Reduced skeletal muscle UCP3 protein content in pre-diabetic subjects and type 2 diabetic patients: restoration by rosiglitazone treatment.

0021-972X/06/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 91(4):1520–1525

doi: 10.1210/jc.2005-1572

Reduced Skeletal Muscle Uncoupling Protein-3 Content in Prediabetic Subjects and Type 2 Diabetic Patients: Restoration by Rosiglitazone Treatment

Patrick Schrauwen, Marco Mensink, Gert Schaart, Esther Moonen-Kornips, Jean-Pierre Sels, Ellen E. Blaak, Aaron P. Russell, and Matthijs K. C. Hesselink

Departments of Human Biology (P.S., M.M., E.M.-K., E.E.B.) and Movement Sciences (G.S., E.M.-K., M.K.C.H.), Maastricht University, NL-6200 MD Maastricht, The Netherlands; Department of Endocrinology (J.-P.S.), Academic Hospital Maastricht, 6229 HX Maastricht, The Netherlands; and Clinique Romande de Readaptation SUVA Care (A.P.R.), 1951 Sion, Switzerland

Context: The mitochondrial uncoupling protein-3 (UCP3) has been implicated in the protection of the mitochondrial matrix against lipid-induced mitochondrial damage. Recent evidence points toward mitochondrial aberrations as a major contributor to the development of insulin resistance and diabetes, and UCP3 is reduced in diabetes.

Objective: We compared skeletal muscle UCP3 protein levels in prediabetic subjects [*i.e.* impaired glucose tolerance (IGT)], diabetic patients, and healthy controls and examined whether rosiglitazone treatment was able to restore UCP3.

Patients, Design, Intervention: Ten middle-aged obese men with type 2 diabetes mellitus [age, 61.4 ± 3.1 yr; body mass index (BMI), 29.8 ± 2.9 kg/m^2], nine IGT subjects (age, 59.0 ± 6.6 yr; BMI, 29.7 ± 3.0 kg/m^2), and 10 age- and BMI-matched healthy controls (age, 57.3 ± 7.4 yr; BMI, 30.1 ± 3.9 kg/m^2) participated in this study. After baseline comparisons, diabetic patients received rosiglitazone (2 × 4 mg/d) for 8 wk.

Main Outcome Measures: Muscle biopsies were sampled to determine UCP3 and mitochondrial protein (complex I–V) content.

Results: UCP3 protein content was significantly lower in prediabetic IGT subjects and in diabetic patients compared with healthy controls (39.0 ± 28.5, 47.2 ± 24.7, and 72.0 ± 23.7 arbitrary units, respectively; $P < 0.05$), whereas the levels of the mitochondrial protein complex I–V were similar between groups. Rosiglitazone treatment for 8 wk significantly increased insulin sensitivity and muscle UCP3 content (from 53.2 ± 29.9 to 66.3 ± 30.9 arbitrary units; $P < 0.05$).

Conclusion: We show that UCP3 protein content is reduced in prediabetic subjects and type 2 diabetic patients. Eight weeks of rosiglitazone treatment restores skeletal muscle UCP3 protein in diabetic patients. **(*J Clin Endocrinol Metab* 91: 1520–1525, 2006)**

IN THE EARLY years after the discovery of human uncoupling protein-3 (UCP3) in 1997, the physiological function of UCP3 was thought to be in the regulation of energy metabolism and body weight. However, 8 yr later, data gathered in human experiments and (transgenic) animals have revealed that the function of skeletal muscle UCP3 is most likely related to fatty acid metabolism and/or reactive oxygen species (ROS) production. Based on the available literature on the regulation of UCP3, we have recently hypothesized that skeletal muscle UCP3 protects mitochondria against lipid-induced mitochondrial damage (1). Thus, UCP3 is consistently up-regulated in conditions in which, because of exceeding the oxidative capacity, fatty acids accumulate in the sarcoplasm (2). On the other hand, UCP3 is down-regulated when fat oxidative capacity is improved (2). In conditions where fatty acids accumulate in the sarcoplasm, the load of fatty acids on the mitochondrial membranes increases and, because membranes are lipid bilayers, fatty acids can enter the mitochondrial matrix via the "flip-flop" mechanism (3). The mitochondrial matrix is also the site where ROS are formed, and fatty acids present in the matrix or in the mitochondrial inner membranes would be highly susceptible to become peroxidized by ROS. Because the resulting lipid peroxides are highly reactive (4) and can cause damage to mitochondrial RNA, DNA, and mitochondrial structures, it is important to keep the import of fatty acids into the matrix and/or the level of ROS production within limits, especially under conditions of high fatty acid availability. To date, there is no consensus regarding the exact physiological function of UCP3. Nevertheless, several independent groups hypothesized that skeletal muscle UCP3 could play a crucial role in preventing such lipid-induced mitochondrial damage albeit via different mechanisms. Thus, we (2) and Himms-Hagen and Harper (5) have postulated the hypothesis that UCP3 exports nonmetabolizable fatty acids from the mitochondrial matrix to prevent them from being peroxidized, albeit that the source of the mitochondrial fatty acids is different in these two hypothesis. In addition, Goglia and Skulachev (6) have suggested that UCP3 exports lipid peroxides from the mitochondrial matrix before they can damage the oxidative machinery in the matrix. Alternatively, Brand and colleagues

First Published Online December 29, 2005

Abbreviations: AU, Arbitrary unit; BMI, body mass index; EGP, endogenous glucose production; FFA, free fatty acid; HbA1c, glycosylated hemoglobin; IGT, impaired glucose tolerance; MCR, metabolic clearance rate; PPARγ, peroxisome proliferator-activated receptor γ; ROS, reactive oxygen species; TZD, thiazolidinedione; UCP3, uncoupling protein-3.

