Brief Genetics Report

The −866A/A Genotype in the Promoter of the Human Uncoupling Protein 2 Gene Is Associated With Insulin Resistance and Increased Risk of Type 2 Diabetes

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Uncoupling protein (UCP)-2 is a member of the mitochondrial inner membrane carriers that uncouple proton entry in the mitochondrial matrix from ATP synthesis. The −866G/A polymorphism in the UCP2 gene, which enhances its transcriptional activity, was associated with enhanced risk for type 2 diabetes in obese subjects. We addressed the question of whether the −866G/A polymorphism contributes to variation in insulin sensitivity by genotyping 181 nondiabetic offspring of type 2 diabetic patients. Insulin sensitivity, assessed by the hyperinsulinemic-euglycemic clamp, was reduced in −866A/A carriers compared with −866A/G or −866G/G carriers ($P = 0.01$). To directly investigate the correlation between UCP2 expression and insulin resistance, UCP2 mRNA levels were measured by real-time RT-PCR in subcutaneous fat obtained from 100 obese subjects who underwent laparoscopic adjustable gastric banding. UCP2 mRNA expression was significantly correlated with insulin resistance as assessed by the homeostasis model assessment index ($r = 0.27$, $P = 0.007$). We examined the association of the −866A/A genotype in a case-control study including 483 type 2 diabetic subjects and 565 control subjects. The −866A/A genotype was associated with diabetes in women (odds ratio 1.84, 95% CI 1.03–3.28; $P = 0.037$), but not in men. These results indicate that the −866A/A genotype of the UCP2 gene may contribute to diabetes susceptibility by affecting insulin sensitivity. *Diabetes* 53:1905–1910, 2004

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CT, computerized tomography; HOMA, homeostasis model assessment; OGTT, glucose tolerance test; UCP, uncoupling protein; UTR, untranslated region.

The pathophysiology of type 2 diabetes includes two apparently distinct defects, i.e., impairments in insulin action at the level of skeletal muscle, fat, and liver and inadequate compensation by the pancreatic β-cells, ultimately resulting in fasting hyperglycemia (1,2). The pathogenesis of both of these components is generally thought to be multifactorial, involving both genetic susceptibility and environmental factors (3). However, identifying genes that confer susceptibility to type 2 diabetes has proven problematic. Given the significant correlation between obesity and type 2 diabetes, it is plausible that genetic defects in candidate genes involved in the development of obesity may also predispose to type 2 diabetes. One of the many genes that might be involved in the development of obesity as well as diabetes could be uncoupling protein (UCP)-2, a member of the mitochondrial inner membrane carrier family that is expressed in several tissues, including adipose tissue, skeletal muscle, liver, and pancreatic islets (4,5). Like the homologous UCP1, UCP2 mediates mitochondrial proton leak, releasing energy stored within the proton motive force as heat, which ultimately results in a decrease in ATP production. In pancreatic β-cells, an increased expression or activity of UCP2 may contribute to impairing insulin secretion by reducing the ATP-to-ADP ratio, which is required for closure of the membrane ATP-sensitive potassium channel, and subsequent membrane depolarization, influx of calcium, and insulin granule exocytosis. Accordingly, it has been demonstrated that overexpression of UCP2 in rat islets of Langerhans or pancreatic β-cell lines resulted in blunted glucose-stimulated insulin secretion that was associated with reduction in cellular ATP levels (6,7). In addition, $UCP2^{-/-}$ knockout mice are characterized by higher islet ATP levels and increased glucose-stimulated insulin secretion both in vivo and in isolated pancreatic islets (8). However, the putative physiological role of UCP2 as a mitochondrial uncoupler, and thus its relation to thermogenesis, is still debatable. It has been reported (9) that mRNA expression of UCP2 in human skeletal muscle is increased during starvation-induced

weight loss, a well-known condition of suppressed thermogenesis and increased lipid utilization. Furthermore, studies (10–12) in rodents and humans have led to an alternative hypothesis that centers on a primary role for UCP2 in regulating lipids as fuel substrate by switching metabolism from a state of enhanced lipid utilization during starvation to one of reduced lipid utilization during refeeding. Because increased lipid oxidation may impair glucose metabolism (the Randle cycle) (13), increased expression of UCP2 could result in peripheral insulin resistance. In support of this view, UCP2$^{-/-}$ knockout mice showed an increased insulin sensitivity during a hyperglycemic clamp study and appeared to be protected against high-fat diet–induced insulin resistance (8,14).

