Rapid Publication

Uncoupling Protein 3 Is Reduced in Skeletal Muscle of NIDDM Patients

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Two recently described proteins in the mitochondrial uncoupling protein (UCP) family, UCP-2 and UCP-3, have been linked to phenotypes of obesity and NIDDM. We determined the mRNA levels of UCP-2 and UCP-3 in skeletal muscle of NIDDM patients and of healthy control subjects. No difference in the mRNA levels or in the protein expression of UCP-2 was observed between the two groups. In contrast, mRNA levels of UCP-3 were significantly reduced in skeletal muscle of NIDDM patients compared with control subjects. In the NIDDM patients, a positive correlation between UCP-3 expression and whole-body insulin-mediated glucose utilization rate was also noted. These results suggest that UCP-3 regulation may be altered in states of insulin resistance. ***Diabetes*** **47:1528–1531, 1998**

Resting skeletal muscle metabolism is a significant determinant of whole-body energy expenditure (1,2). Uncoupling proteins (UCPs) function to uncouple respiration from oxidative phosphorylation and ATP synthesis, converting fuel into heat (3). A substantial part of the basal metabolic rate derives from leaking of protons across the inner mitochondrial membrane. Three UCPs have been described to date. UCP-1 is primarily expressed in brown adipose tissue (3,4). Transgenic mice in which brown fat is ablated develop obesity and insulin resistance (5). Conversely, UCP-1 overexpression in white adipose tissue in genetically obese A^y mice prevents the development of obesity (6). However, because there is little brown fat present in adult humans, UCP-1 is unlikely to play a major role in controlling human energy metabolism.

In humans, skeletal muscle is an important site of thermogenesis, although the molecular mechanisms controlling this process remain unclear (7). The recently identified proteins, UCP-2 and UCP-3, may be candidates for the proton leak observed in skeletal muscle (8–10). UCP-2 and UCP-3 show 59 and 57% amino acid identity to UCP-1, respectively. In adult humans, UCP-2 is expressed in a large number of tissues, including white adipose tissue and skeletal muscle (8,11), whereas UCP-3 appears to be restricted to skeletal muscle (9,10).

Aberrant UCP function could underlie metabolic defects seen in both obesity and NIDDM. We hypothesized that expression levels of UCP-2 and/or UCP-3 may be decreased in skeletal muscle from insulin-resistant NIDDM patients and thus may potentially be involved in the pathogenesis of peripheral insulin resistance. Competitive reverse transcriptase–polymerase chain reaction (RT-PCR) was used to assess UCP-2 and UCP-3 mRNA levels in skeletal muscle obtained from nine NIDDM patients and eight healthy control subjects. UCP-2 protein expression in protein lysates from skeletal muscle was also determined.

RESEARCH DESIGN AND METHODS

Subject characteristics. The study protocol was reviewed and approved by the institutional ethics committee of the Karolinska Institute, and informed consent was received from all subjects before their participation. The clinical characteristics of the subjects are presented in Table 1. The diabetic group consisted of nine male NIDDM patients whose mean duration of disease was 6.5 years and who ranged from those newly diagnosed to those with a duration of disease of 15 years. Patients were treated with insulin (n = 1), a combination of sulfonylureas and insulin (n = 1), sulfonylureas (n = 4), or diet (n = 3). The control group consisted of eight healthy male subjects. None of the study participants smoked or took any other medication. The subjects were instructed to abstain from any form of strenuous physical activity for 48 h before the experiment. On the day of the test, the subjects reported to the laboratory after an overnight fast, and in the case of the NIDDM patients, before the administration of any antidiabetic medication.

Muscle biopsy. Muscle biopsy specimens were obtained, as described previously (12), under local anesthesia from the vastus lateralis portion of the quadriceps femoris muscle and were immediately placed in liquid nitrogen.

Euglycemic-hyperinsulinemic clamp. Insulin-mediated glucose utilization was determined using the euglycemic-hyperinsulinemic clamp procedure, which has been described in detail previously (13,14). This procedure and the muscle biopsy were performed on separate occasions.

RNA extraction and cDNA synthesis. Muscle biopsy specimens (25–35 mg) were removed from liquid nitrogen and were homogenized, using a Polytron mixer, in 1 ml guanidium thiocyanate–phenol solution (Sigma Tri-Reagent; Sigma, St. Louis, MO), and total RNA was extracted according to the manufacturer's instructions. The integrity of the extracted RNA was verified by gel electrophoresis, and 2 µg of RNA was used as template for subsequent cDNA synthesis in the reverse transcription reaction, using Promega Reverse Transcription System (Promega, Madison, WI) in a 20-µl reaction volume according to the manufacturer's instructions. After synthesis was complete, cDNA was diluted tenfold and stored in aliquots at –20°C.