(7) have suggested that UCP3 directly lowers ROS production after being activated by lipid peroxides. Regardless of the exact function of UCP3, all three hypotheses predict that a lack of skeletal muscle UCP3 would result in lipid-induced oxidative damage to the mitochondria. Indeed, UCP3 ablated mice are characterized by increased levels of muscular lipid peroxidation and oxidative damage to proteins and DNA (8).

Because type 2 diabetic patients are characterized by increased levels of plasma fatty acids and a low fat oxidative capacity (9), high levels of skeletal muscle UCP3 would be anticipated to prevent lipid-induced mitochondrial damage. Consistent with this idea, two studies have shown increased levels of muscular UCP3 mRNA in diabetic subjects (10, 11), but another study found a reduced skeletal muscle UCP3 mRNA expression (12). To further address this issue, we measured UCP3 protein levels in type 2 diabetic subjects and healthy controls and found that UCP3 protein was decreased by approximately 50% in muscle of diabetic patients (13). Given the putative function of UCP3 in the protection against lipid-induced mitochondrial damage, it is interesting to note that insulin-resistant subjects are characterized by increased mitochondrial damage (14) and an increased degree of lipid peroxidation in muscle (15), suggesting that low UCP3 in muscle may be part of the etiology of type 2 diabetes. A prerequisite for a role of UCP3 in mitochondrial damage and dysfunction would be that UCP3 levels are already reduced in the prediabetic state, thereby stressing the role of UCP3 during the progression of the prediabetic state toward overt diabetes. Hence, in this study, we examined UCP3 protein levels in prediabetic subjects, *i.e.* subjects with impaired glucose tolerance (IGT).

At present, thiazolidinediones (TZD) are frequently prescribed to treat type 2 diabetic patients. TZDs lower glucose mainly due to an improved insulin-stimulated glucose disposal into skeletal muscle, either indirectly or directly via peroxisome proliferator-activated receptor γ (PPARγ), the main target for TZDs. A recent paper showed that, in healthy rats, acute ip injections of high dosages of TZD increased mRNA expression of skeletal muscle UCP3 (16). Therefore, the aims of the present study were to confirm the reduced levels of UCP3 mRNA and protein in skeletal muscle of diabetic patients and to examine whether UCP3 is already reduced in the prediabetic state. In addition, we examined whether prolonged treatment with TZD, at therapeutical dosages, can result in a restoration of UCP3 levels in type 2 diabetic patients.

Subjects and Methods

Subjects

Ten middle-aged obese men with type 2 diabetes mellitus and 10 age- and body mass index (BMI)-matched healthy controls participated in this study. For baseline comparison of UCP3 protein levels, both groups were compared with nine glucose intolerant subjects (IGT), which have been described previously (17) (see Table 1 for characteristics). Diabetic subjects were diagnosed with diabetes at least 1 yr before the study, and had well-controlled diabetes. Two subjects were treated with metformin only, four with a sulfonylurea only, and four with metformin and sulfonylurea. IGT was diagnosed with an oral glucose tolerance test according to World Health Organization criteria. Control subjects had normal glucose homeostasis, ax determined by a standard oral glucose tolerance test, and had no family history of diabetes. Before the start of the study, a medical history and physical examination were performed, a resting electrocardiogram was taken, and a fasting blood sample was drawn. Subjects with uncontrolled hypertension, active cardiovascular disease, liver dysfunction (alanine aminotransferase or aspartate aminotransferase >2.5 times upper limit), increased creatinine levels, or on medication known to interfere with the goals of the study (*e.g.* corticosteroids) were excluded from participation. None of the participants performed regular intensive exercise or followed a strict diet or weight reduction program for at least 3 months before the start of the study. 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. Baseline subjects characteristics

	Control	IGT	Diabetic
Age (yr)	57.3 ± 7.4	59.0 ± 6.6	61.4 ± 3.1
BMI (kg/m^2)	30.1 ± 3.9	29.7 ± 3.0	29.8 ± 2.9
VO_2max (ml/kg·min)	33.7 ± 5.3	28.1 ± 3.2[a]	26.8 ± 2.0[a]
Glucose (mmol/liter)	5.8 ± 0.4	6.2 ± 1.3	10.1 ± 2.3[a]
HbA1c (%)	NA	NA	7.0 ± 1.0
Duration diabetes (yr)	–	–	5.9 ± 4.1

Data are expressed as mean ± SD. NA, Not available.
[a] $P < 0.05$ compared to control.