Recently, it has been reported (15,16) that a common −866G/A polymorphism in the promoter of the human UCP2 gene, which enhances its transcriptional activity, resulting in increased UCP2 mRNA levels in human fat cells, is associated with a reduced risk of obesity but increased risk of type 2 diabetes in obese middle-aged subjects.

In the present study, the possible association of the common −866G/A polymorphism in the promoter of the human UCP2 gene with insulin resistance was evaluated in a cohort of 181 Italian nondiabetic offspring with only one parent affected by type 2 diabetes. Because these individuals have a 30–40% lifetime risk of developing diabetes, they represent a valuable model to study the pathophysiological impact of polymorphisms in candidate genes without the confounding effect of hyperglycemia (17). The clinical and laboratory features of the study subjects are shown in Table 1. Of these subjects, 75 (41.4%) were −866G/G homozygous, 84 (46.4%) were −866G/A heterozygous, and the remainder (12.2%) were −866A/A homozygous. The genotype distribution was in Hardy-Weinberg equilibrium. No significant differences in age, sex, BMI, waist-to-hip ratio, fasting plasma glucose levels, fasting plasma insulin concentrations, triglycerides, and total and HDL cholesterol were observed among the three genotypes (Table 1). By contrast, insulin sensitivity, assessed as whole-body glucose disposal by the gold standard hyperinsulinemic-euglycemic clamp, was significantly reduced in offspring of type 2 diabetic patients carrying the −866A/A genotype as compared with both −866A/G and −866G/G genotypes ($P = 0.01$). No significant differences in insulin sensitivity were observed between offspring carrying the −866A/G genotype and those carrying the −866G/G genotype, thus suggesting a recessive effect of the polymorphism. These differences remained significant ($P = 0.037$) after adjusting for sex, age, and BMI. Also, the glucose clamp–derived insulin sensitivity index was significantly reduced in offspring of type 2 diabetic patients carrying the −866A/A genotype as compared with both −866A/G and −866G/G genotypes ($P = 0.01$). These differences remained significant ($P = 0.015$) after adjusting for sex, age, and BMI. These data are consistent with those reported by Krempler et al. (16), who showed that obese nondiabetic subjects carrying the −866A/A genotype have reduced insulin sensitivity as compared with those who were −866G/G homozygous and −866G/A heterozygous, although these differences did not reach statistical significance, likely due to the small number of subjects exam-

TABLE 1
Clinical and biochemical characteristics of offspring of type 2 diabetic patients according to the UCP2 genotypes

