found between PPAR- α mRNA and PGC-1 α (Fig. 4D), PGC-1 β ($R = 0.50$; $P < 0.001$; data not shown), steroid receptor coactivator-1 ($R = 0.75$; $P < 0.0001$; data not shown), and nuclear receptor coactivator-2 ($R = 0.75$; $P < 0.0001$; data not shown) but not with ADRB3 (data not shown), UCP-1 (data not shown), or mitochondrial copy number (data not shown).

DISCUSSION

The antidiabetic effects of TZDs are well established, although the full spectrum of actions is not fully understood, especially when the effects on mitochondrial function are considered. There are several important findings from our study testing the in vivo effect of pioglitazone on mitochondria and genes involved in fatty acid metabolism in subcutaneous adipose tissue from diabetic patients. First, using quantitative real-time PCR, we observed a significantly higher mitochondrial copy number per cell in adipose tissue obtained from healthy young control subjects compared with diabetic patients. Furthermore, treatment of diabetic individuals with pioglitazone restored the reduced level of mitochondrial number to that observed in

nondiabetic control subjects. It has been previously reported that the real-time PCR method used in our experiment is highly sensitive to determine the mitochondrial copy number in several tissue including adipocytes (23,27). We found that the increase in mitochondrial DNA was mirrored by an increase in the citrate synthase activity, a mitochondrial protein encoded by a nuclear gene that is widely recognized as a marker of mitochondrial activity and correlated to mitochondrial number in human muscle (28). Previous studies (29) in rodent adipose tissue showed a similar increase in citrate synthase activity with pioglitazone. The relationship between mtDNA copy number and citrate synthase activity is quite variable across tissues, as the mtDNA content and the activity of mitochondrial machinery represent structural and functional aspects of the organelle (30). Although the activity of mitochondrial enzymes and mitochondrial number are generally linked, activity of the enzymes is determined by more than just the mass of the enzymes, adding complexity to the regulation of ATP synthesis and fat oxidation.

Our data are consistent with results from in vivo experiments performed on rat and dogs treated with TZD (31). Morphological studies confirmed a higher number of mitochondria per perirenal adipocyte in both species after TZD treatment (31). In another in vitro experiment, 3T3-L1 adipocytes exposed 48 h to PPAR- γ agonists increased mitochondrial density and altered mitochondrial morphology toward brown adipocytes with concomitant increased expression of several mitochondrial proteins (7,32). Interestingly, in our in vivo study as well as in white adipose tissue tested in vitro (8,9), the expression of UCP-1, a gene whose expression is typically restricted to brown adipocytes, increased after TZD.

The molecular mechanisms involved in mitochondrial biogenesis in fat tissue have only been partially elucidated.

Physiological studies (14,17) imply that PGC-1 α has a principal role in this process in muscle. In humans, cultured in vitro, the overexpression of PGC-1 α or the addition of TZD increased mitochondrial number (7,20). Alternately, preexisting "quiescent" brown adipocytes may have increased their mitochondria number (33).

These observations are important from several perspectives. First, Semple et al. (4) observed a threefold reduction in PGC-1 α mRNA in adipose tissue of morbidly obese patients, suggesting that mitochondrial number might also be reduced in severe obesity. Our study, performed in less severely obese type 2 diabetic subjects underscores the possibility that reduced PGC-1 α and mitochondrial biogenesis are primary defects in obesity and insulin resistance. One shortcoming of these studies is that our nondiabetic control group was slightly leaner and significantly younger than the diabetic subjects. In parallel to these findings in skeletal muscle, nuclear genes encoding mitochondrial oxidative phosphorylation are downregulated in the muscle of individuals with diabetes or a family history of diabetes (1,2,34). The present data demonstrate that pioglitazone increases PGC-1 α expression and mitochondrial copy number. Second, an increase in mitochondrial number per cell might increase aerobic metabolism. Large adipocytes are known to use glycolytic pathways, as evidenced by an increased production of lactate in both in vitro and in vivo experiments (35,36). It has been reported that lactate production is increased in obesity (37). In one study (38), plasma lactate tended to decrease after TZD treatment, but in our study we did not observe any changes in fasting lactate (data not shown). Lastly, changes observed in expression of genes involved in mitochondrial biogenesis were mirrored by an increase in the expression of genes required for fat oxidation (PPAR- α , CPT-1, MLYCD, and MCAD).

