Decreased Mitochondrial Proton Leak and Reduced Expression of Uncoupling Protein 3 in Skeletal Muscle of Obese Diet-Resistant Women

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Weight loss in response to caloric restriction is variable. Because skeletal muscle mitochondrial proton leak may account for a large proportion of resting metabolic rate, we compared proton leak in diet-resistant and diet-responsive overweight women and compared the expression and gene characteristics of uncoupling protein (UCP)2 and UCP3. Of 1,129 overweight women who completed the University of Ottawa Weight Management Clinic program, 353 met compliance criteria and were free of medical conditions that could affect weight loss. Subjects were ranked according to percent body weight loss during the first 6 weeks of a 900-kcal meal replacement protocol. The highest and lowest quintiles of weight loss were defined as diet responsive and diet resistant, respectively. After body weight had been stable for at least 10 weeks, 12 of 70 subjects from each group consented to muscle biopsy and blood sampling for determinations of proton leak, UCP mRNA expression, and genetic studies. Despite similar baseline weight and age, weight loss was 43% greater, mitochondrial proton leak–dependent (state 4) respiration was 51% higher ($P = 0.0062$), and expression of UCP3 mRNA abundance was 25% greater ($P < 0.001$) in diet-responsive than in diet-resistant subjects. There were no differences in UCP2 mRNA abundance. None of the known polymorphisms in UCP3 or its 5′ flanking sequence were associated with weight loss or UCP3 mRNA abundance. Thus, proton leak and the expression of UCP3 correlate with weight loss success and may be candidates for pharmacological regulation of fat oxidation in obese diet-resistant subjects. *Diabetes* 51: 2459–2466, 2002

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Δp, protonmotive force; PPAR, peroxisome proliferator–activated receptor; PPRE, peroxisome proliferator response element; RMR, resting metabolic rate; TRE, thyroid response element; UCP, uncoupling protein.

At the Weight Management Program at the University of Ottawa, we have documented a 10-fold variation in the rate of weight loss in 353 highly compliant women on a standard exercise program and standard 900-kcal meal replacement protocol. These women were ranked according to percent body weight loss, and highest and lowest quintiles were defined as diet responsive and diet resistant, respectively. Regression analyses demonstrated that the known variables regulating energy requirements, including initial weight, age, and plasma free triiodothyronine (T3) concentrations, accounted for only half of this variability (1), leading us to search for novel molecular determinants of weight loss success.

Variable responses to overfeeding have been reported. Rodent studies have demonstrated that genetic factors not only regulate weight gain in response to high-fat high-calorie diets but also determine the susceptibility to obesity when energy intake is controlled (2). In response to the ingestion of hypercaloric diets, the majority of subjects gain less weight than anticipated, and a process of adaptive thermogenesis appears to play a role in the defense against obesity (3). Several studies have demonstrated marked interindividual variability in the susceptibility to weight gain in response to overfeeding (4), and identical twins show marked similarity in this regard, suggesting an important genetic contribution to the ability of individuals to dispose of excess ingested energy (5,6).

The ability to lose weight on an energy-restricted diet has been less extensively investigated, but monozygotic twin studies have also shown greater interpair than intrapair variation in weight loss (7,8). Discrete gene sets may prevent or facilitate obesity in humans by influencing food intake (e.g., leptin), by altering the ability of skeletal muscle to dispose of excess energy (e.g., uncoupling proteins [UCPs]), or by influencing the capacity of the adipocyte to accumulate triglyceride (e.g., CD-36, perilipin). We focused on skeletal muscle because of its importance in whole-body resting energy expenditure (9) and specifically on mitochondrial proton leak because of its importance in regulating energy expenditure in skeletal muscle in rodents (10). UCP3 was of interest because of its expression in skeletal muscle and because mice bearing the human transgene for UCP3 are hyperphagic but lean

compared with wild-type mice (11), suggesting that UCP3 expression and function may regulate energy balance.

We demonstrate significantly less mitochondrial proton leak and lower UCP3 mRNA levels in skeletal muscle of diet-resistant patients than in diet-responsive patients on a controlled dietary regimen. We also present an extensive genetic screening of key 5′ flanking region transcriptional regulatory sequences and of all previously reported variations in UCP3 gene for this subset of patients.

RESEARCH DESIGN AND METHODS

Clinical protocol. The study was approved by the Human Research Ethics Committee of the Ottawa Hospital. Rate of weight loss was evaluated in the first 6 weeks of a 900-kcal meal replacement component of a commercially available meal replacement/lifestyle modification program (Optifast 900; Novartis Nutrition, Whitby, ON, Canada).

Data management. Clinical information, including medical history, medication, weekly compliance assessments, anthropomorphic measurements, physicians' notes, and laboratory values, was collected (12,13). Data were verified by periodic chart review. Exclusions and rate of weight loss percentiles were derived using software developed in the clinic (14).

