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ORIGINAL ARTICLE

Improved skeletal muscle oxidative enzyme activity and restoration of PGC-1α and PPARβ/δ gene expression upon rosiglitazone treatment in obese patients with type 2 diabetes mellitus

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Objective: To examine whether rosiglitazone alters gene expression of some key genes involved in mitochondrial biogenesis and oxidative capacity in skeletal muscle of type 2 diabetic patients, and whether this is associated with alterations in skeletal muscle oxidative capacity and lipid content.
Design: Skeletal muscle gene expression, mitochondrial protein content, oxidative capacity and lipid accumulation were measured in muscle biopsies obtained from diabetic patients, before and after 8 weeks of rosiglitazone treatment, and matched controls. Furthermore, whole-body insulin sensitivity and substrate utilization were assessed.
Subjects: Ten obese type 2 diabetic patients and 10 obese normoglycemic controls matched for age and BMI.
Methods: Gene expression and mitochondrial protein content of complexes I–V of the respiratory chain were measured by quantitative polymerase chain reaction and Western blotting, respectively. Histochemical staining was used to quantify lipid accumulation and complex II succinate dehydrogenase (SDH) activity. Insulin sensitivity and substrate utilization were measured during a hyperinsulinemic–euglycemic clamp with indirect calorimetry.
Results: Skeletal-muscle mRNA of PGC-1α and PPARβ/δ – but not of other genes involved in glucose, fat and oxidative metabolism – was significantly lower in diabetic patients ($P<0.01$). Rosiglitazone significantly increased PGC-1α (~2.2-fold, $P<0.01$) and PPARβ/δ (~2.6-fold, $P<0.01$), in parallel with an increase in insulin sensitivity, SDH activity and metabolic flexibility ($P<0.01$). Surprisingly, none of the measured mitochondrial proteins was reduced in type 2 diabetic patients, nor affected by rosiglitazone treatment. No alterations were seen in muscular fat accumulation upon treatment.
Conclusion: These results suggest that the insulin-sensitizing effect of rosiglitazone may involve an effect on muscular oxidative capacity, via PGC-1α and PPARβ/δ, independent of mitochondrial protein content and/or changes in intramyocellular lipid.
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Keywords: insulin resistance; skeletal muscle; intramyocellular lipid; PGC-1α; thiazolidinediones; metabolic flexibility

Introduction

Research conducted in the last two decades has revealed skeletal-muscle insulin resistance as one of the earliest hallmarks in the development of type 2 diabetes mellitus. Recent studies have implicated a decreased expression of peroxisome-proliferator activated receptor (PPAR)-γ coactivator-1α (PGC-1α) in the development of muscular insulin resistance.[1,2] PGC-1α is involved in mitochondrial biogenesis by supporting the transcriptional activity of nuclear respiratory factors, which regulate the transcription of genes involved in oxidative metabolism. Therefore, as a result of the reduced levels of PGC-1α in insulin-resistant states, a reduction in mitochondrial function was anticipated. Indeed, it has been shown that type 2 diabetic patients have less and smaller mitochondria compared to control subjects.[3] In addition, mitochondrial dysfunction has been

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reported in insulin-resistant prediabetic subjects[4] as well as in insulin-resistant elderly.[5] Together with high circulating levels of fatty acids, mitochondrial dysfunction could contribute to excessive lipid accumulation in skeletal muscle, which has been causally related to the development of insulin resistance.[6]

At present, muscular insulin sensitivity can be improved by the insulin-sensitizing thiazolidinediones (TZDs), which belong to the class of PPARγ agonists. Recent studies indicated that TZDs can cause significant improvements in mitochondrial function and morphology in white adipose tissue of both animals and humans.[7–9] Thus, rosiglitazone upregulated a set of adipose tissue genes encoding mitochondrial proteins, including PGC-1α, accompanied by an increase in mitochondrial mass and changes in mitochondrial structure.[8] Whether a similar effect of rosiglitazone is present in skeletal muscle is so far unknown. Therefore, the aim of the present study was to examine – in skeletal muscle of type 2 diabetic patients – the effect of rosiglitazone treatment on gene expression of some key genes involved in mitochondrial biogenesis (i.e., PGC-1α and PPARβ/δ) and (controlling) lipid oxidation and glucose metabolism (i.e., PPARα, HSL, PDK4, ACAA1). In addition, we evaluated whether this was associated with changes in skeletal-muscle oxidative capacity and skeletal-muscle lipid content.

Methods

Subjects

Ten middle-aged obese men with type 2 diabetes mellitus and 10 age- and BMI-matched control subjects participated in this study (for clinical and metabolic characteristics, see Table 1). Diabetes was diagnosed at least 1 year before the start of the study, and was well controlled. One subject was treated with diet only, two with metformin, five with sulfonylurea and two with metformin and sulfonylurea. Control subjects had normal glucose tolerance, as measured with an oral glucose tolerance test, and had no family history of diabetes. Before the start of the study, a medical history and physical examination was performed, and a resting ECG was taken. Subjects had no major health problems beside their diabetes, and did not use any medication known to interfere with the results of the study. In addition, we used regular performance of intensive exercise or adherence to a strict diet or weight reduction program during the year preceding the study as exclusion criteria. The Medical Ethical Review Committee of Maastricht University approved the study protocol, and all subjects gave their written informed consent before the start of the study.

