

# Expression of *Mfn2*, the Charcot-Marie-Tooth Neuropathy Type 2A Gene, in Human Skeletal Muscle Effects of Type 2 Diabetes, Obesity, Weight Loss, and the Regulatory Role of Tumor Necrosis Factor $\alpha$ and Interleukin-6

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The primary gene mutated in Charcot-Marie-Tooth type 2A is mitofusin-2 (*Mfn2*). *Mfn2* encodes a mitochondrial protein that participates in the maintenance of the mitochondrial network and that regulates mitochondrial metabolism and intracellular signaling. The potential for regulation of human *Mfn2* gene expression in vivo is largely unknown. Based on the presence of mitochondrial dysfunction in insulin-resistant conditions, we have examined whether *Mfn2* expression is dysregulated in skeletal muscle from obese or nonobese type 2 diabetic subjects, whether muscle *Mfn2* expression is regulated by body weight loss, and the potential regulatory role of tumor necrosis factor (TNF) $\alpha$  or interleukin-6. We show that mRNA concentration of *Mfn2* is decreased in skeletal muscle from both male and female obese subjects. Muscle *Mfn2* expression was also reduced in lean or in obese type 2 diabetic patients. There was a strong negative correlation between the *Mfn2* expression and the BMI in nondiabetic and type 2 diabetic subjects. A positive correlation between the *Mfn2* expression and the insulin sensitivity was also detected in nondiabetic and type 2 diabetic subjects. To

determine the effect of weight loss on *Mfn2* mRNA expression, six morbidly obese subjects were subjected to weight loss by bilio-pancreatic diversion. Mean expression of muscle *Mfn2* mRNA increased threefold after reduction in body weight, and a positive correlation between muscle *Mfn2* expression and insulin sensitivity was again detected. In vitro experiments revealed an inhibitory effect of TNF $\alpha$  or interleukin-6 on *Mfn2* expression in cultured cells. We conclude that body weight loss upregulates the expression of *Mfn2* mRNA in skeletal muscle of obese humans, type 2 diabetes downregulates the expression of *Mfn2* mRNA in skeletal muscle, *Mfn2* expression in skeletal muscle is directly proportional to insulin sensitivity and is inversely proportional to the BMI, TNF $\alpha$  and interleukin-6 downregulate *Mfn2* expression and may participate in the dysregulation of *Mfn2* expression in obesity or type 2 diabetes, and the in vivo modulation of *Mfn2* mRNA levels is an additional level of regulation for the control of muscle metabolism and could provide a molecular mechanism for alterations in mitochondrial function in obesity or type 2 diabetes. *Diabetes* 54: 2685–2693, 2005

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BPD, bilio-pancreatic diversion; *Mfn2*, mitofusin-2; TNF, tumor necrosis factor.

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**M**itochondrial metabolism is altered in skeletal muscle during insulin-resistant states such as type 2 diabetes or obesity. Human type 2 diabetes is associated with reduced capacity to oxidize glucose in the presence of insulin and, more importantly, to oxidize fatty acid in various conditions (1,2). A significant decrease in gene expression of subunits participating in complexes I–IV of the mitochondrial electron transport chain was observed in skeletal muscle of type 2 diabetic patients (3,4). Reduced glucose oxidation, decreased pyruvate dehydrogenase activity, and enhanced pyruvate dehydrogenase kinase have also been evidenced in skeletal muscle from diabetic rats (5–7). Similarly, experimental diabetes in rats reduces gene expression of

various subunits of complexes I, III, and IV (8). In keeping with these observations, the respiratory chain is depressed in skeletal muscle from type 2 diabetic patients (9), and oxidative phosphorylation activity is reduced in heart and skeletal muscle in diabetic rats (10,11).

Obesity is also characterized by metabolic alterations that involve mitochondrial defects causing reduced oxidation in skeletal muscle (12,13). In some animal models of obesity such as in obese Zucker rats or *ob/ob* mice, skeletal muscle shows a metabolic profile characterized by reduced glucose uptake and glucose oxidation, altered partitioning of fatty acids that are incorporated into triglycerides, and reduced oxygen consumption (14–18). However, and although deficient muscle substrate oxidation capacity is observed during obesity, there is no alteration in mitochondrial activities of pivotal enzymes such as citrate synthase, succinate dehydrogenase, or  $\beta$ -hydroxyacetyl-CoA dehydrogenase (19–21).

In addition to, or linked to, functional defects of mitochondria, alterations in mitochondrial morphology have also been reported in insulin-resistant states. In humans, skeletal muscle mitochondria from type 2 diabetic patients or obese subjects are smaller than in lean subjects and show large vacuoles (9). In addition, skeletal muscle from WBN/Kob diabetic rats show mitochondrial swelling and lysis of mitochondrial cristae (22), and mitochondria from obese Zucker rats show higher mitochondrial surface, lower mitochondrial volume, and a lower extent of the mitochondrial network (23).

Mitofusin-2 (*Mfn2*) participates in the maintenance of mitochondrial tubules or networks in mammalian cells (23–25). *Mfn2* is a nuclear-encoded mitochondrial protein that shows homology with *Drosophila* or yeast Fzo proteins (26,27) and is highly expressed in skeletal muscle, the heart, and the brain (23). *Mfn2* regulates mitochondrial metabolism, and repression of *Mfn2* reduces glucose oxidation, mitochondrial membrane potential, and cell respiration (23,28). In addition, alterations in *Mfn2* expression in muscle cells cause a parallel change in the expression of subunits of OXPHOS system (28). Mutations in *Mfn2* causes Charcot-Marie-Tooth neuropathy type 2A (29), which further strengthens the importance of *Mfn2* in cell function. Recently, it has been reported that *Mfn2* binds Ras and its overexpression inhibits extracellular signal-related kinase/mitogen-activated protein kinase signaling and subsequently arrests cell cycle (30).

Given the key role of *Mfn2* in cellular function, it is relevant to study the *in vivo* regulation of *Mfn2* gene expression in altered physiological states such as type 2 diabetes or in conditions characterized by weight loss. In this study we address this issue by quantitating the expression of *Mfn2* mRNA in human muscle in a variety of physiological and pathophysiological states.