Quantification of mRNA. UCP-2 and UCP-3 mRNA was quantified by reverse transcription followed by competitive RT-PCR, during which known amounts of standard DNA was coamplified with the target cDNA in the same tube. The standard was designed to use the same polymerase chain reaction (PCR) primers as the target but yield a PCR product differing sufficiently in size to allow separation and quantitation of the two amplicons by gel electrophoresis. A multispecific standard with target sequences for UCP-2, UCP-3, and β-µglobin (Fig. 1) was generated using overlap extension and amplification by PCR (15). The standard was

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PCR, polymerase chain reaction; RT-PCR, reverse transcriptase–PCR; UCP, uncoupling protein.

TABLE 1
Subject characteristics

	NIDDM	Control
n	9	8
Age (years)	57 ± 2	55 ± 2
BMI (kg/m^2)	27.3 ± 1.2	26.7 ± 0.8
Glucose utilization (μmol · kg^{-1} · min^{-1})	27.35 ± 4.5*	37.02 ± 3.4
Fasting glucose (mmol/l)	9.1 ± 0.8†	5.3 ± 0.4
Fasting insulin (pmol/l)	97 ± 20‡	36 ± 8
HbA_{1c} (%)	6.9 ± 0.4†	4.6 ± 0.1
Duration of diabetes (years)	6.7 ± 2.1	—

Data are means ± SE. *P < 0.03; †P < 0.001; ‡P < 0.02. P values express significant differences from control subjects.

purified in large quantity and stored as a concentrated stock at –80°C. Working solutions at defined concentrations were prepared by serial dilution, and several aliquots of each dilution were stored at –20°C.

Competitive PCR. Each cDNA was coamplified in the presence of known amounts of the multispecific competitor in a PCR reaction with specific primers for UCP-2, UCP-3, or β-μglobin to establish an equivalence point for each of the three genes. To reduce errors, the same working dilution of competitor was used for the analysis of all three genes, and each determination was carried out on two separately synthesized batches of cDNA from each muscle biopsy. The results were then normalized and presented by reference to the mRNA levels of the constitutively expressed β-μglobin gene (15). The absence of contamination by genomic DNA was checked by omitting reverse transcriptase in the reactions. Furthermore, the PCR primers were designed to hybridize in separate exons, thus yielding a product of a different size when hybridizing to genomic DNA. The primer sequences and the resulting product sizes are given in Table 2. The UCP-3 primer pair recognizes sequences shared by both the long (UCP-3_L) and short (UCP-3_S) forms of UCP-3 transcripts (16).

Western blot analysis. Muscle biopsy specimens were removed from liquid nitrogen and homogenized in ice-cold homogenizing buffer (50 mmol/l HEPES [pH 7.6], 150 mmol/l NaCl, 1% Triton X-100, 1 mmol/l Na_3VO_4, 10 mmol/l NaF, 30 mmol/l $Na_4P_2O_7$, 10% [vol/vol] glycerol, 1 mmol/l benzamidine, 1 mmol/l dithiothreitol, 10 μg/ml leupeptin, 1 mmol/l phenylmethylsulfonyl fluoride), and lysates cleared by centrifugation at 15,000*g* for 10 min (4°C). Protein was determined using a commercial kit (Bio-Rad, Richmond, CA). Aliquots (30 μg) of the supernatant were solubilized in Laemmli sample buffer, separated by SDS–polyacrylamide gel electrophoresis (10% resolving gel), and transferred to nitrocellulose membranes. Immunodetection of UCP-2 protein was performed using a polyclonal UCP-2 antibody. The antibody was raised against a peptide based on the NH_2-terminal of UCP-2. After incubation with secondary antibody, the nitrocellulose membrane was placed in a phosphoimager cassette for 24 h, after which the UCP-2 protein was visualized by phosphoimaging and quantified by densitometry.

Statistical analysis. Data are presented as means ± SE. Statistical differences were determined using Student's unpaired t test. Significance of correlations was determined by simple regression analysis.

RESULTS

Age and BMI were similar between the two groups (Table 1). Glycemic control, as evaluated by HbA_{1c}, was moderate (6.9 ± 0.4%). The normal range for HbA_{1c} in our laboratory is <5.2%. The mean peripheral glucose utilization rate achieved during steady-state hyperinsulinemia was reduced by 26% in the NIDDM patient group compared with the control group (Table 1). Mean steady-state serum insulin and plasma glucose levels during the last 100 min of the clamp were similar between the two groups (547 ± 67 vs. 516 ± 53 pmol/l for insulin and 5.9 ± 0.2 vs. 5.2 ± 0.1 mmol/l for glucose in NIDDM patients vs. control subjects, respectively; NS).