Selection of patients. From September 1992 to May 2000, 1,129 female subjects, aged 18–65 years with BMI 30–50 kg/m^2, completed the weight management program. Because compliance is a prominent reason for poor response to diet, every effort was made to remove noncompliant patients. Selection of compliant patients was maximized by studying subjects in the first 6 weeks of an 8- or 12-month weight management program with substantial cost to the patient ($1,000–1,700) and by excluding those who did not meet the compliance criteria given in Fig. 1. Patients with conditions or drug use that might affect weight loss were excluded based on the medical criteria given in Fig. 1. As a result, 353 highly compliant patients remained in the study group.

Compliant subjects were invited to participate in the substudy if they were in the highest or lowest quintile of rate of weight loss in the initial 6 weeks of the program. A total of 12 volunteers from each of the 71 diet-responsive and 70 diet-resistant patients (upper and lower quintiles), who were matched for age and initial body weight, gave informed consent to participate in the study.

Muscle biopsy. Muscle biopsies were delayed until several months after meal replacement completion and after a period of 4 weeks of weight stabilization. An open biopsy (3 g) of the rectus femoris was obtained from 12 diet-responsive and 12 diet-resistant patients, matched for initial weight and age. Figure 2 shows the characteristics of weight loss of the biopsied patients. Biopsies were obtained in the fasting state between 7:00 and 9:00 A.M., using local lidocaine anesthesia. One diet-responsive and one diet-resistant patient were sampled on a single morning, and the order of collection from the two groups was randomized. Approximately 50 mg tissue was immediately frozen and stored at −80°C for RNA analyses, and the remaining sample was used for mitochondrial isolation.

Laboratory analyses

Mitochondrial proton leak. Muscle was immediately placed in ice-cold medium (100 mmol/l sucrose, 10 mmol/l EDTA, 100 mmol/l Tris-HCl, and 46 mmol/l KCl; pH 7.4). Mitochondria were isolated as follows: muscle was minced in cold medium containing defatted BSA (0.5% wt/vol) to bind fatty acids liberated during tissue fractionation. The protease, Nagarse XXVII (Sigma, St. Louis, MO), was added at a final concentration of 0.8 units/ml, and the suspension was incubated for 2 min at room temperature. The minced tissue was further disrupted in a Potter Elvjehm homogenizer set at low speed. The homogenate was spun at 750*g* for 10 min at 4°C. The supernatant was then spun at 10,000*g* for 10 min at 4°C, and mitochondria were resuspended in isolation medium (without BSA) and spun again. Mitochondria were resuspended in 0.175 ml BSA-free isolation medium. Protein concentration was assayed using a modified Lowry protocol, using BSA as the reference standard. Assessments of the overall kinetics of proton leak reactions require simultaneous determinations of oxygen consumption and mitochondrial membrane potential (15). Oxygen consumption was measured using a Clark-type oxygen electrode with the chamber maintained at 37°C and magnetically stirred. Mitochondria (0.5 mg protein/ml) were incubated in 1.0 ml suspension medium (120 mmol/l KCl, 20 mmol/l sucrose, 20 mmol/l glucose, 10 mmol/l KH_2PO_4, 5 mmol/l HEPES, 2 mmol/l $MgCl_2$, and 1 mmol/l EGTA; pH 7.2), and respiration was fueled by 10 mmol/l succinate. Titrations were carried out in the presence of 80 ng nigericin/ml to convert mitochondrial-ΔpH to voltage units and in the presence of 5.0 μmol/l rotenone to prevent oxidation of any endogenous NAD-linked substrates. State 4 (nonphosphorylating) respiration was achieved with maximal amounts of the ATP synthase inhibitor oligomycin (8 μg/mg protein). Protonmotive force (Δp; mV) was determined using a $TPMP^+$ (methyltriphenylphosphonium)-sensitive electrode. Electrode calibration and calculation of Δp were carried out as previously described (16,17).