Study design

In glucose intolerant subjects (IGT), only skeletal muscle UCP3 protein content was determined and compared with the diabetic patients and healthy controls. For other measurements, the healthy subjects served as baseline controls to the diabetic patients and were not treated with rosiglitazone. In the diabetic patients, any prior anti-diabetes medication was discontinued 14 d before the baseline experimental trial. After baseline measurements, diabetic subjects received rosiglitazone (2 × 4 mg/d) for 8 wk, after which all measurements were repeated. Unfortunately, two patients did not complete the trial due to mild gastrointestinal complaints (n = 1) and uncontrolled hyperglycemia (n = 1). Therefore, all data on the effect of rosiglitazone is on n = 8 only.

Muscle biopsies

After an overnight fast, a percutaneous needle muscle biopsy of the vastus lateralis muscle was taken, according to the technique by Bergström *et al.* (18). The tissue specimen was frozen immediately in isopentane kept at its melting point in liquid nitrogen and stored at −80 C. For diabetic patients and controls, muscle biopsy was taken before the clamp.

Hyperinsulinemic-euglycemic clamp

At 0800 h, after an overnight fast, subjects report to the laboratory by car or by bus. After taking a muscle biopsy, cannulas were inserted, one into each antecubital vein for the infusion of tracer, insulin, and glucose, and a third one was inserted retrogradely into a superficial dorsal hand vein for arterialized blood sampling. After a first blood sample, a primed constant infusion of 6,6-$^2H_2$-glucose was initiated (0.04 mg/kg·min) for 300 min; blood was sampled at t = 90, 100, 110, and 120 min and substrate oxidation was measured by indirect calorimetry (Omnical, Maastricht, The Netherlands). At t = 120 min, a primed constant infusion of insulin (Actrapid; Bagsvaerd, Novo Nordisk, Denmark) was started (40 mU/m^2·min), and glucose levels were clamped by variable coinfusion of 20% glucose with tracer added (controls: 5.6 ± 0.3 mmol/liter; diabetic subjects: 6.5 ± 1.3 and 6.7 ± 1.9 mmol/liter before and after, respectively). At t = 270, 280, 290, and 300 min, blood was again sampled and indirect calorimetry was performed.

Metabolic flexibility

Both in the basal state and during insulin stimulation, substrate oxidation was measured by indirect calorimetry, and carbohydrate and fat oxidation was calculated. In healthy subjects, fat oxidation is the predominant source contributing to energy expenditure in the fasted

state, whereas insulin will switch substrate use to carbohydrate oxidation. This flexibility to switch from predominantly fat oxidation to glucose oxidation is termed "metabolic flexibility". Here we measured the stimulating effect of insulin on glucose oxidation and the suppressing effect of insulin on fat oxidation in control subjects and in type 2 diabetic patients as a measure of metabolic flexibility, and these measurements were repeated in type 2 diabetic patients treated with rosiglitazone for 8 wk.

Calculations

To calculate plasma glucose rate of appearance and rate of disappearance, Steele's single pool non-steady-state equations, adapted for use with stable isotopes, were used, as no isotopic steady-state was present. Volume of distribution was assumed to be 0.160 liter·kg^{-1} for glucose. The glucose metabolic clearance rate (MCR) equals the rate of disappearance divided by the steady-state plasma glucose concentration. Endogenous glucose production (EGP) was calculated as rate of appearance minus the exogenous glucose infusion rate. Total carbohydrate and lipid oxidation were calculated according to the table of nonprotein respiratory quotient.

Muscle analysis

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, Amsterdam, The Netherlands). Quantitative PCR was performed using an MX3000p thermal cycler system and Brilliant SYBER Green QPCR Master Mix (Stratagene). The PCR conditions consisted of one denaturing cycle at 90 C for 10 min followed by 40 cycles, consisting of denaturing at 90 C for 30 sec, annealing at the predetermined temperature for 60 sec, and elongation at 72 C for 60 sec. At the end of the PCR, the samples were subjected to a melting curve analysis. To control for any variations due to efficiencies of the RT and PCR, acidic ribosomal phosphoprotein PO (36B4) was used as internal control. 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 UCP3 is calculated using the expression $2^{-\Delta CT}$ and reported in arbitrary units (AU). PCR runs were performed in triplicate.

For UCP3 protein determination, muscle biopsies were homogenized in ice-cold Tris-EDTA buffer at pH 7.4, and then the homogenates were sonicated for 15 sec. 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). After gel electrophoresis, the gel was scanned, and the optical density of the 43-kDa band, previously immuno-identified 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) as described previously (19). We used a rabbit polyclonal UCP3 antibody (code 1331; kindly provided by L. J. Slieker, Eli Lilly, Indianapolis, IN) prepared against a 20-amino acid peptide (human sequence amino acid 147–166). For a detailed description of the selectivity and specificity checks see previous reports (19, 20). Cytochrome *c* level, as a marker of mitochondrial content, was measured comparably using a rabbit polyclonal cytochrome *c* antibody (BD PharMingen, Woerden, NL). 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 (COXII), and the α subunit of the F1F0 ATP synthase (complex V) were measured using monoclonal antibodies (MitoSciences, Eugene, OR). All proteins were expressed as AU.

Blood samples

Blood was collected in tubes containing EDTA. Plasma was immediately centrifuged at high speed, frozen in liquid nitrogen and stored at −80 C for later analyses. Insulin concentrations were measured using RIA (Linco Research, St. Charles, MO). Free fatty acids (FFA) were determined using the Wako Nefa C test kit (Wako Chemicals, Neuss, Germany), and plasma glucose was determined using the hexokinase method (LaRoche, Basel, Switzerland).