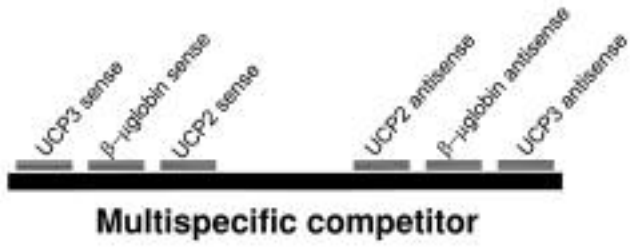

FIG. 1. Schematic outline of the organization of the multispecific competitor. The sequence of primers and the resulting product sizes are given in Table 2.

In human skeletal muscle, UCP-3 was more abundant than UCP-2. A significant degree of variability in the mRNA level of UCP-3 was observed in the skeletal muscle of healthy individuals (Fig. 2). However, mRNA levels of UCP-3 were reduced in the skeletal muscle of NIDDM patients compared with control subjects (41% reduction, P < 0.005) (Fig. 2). The skeletal muscle of NIDDM patients did not differ from that of control subjects in that both had a similar abundance of UCP-2 mRNA (Fig. 3*A*) and protein expression (Fig. 3*B*).

No correlation was observed between BMI and UCP-3 expression levels in skeletal muscle. In all subjects, a weak negative correlation between BMI and UCP-2 expression in skeletal muscle was observed (r = 0.59, P < 0.05; data not shown). Interestingly, we observed a positive correlation between UCP-3 expression level in skeletal muscle and insulin sensitivity as determined by the glucose utilization rate during the hyperinsulinemic-euglycemic clamp in the NIDDM patients (r = 0.66, P < 0.01) (Fig. 2, inset). No such relationship was evident in healthy control subjects.

DISCUSSION

Both UCP-2 and UCP-3 map to regions of human chromosome 11 and mouse chromosome 7, loci that are associated with hyperinsulinemia and obesity (8). Thus, alterations in the regulation of UCP-2 and UCP-3 could potentially occur in both obesity and NIDDM. The coding region of the UCP-2 gene has been examined in two recent studies of large cohorts of obese subjects, and in neither study have any disease-associated polymorphisms or mutations been reported (17,18). The UCPs appear to be under complex regulation. Thiazolidinedione treatment in human brown preadipocytes

TABLE 2
Primer sequences and resulting product sizes

Name	Sense primer	Antisense primer	Size of PCR product: Gene	Size of PCR product: Competitor
β-μglobin	5′ CCA GCA GAG AAT GGA AAG TC 3′	5′ GAT GCT GCT TAC ATG TCT CG 3′	269	309
UCP-2	5′ GCT CGT AAT GCC ATT GTC A 3′	5′ ACA GTG GCC AGC GCT ACT GTA 3′	220	269
UCP-3	5′ ATA CAG CGG GAC TAT GGA C 3′	5′ GAA GTA CTG GCC TGG AGG TG 3′	288	400

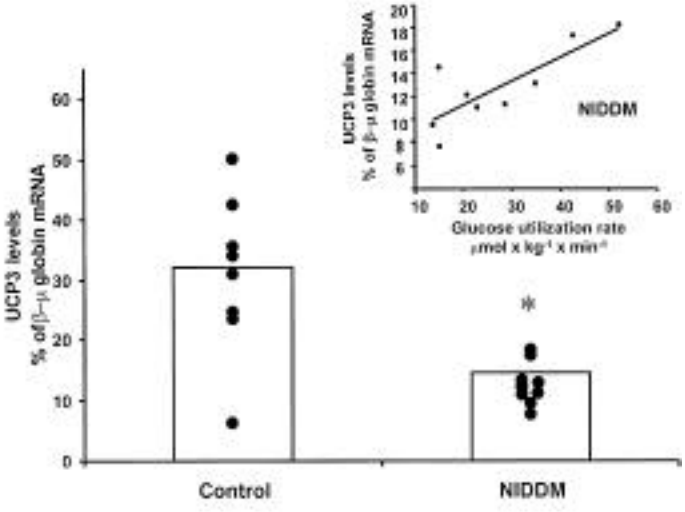

FIG. 2. Individual mRNA levels of UCP-3 in skeletal muscle from NIDDM patients and from control subjects. Results are reported as a percentage of β-μglobin mRNA. The superimposed bars show means ± SE (31.04 ± 4.70 and 12.74 ± 1.15 for control vs. NIDDM subjects, respectively). *P < 0.005 versus control values. Inset shows correlation between UCP-3 mRNA levels and insulin-mediated glucose utilization observed in NIDDM patients (P < 0.01).

