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Uncoupling protein-2 polymorphisms in type 2 diabetes, obesity, and insulin secretion

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Wang, Hua, Winston S. Chu, Tong Lu, Sandra J. Hasstedt, Philip A. Kern, and Steven C. Elbein. Uncoupling protein-2 polymorphisms in type 2 diabetes, obesity, and insulin secretion. *Am J Physiol Endocrinol Metab* 286: E1–E7, 2004. First published August 12, 2003; 10.1152/ajpendo.00231.2003.—The onset of type 2 diabetes (T2DM) is preceded by obesity, insulin resistance, and impaired β-cell function. Uncoupling protein-2 (UCP2) is a widely expressed inner mitochondrial membrane protein. Common polymorphisms of the UCP2 gene have been implicated in diabetes, in obesity, and with changes in UCP2 mRNA levels. We tested the hypothesis that common UCP2 variants influence T2DM susceptibility in four parallel studies of separate populations. We typed the −866 promoter (G/A) variant, a nonsynonymous (Ala55Val or A55V) single-nucleotide polymorphism in exon 4, and a 45-nt insertion in the 3′-untranslated (3′UTR) region. Study populations included a case-control population study, a family-based association study, and a metabolic study of individuals who had been characterized for insulin sensitivity and secretion. To evaluate UCP2 mRNA levels, we examined a fourth population of subjects, who had undergone subcutaneous fat biopsy. All three variants showed a trend to an association with T2DM ($P = 0.05$ to 0.07) in the population but not the family-based association study. The 3′ insertion/deletion (3′UTR I/D) variant was associated with body mass index (BMI, $P = 0.035$) among nondiabetic family members. Haplotype combinations were significantly associated with BMI ($P = 0.028$), triglyceride levels ($P = 0.026$), and fasting insulin ($P = 0.029$); highest values for the three traits were observed in individuals with the heterozygous combination GVI/AVD. In the metabolic study, all three variants were associated with an index of β-cell compensation for insulin sensitivity (disposition index), particularly in interaction with family membership ($P < 0.000001$). Individuals homozygous for the −866 A allele had decreased adipose mRNA levels relative to GG homozygous individuals ($P = 0.009$), but the 3′UTR I/D variant had no impact on mRNA levels. We confirm modest effects of UCP2 variants on BMI and T2DM and show significant effects on insulin secretion in interaction with family-specific factors. However, the associated allele and the effects on gene expression are opposite to those reported previously.

association study; single nucleotide polymorphism; insulin sensitivity; gene expression

OBESITY, IMPAIRED INSULIN SECRETION, and a family history of type 2 diabetes (T2DM) are all risk factors for T2DM in humans (37). Both obesity and insulin secretion show high heritabilities (1, 15, 23), thus suggesting a genetic contribution to these traits. This contribution, in turn, would increase diabetes risk. Uncoupling proteins (UCP) are attractive candidates for that genetic contribution. UCP belong to the family of mitochondrial transporter proteins that uncouple the transport of protons across the inner mitochondrial membrane from electron transport and the synthesis of ATP from ADP (11, 33), hence generating heat rather than energy. UCP1, the first member identified, is expressed primarily in brown adipose tissue and is responsible for nonshivering thermogenesis in infants and small mammals (11, 33), but the role in adult humans is uncertain (4, 33). In contrast, UCP2 is expressed widely, including in white adipose tissue, skeletal muscle, pancreatic islets, and the central nervous system (11, 20, 33). Although the function of UCP2 is controversial (21), in animal and human studies UCP2 mRNA levels were correlated with plasma free fatty acid concentrations (3, 4). Thus UCP2 may play an important role in lipid metabolism (11, 33) as well as in energy expenditure. Furthermore, in mouse models, overexpression of UCP2 in pancreatic β-cells decreased insulin secretion (9, 11), whereas absent UCP2 increased insulin secretion (38). These effects may be secondary to altered β-cell lipid metabolism or to reduced ATP production with impaired glucose-stimulated insulin secretion (38). Together, these data suggest that altered UCP2 expression or action could explain the key defects that precede T2DM: disordered lipid metabolism, impaired insulin secretion, and dysfunctional weight homeostasis.