	−866G/G	−866A/G	−866A/A	*P*	55A/A	55A/V	55V/V	*P*	3′UTR D/D	3′UTR D/I	3′UTR I/I	*P*
n (M/F)	50/25	61/23	16/6	0.68	46/21	62/26	19/7	0.91	58/27	56/24	13/3	0.57
Age (years)	36.9 ± 10.2	36.7 ± 10.7	41.0 ± 10.1	0.11	36.1 ± 9.9	36.8 ± 10.5	42.8 ± 10.5	0.02	36.4 ± 10.1	37.7 ± 10.7	40.8 ± 11.1	0.28
BMI (kg/m^2)	27.3 ± 5.9	27.7 ± 5.7	29.6 ± 6.4	0.21	26.8 ± 6.1	28.3 ± 6.1	28.6 ± 5.0	0.24	27.2 ± 5.8	28.2 ± 6.2	28.8 ± 5.1	0.46
Waist-to-hip ratio	0.85 ± 0.11	0.86 ± 0.10	0.88 ± 0.11	0.38	0.85 ± 0.12	0.86 ± 0.10	0.89 ± 0.10	0.27	0.85 ± 0.10	0.86 ± 0.12	0.87 ± 0.10	0.74
Fasting glucose (mg/dl)	88 ± 11	90 ± 12	90 ± 13	0.56	89 ± 12	89 ± 11	91 ± 13	0.79	90 ± 11	90 ± 12	88 ± 14	0.89
Fasting insulin (μU/ml)	8.9 ± 4.8	9.5 ± 4.8	11.1 ± 6.2	0.20	8.4 ± 4.6	9.9 ± 5.1	10.3 ± 5.0	0.12	9.1 ± 4.8	9.9 ± 5.3	8.7 ± 4.1	0.44
Cholesterol (mg/dl)	198 ± 34	187 ± 39	202 ± 38	0.10	194 ± 30	194 ± 42	194 ± 36	0.99	96 ± 32	195 ± 43	184 ± 30	0.52
HDL (mg/dl)	56 ± 15	53 ± 12	53 ± 12	0.26	55 ± 15	54 ± 12	53 ± 12	0.69	54 ± 14	54 ± 13	53 ± 12	0.86
Triglycerides (mg/dl)	103 ± 50	102 ± 50	107 ± 53	0.93	96 ± 44	108 ± 51	96 ± 55	0.37	101 ± 47	110 ± 53	92 ± 44	0.29
Glucose disposal (mg · kg^{-1} · min^{-1})	8.7 ± 3.0*	8.3 ± 2.5†	6.6 ± 3.0	0.01	8.8 ± 3.0	8.1 ± 2.6	7.4 ± 2.9	0.09	8.8 ± 2.9	7.9 ± 2.7	7.3 ± 2.8	0.07
Insulin sensitivity index (μg · kg^{-1} · min^{-1}/[μU/ml])	1.42 ± 0.94‡	1.27 ± 0.85§	0.76 ± 0.40	0.01	1.35 ± 0.98	1.29 ± 0.86	0.95 ± 0.51	0.32	1.33 ± 0.90	1.29 ± 0.89	0.82 ± 0.39	0.25

Data are means ± SD. Group differences of continuous variables were compared using ANOVA. Categorical variables were compared by χ^2 test. *$P < 0.0$ vs. −866A/A, and †$P < 0.046$ vs. −866A/A genotype after Bonferroni correction; ‡$P < 0.01$ vs. −866A/A, and §$P < 0.05$ vs. −866A/A genotype after Bonferroni correction.

ined. While this work was in preparation, Le Fur et al. (18) reported that obese children carrying the −866A/A genotype exhibit similar insulin sensitivity, as estimated by area under the curves during an oral glucose tolerance test, compared with the other two genotypes. We have no direct explanation for this discrepancy. Obviously, differences in the genetic background influencing sensitivity and insulin secretion between juvenile and adulthood forms of obesity ("early-onset obesity" versus "late-onset obesity") may account for these differences. According to this line of reasoning, in middle-aged adults the −866A/A genotype was found by us and others to be associated with variations in insulin sensitivity and insulin secretion (16,19), whereas no differences in these parameters were observed in children with juvenile obesity (18). There is evidence that insulin secretion is significantly lower in homozygous carriers of the −866A/A genotype compared with −866G/A heterozygous and −866A/A homozygous carriers (16,19). To determine whether the −866G/A polymorphism affected the capability to compensate for insulin resistance, we compared the disposition index, calculated as the product of the glucose disposal measured during hyperinsulinemic-euglycemic clamp, and the insulin secretion estimated by log-transformed homeostasis model assessment (HOMA) index among genotype groups. After adjusting for sex, age, and BMI, carriers of the −866A/A genotype showed a significantly lower disposition index (32.1 ± 14.8) compared with carriers of either −866G/G or −866A/G (41.7 ± 12.9 and 40.7 ± 13.1, respectively; $P < 0.045$).

It has been reported (15,20) that the −866G/A polymorphism is in linkage disequilibrium with two other common polymorphisms of the UCP2 gene, the 55A/V variant and the 3′-untranslated region (UTR) I/D variant. We therefore genotyped our offspring cohort to investigate the possible association of these two common polymorphisms of the UCP2 gene with insulin resistance. As shown in Table 1, although subjects carrying the 55V/V genotype or the 3′-UTR I/I tended to have lower insulin sensitivity, no significant differences in clinical and biochemical characteristics were observed among the genotypes. Of the 12 haplotypes observed, only 5 have a frequency of >5%, accounting for 83.6% of all of the observed haplotypes. As shown in Table 2, no significant associations between clinical and biochemical variables and haplotype combinations were observed.