Statistics

Data are presented as mean ± SD. Selected parameters were compared in healthy controls and diabetic patients using Student's t tests. Results before and after treatment were analyzed using a paired Student's *t* test. Association between variables was evaluated with regression analysis. Statistical significance was set at $P < 0.05$.

Results

Blood parameters

During the 2-wk wash-out period before the start of the first experimental clamp, fasting plasma glucose and glycosylated hemoglobin (HbA1c) levels increased in the diabetic subjects (glucose 7.6 ± 1.3 to 10.1 ± 2.3 mmol/liter, $P < 0.01$; HbA1c 7.0 ± 0.8 to 7.2 ± 0.8%, $P < 0.05$). By definition, type 2 diabetic subjects had significantly higher fasting plasma glucose (10.1 ± 2.3 *vs.* 5.8 ± 0.4 mmol/liter, $P = 0.01$) and insulin levels (17.9 ± 6.6 *vs.* 12.3 ± 2.7 mU/liter, $P < 0.05$) compared with healthy controls. No difference was observed in fasting plasma FFA level (563 ± 157 *vs.* 511 ± 144 μmol/liter, $P = 0.33$). After 8 wk of treatment with rosiglitazone, fasting insulin tended to decrease in type 2 diabetic subjects (from 17.9 ± 6.6 to 13.8 ± 6.6 mU/liter, $P = 0.07$), but plasma glucose (10.0 ± 3.0 to 8.5 ± 1.6 mmol/liter, $P = 0.17$) and HbA1c (7.4 ± 1.0 to 7.8 ± 1.2%, $P = 0.17$) were not significantly affected. No changes were observed in body weight (from 91.8 ± 7.6 to 92.5 ± 8.3 kg, $P = 0.52$). Plasma FFA levels were significantly decreased after 8 wk of rosiglitazone treatment (from 569 ± 165 to 456 ± 145 μmol/liter, $P < 0.05$).

Insulin sensitivity and metabolic flexibility

As expected, insulin sensitivity (MCR) was significantly lower in type 2 diabetic subjects compared with healthy controls (3.30 ± 1.66 *vs.* 5.22 ± 1.95 ml/kg·min in diabetic patients and controls, respectively, $P < 0.05$). Eight weeks of rosiglitazone treatment increased the MCR from 3.36 ± 1.88 to 4.19 ± 1.79 ml/kg·min (Fig. 1; $P = 0.06$), indicating restoration of insulin sensitivity. EGP was increased in the diabetic patients in the basal state (13.1 ± 4.1 *vs.* 8.4 ± 1.4 μmol/kg·min for diabetes and control, respectively; $P < 0.01$), but not during the clamp (3.4 ± 1.4 *vs.* 3.6 ± 2.6 μmol/kg·min, respectively; $P = 0.85$). Rosiglitazone treatment resulted in a reduced basal EGP (13.2 ± 4.5 to 8.7 ± 1.8 μmol/kg·min; $P < 0.05$), whereas clamping EGP was not significantly altered (3.3 ± 1.5 to 1.5 ± 2.3 μmol/kg·min; $P = 0.16$). Metabolic flexibility, *i.e.* the capacity to switch from predominantly fat oxidation in the fasting state to glucose oxidation in the insulin-stimulated state was also reduced in type 2 diabetic patients compared with healthy controls. Thus, the stimulating effect of insulin on glucose oxidation was significantly lower in type 2 diabetic patients *vs.* controls (+3.3 ± 2.4 *vs.* +6.0 ± 3.0 μmol/kg·min in diabetic patients and controls, respectively; $P < 0.05$), whereas insulin suppression of lipid oxidation was also significantly lower in diabetic subjects (−0.22 ± 0.16 *vs.* −0.47 ± 0.26 μmol/kg·min in diabetic patients and controls, respectively; $P < 0.05$).

Rosiglitazone treatment improved metabolic flexibility; the stimulating effect of insulin on glucose oxidation was

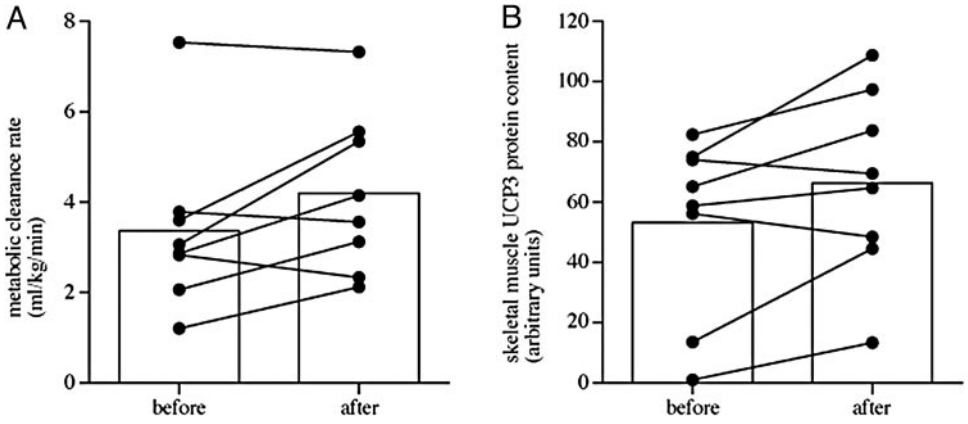

FIG. 1. Insulin sensitivity (A; MCR in milliliters per kilogram per minute) and skeletal muscle UCP3 protein content (B; AU) before and after 8 wk of rosiglitazone treatment in diabetic subjects (n = 8). Both MCR ($P = 0.06$) and UCP3 ($P < 0.05$) were increased after treatment.