That PPAR- α would be correlated to the expression of genes involved in fat oxidation is not unexpected (39,40). PPAR- α agonists increase the transcription of a variety of genes that are associated with decreased lipid synthesis and increased fat oxidation (39,40). One controversial in vitro study (41) demonstrated that pioglitazone can directly activate PPAR- α . TZDs increased the expression in vivo of genes in the fatty acid oxidation pathway in rodents (42,43). Another possibility to explain the observed increase in PPAR- α target genes is that the upregulation of PGC-1 α expression, observed in our study after TZD treatment, could induce expression or coactivate PPAR- α . Vega et al. (16) suggest that PGC-1 α acts as a coactivator of PPAR- α in the transcriptional control of mitochondrial fatty acid oxidation.

PPAR- γ ligands increase fatty acid uptake by human muscle in vitro (44) and increase fat oxidation in cultured human and rodent myotubes (45,46). The opposite effect was observed in vivo (42) and in vitro (47). There is no evidence that TZDs influence whole-body fatty acid oxidation in humans. PPAR- γ agonists increase flux of fatty acids into adipocytes and away from muscle (21). Sequestration of lipid in adipose tissue could explain why we (48) and others (49) find that whole-body resting or postprandial fat oxidation does not change, even though genes required for fatty acid oxidation increase. Alternately,

TZDs could change a metabolic flexibility and the dynamic changes in substrate oxidation throughout the day.

To summarize, we observed an increase in PGC-1 α and PPAR- α expression after pioglitazone treatment. Based on the literature and these results, we speculate that pioglitazone, by increasing transcription of PGC-1 α , upregulates the mitochondrial machinery in adipose tissue. Given the data at hand, we cannot determine which of these factors plays the primary function in mitochondrial biogenesis. We can only speculate that pioglitazone, by activation of PGC-1 α transcription, may coordinately activate mitochondrial biogenesis and PPAR- α /fatty acid oxidation pathways. Alternatively, pioglitazone as a weak agonist of PPAR- α might induce expression of downstream genes involved in fatty acid oxidation. Finally, all of these may happen independently and need to be explored in further experiments in vivo.

In conclusion, these data suggest that pioglitazone increases PGC-1 α expression and mtDNA copy number and may enhance the oxidative capacity of white adipose tissue. It is noteworthy that the increase in mitochondrial fatty acid oxidation capacity is occurring in parallel with an increased lipid accumulation in the adipocytes (21). TZD-treated subjects gain weight, indicating storage of lipid in adipocytes, as previously published (48), and the balance of fat storage versus oxidation tips toward storage. Only if there is an increased demand for ATP or if an uncoupling process is initiated, can the increased amount of enzymes and mitochondrial machinery result in increased metabolism. Lastly, pioglitazone increases mitochondrial copy number to levels seen in healthy young control subjects and increases the expression of genes required for fatty acid oxidation.

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REFERENCES

- Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D, Groop LC: PGC-1 α -responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* 34:267-273, 2003
- Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S, Miyazaki Y, Kohane I, Costello M, Saccone R, Landaker EJ, Goldfine AB, Mun E, DeFronzo R, Finlayson J, Kahn CR, Mandarino LJ: Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: potential role of PGC1 and NRF1. *Proc Natl Acad Sci U S A* 100:8466-8471, 2003
- Hammarstedt A, Jansson PA, Wesslau C, Yang X, Smith U: Reduced expression of PGC-1 and insulin-signaling molecules in adipose tissue is associated with insulin resistance. *Biochem Biophys Res Commun* 301: 578-582, 2003
- Semple RK, Crowley VC, Sewter CP, Laudes M, Christodoulides C, Considine RV, Vidal-Puig A, O'Rahilly S: Expression of the thermogenic nuclear hormone receptor coactivator PGC-1 α is reduced in the adipose tissue of morbidly obese subjects. *Int J Obes Relat Metab Disord* 28:176-179, 2004
- Mootha VK, Handschin C, Arlow D, Xie X, St Pierre J, Sihag S, Yang W, Altshuler D, Puigserver P, Patterson N, Willy PJ, Schulman IG, Heyman RA,