Next, we directly investigated whether there was a significant correlation between UCP2 expression in insulin target tissue and insulin resistance. To this aim, we examined UCP2 mRNA expression by real-time RT-PCR in abdominal subcutaneous fat obtained from a large cohort consisting of 100 unrelated obese individuals who underwent laparoscopic adjustable gastric banding. We found a significant correlation between adipose UCP2 mRNA expression and insulin resistance assessed by HOMA ($r = 0.27$, $P = 0.007$). The correlation remained significant after correction for age, sex, and BMI ($r = 0.22$, $P = 0.03$). There was no significant relationship between UCP2 mRNA levels and age, BMI, waist, waist-to-hip ratio, fasting and 2-h postload plasma glucose levels, HbA_{1c}, triglycerides, and total and HDL cholesterol concentrations. There was no significant relationship between UCP2 mRNA levels

TABLE 2
Clinical and biochemical characteristics of offspring of type 2 diabetic patients according to the UCP2 haplotypes

	GAD/GAD	GAD/AAD	GAD/GAI	GAD/GVD	GAD/AVD	GAD/GVI	GAD/AVI	GVD/AVI	AVI/GVI	AVI/AAD	AVI/AVD	AVI/AVI	*P*
n (M/F)	18/38	3/4	0/4	1/3	4/12	6/6	15/39	0/3	1/2	0/2	4/3	2/11	0.52
Age (years)	35 ± 9	38 ± 11	38 ± 14	43 ± 15	35 ± 10	39 ± 11	36 ± 10	41 ± 5	46 ± 17	36 ± 4	48 ± 9	39 ± 9	0.18
BMI (kg/m^2)	26.9 ± 6.2	24.5 ± 4.2	29.1 ± 7	31.4 ± 1.5	28.2 ± 5.3	25.9 ± 4.8	28.3 ± 6.2	25.6 ± 4.4	27.3 ± 3.2	34.2 ± 12.5	29.5 ± 5.2	29.1 ± 5.5	0.48
Waist-to-hip ratio	0.84 ± 0.11	0.87 ± 0.07	0.88 ± 0.32	0.90 ± 0.02	0.86 ± 0.03	0.83 ± 0.07	0.86 ± 0.12	0.85 ± 0.08	0.96 ± 0.12	0.86 ± 0.10	0.94 ± 0.13	0.85 ± 0.08	0.66
Fasting glucose (mg/dl)	89 ± 11	90 ± 15	84 ± 9	88 ± 5	91 ± 12	85 ± 6	90 ± 12	87 ± 4	100 ± 18	81 ± 14	99 ± 10	86 ± 12	0.34
Fasting insulin (μU/ml)	8.7 ± 4.9	6.3 ± 0.5	6.9 ± 2.0	12.5 ± 3.8	10.2 ± 5.0	8.7 ± 4.6	9.6 ± 4.6	13.9 ± 8.0	5.3 ± 1.7	18.9 ± 17.0	12.1 ± 4.5	9.3 ± 4.1	0.06
Cholesterol (mg/dl)	195 ± 30	180 ± 33	199 ± 30	203 ± 16	197 ± 36	205 ± 53	188 ± 42	187 ± 35	167 ± 9	237 ± 38	226 ± 38	188 ± 33	0.33
HDL (mg/dl)	55 ± 16	54 ± 15	61 ± 11	50 ± 9	52 ± 11	62 ± 15	52 ± 12	57 ± 17	56 ± 8	61 ± 2	54 ± 12	52 ± 13	0.78
Triglycerides (mg/dl)	98 ± 45	105 ± 43	66 ± 21	114 ± 45	102 ± 56	132 ± 65	103 ± 45	123 ± 47	111 ± 60	116 ± 94	147 ± 50	87 ± 40	0.28
Glucose disposal (mg · kg^{-1} · min^{-1})	8.7 ± 3.0	9.6 ± 2.7	9.2 ± 4.6	8.1 ± 2.2	8.7 ± 3.0	8.8 ± 3.0	8.0 ± 2.4	9.7 ± 3.6	8.2 ± 2.6	3.7 ± 2.3	6.6 ± 2.9	7.2 ± 2.9	0.15
ISI (μg · kg^{-1} · min^{-1}/ [μU/ml]	1.46 ± 1.03	0.75 ± 0.06	0.84 ± 0.67	0.77 ± 0.05	1.12 ± 0.61	1.61 ± 0.80	1.33 ± 0.96	1.86 ± 0.50	0.80 ± 0.09	0.10 ± 0.08	0.78 ± 0.28	0.82 ± 0.39	0.37