Three common polymorphisms have been described in the UCP2 gene and have been variably associated with altered body mass index (BMI), changes in energy expenditure, and maintenance of body weight after overfeeding (11, 19). Most studies have examined individually either the amino acid substitution of valine (V) for alanine (A) in exon 4 (Ala55Val or A55V) or the 45-bp insertion/deletion variant in the 3′-untranslated region (3′UTR I/D) of exon 8 (11). Results of these studies have been variable (11), with some, but not all, studies showing an association with obesity and energy expenditure (11, 19). More recently, Esterbauer et al. (19) demonstrated an altered ratio of the 3′UTR I/D alleles in human adipose tissue. They identified a common G/A polymorphism of the UCP2 promoter region (−866), which accounted for 71% of the altered expression ratio between the insertion and deletion alleles. The minor (A) allele of the −866 promoter variant enhanced adipose mRNA expression in vivo and in reporter gene constructs expressed in a human adipocyte cell line and was associated with a modest reduction in obesity prevalence in Austrian Caucasians (19). However, the I/D transcript ratio among those homozygous for the common

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promoter allele suggested an independent role for the 3′UTR I/D variant in mRNA stability (19). Interestingly, the −866 variant lies in a region with putative binding sites for two β-cell transcription factors: insulin promoter factor 1 and paired box-containing 6 protein. Esterbauer et al. demonstrated strong but incomplete linkage disequilibrium between the −866 variant and the 3′UTR I/D polymorphism but did not examine the A55V variant. In their population association study, the −866 variant was associated with obesity, and a haplotype of the −866 G/A variant and the 45-nucleotide 3′UTR I/D variant showed significant associations with BMI (19).

On the basis of the previous reports that the −866 variant altered mRNA levels, that the 3′UTR I/D variant might alter mRNA stability, and that the A55V variant is associated with obesity, we hypothesized that together these variants would alter the risk of T2DM by altering adipocyte mRNA levels, impairing the insulin-secretory response to insulin resistance, and increasing BMI. To test these hypotheses, we conducted four parallel studies in two populations. Because case-control studies offer the best power to detect small gene effects (32), we tested each individual variant for an association with diabetes in unrelated individuals. We sought to confirm and extend these findings to haplotypes and quantitative traits with a large, family-based association study of the same population. To determine the role of UCP2 variants on insulin secretion and β-cell compensation, we examined a subset of family members who had undergone tolbutamide-modified, frequently sampled intravenous glucose tolerance tests (FSIGT). Finally, we sought to replicate earlier reports of altered mRNA levels by measuring adipocyte UCP2 mRNA levels in 36 subjects who had undergone fat biopsies and who were characterized for the −866 and 3′UTR I/D variant.

MATERIALS AND METHODS

Experimental subjects. The first three studies were conducted on individuals of Northern European ancestry ascertained in Utah (16). *Study 1*, the case-control study (Table 1), comprised 131 unrelated diabetic individuals selected from two sources: unrelated members (youngest onset diabetic individual) of families ascertained for at least two diabetic siblings (70 subjects) and unrelated diabetic members of other families from the same population who had at least two diabetic first- or second-degree relatives but for whom other family members were not available for study (61 subjects). Control subjects (118 subjects) were also of Northern European ancestry, had normal oral glucose tolerance tests (OGTT), and had no family history of T2DM. Although case-control study designs offer the highest power to search for disease genes (32), they may be subject to spurious associations due to population stratification. We supplemented the case-control study with a family-based association study, which is not subject to such confounders. These studies also permitted us to establish individual haplotypes and thus to test for haplotype effects. Additionally, we were able to test for associations with the quantitative traits of BMI- and OGTT-based measures of insulin sensitivity (S_I) and secretion in up to 698 members of 63 families, including 283 individuals with T2DM and 388 unaffected individuals (12, 14). Nondiabetic family members underwent 75-g OGTTs with measurement of insulin as well as standard anthropometric measures. The age of diagnosis of diabetic family members was 49.2 ± 12.4 yr, and the mean age of testing of nondiabetic subjects was 43.2 ± 15.9 yr (means ± SD). Because of missing genotype and phenotype data, for the present study the maximum number of individuals for any trait was 661 (BMI). To more accurately assess the influence of UCP2 variants on S_I and insulin secretion, we also analyzed the UCP2 variants in a subset of the full families (*study 3*, 125 nondiabetic individuals) who had undergone tolbutamide-modified FSIGT for determination of S_I and the acute insulin response to the initial glucose bolus (AIR_G) (15, 18). All subjects participating in *studies 1–3* provided written, informed consent under a protocol approved by the University of Utah Institutional Review Board.

Table 1. *Characteristics of case-control study population*

Group	Number	Male/ Female	Mean BMI, kg/m^2	Mean Age, yr
T2DM, family members	70	40/30	31.5±5.8	62.6±10.5
T2DM, population sample	61	48/13	27.6±18.9	62.3±12.3
Nondiabetic controls	118	54/64	28.0±6.4	55.6±12.8

Characteristics of the population for *study 1*. Mean values are expressed ± SD. BMI, body mass index; T2DM, type 2 diabetes mellitus.