- Lander ES, Spiegelman BM: ERRalpha and Galpha/b specify PGC-1alpha-dependent oxidative phosphorylation gene expression that is altered in diabetic muscle. *Proc Natl Acad Sci U S A* 101:6570–6575, 2004
6. Jove M, Salla J, Planavila A, Cabrero A, Michalik L, Wahli W, Laguna JC, Vazquez-Carrera M: Impaired expression of NADH dehydrogenase subunit 1 and PPARgamma coactivator-1 in skeletal muscle of ZDF rats: restoration by troglitazone. *J Lipid Res* 45:113–123, 2004
 7. Wilson-Fritch L, Burkart A, Bell G, Mendelson K, Leszyk J, Nicoloso S, Czech M, Corvera S: Mitochondrial biogenesis and remodeling during adipogenesis and in response to the insulin sensitizer rosiglitazone. *Mol Cell Biol* 23:1085–1094, 2003
 8. Fukui Y, Masui S, Osada S, Umesono K, Motojima K: A new thiazolidinedione, NC-2100, which is a weak PPAR-gamma activator, exhibits potent antidiabetic effects and induces uncoupling protein 1 in white adipose tissue of KKAY obese mice. *Diabetes* 49:759–767, 2000
 9. Digby JE, Montague CT, Sewter CP, Sanders L, Wilkison WO, O'Rahilly S, Prins JB: Thiazolidinedione exposure increases the expression of uncoupling protein 1 in cultured human preadipocytes. *Diabetes* 47:138–141, 1998
 10. Falkenberg M, Gaspari M, Rantanen A, Trifunovic A, Larsson NG, Gustafsson CM: Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA. *Nat Genet* 31:289–294, 2002
 11. Virbasius CA, Virbasius JV, Scarpulla RC: NRF-1, an activator involved in nuclear-mitochondrial interactions, utilizes a new DNA-binding domain conserved in a family of developmental regulators. *Genes Dev* 7:2431–2445, 1993
 12. Schreiber SN, Emter R, Hock MB, Knutti D, Cardenas J, Podvenc M, Oakeley EJ, Kralli A: The estrogen-related receptor alpha (ERRalpha) functions in PPARgamma coactivator 1alpha (PGC-1alpha)-induced mitochondrial biogenesis. *Proc Natl Acad Sci U S A* 101:6472–6477, 2004
 13. Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM: A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* 92:829–839, 1998
 14. Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, Spiegelman BM: Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* 98:115–124, 1999
 15. Lin J, Wu H, Tarr PT, Zhang CY, Wu Z, Boss O, Michael LF, Puigserver P, Isotani E, Olson EN, Lowell BB, Bassel-Duby R, Spiegelman BM: Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. *Nature* 418:797–801, 2002
 16. Vega RB, Huss JM, Kelly DP: The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor alpha in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. *Mol Cell Biol* 20:1868–1876, 2000
 17. Puigserver P, Spiegelman BM: Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator. *Endocr Rev* 24:78–90, 2003
 18. Terada S, Tabata I: Effects of acute bouts of running and swimming exercise on PGC-1alpha protein expression in rat epitrochlearis and soleus muscle. *Am J Physiol Endocrinol Metab* 286:E208–E216, 2004
 19. Tunstall RJ, Mehan KA, Wadley GD, Collier GR, Bonen A, Hargreaves M, Cameron-Smith D: Exercise training increases lipid metabolism gene expression in human skeletal muscle. *Am J Physiol Endocrinol Metab* 283:E66–E72, 2002
 20. Tiraby C, Langin D: Conversion from white to brown adipocytes: a strategy for the control of fat mass? *Trends Endocrinol Metab* 14:439–441, 2003