Table 1 Subject characteristics

	Control	*Diabetes*	
		Before	*After*
Age	57.4±7.6	61.8±3.7	—
Body weight (kg)	92.7±13.3	90.2±9.1	90.8±10.0
Body mass index (kg/m^2)	30.3±4.1	30.2±3.3	30.4±3.4
V_{O2max} (ml/kg/min)	33.7±5.3	26.5±3.8**	—
Glucose (mmol/l)	5.8±0.4	9.8±2.2**[a]	8.3±1.4
HbA_{1c} (%)	—	7.3±0.7	7.7±1.1
S_i	0.044±0.028	0.012±0.019**	0.032±0.019[##]
γ-GT (U/l)	29.6±14.3	43.4±35	22.8±11.2[#]

Abbreviations: S_i, insulin sensitivity index; γ-GT, γ-glutamyltranspeptidase. Data are mean±s.d. **$P<0.01$ diabetics vs control, [#]$P<0.05$, [##]$P<0.01$ after vs before treatment. [a]After 2-week cessation of any pre-existing antidiabetic medication.

Study design

Fourteen days before the experimental trial any prior antidiabetic medication was discontinued. Subjects were asked to refrain from any (exhaustive) physical activity the last 3 days preceding the experimental trial and to consume a diet according to the Dutch guidelines for a healthy diet. After baseline measurements, diabetic subjects received rosiglitazone (2×4 mg per day) for 8 weeks. At the end of this 8-week period, all measurements were repeated. During this treatment period, subjects visited the laboratory every 2 weeks for measurement of their fasting glucose and to check compliance.

Hyperinsulinemic euglycemic clamp

On the morning of the test day, subjects reported to the University in the fasted state. First a percutaneous muscle biopsy of the vastus lateralis muscle was taken, according to the technique by Bergström *et al*.[10] The tissue specimen was immediately frozen in melting isopentane and stored at −80°C until further analyses. Thereafter, three Teflon catheters were inserted, one into an antecubital vein for the infusion of tracer, one into an antecubital vein of the contra lateral arm for simultaneous infusion of insulin and glucose, and a third one retrograde into a superficial dorsal hand vein for arterialized blood sampling. After taking fasting blood samples, a primed constant infusion of (6,6-$^2H_2$)glucose was initiated (0.04 mg/kg/min). After 90 min, to allow isotopic equilibration, four blood samples were drawn ($t=90$, 100, 110 and 120 min). Also, during the last 30 min of this basal period, substrate oxidation was measured using indirect calorimetry (Omnical, Maastricht, The Netherlands). At $t=120$ min, a primed constant infusion of regular insulin (Actrapid, NovoNordisk, Denmark) was started (40 mU/m^2/min). Plasma glucose levels were clamped by variable coinfusion of 20% glucose with tracer added ('hot'-glucose infusion). At regular time points, blood was sampled for immediate determination of plasma glucose concentration. Because of the high fasting glucose levels of some diabetic patients, we did not intend to clamp controls and patients at the same glucose levels, but allowed glucose levels around 6.5 in diabetic patients to prevent hypoglycemic reactions. As a result, clamped glucose was higher in

Table 2 Primer sequences and annealing temperatures

Gene	*GenBank abbreviation*[a]	*Forward 5′–3′*	*Reverse 5′–3′*	*Temperature (°C)*
PGC-1α	PPARGC1A	GGT CTC TCC TTG CAG CAC AAG	CTG GGA TGA CCG AAG TGC TT	60
PPARα	PPARA	AGC TTT GGC TTT ACG GAA TAC CA	CCA CAG GAT AAG TCA CCG AGG A	60
PPARβ/δ	PPARD	ATG GAG CAG CCA CAG GAG GAA GCC	GCA TGA GGC CCC GTC ACA GC	58
HSL	LIPE	ACG TGC GCA CAA TGA CAC A	TGG CTC GAG AAG AAG GCT ATG	60
ACAA1	ACAA1	GCA CAC CTG GTT CAGC GAA TTA A	TGG CCA CCTR CCA TTG CA	60
PDK4	PDK4	CCC GAG AGG TGG AGC ATT T	GCA TTT TCT GAA CCA AAG TCC AGT A	60
MnSOD	SOD2	CTT CAG CCT GCA CTG AAG TTC AAT	CTG AAG GTA GTA AGC GTG CTC CC	56
36B4	RPLPO	GTG ATG TGC AGC TGA TCA AGA CT	GAT GAC CAG CCC AAA GGA GA	60