## RESEARCH DESIGN AND METHODS

To study the expression of *Mfn2* in human skeletal muscle, we took the opportunity of disposing of muscle samples from different cohorts of patients and age-matched control subjects that have been previously obtained in experimental protocols performed at INSERM U449, Lyon, France, or at Karolinska Institutet, Stockholm, Sweden. All subjects were informed of the possible risks of the study and gave their written informed consent. The Ethical Committee of the Hospices Civils de Lyon and of the Karolinska Institutet, respectively, approved the experimental protocols, which were

TABLE 1  
Characteristics of the subjects from the Lyon study

	Lean control	Obese	Type 2 diabetes
Men/women	6/7	6/9	8/10
Age (years)	51 ± 4	43 ± 5	52 ± 2
BMI (kg/m <sup>2</sup> )	23 ± 0.4	33 ± 1†	31 ± 1†
Glucose (mmol/l)	4.9 ± 0.1	4.9 ± 0.1	12.1 ± 0.7†
Insulin (pmol/l)	35 ± 2	67 ± 9*	64 ± 6*
Free fatty acid (mmol/l)	394 ± 46	574 ± 47*	670 ± 45†
Triglycerides (mmol/l)	0.6 ± 0.1	1.0 ± 0.1*	1.8 ± 0.3*
Glucose disposal rate (during the clamp) (mg · kg <sup>-1</sup> · min <sup>-1</sup> )	9.9 ± 1.1	4.9 ± 0.8†	3.6 ± 0.4†

Data are means ± SD, unless otherwise indicated. \* and † indicate statistically significant differences compared with the control groups, at  $P < 0.01$  and  $P < 0.001$ , respectively.

performed according to the principles of the Helsinki Declaration and the French legislation (for the Lyon studies).

The studies performed at Lyon included three groups of age-matched subjects including nondiabetic obese subjects (with normal glucose tolerance as assessed by a 75-g glucose load), obese type 2 diabetic patients (HbA<sub>1c</sub> 10.2 ± 0.3%), and healthy lean subjects. Table 1 presents the metabolic characteristics of the 49 subjects (20 men and 29 women) included in the Lyon studies. None of the healthy lean volunteers had impaired glucose tolerance or a familial or personal history of diabetes, obesity, dyslipidemia, or hypertension. The type 2 diabetic patients interrupted, under medical control, their usual treatment with oral antidiabetic agents at least 5 days before the investigation. The metabolic data of some of these subjects have been presented in previous studies (31,32). To characterize insulin action on glucose metabolism and on *Mfn2* expression, the subjects underwent a 3-h euglycemic-hyperinsulinemic (2 mU · kg<sup>-1</sup> · min<sup>-1</sup>) clamp, as described previously (33).

The study performed at the Karolinska Institutet involved eight nonobese type 2 diabetic men and nine age-matched and BMI-matched male healthy volunteers. Their anthropometric and metabolic characteristics are shown in Table 2. Insulin sensitivity of glucose metabolism was assessed by the mean of a 2-h euglycemic-hyperinsulinemic clamp, as previously described (34,35).

All studies were performed after an overnight fast. Percutaneous biopsies of the vastus lateralis muscle were obtained in the basal state under local anesthesia, as described elsewhere (31,35). For some subjects of the Lyon studies (see RESULTS), additional muscle biopsies were taken at the end of the hyperinsulinemic clamp, as described previously (33). Tissue samples were immediately frozen in liquid nitrogen and stored at -80°C for further analysis. **Bilio-pancreatic diversion study.** The study groups consisted of six severely (BMI >40 kg/m<sup>2</sup>) obese subjects studied on two separate occasions: before and 2 years after bilio-pancreatic diversion (BPD). None had impaired glucose tolerance, diabetes, or any other endocrine or nonendocrine disease. At the time of the baseline study, all subjects were on an ad libitum diet, with the following average composition: 60% carbohydrate, 30% fat, 10% protein (at

TABLE 2  
Characteristics of the male subjects from the Karolinska study

	Control	Type 2 diabetes
<i>n</i>	9	8
Age (years)	58 ± 2	57 ± 2
BMI (kg/m <sup>2</sup> )	26 ± 1	27 ± 2
Body fat (%)	24 ± 2	23 ± 3
Glucose (mmol/l)	5.1 ± 0.1	7.8 ± 0.9*
Insulin (pmol/l)	97 ± 53	113 ± 50
Free fatty acid (mmol/l)	513 ± 53	589 ± 96
Triglycerides (mmol/l)	1.3 ± 0.1	1.3 ± 0.2
Glucose disposal rate (during the clamp) (mg · kg <sup>-1</sup> · min <sup>-1</sup> )	7.5 ± 1.1	5.2 ± 1.1*

Data are means ± SD, unless otherwise indicated. \*Statistically significant differences compared with the control groups at  $P < 0.05$ .

least 1 g/kg body wt). This dietary regimen was maintained for 1 week before the study. The nature and purpose of the investigation were explained to all subjects before they agreed to participate in the study, which complied with the guidelines of the hospital ethics committee of Catholic University of Rome. The nature and purpose of the study were carefully explained to all subjects before obtaining their written consent to participate. BPD is an essentially malabsorptive surgical procedure and was performed as reported (36). Anthropometric parameters were determined at 0 and  $18 \pm 2$  months after surgery. Fat-free mass (in kilograms) and fat mass were determined as reported (36).

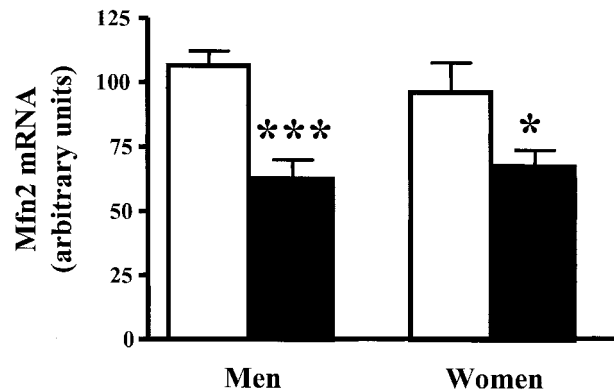
Subjects were instructed not to perform strenuous physical exercise for 48 h before the muscle biopsy procedure. Biopsies were obtained in fasting conditions from the middle region of the quadriceps muscles by a percutaneous needle before and after BPD surgical treatment. Muscle specimens were trimmed accurately free of fat, immediately frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ .

**Cell culture.** For the culture of human skeletal muscle cells, skeletal muscle biopsies (1–4 g) were obtained from healthy lean subjects undergoing orthopedic surgical intervention (1 man and 2 women, age  $51 \pm 6$  years, BMI  $23.3 \pm 1.5$  kg/m<sup>2</sup>). The apparent connective and fat tissues were removed, and the muscle was minced before being incubated for 30 min in 5 ml of trypsin-EDTA (Invitrogen, Carlsbad, CA) at  $37^{\circ}\text{C}$  under agitation. After being centrifuged (150g), the pellet was rinsed several times in PBS and filtered on a 70- $\mu\text{m}$  filter before being plated. Cells were first preplated for 1 h to eliminate rapidly adherent fibroblasts. The remaining cells were then cultured in a Primaria flask (Falcon; Becton Dickinson, Bedford, MA) in a growth medium composed of HAM F-10 supplemented with 2% Ultrosor G (BioSepra, Cergy-Saint-Christophe, France), 2% fetal bovine serum (FBS; Invitrogen), and 1% antibiotics (Invitrogen). After proliferation, the myoblasts were selected using a monoclonal antibody (5.1H11) combined with paramagnetic polystyrene beads (CELLlection Pan Mouse IgG kit; Dynal Beads, Oslo, Norway), according to the procedure described by Lequerica et al. (37). This antibody is specific for a surface antigen expressed on human myogenic cells (38) and was produced from a hybridoma (University of Iowa, Iowa City, IA). The selected cells were cultured as reported (39).

NH3T3 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 units/ml streptomycin, and 100 units/ml penicillin at  $37^{\circ}\text{C}$  in an atmosphere of 5% CO<sub>2</sub>/95% air. These cells were treated with tumor necrosis factor (TNF) $\alpha$  (20 ng/ml) for 6–12 h at  $37^{\circ}\text{C}$ . At this time, the attached cells were harvested and centrifuged and the isolation of RNA was performed using RNeasy Kit (Qiagen).

**Total RNA preparation and quantification of mRNA levels.** Total RNA extraction and treatment with DNase I were performed with RNeasy mini kit (Qiagen). Total RNA from muscle or cell samples was stored at  $-80^{\circ}\text{C}$  until further assay. RNA concentration was determined by spectrophotometry at an absorbance of 260 nm. RNA integrity was assessed by agarose gel electrophoresis and ethidium bromide staining. Quantification of the mRNAs was done by RT-cPCR (RT competitive PCR) or by real-time PCR. RT-cPCR relies on the addition of known amounts of a competitor DNA molecule in the PCR to standardize the amplification process (40). Reverse transcription to synthesize complementary DNA was carried out using Sensiscrip RT kit (Qiagen). In RT-cPCR assays, reverse transcription was performed from 100 ng total RNA in the presence of specific Mfn2 antisense primer under conditions that warrant optimal synthesis of first strand cDNA, as described (23). The primers used for the RT-cPCR were 5'-ATGCATCCCCACTTAAGCAC-3' for the sense primer and 5'-CCAGAGGGCAGAACTTTGTGTC-3' for the antisense primer (23). In real-time PCR assays, quantification of PCR products was performed by measuring fluorescence from the progressive binding of SYBR green I dye to double-stranded DNA using the ABI Prism 7700 sequence detection system (Applied Biosystems). The relative quantification value of PCR transcripts was calculated by using the manufacturer's protocol (comparative Ct method) with normalization to cyclophilin as endogenous control. The sets of primers that we used were as follows: Mfn2 (5'-CCCCTTGCTTTATGCTGATGTT-3' and 5'-TTTTGGGAGAGGTGTTGCTTATTT-3'), Cyclophilin (5'-CAAATGCTGACCAACACAA-3' and 5'-CCTCCACAATATTCATGCCTTCT-3'), ARP (5'-AAGCGCTCCTGGCATTGTCT-3' and 5'-CCGACGGGGCAGCAGTGGT-3'), hypoxanthine-guanine phosphoribosyltransferase-1 (5'-TTGCTGACCTGC TGGATTAC-3' and 5'-AGTTGAGAGATCATCTCCAC-3'), citrate synthase (5'-CCATCCACAGTGACCATGAG-3' and 5'-CTTTGCCAACTTCTCTCTGC-3'), Cytochrome C Oxidase (5'-CGCCTGATACCTGGCATTTTGT-3' and 5'-TTTTTT TTTTTTTTTTTTTTAAAGACC-3'), porin (5'-ACGTGGACTGAAGCTGAC CT-3' and 5'-CCAAACTCTGTCCCGTCATT-3'), and GLUT4 (5'-CTTCGAGAC AGCAGGGGTAG-3' and 5'-AGGAGCAGAGCCACAGTCAT-3').

**Statistical analysis.** All data in text and figures are presented as means  $\pm$  SE. Statistical significance of the results was determined using Student's *t* test when comparing groups of subjects. Student's test for paired values was used



**FIG. 1.** Obesity reduces muscle *Mfn2* expression in both men and women. Total RNA was purified lean (10 women and 6 men) or obese (9 women and 6 men) subjects. *Mfn2* mRNA was quantified by real-time PCR and data were expressed as percentage of the values measured in the different groups of control subjects. Data are means  $\pm$  SE; \* and \*\*\* indicate statistically significant differences compared with the lean groups, at  $P < 0.05$  and  $P < 0.001$ , respectively.

when comparing mRNA levels before and after clamp in the same group of subjects. Correlations were analyzed using Pearson's test.