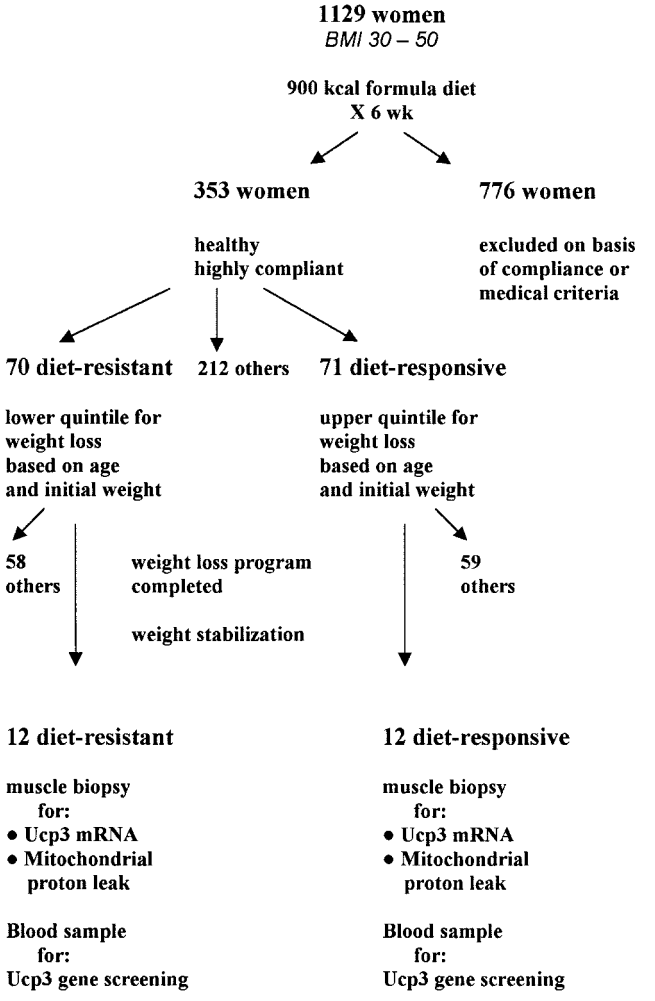

FIG. 1. Study protocol. Compliance criteria for exclusion included physician notes expressing concern about self-reported compliance (38 excluded), completion of <75% of the 16 or 26 visits in the weekly part of the program (512), absent for more than two of the six visits in the study segment (10), and inadequate completion of the lab testing protocol (50). Medical criteria for exclusion included thyroid-stimulating hormone out of normal range at week 1 or 13 (30), diabetes treated with insulin or oral hypoglycemic agents (20), smoker (54), surgery during study segment (3), obstructive sleep apnea (21), congestive heart failure (2), wheelchair-bound (2), previous surgery for weight control (0), cancer diagnosed at any time during program (1), regular use of tricyclic antidepressants at doses >50 mg per day, e.g., oral glucocorticoids, antipsychotic medication, β-blockers, or lithium (6), and regular use of fluoxetine at doses >30 mg per day, e.g., appetite suppressants or orlistat (27).

Relative-quantitative RT-PCR. Muscle biopsy specimens were thawed and homogenized in 1.0 ml Tri-Reagent (Bio/Can, Mississauga, Canada) at 10,000 rpm for 1 min using a Virtashear homogenizer. The samples were stored at −20°C overnight, and RNA was isolated according to Tri-Reagent manufacturer's protocol. RNA concentration was determined spectrophotometrically using optical density (260/280 nm ratio), and sample quality was confirmed by agarose gel electrophoresis. Relative-quantitative RT-PCR was performed using the Quantum RNA 18S Internal Standards kit from Ambion (Austin, TX). This kit has been previously shown to accurately determine relative changes in gene expression between samples (18). Briefly, 2.5 μg total RNA was used to synthesize first-strand cDNA using 10 μmol/l random decamer primers (Ambion) and 200 units Moloney murine leukemia virus (MMLV) reverse transcriptase (Life Technologies, Burlington, Canada), with incubation at 42°C for 1 h. UCP3 primers (UCP3 forward 5′-CCTCGTTACCTTTCCACTGG-3′ and UCP3 reverse 5′-GGCAGAGACAAAGTGGCAGG-3′) were designed from human UCP3 cDNA sequence information to amplify a 615-bp sequence common

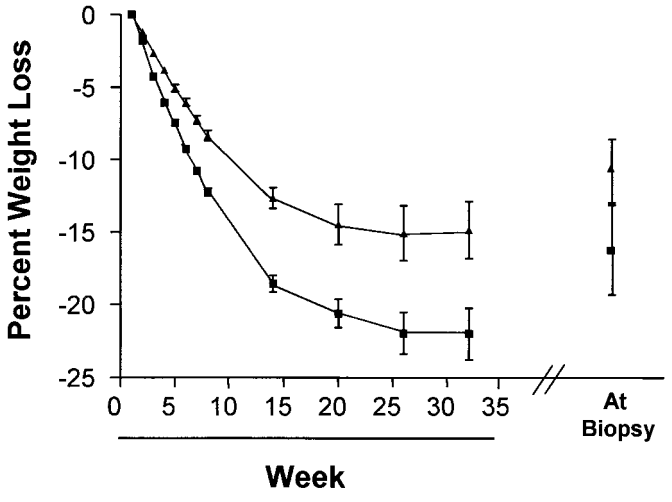

FIG. 2. Rate of weight loss in diet-responsive and diet-resistant patients undergoing muscle biopsy. Percent weight loss for diet-responsive (■) and diet-resistant (▲) patients is shown for the time of entry into the program (week 0), over the subsequent 32 weeks, and at the time of biopsy.