Data are means ± SD. Group differences of continuous variables were compared using ANOVA. Categorical variables were compared by χ^2 test. Genotype combinations are in order as follows: −866G/A, 55A/V, and 3′UTR I/D. ISI, insulin sensitivity index.

TABLE 3
Clinical and biochemical characteristics of the case-control cohort

	Nondiabetic control subjects					Diabetic patients				
	All	−866G/G	−866G/A	−866A/A	*P*	All	−866G/G	−866G/A	−866A/A	*P*
n (M/F)	235/330	97/150	110/156	26/24	—	241/242	107/115	104/93	30/34	—
Age (years)	53 ± 12	53 ± 12	52 ± 12	54 ± 10	0.69	62 ± 10*	62 ± 11	63 ± 10	62 ± 11	0.53
Age at diagnosis (years)	—	—	—	—	—	52 ± 10	52 ± 10	51 ± 10	52 ± 10	0.46
BMI (kg/m^2)	29.8 ± 6.4	29.8 ± 6.2	30.1 ± 6.9	28.8 ± 4.2	0.43	30.1 ± 6.0	30.0 ± 5.7	29.9 ± 5.8	31.0 ± 7.3	0.41
Fasting glucose (mg/dl)	92 ± 11	92 ± 12	92 ± 11	92 ± 8	0.78	163 ± 73*	158 ± 68	169 ± 76	161 ± 83	0.35
HbA_{1c} (%)	—	—	—	—	—	7.5 ± 2.4	7.5 ± 2.6	7.5 ± 2.3	7.6 ± 2.4	0.94
Total cholesterol (mg/dl)	208 ± 43	209 ± 46	208 ± 41	199 ± 34	0.28	207 ± 44	210 ± 44	203 ± 47	208 ± 39	0.27
HDL cholesterol (mg/dl)	52 ± 14	52 ± 13	52 ± 14	51 ± 12	0.76	45 ± 14*	45 ± 13	46 ± 16	46 ± 13	0.79
Triglycerides (mg/dl)	132 ± 71	133 ± 76	134 ± 70	115 ± 53	0.18	165 ± 99*	163 ± 93	172 ± 110	152 ± 89	0.38

Data are means ± SD. Differences between the whole cohort of nondiabetic control subjects and diabetic patients were compared using unpaired Student's *t* test. Genotype differences of continuous variables were compared using ANOVA. Categorical variables were compared by χ^2 test. *$P < 0.0001$ vs. nondiabetic control subjects.

and ultrasound thickness of visceral or subcutaneous adipose tissue measured in 60 of the 100 obese individuals studied. Furthermore, there was no significant relationship between UCP2 mRNA levels and computerized tomography (CT) scan thickness of visceral adipose tissue, CT scan thickness of subcutaneous adipose tissue, CT scan area of visceral adipose tissue, and CT scan area of subcutaneous adipose tissue measured in 32 of the 100 obese individuals. These data are consistent with those of some, but not all, reports (9,21,22) showing an increased expression of UCP2 in adipose tissue of insulin-resistant subjects.