[a]ACAA1 (NM001607), acetyl-coenzyme A acyltransferase 1; LIPE (NM005357), hormonesensitive lipase; PDK4 (NM002612), pyruvate dehydrogenase kinase, isozyme 4; PPARA (AY206718), peroxisome proliferative activated receptor, α; PPARD (NM006238), peroxisome proliferative activated receptor, δ; PPARGC1A (RefSeq: NM013261), peroxisome proliferative activated receptor, γ, coactivator 1 α; RPLPO (BC019014), ribosomal protein, large, PO; SOD2 (NM000636), superoxide dismutase, mitochondrial.

diabetic patients (5.6 ± 0.3 vs 6.6 ± 1.3 mmol/l for obese control and diabetics; $P<0.05$); however, it was comparable before and after 8 weeks rosiglitazone in diabetic patients ($P=0.85$). Two-and-a-half hours after the start of the insulin infusion, four blood samples were taken ($t=270$, 280, 290 and 300 min) and indirect calorimetry was performed. Thereafter, insulin infusion was ended while glucose infusion was continued to prevent hypoglycemia, and subjects were offered a lunch.

Maximal aerobic capacity

An incremental bicycle-exercise test was performed to measure maximal oxygen consumption (VO_{2max}). After a warming-up period, power output was increased every 2.5 min until exhaustion was reached. The VO_{2max} test was performed within 1 week of the (initial) clamp. We did not repeat this test after rosiglitazone treatment.

Plasma assays

For determination of free fatty acids, glucose and insulin blood was collected in tubes containing ethylenediaminetetraacetic acid (EDTA). Blood was immediately centrifuged at high speed and plasma was frozen in liquid nitrogen and stored at −80°C until assayed. Plasma nonesterified fatty acids (NEFA) and glucose were measured with enzymatic assays automated on a Cobas Fara/Mira (NEFA: Wako Nefa C testkit, Wako chemicals, Neuss, Germany; glucose: hexokinase method, LaRoche, Basel, Switzerland). Insulin concentration was determined using a radioimmune assay (Linco Research, St Charles, USA). Isotopic enrichment of plasma glucose was determined by electron ionization gas chromatography–mass spectrometry, and expressed as tracer–tracee ratio (TTR).

Skeletal-muscle gene expression

Total RNA was extracted from skeletal-muscle biopsies using Trizol reagent (Invitrogen, Breda, The Netherlands). One microgram of RNA was reverse-transcribed to cDNA using random hexomer primers and a stratascript enzyme (Stratagene, The Netherlands). Reverse transcription and quantitative PCR (qPCR) were performed using an MX3000p thermal cycler system and Brilliant® SYBER Green QPCR Master Mix (Stratagene, The Netherlands). The PCR conditions for all genes consisted of one denaturing cycle at 90°C for 10 min followed by 40 cycles, consisting of denaturing at 90°C for 30 s, annealing at the pre-determined temperature (see Table 2) for 60 s and elongation at 72°C for 60 s. At the end of the PCR, the samples were subjected to a melting curve analysis. To control for any variations because of the efficiencies of the reverse transcription and PCR, acidic ribosomal phosphoprotein PO (36B4) was used as an internal control (CON: 9.3 ± 3.0 AU; DM: 10.5 ± 2.4 and 11.3 ± 3.6 AU before and after rosiglitazone, respectively; $P=$n.s.). The number of cycles at which the best-fit line through the log-linear portion of each amplified curve intersects the noise band is inversely proportional to the log copy number.[11] This value is referred to as the critical threshold (C_T) value. The ΔC_T was calculated by subtracting the C_T for 36B4 from the C_T for the gene of interest. The relative expression of the gene of interest is calculated using the expression $2^{-\Delta C_T}$ and reported as arbitrary units. All PCR runs were performed in triplicate. The analyzed genes, including Genbank abbreviations, RefSeq and PCR primer sequences, are provided in Table 2.

Skeletal-muscle protein content

To evaluate whether alterations in gene expression were associated with changes in mitochondrial protein mass, protein content of randomly selected subunits of complexes I–V of the respiratory chain was measured. Muscle biopsies were homogenized in ice-cold Tris–EDTA buffer at pH 7.4, and then the homogenates were sonicated for 15 s. Subsequently, two volumes of each skeletal-muscle homogenate and one volume of SDS-sample buffer were boiled for 4 min. Next, 13% polyacrylamide gels containing 0.1% SDS were loaded with equal amounts of protein from each sample, and

electrophoresis was performed using a Mini-Protean 3 Electrophoresis Cell (Bio-Rad Laboratories, Hercules, CA, USA). After gel electrophoreses, the gel was scanned, and the optical density of the 43-kDa band, previously immunoidentified to represent actin, was assessed. Then, a second gel was prepared and loaded with the sample volume (which had been recalculated based on the optical density of the actin band), after which Western blotting was performed using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories). The ND6 subunit of complex I, the 30-kDa Ip subunit of complex II, the 47-kDa core protein 2 of complex III, subunit II of cytochrome *c* oxidase (complex IV) and the α subunit of the F1F0 ATP synthase (complex V) were measured using monoclonal antibodies (MitoSciences, OR, USA). All proteins were expressed as arbitrary units (AU).