to both known splice variants of the UCP3 mRNA. A cycle number of 31 was determined to be within the linear range of PCR and was used for all subsequent PCR. The 18S primer:competimer ratio of 1:4 was experimentally determined so that the UCP3 and 18S PCR products were amplified to give similar yields that could be compared between samples. Multiplex PCR was performed on 1 μl of the RT reaction using 20 pmol of each UCP3 primer and 4 μl of 18S primer/competimer mix with the following PCR conditions: 1 cycle × 95°C for 3 min, 31 cycles × 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min. UCP2 mRNA levels were also measured using the following primers: UCP2 forward 5′-GACCTATGACCTCATCTCCTG-3′ and UCP2 reverse 5′-ATAGGTGACGAACATCACCACG-3′. A cycle number of 26 was determined to be within the linear range of amplification, and a 18S primer:competimer ratio of 1.5:8.5 was found to be optimal. Multiplex PCR was performed on 1 μl of the RT reaction using 20 pmol of each UCP2 primer and 2 μl of 18S primer/competimer mix with the following PCR conditions: 1 cycle × 95°C for 3 min, 26 cycles × 95°C for 30 s, 48°C for 30 s, and 72°C for 30 s. Cocktails containing all shared components were used to reduce variation between samples. PCR products were run on 1% agarose gels and visualized with ethidium bromide staining. Band intensities were measured using the Chemi-Doc apparatus and Quantity One software (Bio-Rad, Mississauga, Canada). Relative intensity was calculated by dividing the intensity of the 615-bp band corresponding to the UCP3 message or the 279-bp band corresponding to UCP2 message by the 418-bp band corresponding to the 18S message. To compare ratios from different RT reactions, one sample was always used as an internal control and all relative mRNA levels were normalized to this internal control.

Genetic screening of potential 5′ flanking region transcriptional regulatory sequences and of all previously reported variations in UCP3 gene. Genomic DNA was prepared from white blood cells using the QIAamp DNA Blood Mini Kit (Qiagen). Regions of interest were amplified by PCR using oligonucleotide primers chosen in published sequences (GenBank accession no. AF127916 for promoter region and AF050113 for introns/exons) in appropriate conditions (Table 1). The PCR conditions were as follows: final volume 25 μl: 100 ng DNA per well, 200 μmol/l dNTP, 0.5 μmol/l primers, 20 mmol/l Tris-HCl (pH 8.4), 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, and 1 unit recombinant *Taq* DNA polymerase (Life Technologies). PCRs were run for 30 cycles: 1 min at 94°C, 1 min at the indicated annealing temperature (Table 1), and 1 min at 72°C. The sequencing of PCR products was performed by the dideoxynucleotide chain termination method with fluorescent dideoxynucleotides on an Applied Biosystems DNA sequencer (ABI Prism 310 Genetic Analyzer).

Statistical analyses. Data are expressed as means ± SE. Student's *t* tests were used for comparisons between two independent means. Relationships between continuous variables were determined using linear regression analyses in SAS version 8.0 (SAS Institute, Cary, NC). Analyses of the overall kinetics of the curve of best fit for proton leak data were performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA). Allele frequencies were deduced from the genotype frequencies. Hardy-Weinberg equilibrium was tested using χ^2 with 1 df and Fisher's exact tests. Pairwise linkage-disequilibrium coefficient (D′) was calculated from haplotype frequencies estimated by a maximum likelihood method based on log-linear model analysis (19). Differences in the distribution of genotypes between two groups were compared by χ^2 with 2 df and Fisher's exact tests. $P \leq 0.05$ was considered statistically significant.

RESULTS

Baseline parameters of the study subjects are described in Table 2. The results demonstrate that the biopsied subjects in the diet-responsive and diet-resistant groups are representative of their overall cohorts of subjects in the diet-responsive and diet-resistant quintiles. In the biopsied subjects, there were no significant differences between the groups in terms of age at program entry, average number of parents overweight, percentage of overweight subjects with obesity onset after 20 years of age, initial BMI, or initial body weight.

Characteristics of weight loss over 6 weeks of the meal replacement program and at the time of muscle biopsy are described in Table 3 for diet-responsive and diet-resistant groups. Despite similar baseline age, BMI, and weight, the mean weight loss after 6 weeks was 43% greater in the biopsied diet-responsive groups than in the diet-resistant groups ($P = 0.002$). The percentage of initial body weight lost over this period was also 59% higher in the responsive than in the resistant group.

Body weight at the time of the muscle biopsy did not differ significantly between the two groups ($P = 0.10$). The period between the completion of the weight loss program and the muscle biopsy was 22.1 ± 4.1 and 18.7 ± 5.0 months (means ± SE) for the responsive and resistant groups, respectively (NS).