We have recently reported (19) that insulin secretion was significantly lower in homozygous glucose-tolerant carriers of the −866A/A genotype compared with −866G/A heterozygous and −866A/A homozygous carriers. Furthermore, pancreatic islets isolated from −866A/A homozygous nondiabetic individuals showed lower insulin secretion in response to glucose stimulation compared with −866G/G and −866G/A carriers, thus supporting the possibility that the common −866G/A polymorphism in the UCP2 gene may contribute to type 2 diabetes by affecting insulin secretion (19). Because both insulin resistance and impaired compensatory insulin secretion play essential roles in the pathogenesis of type 2 diabetes, we next tested whether the −866A/A genotype is associated with type 2 diabetes in a cohort consisting of 483 type 2 diabetic patients and 565 nondiabetic control subjects. The clinical characteristics of this cohort are shown in Table 3. Hardy-Weinberg expectations were fulfilled in both nondiabetic control subjects and diabetic patients (Table 4). The frequency of the −866A/A genotype was 13.3% among diabetic patients and 9.2% among nondiabetic control subjects. Thus, applying a recessive model,

TABLE 4
−866G/A UCP2 polymorphism and associated risk for type 2 diabetes

	n	%	*P*	OR (95% CI) (univariate analysis)	*P**	OR (95% CI) (multivariate analysis)	*P*†
Whole cohort of diabetic patients							
−866G/G	222	46.0	—				
−866G/A	197	40.7	—				
−866A/A	64	13.3	—				
Whole cohort of nondiabetic control subjects							
−866G/G	247	43.7	—	1.00			
−866G/A	260	47.1	—	0.82 (0.63–1.06)	0.13		
−866A/A	52	9.2	0.037‡	1.36 (0.90–2.05)	0.13		
Diabetic women							
−866G/G	115	47.5	—				
−866G/A	93	38.4	—				
−866A/A	34	14.0	—				
Nondiabetic women							
−866G/G	150	45.5	—	1.00		1.00	
−866G/A	156	47.3	—	0.78 (0.54–1.11)	0.16	0.77 (0.54–1.10)	0.15
−866A/A	24	7.3	0.011§	1.84 (1.03–3.28)	0.037	1.82 (1.02–3.24)	0.042

*P values and their OR for genotypes were calculated by logistic regression analysis; †P values for multivariate analyses were adjusted for age and BMI; ‡P value for the at-risk allele recessive model, assigning numbers to the genotypes of 0, 0, and 1 for G/G, G/A, and A/A, respectively, by χ^2 test; §P value for overall comparison between diabetic women and nondiabetic women by χ^2 test.

the −866A/A genotype was significantly associated with type 2 diabetes ($P = 0.037$), with carriers having a relative risk of 1.5 (95% CI 1.02–2.22). Sex significantly influenced the results. When analysis was carried out in women, the −866A/A genotype was more prevalent in the diabetic group (14.0%) than in the nondiabetic control subjects (7.3%) (odds ratio [OR] 1.8, 95% CI 1.03–3.28; $P = 0.037$) (Table 4). Adjustment for age and BMI in a multivariate logistic regression analysis did not change this association (1.8, 1.02–3.24; $P = 0.042$) (Table 4). Estimated risks of G/G homozygous and G/A heterozygous subjects did not differ, which is consistent with a recessive effect of the "at-risk" allele A. In men, the difference in genotype distribution between diabetic patients (12.4%) and nondiabetic control subjects (11.9%) was not statistically significant ($P = 0.72$). These results suggest that the −866A/A genotype may confer increased risk to develop type 2 diabetes in a sex-specific fashion, as previously reported (16) in a cohort of obese middle-aged subjects.

In conclusion, our results suggest that the −866A/A genotype in the promoter of the UCP2 gene is associated with insulin resistance in individuals with genetic predisposition for type 2 diabetes. These findings, coupled with previous data reported by us and other groups (16,19) showing that −866A/A homozygous carriers have impaired insulin secretion, suggest that this polymorphism may contribute to type 2 diabetes by affecting both β-cell function and insulin action. Our results in diabetic women are consistent with this idea and support concepts that are emerging from studies (8,12,14) in animal models. Allele-specific enhancement of UCP2 expression in β-cells would decrease ATP synthesis by mitochondrial uncoupling and, thereby, glucose-stimulated insulin secretion. On the other hand, because ATP is required for many cellular functions, including insulin signaling cascade and translocation of glucose transporters from the intracellular pool to the plasma membrane, allele-specific enhancement of UCP2 expression in skeletal muscle and adipose tissue could result in impaired insulin action. Moreover, in light of increasing evidence of the role of UCP2 as a regulator of lipid metabolism, it is possible that increased UCP2 expression may interfere with glucose metabolism by favoring lipid oxidation according to Randle's hypothesis (13). However, this possibility seems in contrast with a recent report (18) showing that obese children carrying the −866A/A genotype exhibit increased carbohydrate oxidation and decreased lipid oxidation compared with −866G/G homozygous and −866G/A heterozygous carriers despite comparable glucose, free fatty acid, and insulin levels.