significantly greater after rosiglitazone treatment (from +3.4 ± 2.6 to +5.3 ± 2.3 μmol/kg·min; $P < 0.05$), whereas insulin suppression of lipid oxidation was also significantly improved by rosiglitazone (−0.23 ± 0.18 to −0.36 ± 0.15 μmol/min; $P < 0.05$). The improved metabolic flexibility is also indicated by the more pronounced increase in respiratory quotient after rosiglitazone treatment, during transition from the basal to the insulin-stimulated state (from 0.84 ± 0.05 to 0.88 ± 0.06 before treatment, *vs.* from 0.84 ± 0.02 to 0.91 ± 0.03 after 8 wk rosiglitazone; $P < 0.05$).

UCP3 and mitochondrial protein levels

UCP3 mRNA expression was reduced by 42% in type 2 diabetic patients compared with healthy, age- and BMI-matched controls (1.59 ± 1.1 *vs.* 2.75 ± 0.93 AU in diabetic patients and controls, respectively; $P < 0.05$). This corresponds well with the approximately 35% reduction in UCP3 protein content in type 2 diabetic patients compared with healthy, age- and BMI-matched controls (47.2 ± 24.7 *vs.* 72.0 ± 23.7 AU in diabetic patients and controls, respectively; $P < 0.05$; Fig. 2). Importantly, no significant differences in protein levels of any of the other mitochondrial proteins examined (complex I–V and cytochrome *c*) were observed between type 2 diabetic patients and healthy controls (Table 2). In addition, we also report that UCP3 protein content is already significantly reduced by approximately 46% in prediabetic subjects (IGT) compared with age- and BMI-matched healthy controls (39.0 ± 28.5 *vs.* 72.0 ± 23.7 AU; $P < 0.05$; Fig. 2), whereas cytochrome *c* levels were not significantly different between prediabetic subjects (IGT) and healthy controls (33.6 ± 9.5 *vs.* 41.0 ± 25.1 AU; $P = 0.49$).

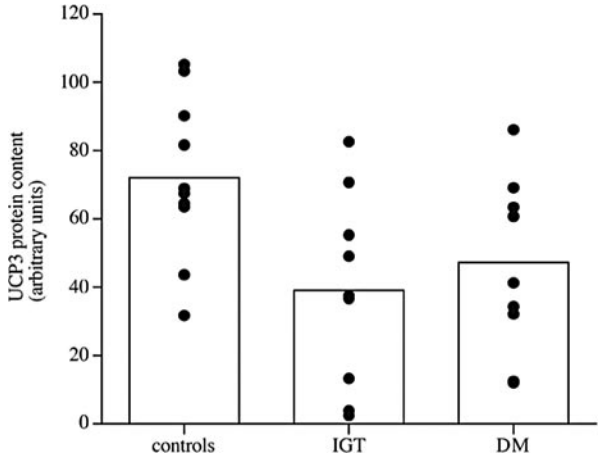

FIG. 2. Skeletal muscle UCP3 protein content (AU) in healthy controls (n = 10), prediabetic IGT subjects (n = 9), and type 2 diabetic patients (n = 10). UCP3 was lower in IGT and DM compared to controls ($P < 0.05$).

Eight weeks of rosiglitazone treatment tended to increase the mRNA expression of UCP3 by 37% (from 1.52 ± 1.04 to 2.09 ± 1.25 AU; $P = 0.10$), whereas this effect was statistically significant at the UCP3 protein level (from 53.2 ± 29.9 to 66.3 ± 30.9 AU; $P < 0.05$; Fig. 1). Again, no effect of rosiglitazone treatment on protein levels of the other mitochondrial proteins (complex I to V and cytochrome *c*) was observed (Table 3). There were no correlations between plasma FFA levels, insulin sensitivity, metabolic flexibility, and UCP3 protein levels at any time point.

Discussion

Based on the putative functions of UCP3 and its association with fatty acid metabolism, increased levels of skeletal muscle UCP3 protein are anticipated in diabetic subjects who are characterized by high plasma fatty acid levels and low fat oxidative capacity (9). However, here we confirmed our previous finding (13) that type 2 diabetic patients have reduced levels of skeletal muscle UCP3 mRNA and protein when compared with age- and BMI-matched healthy controls. Importantly, the difference in UCP3 protein was not simply due to a reduced mitochondrial content, as indicated by the similar levels of mitochondrial proteins of complex I–V in our (relatively "healthy") diabetic patients and nondiabetic controls. The reduction of UCP3 in skeletal muscle of diabetic

TABLE 2. UCP3 and mitochondrial protein complex I–V in control (n = 10) and diabetic (n = 10) subjects at baseline

	Control	Diabetic	*P*
UCP3	72.0 ± 23.7	47.2 ± 24.7	0.035
Cytochrome *c*	39.6 ± 17.7	41.0 ± 25.1	0.96
Complex I	2.7 ± 1.9	1.8 ± 0.8	0.18
Complex II	4.7 ± 2.3	4.6 ± 1.9	0.95
Complex III	44.3 ± 16.6	39.7 ± 0.56	0.56
Complex IV	15.3 ± 8.5	12.9 ± 5.8	0.46
Complex V	48.7 ± 17.5	43.8 ± 16	0.52

Data are expressed as mean ± SD.