Part of the data on mitochondrial protein content was published earlier.[12] However, we choose to present the data of mitochondrial protein content once more, as the topic of both papers is quite different, but both benefit from including the mitochondrial protein data. In our previously published paper, we presented the mitochondrial protein data to demonstrate that the observed upregulation of UCP3 protein was independent of an increase in mitochondrial protein content.[12] The present paper is focused on the metabolic/molecular effects of TZD treatment and it is hypothesized that an increased expression of genes involved in mitochondrial biogenesis – as reported in white adipose tissue – could be one of the mechanisms underlying the beneficial effects of TZDs. The presentation of data on mitochondrial protein content is important to address this question, as it improves the interpretation of the gene expression data.

Histological assays

Intramyocellular lipid (IMCL) was quantified pre- and post-treatment with rosiglitazone. For determination of IMCL, cryosections were processed for Oil Red O staining combined with immunolabeling of the basal membrane marker laminin (Sigma-Aldrich, St Louis, MO, USA, 1:50 dilution in PBS) and a monoclonal antibody raised against adult human slow myosin heavy chain (A4.951, developed by Dr Blau[13]), to allow quantification of IMCL per fiber type (for a detailed description see Koopman *et al.*[14]). The area fraction was computed by dividing the area covered by lipid droplets by the cell surface of the measured myocytes. The mean area fraction thus reflects the percentage of the total measured cell surface covered by lipid droplets.

Succinate dehydrogenase (SDH) activity analysis in combination with immunofluorescence assays was performed to examine mitochondrial complex II activity pre- and post-rosiglitazone treatment. Briefly, cryosections were incubated in a 0.2 M sodium phosphate buffer containing 0.1 M succinic acid (Sigma-Aldrich, St Louis, MO, USA) and 1.2 mM nitro-blue tetrazolium (Sigma-Aldrich, St Louis, MO, USA). Incubation was performed at a strictly controlled temperature of 37°C for the duration of 60 min. Next, sections were shortly rinsed with deionized water followed by three successive exchanges in 30, 60 and 90% acetone in deionized water, respectively. SDH-stained sections were air-dried for 15 min. Thereafter, sections were incubated for 30 min with the laminin-antibody to visualize the cell membranes, and stained sections were embedded in Mowiol. Following this procedure, sites of high SDH activity are colored gray-blue, whereas low SDH activity results in a pale color. The bright-field image of SDH activity was converted into 8-bits gray scale after which background-corrected integral optical density of every pixel in individual muscle cells was measured using a semi-automatic macro and used as a semiquantative read-out for SDH activity. Similar methods have previously been shown to correlate well with whole-muscle homogenate spectrophotometrical analysis of SDH activity.[15,16]

Calculations

To calculate plasma glucose rate of appearance (R_a) and rate of disappearance (R_d), Steele's single-pool nonsteady-state equations adapted for use with stable isotopes were used, as no isotopic or glycemic steady state was present. Volume of distribution was assumed to be 0.160 l/kg for glucose. Whole-body insulin sensitivity (S_i) was calculated according to Bergman *et al.*,[17] taking differences in insulin and glucose levels into account: $S_i = \Delta R_d/(\Delta\text{insulin}^*\text{clamping glucose})$, where Δ represents the change from the basal state to the insulin-stimulated condition. Endogenous glucose production (EGP) was calculated as R_a minus the exogenous glucose infusion rate. From the recorded VO_2 and V_CO_2, total carbohydrate and fat oxidation were calculated.[18] Non-oxidative glucose disposal (NOGD), indicating glycogen formation, was obtained by subtracting the rate of carbohydrate oxidation from the R_d value.

Statistics

Data are presented as mean ± standard deviation (s.d.). Substrate fluxes are expressed as μmol/kg/min Statistics were performed with Statview 5.0 for Macintosh. When not normally distributed, data were log-transformed before analyzing (i.e., S_i, IMCL type II fiber). Differences between groups were compared using an unpaired Student's *t*-test; the treatment effect of rosiglitazone was analyzed using a paired Student's *t*-test. Association between variables was evaluated with regression analysis. Statistical significance was set at *P*-value <0.05.

Results

Subjects

Two weeks of discontinuation of their antihyperglycemic medication increased the plasma glucose levels in diabetic

patients from 7. 8±1.2 to 9.8±2.2 mmol/l, indicating that at least the major part of the antihyperglycemic effect of the patients' original medication has disappeared. By definition, fasting glucose was higher and insulin sensitivity (S_i) was lower in diabetic patients (Table 1; $P<0.01$). Furthermore, aerobic capacity (i.e., VO_{2max}) was lower in the patients with diabetes compared to the obese control subjects (33.7±5.3 vs 26.5±3.8 ml/kg/min, in obese controls vs diabetic patients; $P<0.05$). After 8 weeks of TZD treatment, fasting plasma glucose was reduced, although not statistically significant ($P=0.08$), whereas HbA_{1c} levels remained unchanged. Insulin sensitivity improved markedly upon rosiglitazone treatment (S_i: 0.012±0.019 to 0.032±0.019; $P<0.01$). TZD treatment did not increase body mass (90.2±9.1 to 90.8±10.0 kg; $P=0.16$).