The overall kinetics of mitochondrial proton leak reactions were significantly different between the diet-responsive and diet-resistant groups (Fig. 3). Nonphosphorylating (state 4) respiration was significantly lower in mitochondria from diet-resistant patients than in mitochondria from diet-responsive patients (121.1 ± 15.0 vs. 183.3 ± 14.0 $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, $P = 0.0062$). The equations for the lines of best fit were generated using computer software (see RESEARCH DESIGN AND METHODS), and the R^2 values for goodness of fit were 0.9049 and 0.9985 for diet-resistant and diet-responsive groups, respectively. Mitochondrial oxygen consumption at any given value of Δp was lower for diet-resistant than for diet-responsive patients. For example, at 150 mV, leak-dependent oxygen consumption was ~51% higher in the diet-responsive group than in the diet-resistant group.

UCP3 mRNA abundance in skeletal muscle was 25% greater in the diet-responsive group than in the diet-resistant group ($P < 0.001$) (Fig. 4). There were no differences in UCP2 mRNA abundance (results not shown). Skeletal muscle UCP3 mRNA was not significantly correlated with age, weight, or BMI at the time of study.

In the 5′ flanking region, the three putative peroxisome proliferator response elements (PPREs), PPRE1 (−1,677 to −1,690), PPRE2 (−456 to −468), and PPRE3 (−242 to −254), and the only thyroid response element (TRE) (−3,543 to −3,528) were screened for variation by PCR sequencing in the subset of 24 individuals. Nucleotides were numbered from the first "atg." Sequences were identical to the published sequence (GenBank accession

TABLE 1
Oligonucleotide primers and PCR conditions for amplification of potential 5′ flanking region transcriptional regulatory sequences and of all previously reported variations in UCP3 gene

Fragment or genetic variant	Primer sequence	Annealing temperature (°C)
TRE	F: 5′-AGGTTGGTCCAGATGACAGT-3′ R: 5′-TGGGTTCAAGTGATTCTCCT-3′	58
PPRE1	F: 5′-TGCAATTTACTGCATCACCT-3′ R: 5′-GGAGTTAACTGGGTAGGGCA-3′	58
PPRE2	F: 5′-ATCCTAATAGTACCTATCTC-3′ R: 5′-ATCTGGTTCAGTCCTCTTGA-3′	48
PPRE3	F: 5′-GCGTCCACAGCTTAAAGGAG-3′ R: 5′-TCTTACCTGTGAGTCCTGCC-3′	52
−439Ains	F: 5′-GGGAACACAGCAAGACCT-3′ R: 5′-TTGACTTGGGGTGCTTTA-3′	50
−155C/T	F: 5′-AAGCGTCCACAGCTTAAA-3′ R: 5′-GGCAGGGGCAGCACAGGG-3′	53
−55C/T	F: 5′-AAGCGTCCACAGCTTAAA-3′ R: 5′-GGCAGGGGCAGCACAGGG-3′	53
+5G/A	F: 5′-AAGCGTCCACAGCTTAAA-3′ R: 5′-GGCAGGGGCAGCACAGGG-3′	53
V9M	F: 5′-GCCAGGCCAGACATCACT-3′ R: 5′-TGGGGGATATGGGAGAGA-3′	58
R70W	F: 5′-CTTGCCTTCCCATCTGAGTC-3′ R: 5′-GTTCAGGGACCTGGTCACTC-3′	53
A83A	F: 5′-CTTGCCTTCCCATCTGAGTC-3′ R: 5′-GTTCAGGGACCTGGTCACTC-3′	53
G84S	F: 5′-CTTGCCTTCCCATCTGAGTC-3′ R: 5′-GTTCAGGGACCTGGTCACTC-3′	53
Y99Y	F: 5′-CTTGCCTTCCCATCTGAGTC-3′ R: 5′-GTTCAGGGACCTGGTCACTC-3′	53
V102I	F: 5′-CTTGCCTTCCCATCTGAGTC-3′ R: 5′-GTTCAGGGACCTGGTCACTC-3′	53
R143X	F: 5′-GAGTGGACATCAAGGAAGCT-3′ R: 5′-CAGTCTGAGGGGAGGGAAAG-3′	57
Intron4C/T	F: 5′-GAGTGGACATCAAGGAAGCT-3′ R: 5′-CAGTCTGAGGGGAGGGAAAG-3′	57
Y210Y	F: 5′-CAGCCAGGGCATCCATTT-3′ R: 5′-TGCCCACTCCACGGAGTT-3′	48
IVS6+1G/A	F: 5′-GGGCACTGTGAGAGATATGGA-3′ R: 5′-CGCTAGCCACATTCGAAAAGA-3′	57
R308W	F: 5′-ATCTGTTGTGGTCCCCTA-3′ R: 5′-CACCGTTTTCTTCCATTC-3′	48

no. AF127916 for promoter region and AF050113 for introns/exons) for all 24 studied individuals. We also screened all previously described variations in human UCP3 for variation by PCR sequencing: −439Ains (20), −155C/T (20), −55C/T (21), +5G/A (20), V9M (22), R70W (23), A83A (22), G84S (24), Y99Y (22), V102I (25), R143X (25), Intron4C/T (22), Y210Y (22), IVS6 + 1G/A (25), and R308W (22). We did not find any variation compared with the wild sequence at these positions, except for the three polymorphisms 55C/T, Y99Y, and Y210Y. For these three polymorphisms, allele frequencies in the two groups were consistent with Hardy-Weinberg equilibrium. Allele frequencies in our population (Table 4) were similar to published allele frequencies in populations of obese subjects (for −55C/T see Otabe et al. [20] and for Y99Y and Y210Y see Otabe et al. [22]). The polymorphisms −55C/T