The present findings in an Italian population need to be replicated in independent studies to determine whether the −866G/A UCP2 promoter polymorphism influences insulin secretion and action and whether it is truly implicated in the development of type 2 diabetes in individuals at risk. We cannot exclude the possibility that the −866G/A polymorphism of UCP2 is not itself responsible for the observed association with impaired peripheral insulin action, but instead it is in linkage disequilibrium with an unknown causative variant in a distal regulatory site or with an unidentified causative polymorphism in a gene different from, but close to, the UCP2 gene.

RESEARCH DESIGN AND METHODS

Clinical characteristics of the groups studied are provided in Tables 1–3.

Cohort 1. Unrelated offspring ($n = 181$) of type 2 diabetic patients were consecutively recruited at the Department of Internal Medicine of the University of Rome-Tor Vergata according to the following inclusion criteria (17): the eldest offspring in each family with only one parent affected by type 2 diabetes, absence of diabetes or impaired glucose tolerance (fasting plasma glucose <110 mg/dl and 2-h plasma glucose <140 mg/dl), and absence of diseases able to modify glucose metabolism. On the first day, after a 12-h fast, all subjects underwent anthropometrical evaluation, including BMI and waist-to-hip ratio. A 75-g oral glucose tolerance test was performed with 0-, 30-, 60-, 90-, and 120-min sampling for plasma glucose. On the second day, at 0800, after a 12-h overnight fast, subjects underwent a euglycemic-hyperinsulinemic clamp study. Insulin (Humulin; Eli Lilly, Indianapolis, IN) was given as a primed continuous infusion targeted to produce plasma insulin levels of ~420 pmol/l. Thereafter, the insulin infusion rate was fixed at 40 $mU \cdot m^{-2} \cdot min^{-1}$. The blood glucose level was maintained constant throughout the study by infusing 20% glucose at various rates according to blood glucose measurements performed at 5-min intervals. The glucose clamp–derived insulin sensitivity index was defined as $M/(G \times \Delta I)$ corrected for body weight, where M is the steady-state glucose infusion rate (in milligrams per minute), G is the steady-state blood glucose concentration (in milligrams per deciliter), and ΔI is the difference between basal and steady-state plasma insulin concentrations (in microunits per milliliter). Insulin secretion was estimated by the HOMA index (23).

Cohort 2. Abdominal subcutaneous adipose tissue samples (1.5–3 g) were obtained from 100 patients with morbid obesity, i.e., grade 3 obesity according to World Health Organization criteria (24), during laparoscopic adjustable gastric banding at the Istituto San Raffaele, Milan, Italy. Patients with morbid obesity were eligible for laparoscopic adjustable gastric banding when fulfilling the following criteria: age 18–66 years inclusive, BMI >40 kg/m^2 or >35.0 in the presence of comorbidities (25), and history of at least two previous attempts to lose weight with dietary and medical measures followed by relapse of obesity. Exclusion criteria were obesity secondary to endocrinopathies (Cushing's disease or syndrome or hypothyroidism), gastrointestinal inflammatory diseases, risk of upper gastrointestinal bleeding, pregnancy, alcohol or drug addiction, and previous or current malignancies. Two 75-g oral glucose tolerance tests were performed in all subjects who were classified as having normal glucose tolerance, impaired fasting glucose, impaired glucose tolerance, or type 2 diabetes according to the American Diabetes Association criteria (26). Waist circumference and waist-to-hip ratio were evaluated, and an ultrasound and CT scan evaluation of visceral and subcutaneous adipose tissue were performed as previously reported (25). Insulin sensitivity was estimated by using the HOMA index (23). The fat samples were immediately frozen in liquid nitrogen and stored at −70°C for later analyses. Clinical and biochemical characteristics of obese subjects who underwent laparoscopic adjustable gastric banding were sex (17 men and 83 women), age (39.5 ± 10.4 years), BMI (44.6 ± 6.6 kg/m^2), fasting glucose (113 ± 36 mg/dl), 2-h glucose (153 ± 71 mg/dl), fasting insulin (20 ± 11 mU/ml), 2-h insulin (75 ± 52 μU/ml), HbA_{1c} (6.2 ± 1.4%), total cholesterol (208 ± 42 mg/dl), HDL cholesterol (50 ± 16 mg/dl), triglycerides (160 ± 104 mg/dl), HOMA (5.1 ± 3.3), and glucose tolerance status (56 with normal glucose tolerance, 6 with impaired fasting glucose, 24 with impaired glucose tolerance, and 14 with diabetes).