TABLE 3. UCP3 and mitochondrial protein complex I–V before and after TZD treatment in diabetic patients (n = 8)

	Before	After	*P*
UCP3	53.2 ± 29.9	66.3 ± 30.9	0.043
Cytochrome *c*	45.7 ± 22.3	44.4 ± 16.3	0.85
Complex I	2.0 ± 0.8	1.3 ± 1.1	0.15
Complex II	4.6 ± 1.91	3.5 ± 2.6	0.38
Complex III	41.4 ± 20.5	37.1 ± 27.7	0.52
Complex IV	14.0 ± 5.9	11.2 ± 7.2	0.31
Complex V	42.5 ± 17.6	45.0 ± 21.4	0.69

Data are expressed as mean ± SD.

patients was accompanied by decreased insulin sensitivity and reduced metabolic flexibility, *i.e.* a reduced capacity to switch from predominantly fat oxidation in the fasting state to glucose oxidation in the insulin-stimulated state. Importantly, we also show that a reduction to a similar extent of UCP3 protein content can be observed in glucose-intolerant subjects, which are generally regarded as prediabetic subjects. In addition, we show that rosiglitazone treatment results in an up-regulation of UCP3 protein content by, on average 25%, accompanied by increased insulin sensitivity and improved metabolic flexibility. To the best of our knowledge, this is the first time that reduced UCP3 protein content in prediabetic subjects and/or an up-regulation of UCP3 protein content in type 2 diabetic patients by a therapeutic treatment has been shown and it suggests that a reduced level of UCP3 in type 2 diabetes is indeed part of the etiology of the disease.

The reduction of skeletal muscle UCP3 protein content in type 2 diabetic patients is remarkable and cannot simply be explained by the physiological regulation of UCP3 protein. Thus, skeletal muscle UCP3 protein levels are inversely related to fat oxidative capacity (21), are low in type 1 muscle fibers (22), and are reduced by endurance training (23, 24). On the other hand, UCP3 is up-regulated by increased levels of plasma fatty acids (25) and high-fat diets (19), and is high in type 2 muscle fibers (22) and in patients with decreased fat oxidative capacity (26). Based on all these and other observations, an increased level of UCP3 protein content would be expected in type 2 diabetic patients. Therefore, the reduction of UCP3 in type 2 diabetic patients may reflect a pathological condition, which is further stressed by the finding that rosiglitazone treatment, which improved metabolic characteristics in these patients, restored UCP3 protein levels. However, it cannot be excluded that other factors, such as hyperinsulinemia or hyperglycemia, are responsible for the reduced levels of UCP3 in diabetes. To this end, we determined UCP3 protein content in subjects who are prone to develop diabetes. Full-blown type 2 diabetes is preceded by a long-term state of IGT; up to 60% of people who develop diabetes have IGT 5 yr before the diagnosis of type 2 diabetes (27). We found that UCP3, but not cytochrome *c* protein content, was reduced to the same extent in IGT and type 2 diabetic subjects when compared with age- and BMI-matched controls. This indicates that low UCP3 levels are already present in a population at risk for developing diabetes suggesting that UCP3 is indeed involved in the etiology of type 2 diabetes mellitus.

As mentioned above, one of the most potent stimulators of UCP3 expression is the level of plasma FFA. Acute fasting and intralipid infusion both rapidly up-regulated UCP3 (28). Also in the recent study of Brunmair *et al.* (16), showing that acute injection of rosiglitazone in muscle of rats induced UCP3 mRNA expression, plasma fatty acids were elevated, which might partly have explained the effect of rosiglitazone on UCP3. In the present human study, however, plasma fatty acids levels were significantly reduced by rosiglitazone, most likely due to a TZD-induced improved uptake and storage in white adipose tissue. Therefore, plasma fatty acids could not have been responsible for the up-regulation of UCP3 in the current study. Alternatively, the reduction of plasma glucose and insulin levels after TZD treatment may have been responsible for the up-regulation of UCP3, as hyperglycemia has been shown to down-regulate UCP3 in human neuroblastoma cells (29), but data on glucose/insulin regulation of human skeletal muscle UCP3 is lacking. More likely, rosiglitazone may exert a direct effect on skeletal muscle, despite the fairly low expression of PPARγ in skeletal muscle. The importance of PPARγ in skeletal muscle is underscored by the finding that muscle-specific PPARγ knockout mice develop muscular insulin resistance and do not enhance insulin-stimulated glucose uptake upon TZD treatment (30). In addition, it is of importance to note that the promoter of the UCP3 gene contains a PPAR response element, enabling direct effects of the PPARγ agonist rosiglitazone (31, 32).