## RESULTS

**Effect of obesity and type 2 diabetes on *Mfn2* expression.** First, we explored the hypothesis that obesity or type 2 diabetes are associated with abnormal muscle expression of *Mfn2*. The characteristics of the subjects involved in those studies are presented in Tables 1 and 2. Nondiabetic obese subjects showed high plasma insulin levels and moderate increases in plasma concentrations of nonsterified fatty acids and triglycerides (Table 1). Type 2 diabetic obese subjects from the Lyon study showed higher plasma concentrations of glucose, insulin, nonsterified fatty acids, and triglycerides than lean control subjects (Table 1). Insulin-stimulated glucose disposal measured during a 3-h euglycemic-hyperinsulinemic clamp was markedly lower in obese type 2 diabetic and nondiabetic subjects when compared with the lean controls (Table 1), indicating a state of insulin resistance. In keeping with previous reports (9,41), glucose oxidation during the insulin clamp was significantly reduced in obese subjects and in type 2 diabetic patients (data not shown).

The type 2 diabetic patients included in the Karolinska Institutet study were not obese and were moderately hyperglycemic (Table 2). They did not show any significant increase in plasma concentrations of fatty acids or triglycerides when compared with healthy control subjects matched for age and BMI (Table 2). Insulin-stimulated glucose disposal assayed by an euglycemic-hyperinsulinemic clamp was lower in type 2 diabetic patients as compared with the control subjects indicating insulin resistance (Table 2).

The muscle expression of *Mfn2* was initially studied in total RNA from skeletal muscle of 16 lean subjects (10 women and 6 men) and 15 obese subjects (9 women and 6 men). *Mfn2* mRNA levels were measured by real-time PCR and normalized for differences in RNA input. In keeping with previous studies (23), muscle expression of *Mfn2* mRNA was clearly reduced in human obesity, and the decrease was found in both males ( $P = 0.0007$ ) (41% reduction) and females ( $P = 0.049$ ) (30% reduction) (Fig. 1).



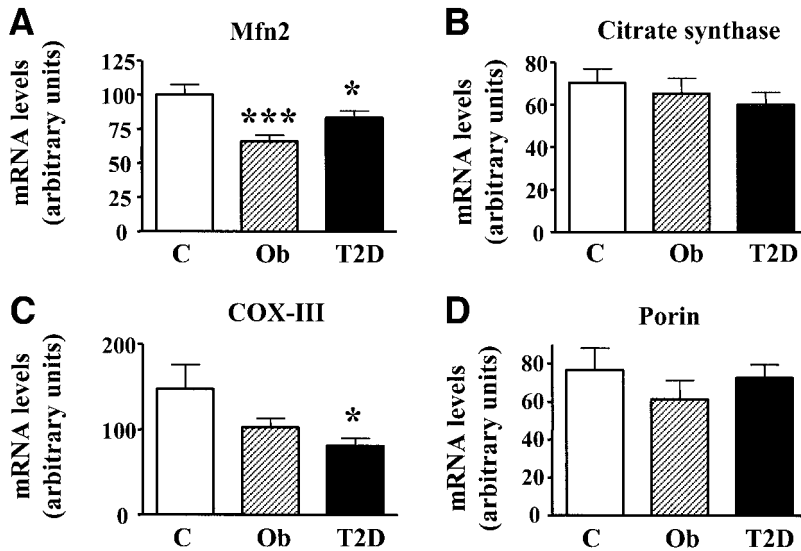


FIG. 2. Type 2 diabetes alters *Mfn2* expression in skeletal muscle. Total RNA was purified from skeletal muscle from middle-aged healthy subjects (C), nondiabetic obese subjects (Ob), and type 2 diabetic patients (T2D). The levels of mRNA corresponding to *Mfn2* (A), citrate synthase (B), COX-III (C), and porin (D) were quantified by real-time PCR, and data were expressed as arbitrary units. Data are means  $\pm$  SE; \* and \*\*\* indicate statistically significant differences compared with the healthy control groups, at  $P < 0.05$  and  $P < 0.001$ , respectively.

Type 2 diabetic patients also showed a reduction in *Mfn2* expression in skeletal muscle compared with lean control subjects (83% of control values,  $P = 0.05$ ) (Fig. 2A). COX-III mRNA were also markedly reduced in skeletal muscle from type 2 diabetic patients, whereas citrate synthase mRNA levels were only moderately repressed (Fig. 2). Under these conditions, porin mRNA remained unaltered in muscle from type 2 diabetic patients (Fig. 2A). Based on these results, we also analyzed whether the effect of diabetes on *Mfn2* expression was similar in male and in female subjects. Type 2 diabetes also showed a reduction in *Mfn2* expression in muscle compared with lean control subjects (70% of control values,  $P = 0.005$ ) (data not shown). In contrast, *Mfn2* expression in female type 2 diabetic patients was similar to control subjects and was markedly greater than in nondiabetic obese women (data not shown).

Interestingly, there was a strong negative correlation ( $r = 0.496$ ,  $P = 0.0003$ ) between the levels of *Mfn2* mRNA and the BMI of control, obese, or type 2 diabetic patients regardless of sex (Fig. 3A). In addition, a positive correlation ( $r = 0.565$ ,  $P = 0.002$ ) between the levels of *Mfn2* mRNA and the insulin sensitivity assessed as glucose disposal rates during the insulin clamp was also detected (Fig. 3B).

Nonobese type 2 diabetic men of the Karolinska study also showed a significant reduction in *Mfn2* mRNA levels in skeletal muscle (Fig. 4A). Under these conditions, type 2 diabetes caused a moderate reduction in the abundance of COX-III mRNA levels (Fig. 4A), whereas citrate synthase or GLUT4 mRNA remained unaltered (data not shown). Analysis of these subjects also revealed the existence of a negative correlation ( $r = 0.539$ ,  $P = 0.046$ ) between the levels of *Mfn2* mRNA and the BMI of these patients (Fig. 4B) and a positive correlation ( $r = 0.542$ ,  $P = 0.045$ ) between *Mfn2* mRNA levels and glucose disposal rates estimated during the insulin clamp (Fig. 4C).