Circulating metabolites

Basal plasma insulin concentration was higher in diabetic patients compared to obese controls ($P<0.05$), and decreased upon rosiglitazone treatment (20.5±11.2 to 14.5±6.4 mU/l; $P<0.05$). Despite a similar infusion rate, plasma insulin levels during the clamp were lower after rosiglitazone treatment in diabetic patients (Table 3; $P<0.05$), possibly reflecting improved clearance of insulin or an improved autofeedback inhibition of insulin secretion by the β-cell. Basal plasma NEFA levels were comparable between diabetic patients and obese controls, however, insulin-suppression of NEFA levels was higher in obese controls compared to diabetic subjects, resulting in lower circulating NEFA during the clamp (Table 3, $P<0.01$). Eight weeks of rosiglitazone treatment significantly decreased plasma NEFA in the basal state as well as during the clamp ($P<0.01$, Table 3).

Glucose metabolism

Insulin-stimulated glucose uptake was considerably lower in diabetic patients compared to healthy obese controls (ΔR_d: 6.8±9. 2 vs 20.6±10.6 μmol/kg/min, respectively; $P<0.01$). This was accounted for by both a lower oxidative and nonoxidative glucose disposal (Table 3). Eight weeks of rosiglitazone treatment significantly improved insulin-stimulated glucose uptake, even in the presence of lower circulating levels of insulin ($P<0.01$; Table 3). Again, both oxidative and nonoxidative glucose accounted for the increase in total insulin-stimulated glucose disposal, with the increase in nonoxidative glucose disposal being most pronounced. In two out of 10 patients, there was no improvement in insulin-stimulated glucose uptake after treatment. Endogenous glucose production (i.e., hepatic glucose output) was significantly higher in the basal state in diabetic patients ($P<0.05$), and was reduced to control levels after rosiglitazone treatment ($P<0.05$). TZD reduction of basal EGP closely correlates with the reduction in fasting plasma glucose ($R=0.75$, $P<0.01$). Insulinsuppression of hepatic glucose output was not different between obese controls and diabetic patients. After rosiglitazone treatment, absolute insulin suppression of EGP was lower, owing to the large reduction in basal EGP (Table 3, $P<0.05$). However, expressed as relative suppression, it was not different (−65±23 vs −79±27%, before and after treatment, respectively; $P=$NS).

Table 3 Substrate kinetics before and after rosiglitazone treatment

	Control	*Diabetes*	
		Before	*After*
Plasma glucose (mmol/l)			
Basal	5.9±0.5	9.3±2.4**	8.0±1.5
Clamp	5.6±0.3	6.6±1.3*	6.7±1.6
Plasma insulin (mU/l)			
Basal	12.3±2.7	20.5±11.2*	14.5±6.4$^{\#}$
Clamp	100.5±14.2	115.0±23.7	95.6±13.6$^{\#}$
Plasma NEFA (μmol/l)			
Basal	513±144	590±165	452±140$^{\#\#}$
Clamp	97±51	227±86**	154±71$^{\#\#}$
R_d glucose (μmol/kg/min)			
Basal	8.2±2.0	13.2±4.8**	10.0±2.7
Clamp	28.8±9.7	20.0±8.8*	26.1±6.4$^{\#\#}$
Change	20.6±10.6	6.8±9.2**	16.1±7.0$^{\#\#}$
EGP (μmol/kg/min)			
Basal	8.8±1.4	12.3±4.4*	8.5±1.6$^{\#}$
Clamp	3.6±2.6	3.3±1.8	1.8±2.1
Change	−5.2±2.6	−9.0±4.4*	−6.7±2.1
CHO oxidation (μmol/kg/min)			
Basal	7.1±2.6	9.0±2.9	9.1±1.5
Clamp	12.5±3.4	12.6±3.9	14.8±2.8$^{\#}$
Change	5.4±3.4	3.6±2.3**	5.8±2.2$^{\#}$
NOGD (μmol/kg/min)			
Basal	1.1±3.3	4.1±6.5	1.0±1.9
Clamp	16.3±8.0	7.3±6.9*	11.3±
Change	15.2±10.2	3.2±8.3	10.3±5.5$^{\#\#}$
Lipid oxidation (μmol/kg/min)			
Basal	1.10±0.21	0.89±0.23*	0.82±0.15
Clamp	0.69±0.19	0.63±0.30	0.42±0.18$^{\#}$
Change	−0.41±0.31	−0.26±0.18	−0.40±0.16$^{\#}$

Data are mean±s.d. *$P<0.05$, **$P<0.01$, diabetics vs control, $^{\#}P<0.05$, $^{\#\#}P<0.01$ after vs before treatment. CHO, carbohydrates; EGP, endogenous glucose production; MCR, metabolic clearance rate; NEFA, free fatty acids; NOGD, nonoxidative glucose disposal; R_d, rate of disappearance.