TABLE 2
Baseline characteristics of diet-responsive and diet-resistant weight loss cohorts (quintiles) and biopsied subgroups

	Cohort		Biopsied subjects	
	Diet responsive	Diet resistant	Diet responsive	Diet resistant
n	71	70	12	12
Age at program entry (years)	41.07 (0.96)	45.07 (1.96)	44.0 (1.673)	45.2 (3.38)
Average no. parents overweight	1.16 (0.087)	1.14 (0.101)	1.17 (0.167)	1.41 (0.1486)
Overweight with onset after 20 years of age (%)	28	31.9	50	33.3
Initial BMI (kg/m^2)	37.39 (0.58)	40.61 (1.20)	37.07 (1.50)	41.0 (2.54)
Initial weight (lb)	218.9 (4.09)	240.4 (6.47)	218.50 (9.39)	235.02 (14.70)

Data are means (SE).

TABLE 3
Weight changes in biopsied subgroups

	Weight (lb)		Weight change (lb)		Difference in weight change: responsive versus resistant (lb)	
	Diet responsive	Diet resistant	Diet responsive	Diet resistant	95% CI	*P*
At baseline	218.50 (9.38)	235.02 (14.7)				
After 6 weeks meal replacement	191.3 (8.26)	216.0 (13.25)	27.1 (1.20)	19.3 (1.49)	8.2 (4.11–12.29)	0.0018
At biopsy	184.8 (9.53)	209.8 (19.1)	33.7 (7.97)	25.2 (6.99)	8.5 (−13.44 to 30.51)	0.3709

Data are means (SE).

and Y99Y were completely concordant (i.e., same allele frequencies and complete positive linkage disequilibrium). There was no association between the −55C/T or Y99Y polymorphisms and the Y210Y polymorphism (D′ = 0.20). Allele frequencies and distribution of genotypes are indicated in Table 4. Genotype distributions were similar in the diet-resistant and diet-responsive groups (Table 4).

DISCUSSION

Skeletal muscle mitochondrial proton leak has not previously been measured in humans because the biopsy procedure is invasive and the analyses are technically difficult. Our data provide strong support for the hypothesis of Brand and colleagues (10,15) that proton leak is the single most important determinant of mammalian energy expenditure. Importantly, these findings also suggest that mitochondrial leak differs according to weight loss success in response to energy-restricted diets and that leak variation is correlated with differences in UCP3 expression.

Although the mechanism of proton leak is unknown, there is abundant evidence that leak accounts for 20–25% of resting metabolic rate (RMR) in rodents (10). Remarkably, proton leak is estimated to account for ~52% of the resting muscle oxygen consumption in studies of intact skeletal muscle in the rat (10). Leak was thus proposed as an important factor contributing to RMR (15), and studies in other mammals support this hypothesis (26). Thyroid hormones are the only endocrine factors known to regulate leak (27,28). Thus, if muscle energy expenditure accounts for ~20% of standard metabolic rate (rev. in 29), our observed differences in maximal mitochondrial proton leak respiration could conceivably reflect a 5% difference in standard metabolic rate between diet-responsive and diet-resistant individuals.

In addition to providing the first determinations of mitochondrial proton leak in human muscle, these studies demonstrate, for the first time, differences in proton leak and muscle UCP3 mRNA in obese subjects exhibiting fast or slow rates of weight loss in response to a precise hypocaloric regimen. The strengths of the study relate to the rigorous dietary protocol, careful selection of subjects and matching of the study groups, and the simultaneous study of UCP3 function, mRNA expression, and genetic variation. Subjects were identified on the basis of weight loss success in the first 6 weeks of the program, when the dietary regimen was rigorously controlled for all subjects. We excluded 776 of the 1,129 participants in order to define a highly compliant cohort without medical condi-