**Effect of weight loss on *Mfn2* expression.** The BPD surgical approach and the subsequent effect on weight reduction allowed us to study a group of six obese subjects (five women and one man) after a substantial, long-lasting body weight decrease. The average preoperative BMI was  $56 \pm 5$  kg/m<sup>2</sup>, and BMI was dramatically

reduced after BPD to  $31 \pm 2$  kg/m<sup>2</sup> (Fig. 5B). A significant decrease in fat-free mass was observed together with a decrease in fat mass (data not shown). These body weight changes secondary to BPD were associated with increased insulin sensitivity and glucose oxidation (36). In parallel to the observed decline in BMI, fat-free mass, fat mass, and enhanced insulin sensitivity, we found a threefold greater

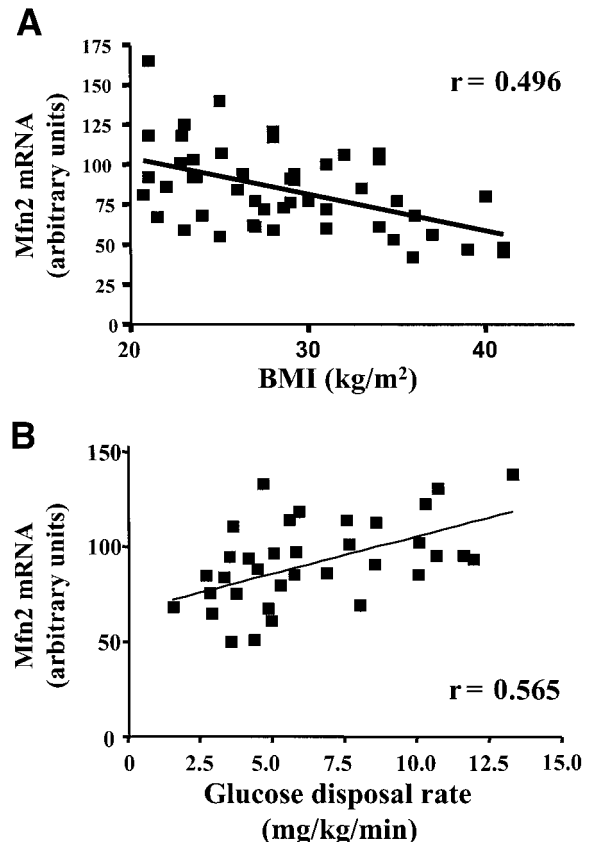
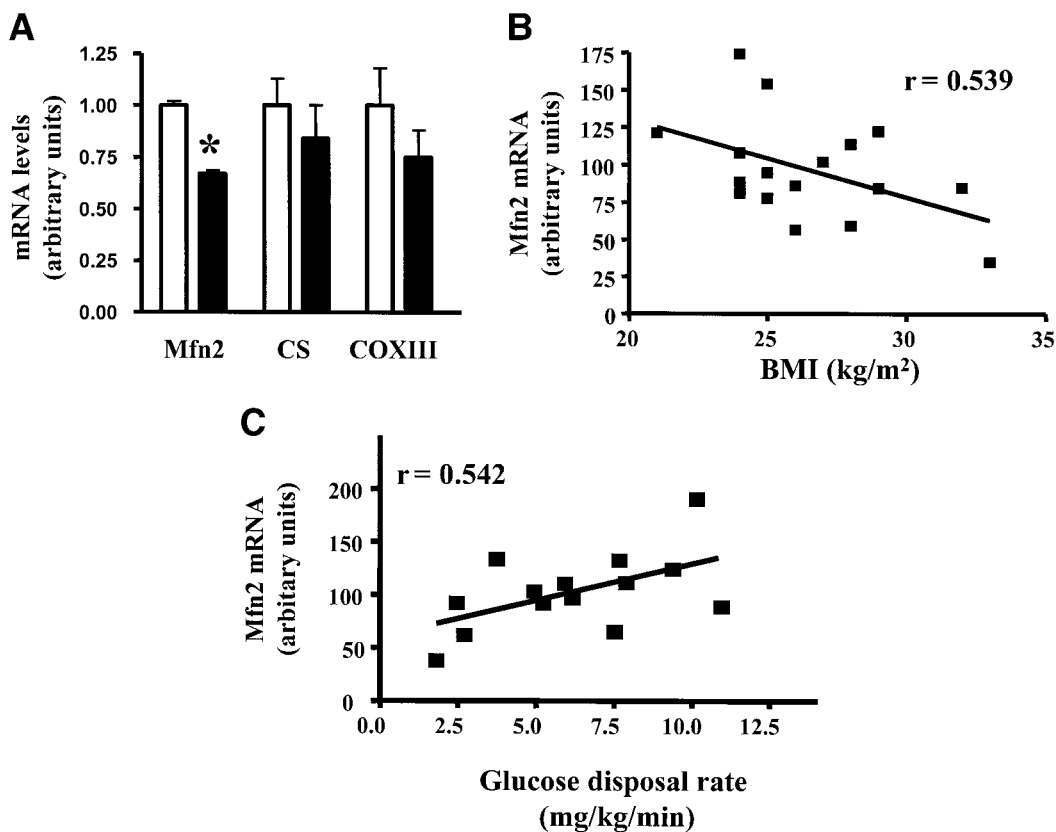


FIG. 3. Muscle *Mfn2* mRNA levels in skeletal muscle are inversely proportional to BMI and directly proportional to insulin sensitivity. Data from Fig. 2A were plotted against BMI (A) or glucose disposal rates measured during the insulin clamp (B). Pearson correlation analysis gave  $r = 0.496$  and  $P = 0.0003$  for A and  $r = 0.565$  and  $P = 0.002$  for B.



**FIG. 4.** Type 2 diabetes, not associated with obesity, alters *Mfn2* expression in skeletal muscle. **A:** Total RNA was purified from skeletal muscle from middle-aged healthy subjects and nonobese type 2 diabetic patients. Levels of mRNA corresponding to *Mfn2*, citrate synthase (CS), or COX-III were quantified by real-time PCR, and data were expressed as percentage of the values measured in the different groups of control subjects. Data are means  $\pm$  SE; \*statistically significant difference compared with the healthy control group, at  $P < 0.05$ . **B:** Correlation between muscle *Mfn2* mRNA levels in skeletal muscle and BMI. Data from **A** were plotted against BMI. Pearson correlation analysis gave  $r = 0.539$  and  $P = 0.046$ . **C:** Correlation between muscle *Mfn2* mRNA levels in skeletal muscle and glucose disposal rates. Data from **A** were plotted against glucose disposal rates measured during the insulin clamp. Pearson correlation analysis gave  $r = 0.542$  and  $P = 0.045$ .

expression of *Mfn2* mRNA in skeletal muscle from subjects after BPD (Fig. 5A) in the absence of changes in cyclophilin mRNA expression. Under these conditions, weight loss also caused a slight increase in the abundance of mRNA for citrate synthase or COX-III, which was not significant due to large variability (Fig. 5A). Analysis of these subjects also revealed the existence of a positive correlation ( $r = 0.927$ ;  $P = 0.0001$ ) between the levels of *Mfn2* mRNA and glucose disposal rates measured during the insulin clamp (Fig. 5C).