 21. Bogacka I, Xie H, Bray GA, Smith SR: The effect of pioglitazone on peroxisome proliferator-activated receptor-gamma target genes related to lipid storage in vivo. *Diabetes Care* 27:1–8, 2004
 22. Smith SR, Gawronska-Kozak B, Janderova L, Nguyen T, Murrell A, Stephens JM, Mynatt RL: Agouti expression in human adipose tissue: functional consequences and increased expression in type 2 diabetes. *Diabetes* 52:2914–2922, 2003
 23. He L, Chinnery PF, Durham SE, Blakely EL, Wardell TM, Borthwick GM, Taylor RW, Turnbull DM: Detection and quantification of mitochondrial DNA deletions in individual cells by real-time PCR. *Nucleic Acid Res* 30:E68, 2002
 24. Ceddia RB, William WN Jr, Lima FB, Flandin P, Curi R, Giacobino JP: Leptin stimulates uncoupling protein-2 mRNA expression and Krebs cycle activity and inhibits lipid synthesis in isolated rat white adipocytes. *Eur J Biochem* 267:5952–5958, 2000
 25. Wang YX, Lee CH, Tiep S, Yu RT, Ham J, Kang H, Evans RM: Peroxisome-proliferator-activated receptor delta activates fat metabolism to prevent obesity. *Cell* 113:159–170, 2003
 26. Picard F, Gehin M, Annicotte J, Rocchi S, Champy MF, O'Malley BW, Chambon P, Auwerx J: SRC-1 and TIF2 control energy balance between white and brown adipose tissues. *Cell* 111:931–941, 2002
 27. Gahan ME, Miller F, Lewin SR, Cherry CL, Hoy JF, Mijch A, Rosenfeldt F, Wesselingh SL: Quantification of mitochondrial DNA in peripheral blood mononuclear cells and subcutaneous fat using real-time polymerase chain reaction. *J Clin Virol* 22:241–247, 2001
 28. Wang H, Hiatt WR, Barstow TJ, Brass EP: Relationships between muscle mitochondrial DNA content, mitochondrial enzyme activity and oxidative capacity in man: alterations with disease. *Eur J Appl Physiol Occup Physiol* 80:22–27, 1999
 29. de Souza CJ, Eckhardt M, Gagen K, Dong M, Chen W, Laurent D, Burkey BF: Effects of pioglitazone on adipose tissue remodeling within the setting of obesity and insulin resistance. *Diabetes* 50:1863–1871, 2001
 30. Van den Bogert C, De Vries H, Holtrop M, Muus P, Dekker HL, Van Galen MJ, Bolhuis PA, Taanman JW: Regulation of the expression of mitochondrial proteins: relationship between mtDNA copy number and cytochrome-C oxidase activity in human cells and tissues. *Biochim Biophys Acta* 1144:177–183, 1993
 31. Toseland CD, Campbell S, Francis I, Bugelski PJ, Mehdi N: Comparison of adipose tissue changes following administration of rosiglitazone in the dog and rat. *Diabetes Obes Metab* 3:163–170, 2001
 32. Lenhard JM, Kliever SA, Paulik MA, Plunkert KD, Lehmann JM, Weiel JE: Effects of troglitazone and metformin on glucose and lipid metabolism: alterations of two distinct molecular pathways. *Biochem Pharmacol* 54:801–808, 1997
 33. Cinti S: Adipocyte differentiation and transdifferentiation: plasticity of the adipose organ. *J Endocrinol Invest* 25:823–835, 2002
 34. Sreekumar R, Halvatsiotis P, Schimke JC, Nair KS: Gene expression profile in skeletal muscle of type 2 diabetes and the effect of insulin treatment. *Diabetes* 51:1913–1920, 2002
 35. Jansson PA, Smith U, Lonnroth P: Evidence for lactate production by human adipose tissue in vivo. *Diabetologia* 33:253–256, 1990