Gene expression and mitochondrial proteins

mRNA content of PGC-1α and PPARβ/δ was significantly reduced in type 2 diabetic patients compared to obese controls ($P<0.05$,. Figure 1a). Interestingly, PGC-1α and PPARβ/δ mRNA increased upon rosiglitazone treatment toward control values (Figure 1a). The increase in expression of PPARβ/δ significantly correlated with the improvement in peripheral glucose disposal ($R^2=0.45$, $P<0.05$) (Figure 2). No

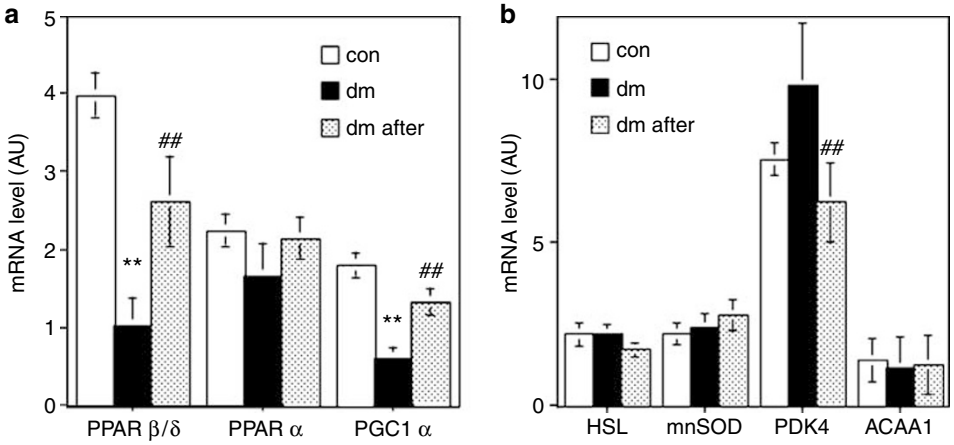

Figure 1 Skeletal-muscle gene expression in control subjects (white bars) and in diabetic subjects before (black bars) and after (dotted bars) 8 weeks of rosiglitazone treatment. (**a**) Cofactors and transcription factors; (**b**) other muscle metabolic genes. Data are mean ± s.d.; $^{**}P<0.01$ diabetes vs control, $^{\#\#}P<0.01$ after vs before treatment.

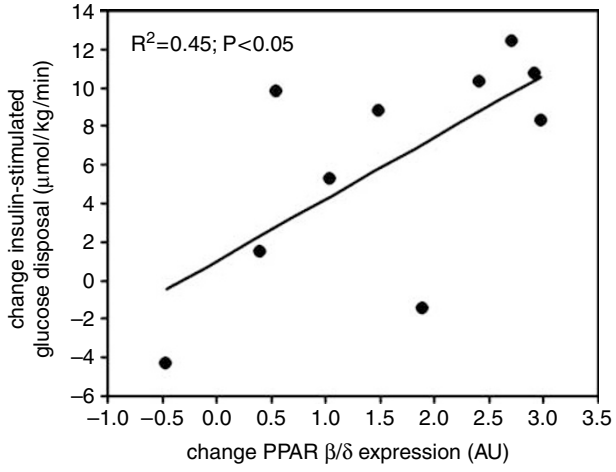

Figure 2 Correlation between skeletal-muscle PPAR β/δ gene expression and the change in insulin-stimulated glucose disposal (R_d) upon 8 weeks of rosiglitazone treatment in diabetic patients.

other significant correlations were observed between alterations in gene expression and measures of glucose and lipid metabolism. Baseline gene expression levels of PDK4, HSL, mnSOD, ACAA1 and PPARα were similar between type 2 diabetic patients and obese controls (Figure 1b). Of these genes, PDK4 mRNA expression decreased after rosiglitazone treatment ($P<0.01$), whereas the other genes were not affected (Figure 1b).

To examine if the rosiglitazone-mediated restoration of PGC-1α and PPARβ/δ, which have been implicated in mitochondrial biogenesis, was reflected by changes in mitochondrial protein content, we measured protein content of randomly selected subunits of complexes I–V of the respiratory chain. Remarkably, we found no significant differences in mitochondrial protein content between type 2 diabetic patients and obese control subjects (complex I: 2.70 ± 1.92 vs 2.01 ± 0.73 AU; complex II: 4.69 ± 2.34 vs 4.60 ± 1.91 AU; complex III: 44.3 ± 16.6 vs 44.4 ± 20.5 AU; complex IV: 15.3 ± 8.5 vs 14.0 ± 5.9 AU; complex V: 48.7 ± 17.5 vs 42.5 ± 17.6 AU, obese control vs diabetics, respectively; P = NS). Rosiglitazone treatment did not alter the content of these proteins (change upon treatment: complex I: −0.7 ± 1.2 AU; complex II: −1.1 ± 3.3 AU; complex III: −4.3 ± 17.7 AU; complex IV: −2.9 ± 7.2 AU; complex V: + 2.5 ± 16.7 AU; P = NS).