**Effect of insulin on *Mfn2* expression in skeletal muscle.** Next, we tested whether variations in insulin action may explain the alterations in muscle *Mfn2* expression. We studied the effect of 3 h of hyperinsulinemia (150 mU/l) during the euglycemic-hyperinsulinemic clamp on *Mfn2* expression in skeletal muscle of subgroups of the subjects involved in the Lyon studies. These subjects consisted of seven type 2 diabetic patients (three women and four men), seven obese nondiabetic subjects (three women and four men), and nine age-matched lean subjects (five women and four men). Hyperinsulinemia was similar in all groups (data not shown). The characteristics of these subgroups of subjects did not differ significantly from the whole groups shown in Table 1 (data not shown).

*Mfn2* mRNA remained unchanged after the insulin clamp in middle-aged healthy lean subjects who served as control subjects for the type 2 diabetic and nondiabetic patients (Fig. 6). No changes in *Mfn2* mRNA levels were detected in nondiabetic obese subjects or in type 2 diabetic patients after 3 h of hyperinsulinemia (Fig. 6). In keeping with these observations, incubation of cultured L6E9 muscle cells in the presence of 100 nmol/l insulin for

2, 5, or 48 h did not alter *Mfn2* expression (data not shown).

**TNF $\alpha$  and interleukin-6 inhibit *Mfn2* gene expression.** Based on the findings that *Mfn2* is repressed in skeletal muscle under conditions such as obesity or type 2 diabetes associated with insulin resistance, next we tested whether TNF $\alpha$  or interleukin-6, factors implicated in the regulation of insulin sensitivity, may control *Mfn2* expression. In one set of studies we selected NIH3T3 cells, since they are highly responsive to TNF $\alpha$  (42) and express endogenous *Mfn2* (Fig. 7). NIH3T3 cells were incubated for 12 h in the absence or presence of 100 nmol/l TNF $\alpha$  and *Mfn2* mRNA levels were subsequently measured by real-time PCR. Measurement of *Mfn2* mRNA levels relative to cyclophilin (Fig. 7A) or to ARP (Fig. 7B) indicate that TNF $\alpha$  caused a  $\sim 30\%$  reduction.

In separate studies, we selected human skeletal muscle cells in culture that are known to respond to interleukin-6 (39). Cells were incubated in the presence of interleukin-6 for different times (ranging from 0 to 4 h) and *Mfn2* mRNA was measured by real-time PCR. Interleukin-6 (1  $\mu$ mol/l) caused a time-dependent reduction in *Mfn2* mRNA levels relative to cyclophilin so exposure to interleukin-6 for 4 h caused a 27% reduction in *Mfn2* mRNA (Fig. 8).

## DISCUSSION

Mutations in *Mfn2* cause Charcot-Marie-Tooth 2A, an autosomal dominant axonal neuropathy (29). *Mfn2* is a mitochondrial protein, highly abundant in skeletal muscle, heart, and brain (23), that regulates the generation of the mitochondrial network in mammalian cells (23–25) so partial or total elimination of *Mfn2* expression causes a

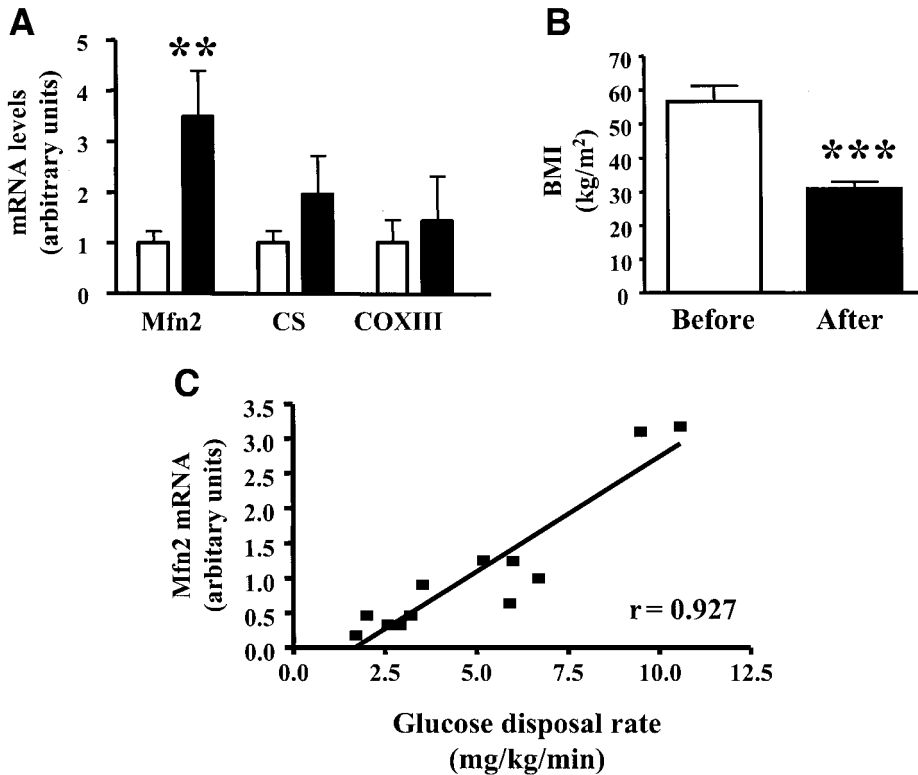


FIG. 5. Weight loss induced by BPD upregulates skeletal muscle *Mfn2* expression. Total RNA was purified from skeletal muscle from morbidly obese patients before and after BPD. Levels of mRNA corresponding to *Mfn2*, citrate synthase (CS), or COX-III were quantified by real-time PCR, and data were expressed as percentage of the values measured in the different groups of control subjects (A). BMI of the subjects is shown in B. Data are means  $\pm$  SE; \*\* and \*\*\* indicate statistically significant differences compared with the group before surgery, at  $P < 0.01$  and  $P < 0.001$ , respectively. C: Correlation between muscle *Mfn2* mRNA levels in skeletal muscle and glucose disposal rates. Data from A were plotted against glucose disposal rates measured during the insulin clamp. Pearson correlation analysis gave  $r = 0.927$  and  $P = 0.0001$ .