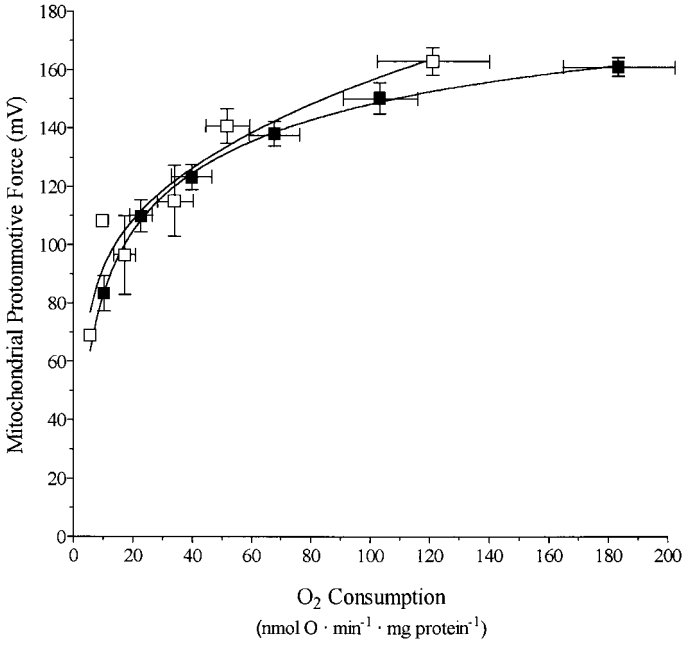

FIG. 3. Overall kinetics of skeletal muscle mitochondrial proton leak reactions in diet-responsive versus diet-resistant patients. State 4 respiration rate (the point on the far right of each curve) is 51% greater in responsive than in resistant patients (P = 0.0062). Closed and open squares represent results from responsive and resistant patients, respectively. Respiration rates over a range of mitochondrial Δp values are greater in the diet-responsive than in the diet-resistant patients.

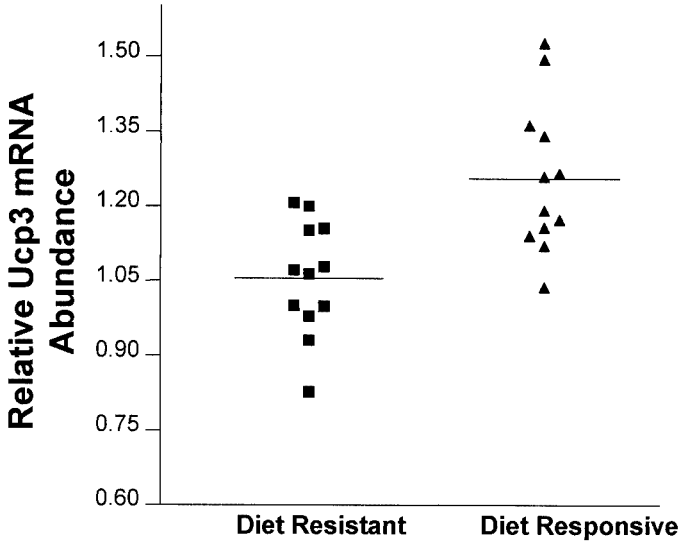

FIG. 4. Skeletal muscle UCP3 mRNA abundance in diet-responsive versus diet-resistant patients. UCP3 mRNA expression was 25% lower in skeletal muscle of subjects in the lowest quintile for the rate of weight loss (diet resistant) (P < 0.001).

TABLE 4
Allele frequencies and genotype distribution of –55/CT, Y99Y, and Y210Y polymorphisms in diet-resistant and diet-responsive groups

	Allele frequency (C/T)	Group	Genotype			*P*
			CC	CT	TT	
−55C/T	0.71/0.29	Diet resistant	5	6	1	0.499
		Diet responsive	7	4	1	
Y99Y	0.29/0.71	Diet resistant	1	6	4	0.315
		Diet responsive	1	4	7	
Y210Y	0.35/0.65	Diet resistant	0	5	6	0.186
		Diet responsive	3	5	4	

tions that might affect weight loss. The two groups that underwent muscle biopsies consisted of 12 subjects in each of the upper and lower quintiles for weight loss and were similar in terms of age, initial weight, and other variables. Wadden et al. (30) and others have demonstrated that RMR decreases in the first few weeks of this weight loss regimen but then returns toward normal despite continued weight loss. Given the invasive nature of this procedure, it was not possible to perform muscle biopsies before and after entry into the weight loss program. To avoid the possible effects of short-term weight loss on UCP3 expression, muscle biopsies were carried out at a later date, after a prolonged period of weight stabilization. Although small differences remained in body weight between the two groups at the time of muscle biopsy, they were not significant. Furthermore, we clearly demonstrated that there was no relationship between body weight or age at the time of biopsy and levels of either UCP3 mRNA or mitochondrial proton leak.