 36. DiGirolamo M, Newby FD, Lovejoy J: Lactate production in adipose tissue: a regulated function with extra-adipose implications. *FASEB J* 6:2405–2412, 1992
 37. Lovejoy J, Newby FD, Gebhart SS, DiGirolamo M: Insulin resistance in obesity is associated with elevated basal lactate levels and diminished lactate appearance following intravenous glucose and insulin. *Metabolism* 41:22–27, 1992
 38. Virtanen KA, Hallsten K, Parkkola R, Janatuinen T, Lonnqvist F, Viljanen T, Ronnema T, Knuuti J, Huupponen R, Lonnroth P, Nuutila P: Differential effects of rosiglitazone and metformin on adipose tissue distribution and glucose uptake in type 2 diabetic subjects. *Diabetes* 52:283–290, 2003
 39. Barger PM, Kelly DP: PPAR signaling in the control of cardiac energy metabolism. *Trends Cardiovasc Med* 10:238–245, 2000
 40. Brandt JM, Djouadi F, Kelly DP: Fatty acids activate transcription of the muscle carnitine palmitoyltransferase I gene in cardiac myocytes via the peroxisome proliferator-activated receptor alpha. *J Biol Chem* 273:23786–23792, 1998
 41. Sakamoto J, Kimura H, Moriyama S, Odaka H, Momose Y, Sugiyama Y, Sawada H: Activation of human peroxisome proliferator-activated receptor (PPAR) subtypes by pioglitazone. *Biochem Biophys Res Commun* 278:704–711, 2000
 42. Way JM, Harrington WW, Brown KK, Gottschalk WK, Sundseth SS, Mansfield TA, Ramachandran RK, Willson TM, Kliever SA: Comprehensive messenger ribonucleic acid profiling reveals that peroxisome proliferator-activated receptor gamma activation has coordinate effects on gene expression in multiple insulin-sensitive tissues. *Endocrinology* 142:1269–1277, 2001
 43. Suzuki A, Yasuno T, Kojo H, Hirosumi J, Mutoh S, Notsu Y: Alteration in expression profiles of a series of diabetes-related genes in db/db mice following treatment with thiazolidinediones. *Jpn J Pharmacol* 84:113–123, 2000
 44. Wilmsen HM, Ciaraldi TP, Carter L, Reehman N, Mudaliar SR, Henry RR: Thiazolidinediones upregulate impaired fatty acid uptake in skeletal muscle of type 2 diabetic subjects. *Am J Physiol Endocrinol Metab* 285:E354–E362, 2003
 45. Cha BS, Ciaraldi TP, Carter L, Nikolouina SE, Mudaliar S, Mukherjee R, Paterniti JR Jr, Henry RR: Peroxisome proliferator-activated receptor (PPAR) gamma and retinoid X receptor (RXR) agonists have complementary effects on glucose and lipid metabolism in human skeletal muscle. *Diabetologia* 44:444–452, 2001
 46. Ide T, Nakazawa T, Mochizuki T, Murakami K: Tissue-specific actions of antidiabetic thiazolidinediones on the reduced fatty acid oxidation in

- skeletal muscle and liver of Zucker diabetic fatty rats. *Metabolism* 49:521–525, 2000
47. Brunmair B, Gras F, Neschen S, Roden M, Wagner L, Waldhausl W, Fornsinn C: Direct thiazolidinedione action on isolated rat skeletal muscle fuel handling is independent of peroxisome proliferator-activated receptor-gamma-mediated changes in gene expression. *Diabetes* 50:2309–2315, 2001
48. Smith SR, de Jonge L, Volaufova J, Li Y, Xie H, Bray GA: Effect of pioglitazone on body composition and energy expenditure: a randomized controlled trial. *Metab Clin Exp* 54:24–32, 2005
49. Mayerson AB, Hundal RS, 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