reduction of the mitochondrial network in cells (23,43). In addition, *Mfn2* controls mitochondrial function (23) and modulates mitochondrial metabolism, and its expression level regulates mitochondrial membrane potential, fuel oxidation, and the OXPHOS system (23,28). Thus, in myoblasts with a limited oxidative capacity, *Mfn2* gain of function causes an increase in the rate of glucose oxidation and a parallel increase in mitochondrial membrane potential, which indicates augmented pyruvate oxidation in mitochondria and enhanced Krebs cycle and oxidative phosphorylation (28). In addition, *Mfn2* repression leads to a decrease in the oxidation rates of glucose, pyruvate, and palmitate, and reduces mitochondrial membrane potential in myotubes (23,28). These observations indicate that pyruvate oxidation,  $\beta$ -oxidation of fatty acids, Krebs cycle, and oxidative phosphorylation are lowered in cells with a reduced expression of *Mfn2*. Mice deficient in *Mfn2* die in midgestation and show a severe disruption of the placental trophoblast giant cell layer (43), which may be explained by a defective energy metabolic situation in giant trophoblast cells that lack *Mfn2*.

Based on these observations, we determined whether changes in *Mfn2* expression may participate in some of the alterations found in metabolic diseases such as obesity or type 2 diabetes. We have previously reported that human obesity is associated with reduced expression of *Mfn2* mRNA and protein in skeletal muscle (23); similarly, *Mfn2* expression is reduced in muscle from obese Zucker rats (23). Here, we have found that skeletal muscle *Mfn2* expression is reduced in obesity, and this affects both men and women to a similar extent. In addition, we have found that *Mfn2* expression in muscle is inversely proportional to the BMI and directly proportional to insulin sensitivity. We have also found that weight loss induced by BPD results in a very substantial increase in *Mfn2* expression in

skeletal muscle. These data indicate that increased adiposity is linked to repression of *Mfn2* mRNA in skeletal muscle and this can be reversed by weight loss.

Our data also indicate that *Mfn2* expression is dysregulated in skeletal muscle from type 2 diabetic patients. In fact, a reduction in *Mfn2* expression was detected in both obese and nonobese type 2 diabetic patients. Low *Mfn2* expression occurred in the presence of reduced expression of the mitochondrial gene COX-III and in the presence of a moderate reduction in citrate synthase mRNA, which suggests the existence of mitochondrial dysfunction. *Mfn2* mRNA expression was decreased in male but not in female patients with type 2 diabetes, and the precise mechanism

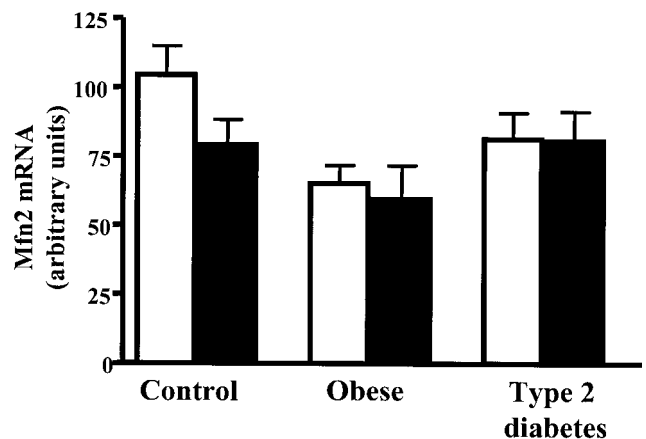


FIG. 6. Effect of insulin on *Mfn2* expression in skeletal muscle. Total RNA was purified from skeletal muscle from middle-aged healthy control subjects, nondiabetic obese subjects, or type 2 diabetic patients. Data are expressed as percentage of the values in the control groups in the basal state (data are means  $\pm$  SE; □: before clamp, ■: after 3 h of euglycemic-hyperinsulinemic clamp). Differences between basal and clamped groups were not statistically significant.

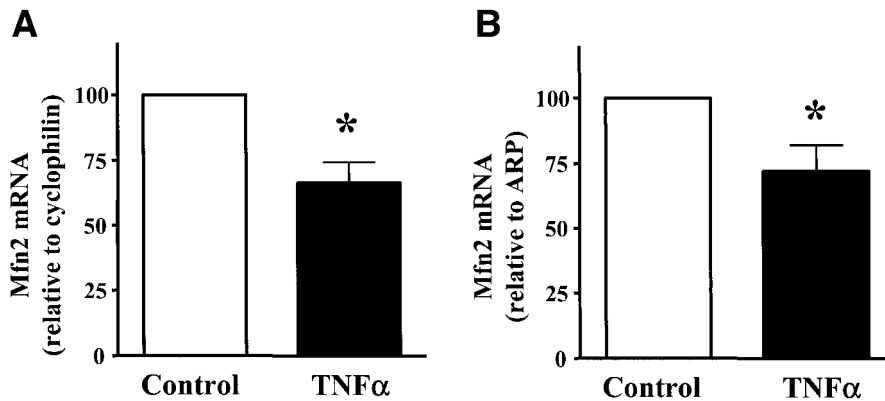


FIG. 7. Effect of TNF $\alpha$  on *Mfn2* expression in NIH-3T3 cells. Cells were incubated in the presence or absence of 100 nmol/l TNF $\alpha$  for 12 h. Total RNA was obtained from cells and *Mfn2* mRNA was quantified by real-time PCR. Data were corrected by cyclophilin (A) or ARP (B) mRNA levels and expressed as percentage of the values measured in control cells. \*Statistically significant differences compared with the control groups, at  $P < 0.05$ .

for these differences is unclear. In any case, sex differences in the effects of type 2 diabetes on gene expression have been previously reported for fatty acid transporter protein 1 in human muscle (44). In all, our data suggest that factors altered in type 2 diabetes in a manner perhaps dependent on sex regulate *Mfn2* expression in skeletal muscle.