In regard to the physiological function of skeletal muscle UCP3, we have recently hypothesized (1) that the reduced level of UCP3 observed in diabetes contributes to the increased mitochondrial damage (14) and decreased mitochondrial function (15) observed in this disease. In support of this is the finding that mice lacking UCP3 are indeed characterized by increased levels of mitochondrial oxidative damage and lipid peroxidation (8). Although we did not examine these parameters directly in the present study, metabolic flexibility—an indicator of oxidative capacity—was significantly reduced in type 2 diabetic subjects compared with age- and BMI-matched healthy controls. In addition, the restoration of UCP3 protein levels after 8 wk of rosiglitazone treatment was accompanied by an enhanced insulin stimulation of glucose oxidation and insulin suppression of fat oxidation, indicating improved metabolic flexibility, which requires proper mitochondrial function. The finding that the reduced UCP3 protein content in skeletal muscle of diabetic patients was accompanied by a reduced insulin sensitivity and decreased metabolic flexibility, and that the TZD-induced restoration of UCP3 was accompanied by improved insulin resistance and increased metabolic flexibility, is indeed compatible with a role of UCP3 in the protection of lipid-induced mitochondrial damage, but clearly future studies are needed to examine whether an up-regulation of UCP3 by TZDs indeed is accompanied by an improved mitochondrial function.

In conclusion, we confirm that skeletal muscle UCP3 protein is reduced in type 2 diabetic patients compared with age- and BMI-matched healthy controls. Moreover, we show for the first time that the reduction in UCP3 protein content can already be found in the prediabetic state. In addition, we showed that rosiglitazone treatment up-regulates UCP3 protein content in skeletal muscle of type 2 diabetic patients. Further studies are required to establish whether a reduction

of UCP3 plays a significant role in the mitochondrial dysfunction that is observed in type 2 diabetes mellitus.

Acknowledgments

Received July 14, 2005. Accepted December 19, 2005.

Address all correspondence and requests for reprints to: Dr. P. Schrauwen, Nutrition and Toxicology Research Institute Maastricht, Department of Human Biology, Maastricht University, P.O. Box 616, NL-6200 MD Maastricht, The Netherlands. E-mail: p.schrauwen@hb.unimaas.nl.

P.S. was supported by a grant from the Dutch Royal Academy of Arts and Sciences. This work was financially supported by a grant from GlaxoSmithKline. M.K.C.H. is supported by a VIDI grant for innovative research from The Netherlands Organization for Scientific Research (Grant 917.66.359).