Cohort 3. Unrelated type 2 diabetic patients ($n = 473$) and unrelated nondiabetic subjects ($n = 542$) were consecutively recruited at the Department of Internal Medicine of the University of Rome-Tor Vergata and at the Department of Experimental and Clinical Medicine of the University Magna Graecia of Catanzaro. All subjects were Caucasian. Type 2 diabetes was diagnosed according to the American Diabetes Association criteria (26). Patients were excluded if they had chronic gastrointestinal diseases associated with malabsorption, chronic pancreatitis, history of any malignant disease, history of alcohol or drug abuse, liver or kidney failure, and treatments able to modify glucose metabolism, such as corticosteroids or estrogens.

All studies were approved by institutional ethics committees, and informed consent was obtained from each subject in accordance with principles of the Declaration of Helsinki.

DNA analysis. Genomic DNA was isolated from peripheral blood according to standard procedures. The −866A/G polymorphism in the promoter of the human UCP2 gene was determined by digesting PCR products with the restriction enzyme *Mlu*I (Invitrogen, Carlsbad, CA), as previously described (19). The 3′-UTR I/D polymorphism was determined by amplification of a 230-bp product, followed by size separation on 2.5% agarose gels as previously described (20). The 55A/V polymorphism was determined by digesting PCR products with the restriction enzyme *Hinc*II (New England Biolabs, Beverly, MA). The primers used were 5′-TAC TGC TAA AGT CCG GTT ACA G-3′ as the

upstream primer and 5′-CAT CAC AC GCG GTA CTA CTG GGC GTT G-3′ as the downstream primer.

Adipose tissue gene expression. Total RNA was extracted using Trizol (Life Technologies, Gaithersburg, MD). The amount of total RNA was quantified spectrophotometrically at 260 nm. The ratio of absorption (260/280 nm) of all preparations was between 1.8 and 2.0. cDNA was prepared using TaqMan reverse transcription reagents, and expression of human UCP-2 mRNA was measured quantitatively by the TaqMan Real-Time PCR technique (Applied Biosystems, Foster City, CA). Ready-to-use primers and fluorescence probes for human UCP-2 and GAPDH genes were purchased from Applied Biosystems (Assay on Demand) and used according to the manufacturer's protocol. The Ct value for every sample was measured in duplicate, and UCP-2 expression levels were determined by a comparative Ct method using GAPDH as endogenous reference (Applied Biosystems).

Statistical analysis. Parametric data are expressed as means ± SD. Nonnormally distributed parameters were logarithmically transformed to approximate a normal distribution. Group differences of continuous variables were compared using ANOVA or unpaired Student's t test. The Bonferroni correction for multiple comparisons was applied. Categorical variables were compared by contingency χ^2 test. All differences were also tested after adjusting for sex, age, and BMI. The Hardy-Weinberg equilibrium between the two genotypes was evaluated by χ^2 test. Relationships between variables were determined by Pearson's correlation coefficient. To assess the extent to which the various genotypes were associated with type 2 diabetes, we estimated ORs and the corresponding 95% CIs by univariate logistic regression analysis. Multivariate ORs from the case-control study were calculated using a logistic regression analysis while adjusting for age and BMI. All tests were two sided, and a P value <0.05 was considered statistically significant. All analyses were performed using SPSS version 10.0 for Windows.

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