Interestingly, insulin resistance associated with obesity and type 2 diabetes is characterized by reduced oxidative capacities in skeletal muscle (1,9,13). Recent studies also support the concept that muscle mitochondrial dysfunction contributes to insulin resistance in these pathologies (9) and in the elderly (45). In this regard, a reduction in *Mfn2* expression in muscle cells reduces glucose oxidation and mitochondrial membrane potential (23,28). Based on these observations, we suggest that a deficit in *Mfn2* protein may participate in the reduced mitochondrial activity in insulin resistant conditions, namely type 2 diabetes and obesity. In this connection, we have detected the existence of a positive correlation between *Mfn2* expression in skeletal muscle and insulin sensitivity assessed by glucose disposal rates measured by the insulin clamp. Further studies are needed to determine the relationship between *Mfn2* and insulin sensitivity in a more direct manner.

As for the nature of the circulating factors that may regulate *Mfn2* expression, we have examined the possible role of TNF $\alpha$ , interleukin-6, and insulin. TNF $\alpha$  is an important factor contributing to obesity-related insulin resistance (46). Several animal models of obesity and insulin resistance show significantly higher levels of TNF $\alpha$  mRNA and protein compared with their lean counterparts (47–49), and neutralization of TNF $\alpha$  with a soluble TNF receptor-IgG fusion protein ameliorates insulin sensitivity (50). Enhanced TNF $\alpha$  expression in obesity has been detected in adipose tissue, muscle, and macrophages. Elevated TNF $\alpha$  mRNA and protein levels have also been detected in human obesity (51,52). In this regard, our data indicate that TNF $\alpha$  rapidly represses *Mfn2* expression in cells.

Interleukin-6 is produced by many cell types including macrophages, adipose cells, and skeletal muscle, and circulating levels are increased in type 2 diabetes (53,54). The biological role of interleukin-6 in glucose homeostasis is not completely understood. Several reports indicate that interleukin-6 interferes with insulin signaling in muscle and hepatic cells (39,55) and causes hepatic and muscle insulin resistance in humans and in mice (39,55–60).

However, there is also evidence that interleukin-6 is produced in, and subsequently released from, contracting muscle (61,62), which causes increased plasma interleukin-6 concentrations (63). In this connection, it has been shown that interleukin-6 contributes to the contraction-induced increase in hepatic glucose production (64). Here, we have demonstrated that exposure to interleukin-6 causes a rapid repression of *Mfn2* gene expression in muscle cells. We propose that TNF $\alpha$  and interleukin-6 are involved in the *in vivo* regulation of *Mfn2*, and this may be relevant to the mechanism of generation of insulin resistance or in the metabolic changes that take place in muscle in the postexercise state.

In contrast, our data do not support a direct role of insulin. Thus, insulin infusion for 3 h did not alter *Mfn2* expression in muscle of middle-aged healthy lean subjects, obese nondiabetic, or type 2 diabetic patients. Similarly, addition of insulin for up to 24 h did not alter *Mfn2* expression in muscle cells in culture. We cannot discard that insulin regulates the long-term *Mfn2* expression.

It is also important to elucidate the nature of the intracellular factors that may be involved in the dysregulation of *Mfn2* in obesity or type 2 diabetes. Recently, it has been reported that the nuclear coactivator peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  is crucial in the regulation of mitochondrial activity and mitochondrial biogenesis (65–67), and the expression of this factor

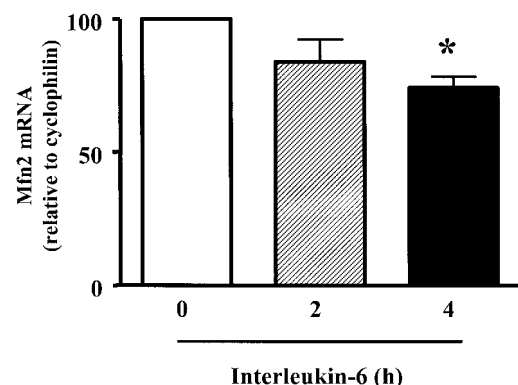


FIG. 8. Effect of interleukin-6 on *Mfn2* expression in human skeletal muscle cells. Cells were incubated in the presence or absence of 1  $\mu$ mol/l interleukin-6 for 2 or 4 h. Total RNA was obtained from cells and *Mfn2* mRNA was quantified by real-time PCR. Data were corrected by cyclophilin mRNA levels and expressed as percentage of the values measured in control cells. \*Statistically significant differences compared with the control group, at  $P < 0.05$ . ANOVA test was statistically significant ( $P = 0.0387$ ).



is downregulated in skeletal muscle during type 2 diabetes (4,68). In this regard, peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  is a potent stimulator of *Mfn2* gene expression and acts through an estrogen-related receptor  $\alpha$ -binding element in the *Mfn2* promoter (F.X.S., M. Liesa, M.P., A.Z., unpublished observations). Based on these data, we propose that some of the conditions in which we find alterations in the expression of *Mfn2* may be also associated with changes in peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  expression in skeletal muscle.

In summary, the present study shows that *Mfn2* expression is regulated in skeletal muscle in humans under conditions associated with changes in insulin sensitivity such as obesity, type 2 diabetes, or body weight loss. Based on the observation that TNF $\alpha$  or interleukin-6 downregulate *Mfn2* expression, these factors may participate in the dysregulation of *Mfn2* expression detected in obesity or type 2 diabetes. Recent studies have demonstrated that *Mfn2* regulates mitochondrial glucose oxidation and cell respiration in muscle and nonmuscle cells in culture (23,28). Based on this, we propose that the in vivo modulation of *Mfn2* mRNA levels is an additional level of regulation for the control of muscle metabolism and could provide a molecular mechanism for alterations in mitochondrial function in obesity or type 2 diabetes.

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