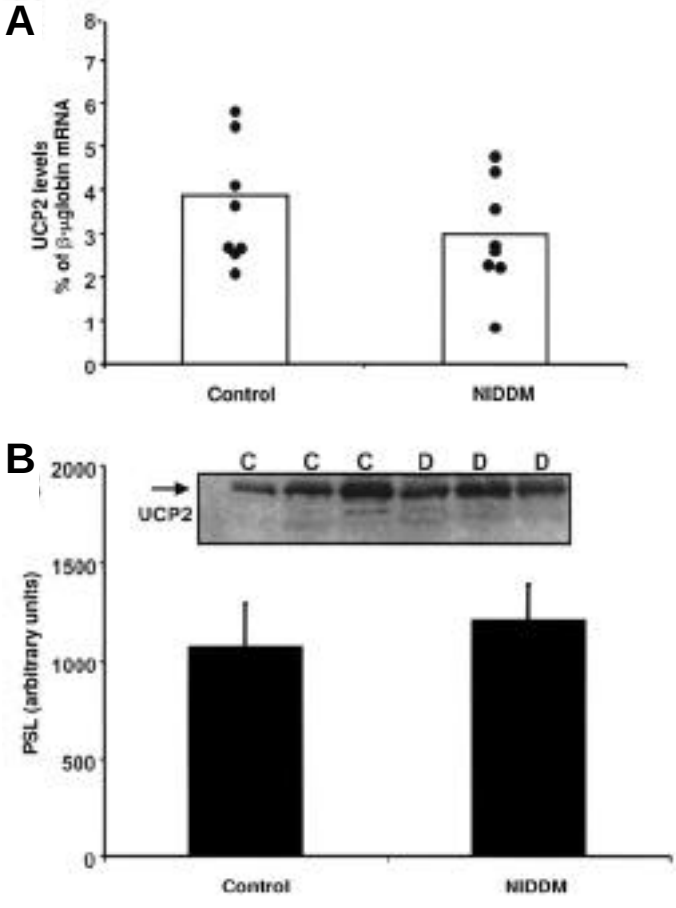

FIG. 3. *A*: Individual mRNA levels of UCP-2 in skeletal muscle from NIDDM patients and healthy control subjects. Results are reported as a percentage of β-μglobin mRNA. The superimposed bars show means ± SE (3.64 ± 0.50 and 2.71 ± 0.47 for control vs. NIDDM subjects, respectively). *B*: UCP-2 protein expression in skeletal muscle from NIDDM and control subjects (means ± SE), expressed in arbitrary phosphoimager units. Inset shows a representative autoradiogram. C, control; D, NIDDM; PSL, photostimulated luminescence.

results in an increase in UCP-1 expression (19). UCP-2 has also been shown to be upregulated by thiazolidinediones in skeletal muscle and adipocyte cell lines (20), and by leptin (21). Hypothyroidism in rats is associated with decreased UCP-3 levels in skeletal muscle, while hyperthyroidism is associated with increased expression of UCP-3 (22). Recently, Millet et al. (23) showed that 5-day calorie restriction leads to upregulation of both UCP-2 and UCP-3 in skeletal muscle, and of UCP-2 in adipose tissue, in both lean and obese human subjects. Interestingly, the induction of gene expression in response to food restriction did not differ between the two groups. Furthermore, no significant difference was noted in UCP-2 and UCP-3 expression levels between the lean and obese subjects studied (23). Similarly, Surwit et al. (24) found that resistance to high-fat feeding–induced obesity in mice is associated with induction of UCP-2 expression in white adipose tissue and UCP-1 expression in brown adipose tissue, but not with changes in UCP-3 expression in muscle. However, a similar high-fat feeding challenge in rats resulted in a twofold increase in UCP-3 expression in skeletal muscle and a 1.6-fold increase in UCP-2 expression in adipose tissue, indicating possible species differences (25).

Here we report that mRNA levels of UCP-3 are reduced in skeletal muscle from NIDDM subjects compared with healthy control subjects. Furthermore, in NIDDM subjects, UCP-3 expression is positively correlated with whole-body insulin-mediated glucose utilization. These results suggest that UCP-3 regulation may be altered in states of insulin resistance. Further studies are needed to investigate whether this defect contributes to the pathogenesis of NIDDM.

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