SDH activity and IMCL

As we found that rosiglitazone treatment improved metabolic flexibility without changes in marker proteins for mitochondrial content, we measured mitochondrial complex II activity by assessing SDH activity before and after rosiglitazone treatment. SDH activity increased significantly from 0.35 ± 0.07 to 0.41 ± 0.10 AU after 8 weeks of rosiglitazone treatment ($P<0.01$) (Figure 3a). No correlations were observed between the improved SDH activity and alterations in glucose or lipid metabolism. In addition, improved metabolic flexibility may result in increased oxidation of stored muscular fat. Therefore, we examined IMCL content before and after rosiglitazone treatment. Eight weeks of rosiglitazone had no effect on skeletal-muscle lipid content, nor in type I fibers (4.30 ± 2.70 vs. 3.58 ± 2.99% before and after, respectively; $P=0.33$), neither in type II fibers (1.68 ± 1.01 vs 1.46 ± 1.51% before and after, respectively; $P=0.11$).

Metabolic flexibility

The insulin-induced increase in respiratory exchange ratio (RER), an indicator of metabolic flexibility, was higher in obese controls compared to diabetic patients; however, this

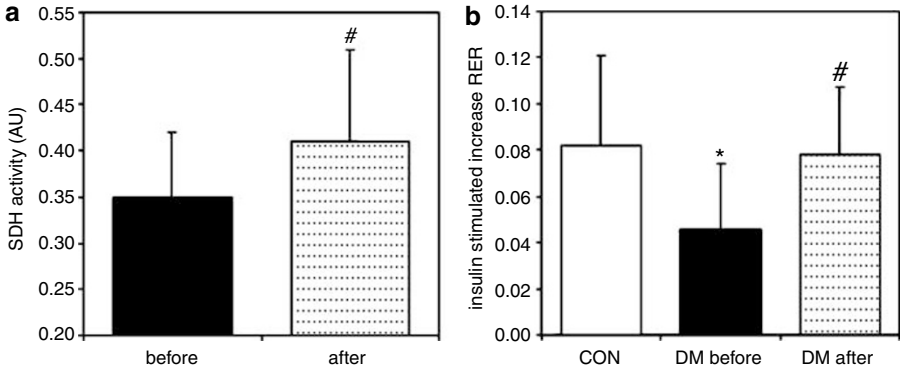

Figure 3 (**a**) Skeletal-muscle SDH activity in diabetic subjects before (black bars) and after (dotted bars) rosiglitazone treatment. (**b**) Metabolic flexibility, that is, the insulin stimulated increase in RER, in control subjects (white bars) and in diabetic subjects before (black bars) and after (dotted bars) 8 weeks of rosiglitazone treatment. Data are mean ± s.d. $^{*}P = 0.06$ diabetes vs control, $^{\#}P < 0.01$ after vs before treatment.

difference was not significant ($+0.08 \pm 0.04$ vs $+0.05 \pm 0.03$, CON and DM respectively; $P = 0.06$). After rosiglitazone treatment, metabolic flexibility was improved, indicated by a larger increase in RER from the basal to the insulin-stimulated state (from $+0.05 \pm 0.03$ to $+0.08 \pm 0.03$; $P < 0.05$) (Figure 3b).

Discussion

Skeletal muscle of insulin-resistant subjects is characterized by a reduced oxidative capacity and a compromised mitochondrial function.[3,4] The transcriptional co-activator PGC-1α is thought to play an important role in this defect.[1] Here we reported reduced expression of PGC-1α and PPARβ/δ in diabetic muscle and showed that 8 weeks of rosiglitazone treatment improves PGC-1α and PPARβ/δ expression. The upregulation of these pivotal players in mitochondrial biogenesis was not reflected in protein content of any of the subunits of complexes I–V of the respiratory chain. Despite similar protein levels, complex II activity (measured as SDH activity) increased significantly upon treatment with rosiglitazone. This increase in complex II activity occurred along with improved metabolic flexibility and improved insulin sensitivity. Interestingly, no significant alterations were seen in intramyocellular lipid content. The latter result supports the idea that an improvement in skeletal-muscle oxidative capacity *per se* may be more important in determining insulin sensitivity than changes in lipid accumulation.[19]