Human UCP3 differs from UCP1 and -2 by its high and selective expression in muscle (31,32), making it an attractive candidate for obesity. UCP1 is expressed exclusively in brown adipose tissue, which is normally present in limited amounts in adults. UCP2 is expressed in most tissues of the body and is thought to play major roles in protection from reactive oxygen species (33) and insulin secretion (34,35). The physiological function of UCP3 is thought to involve the regulation of energy expenditure and/or a switch to fatty acid oxidation (3,36,37). Overexpression of UCP3 or UCP1 in muscle of transgenic mice results in increased energy expenditure, resistance to diet-induced obesity, and increased insulin sensitivity (11,38). Few data are available on the relationship between UCP3 expression and obesity in humans. However, UCP3 mRNA expression was positively related to sleeping metabolic rate in Pima Indians (21). The 5′ flanking region of UCP3 contains a number of potential binding sequences for regulatory factors. Because thyroid hormones are the only endocrine factors known to regulate leak (27,28), we screened for genetic variation in the only putative TRE found in the proximal promoter. We did not find any variation in the TRE sequence. Interestingly, there are three putative PPREs in the 5′ flanking sequence of UCP3. Peroxisome proliferator–activated receptor (PPAR)-α and PPAR-γ1 are expressed in skeletal muscle (39). PPAR ligands include a variety of long-chain fatty acids and their derivatives (40). These PPREs could provide a mechanism by which free fatty acids modulate UCP3 expression. However, we were unable to detect any polymorphic variation in the three putative PPREs in the UCP3 promoter of the subjects studied. Thus, the variation noted in UCP3 mRNA abundance may be related to differences in the availability of long-chain fatty acids or other PPAR ligands rather than to genomic sequence differences.

UCP3 mutations and polymorphisms have been identified in humans (20–25). The functional significance of a few of these UCP3 gene variations has been studied. Argyropoulos et al. (25) showed that in heterozygotes for exon 6 splice donor polymorphism (IVS6 + 1G/A), basal fat oxidation rates were reduced by 50% and the respiratory quotient was markedly increased compared with obese control subjects. However, another study reported that RMR, respiratory quotient, and metabolic response to graded exercise were normal in exon 6 splice donor polymorphism individuals (41). Expression of obese subject mutations R70W and R143X in yeast showed a complete loss and a significant reduction of UCP3 uncoupling activity, respectively (23). On the other hand, expression of V102I and exon 6 splice donor (IVS6 + 1G/A) polymorphisms had no effect on UCP3 uncoupling activity (23). We screened all reported UCP3 mutations and polymorphisms in diet-resistant and diet-responsive obese women. We did not find any association with a particular genotype that may account for the differences in ability to lose weight. However, these statistical results were obtained on a very limited number of patients and therefore may not reflect the small influence of genotype on the ability to lose weight among obese women.

The role of UCPs in mitochondrial proton leak is a subject of significant controversy, and it has not been definitively demonstrated that UCP3 causes the physiological proton leak (42). The literature describing the physiological induction of the UCPs includes a number of paradoxical findings. The most outstanding paradox is the significant increases in UCP2 and UCP3 gene expression during fasting and severe food restriction (43)—situations where energy waste would counter the observed increases in the efficiency of energy metabolism. Cadenas et al. (44) studied the increased expression of UCP3 mRNA and protein resulting from fasting in rats. Although there were significant increases in muscle UCP3 expression, there were no changes in mitochondrial proton leak. Bézaire et al. (45) conducted a similar study in wild-type mice and UCP3 knockout mice. Fasting caused a fourfold increase in UCP3 and a twofold increase in UCP2 in muscle of wild-type mice; however, similar to the findings of Cadenas et al., there were no changes in mitochondrial proton leak reactions. It is also important to note that two independent groups have shown that UCP3 knockout mice are not obese (46,47).

The literature thus demonstrates that changes in UCP3 expression (e.g., with fasting) do not necessarily result in

altered proton leak. The cause(s) of proton leak is complex and not well understood. Additional factors shown to correlate with leak include phospholipid fatty acid composition and inner membrane surface area (42). These factors were not examined in the current study due to the additional amount of tissue that would have been required but will be investigated in future studies.

The physiological function of UCP3 may primarily be associated with energy substrate partitioning and fatty acid metabolism (25,36), and one of the authors of the present study (M.-E.H.) has proposed that UCP3 may play a role in mitochondrial fatty acid efflux to facilitate rapid fatty acid oxidation (43). It is also important that Bézaire et al. (45) document impaired fatty acid oxidation in UCP3 knockout mice compared with wild-type controls, as determined by indirect calorimetry. Regardless of mechanistic details, our findings are consistent with an important role for mitochondrial proton leak and UCP3 in regulating the efficiency of energy metabolism.

Finally, despite the rapid advances in our understanding of the molecular causes of obesity, there has been reluctance in the medical community to accept the premise that large variations in energy requirements exist (48). Many patients experience difficulty in losing weight despite diet adherence. Skeletal muscle UCP3 and proton leak may be candidates for pharmacological upregulation of fatty acid oxidation in these obese diet-resistant subjects. Further studies of the mechanistic aspects of UCP3 function are warranted.

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