References

1. **Schrauwen P, Hesselink MK** 2004 Oxidative capacity, lipotoxicity, and mitochondrial damage in type 2 diabetes. Diabetes 53:1412–1417
2. **Schrauwen P, Saris WH, Hesselink MK** 2001 An alternative function for human uncoupling protein 3: protection of mitochondria against accumulation of nonesterified fatty acids inside the mitochondrial matrix. FASEB J 15:2497–2502
3. **Hamilton JA, Kamp F** 1999 How are free fatty acids transported in membranes? Is it by proteins or by free diffusion through the lipids? Diabetes 48:2255–2269
4. **Yagi K** 1987 Lipid peroxides and human diseases. Chem Phys Lipids 45:337–351
5. **Himms-Hagen J, Harper ME** 2001 Physiological role of UCP3 may be export of fatty acids from mitochondria when fatty acid oxidation predominates: an hypothesis. Exp Biol Med (Maywood) 226:78–84
6. **Goglia F, Skulachev VP** 2003 A function for novel uncoupling proteins: antioxidant defense of mitochondrial matrix by translocating fatty acid peroxides from the inner to the outer membrane leaflet. FASEB J 17:1585–1591
7. **Echtay KS, Esteves TC, Pakay JL, Jekabsons MB, Lambert AJ, Portero-Otin M, Pamplona R, Vidal-Puig AJ, Wang S, Roebuck SJ, Brand MD** 2003 A signalling role for 4-hydroxy-2-nonenal in regulation of mitochondrial uncoupling. EMBO J 22:4103–4110
8. **Brand MD, Pamplona R, Portero-Otin M, Requena JR, Roebuck SJ, Buckingham JA, Clapham JC, Cadenas S** 2002 Oxidative damage and phospholipid fatty acyl composition in skeletal muscle mitochondria from mice underexpressing or overexpressing uncoupling protein 3. Biochem J 368:597–603
9. **McGarry JD** 2002 Banting lecture 2001: dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. Diabetes 51:7–18
10. **Bao S, Kennedy A, Wojciechowski B, Wallace P, Ganaway E, Garvey WT** 1998 Expression of mRNAs encoding uncoupling proteins in human skeletal muscle: effects of obesity and diabetes. Diabetes 47:1935–1940
11. **Vidal H, Langin D, Andreelli F, Millet L, Larrouy D, Laville M** 1999 Lack of skeletal muscle uncoupling protein 2 and 3 mRNA induction during fasting in type-2 diabetic subjects. Am J Physiol Endocrinol Metab 277:E830–E837
12. **Krook A, Digby J, OqRahilly S, Zierath JR, Wallberg-Henriksson H** 1998 Uncoupling protein 3 is reduced in skeletal muscle of NIDDM patients. Diabetes 47:1528–1531
13. **Schrauwen P, Hesselink MK, Blaak EE, Borghouts LB, Schaart G, Saris WH, Keizer HA** 2001 Uncoupling protein 3 content is decreased in skeletal muscle of patients with type 2 diabetes. Diabetes 50:2870–2873
14. **Kelley DE, He J, Menshikova EV, Ritov VB** 2002 Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. Diabetes 51:2944–2950
15. **Russell AP, Gastaldi G, Bobbioni-Harsch E, Arboit P, Gobelet C, Deriaz O, Golay A, Witztum JL, Giacobino JP** 2003 Lipid peroxidation in skeletal muscle of obese as compared to endurance-trained humans: a case of good vs. bad lipids? FEBS Lett 551:104–106
16. **Brunmair B, Gras F, Wagner L, Artwohl M, Zierhut B, Waldhausl W, Furnsinn C** 2004 Expression of uncoupling protein-3 mRNA in rat skeletal muscle is acutely stimulated by thiazolidinediones: an exercise-like effect? Diabetologia 47:1611–1614
17. **Mensink M, Blaak EE, Corpeleijn E, Saris WH, de Bruin TW, Feskens EJ** 2003 Lifestyle intervention according to general recommendations improves glucose tolerance. Obes Res 11:1588–1596
18. **Bergström J, Hermansen L, Hultman E, Saltin B** 1967 Diet, muscle glycogen and physical performance. Acta Physiol Scand 71:140–150
19. **Hesselink MKC, Greenhaff PL, Constantin-Teodosiu D, Hultman E, Saris WHM, Nieuwlaat R, Schaart G, Kornips E, Schrauwen P** 2003 Increased uncoupling protein 3 content does not affect mitochondrial function in human skeletal muscle in vivo. J Clin Invest 111:479–486
20. **Schrauwen P, Schaart G, Saris WH, Slieker LJ, Glatz JF, Vidal H, Blaak EE** 2000 The effect of weight reduction on skeletal muscle UCP2 and UCP3 mRNA expression and UCP3 protein content in type II diabetic subjects. Diabetologia 43:1408–1416
21. **Hoeks J, Hesselink MKC, Van Bilsen M, Schaart G, van der Vusse GJ, Saris WHM, Schrauwen P** 2003 Differential response of UCP3 to medium versus long chain triacylglycerols; manifestation of a functional adaptation. FEBS Lett 555:631–637
22. **Hesselink MKC, Keizer HA, Borghouts LB, Schaart G, Kornips CFP, Slieker LJ, Sloop KW, Saris WHM, Schrauwen P** 2001 Protein expression of UCP3 differs between human type 1, type 2a and type 2b fibers. FASEB J 15:1071–1073
23. **Russell AP, Somm E, Praz M, Crettenand A, Hartley O, Melotti A, Giacobino JP, Muzzin P, Gobelet C, Deriaz O** 2003 UCP3 protein regulation in human skeletal muscle fibre types I, IIa and IIx is dependent on exercise intensity. J Physiol 550:855–861
24. **Schrauwen P, Russell AP, Moonen-Kornips E, Boon N, Hesselink MK** 2005 Effect of 2 weeks of endurance training on uncoupling protein 3 content in untrained human subjects. Acta Physiol Scand 183:273–280
25. **Khalfallah Y, Fages S, Laville M, Langin D, Vidal H** 2000 Regulation of uncoupling protein-2 and uncoupling protein-3 mRNA expression during lipid infusion in human skeletal muscle and subcutaneous adipose tissue. Diabetes 49:25–31
26. **Russell AP, Schrauwen P, Somm E, Gastaldi G, Hesselink MKC, Schaart G, Kornips E, Lo SK, Bufano D, Giacobino J-P, Muzzin P, Ceccon M, Angelini C, Vergani L** 2003 Decreased fatty acid β-oxidation in riboflavin-responsive multiple acylCoA dehydrogenase deficient patients is associated with an increase in UCP3. J Clin Endocrinol Metab 88:5921–5926
27. **Unwin N, Shaw J, Zimmet P, Alberti KG** 2002 Impaired glucose tolerance and impaired fasting glycaemia: the current status on definition and intervention. Diabetes Med 19:708–723
28. **Weigle DS, Selfridge LE, Schwartz MW, Seeley RJ, Cummings DE, Havel PJ, Kuijper JL, BertrandelRio H** 1998 Elevated free fatty acids induce uncoupling protein 3 expression in muscle. A potential explanation for the effect of fasting. Diabetes 47:298–302
29. **Gustafsson H, Soderdahl T, Jonsson G, Bratteng JO, Forsby A** 2004 Insulin-like growth factor type 1 prevents hyperglycemia-induced uncoupling protein 3 down-regulation and oxidative stress. J Neurosci Res 77:285–291
30. **Hevener AL, He W, Barak Y, Le J, Bandyopadhyay G, Olson P, Wilkes J, Evans RM, Olefsky J** 2003 Muscle-specific Pparγ deletion causes insulin resistance. Nat Med 9:1491–1497
31. **Acín A, Rodriguez M, Rique H, Canet E, Boutin JA, Galizzi J-P** 1999 Cloning and characterization of the 5′ flanking region of the human uncoupling protein 3 (UCP3) gene. Biochem Biophys Res Commun 258:278–283
32. **Solanes G, Pedraza N, Iglesias R, Giralt M, Villarroya F** 2003 Functional relationship between MyoD and peroxisome proliferator-activated receptor-dependent regulatory pathways in the control of the human uncoupling protein-3 gene transcription. Mol Endocrinol 17:1944–1958