To examine if the reduced expression and the rosiglitazone-induced upregulation of PGC-1α and PPARβ/δ in type 2 diabetic patients reflected a general effect on muscular oxidative gene expression, we measured as a control some other genes involved in glucose, fat and oxidative metabolism. However, none of these genes was reduced in diabetes or affected by rosiglitazone treatment, suggesting a specific effect of rosiglitazone on the expression levels of PGC-1α and PPARβ/δ. Whether this is the result of a direct effect of rosiglitazone on skeletal muscle, or an indirect effect through alterations in other tissues, cannot be deduced from our study. As PPARγ expression is low in skeletal muscle compared to adipose tissue, an indirect effect can be expected. Rosiglitazone treatment reduced plasma NEFA, most probably through an effect on adipose tissue. As increased circulating NEFA levels are known to downregulate the expression of PGC-1α and other genes involved in oxidative metabolism,[20,21] the rosiglitazone-induced reduction in plasma NEFA could have resulted in the observed upregulation of PGC-1α and PPARβ/δ expression. However, plasma NEFA levels do not explain the lower PGC-1α and PPARβ/δ mRNA levels in the diabetic subjects compared to the healthy obese controls, as plasma NEFA levels were not different between the two subject groups. Alternatively, there is some evidence for a direct effect of rosiglitazone on PGC-1α and PPARβ/δ gene expression in muscle. Rosiglitazone-treated human skeletal myotubes, obtained from healthy subjects, showed an increased PGC-1α expression, which coincided with an improved insulin-stimulated glycogen synthesis.[22] Furthermore, in cultured muscle cells of diabetic patients, a direct effect of TZD treatment on FA uptake and oxidation was shown.[23,24] Moreover, the diabetes-related defect in FA oxidation was localized in the mitochondria, and TZD treatment increased the mitochondrial component of FA oxidation.[24]

As PGC-1α and PPARβ/δ have been implicated in mitochondrial biogenesis, we examined if the reduction in diabetes and restoration by rosiglitazone was reflected in an increase in mitochondrial protein mass. Therefore, we measured the protein content of five subunits of complexes I–V of the respiratory chain. Remarkably, none of these proteins was reduced in our type 2 diabetic patients, nor was affected by rosiglitazone treatment. This may suggest that the downregulation of PGC-1α and PPARβ/δ mRNA species in diabetes, observed in two previous cDNA microarray experiments[1,2] and in our study, does not result in reduction of mitochondrial proteins. Furthermore, our

results suggest that the effect of TZD on mitochondrial content may be different between skeletal muscle and white adipose tissue.[7–9]

Although these findings may question the impact of reduced PGC-1α in the development of type 2 diabetes, it cannot be excluded that the reduced PGC-1α affects mitochondrial functioning, independent of an effect on mitochondrial protein content. In that context, we have shown here that 8 weeks of treatment with rosiglitazone significantly improved metabolic flexibility, which could be considered as an indirect physiological measure of mitochondrial functioning. Although the molecular routes contributing to improved metabolic flexibility are far from understood, it could be argued that optimal metabolic flexibility requires parallel electron input in complex I and complex II of the respiratory chain. Electron input in complex II requires the formation of $FADH_2$, which may originate from β-oxidation, as well as from the Tri Carboxylic Acid cycle-bound SDH reaction. Earlier work revealed decreased SDH activity in type 2 diabetes.[25] Therefore, to investigate if this improved metabolic flexibility after rosiglitazone – despite changes in mitochondrial protein content – could be explained by improved mitochondrial activity, we determined SDH activity before and after rosiglitazone. Rosiglitazone treatment significantly improved mitochondrial complex II activity (i.e., SDH activity). This indicates that rosiglitazone is able to improve mitochondrial oxidative capacity. Whether the restoration of PGC-1α and PPARβ/δ gene expression is involved in the improved metabolic flexibility cannot be deduced from the present study and requires further investigation.

An improved muscular oxidative capacity, together with reduced plasma NEFA levels after rosiglitazone treatment, could improve insulin sensitivity by affecting the accumulation of muscular fat. Therefore, IMCL content in skeletal muscle was examined before and after 8 weeks of rosiglitazone treatment. However, despite the improved oxidative capacity and reduced circulating NEFA, we did not observe a reduction in IMCL content after rosiglitazone treatment. This situation reflects to a certain extent what is observed in trained athletes. Endurance-trained athletes are among the most insulin-sensitive people, despite having high levels of IMCL.[26] This 'athletes paradox' is suggested to be explained by the high oxidative capacity in endurance-trained muscle.[26] One could speculate that the improved (lipid) oxidative capacity upon the administration of rosiglitazone modulated the relationship between IMCL and insulin sensitivity, like in trained athletes, most probably via lowering of lipid intermediates such as diacylglycerol (DAG) and long-chain fatty acyl-CoA (LCFA-CoA). Unfortunately, owing to the limited size of the muscle biopsies, we could not determine the levels of DAG and LCFACoA in the present study, which, therefore, deserves further investigation.

In conclusion, 8 weeks of rosiglitazone treatment improved the expression of PGC-1α and PPARβ/δ in obese type 2 diabetic patients, along with an improved mitochondrial complex II activity and metabolic flexibility. However, mitochondrial protein content was not reduced in type 2 diabetic patients, nor affected by TZD. Also, the improvement in skeletal-muscle oxidative capacity was not associated with a decreased intramyocellular lipid accumulation. These results suggest that the insulin-sensitizing effect of TZDs may involve an effect on muscular oxidative capacity, independent of mitochondrial protein content, and is not dependent on changes in IMCL.

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