Subjects for *study 4* (gene expression) were ascertained for no known diabetes and age between 18 and 65 yr and for either normal glucose tolerance or impaired glucose tolerance on a standard 75-g OGTT. Subjects were in good health and were not taking any medications at the time of study other than hormone replacement therapy. All subjects underwent FSIGT testing and excisional biopsy of subcutaneous fat from the lower abdomen after a 12-h fast under local anesthesia, as described elsewhere (26). After biopsy, fat was frozen immediately at −70°C in liquid nitrogen. The mean age of the subjects was 38 yr (range 26–61 yr), and the mean BMI was 36 kg/m^2 (range 19–65 kg/m^2). Subjects included 27 Caucasian individuals, 2 Asian individuals, and 7 African American individuals. All subjects for *study 4* provided informed consent under a protocol approved by the University of Arkansas for Medical Sciences Human Research Advisory Committee. For all studies, subjects were studied at either the University of Utah General Clinical Research Center or the University of Arkansas for Medical Sciences General Clinical Research Center.

Genetic typing. The 3′UTR I/D variant was genotyped by enzymatic amplification of a 230-bp product followed by size separation on 2% agarose gels and scoring with GeneImagR software (Scanalytics, Boston, MA). The −866 promotor and A55V polymorphisms were typed in the case-control population (*study 1*), FSIGT study population (*study 3*), and gene expression study (*study 4*) by use of an oligonucleotide ligation assay. Primers were labeled with ^{32}P, separated by polyacrylamide gel electrophoresis, detected on a Storm imager (Molecular Dynamics, Sunnyvale, CA), and scored using GeneImagR software (12). Typing of the −866 and A55V variants for all members of 63 families (*study 3*) was performed using a PSQ96 Pyrosequencer according to the manufacturer's methods (Pyrosequencing, Uppsula, Sweden). All methods included ≥10% duplicate samples, and overlap between the full family set and the case-control study permitted cross-validation of the oligonucleotide ligation and Pyrosequencing assays. All variants were in Hardy-Weinberg equilibrium in unrelated subjects. Primer sequences for all methods are available from the authors.

Quantitation of UCP2 mRNA levels. Adipocyte UCP2 mRNA levels were determined by quantitative competitive reverse transcriptase PCR, as described in detail elsewhere (31). Briefly, total RNA was extracted using guanidine thiocyanate (10), and 0.2 μg of total RNA for each sample was added to increasing quantities of a cRNA construct that included the mRNA primer sites but incorporated a 53-bp internal deletion. The 5′ and 3′ primers were GCAGCTTTGAAGAACGGGA and TGGCAGTAGGGGGCACATCTGTGG, respectively, both located within the mRNA coding region but spanning an intron in the UCP2 gene to avoid genomic contamination. After 35 cycles of PCR, bands were separated on 2% agarose, stained with ethidium bromide, visualized using the Stratagene Eagle Eye II Still Video System, and analyzed with EagleSight software (Stratagene, La

Table 2. *Allele frequencies of variants in Caucasian case and control populations*

	T2DM	Control	*P* Value	Relative Risk
−866 Promoter (G allele)	0.67	0.58	0.05	1.43 (0.99, 2.07)
A55V (A55 allele)	0.37	0.45	0.066	0.71 (0.50, 1.03)
3′ UTR I/D (D allele)	0.75	0.68	0.07	1.42 (0.96, 2.11)

A55V, amino acid substitution of valine (V) for alanine (A) in exon 4 (Ala55Val); 3′ UTR I/D, 45-bp insertion/deletion (I/D) variant in the 3′-untranslated region of exon 8.

Jolla, CA). The ratio of UCP2 product to cRNA standard was plotted against the number of copies of cRNA added to yield the equivalence point between cRNA and UCP2 mRNA. Data are expressed as copies $\times 10^6$ of UCP2 mRNA in the sample, normalized to 1 μg total RNA.

Statistical analysis. Allele frequencies for association studies were determined by gene counting, and association of each single nucleotide polymorphism (SNP) with T2DM was tested individually using the Fisher Exact Test [2By2 program (30)]. Because all hypotheses tested were based on previous published reports and because many of the tests were correlated, we did not correct for multiple testing. Two-tailed *P* values are reported. To permit simple allelic association tests, no correction was made for BMI, age, or sex. Pairwise linkage disequilibrium was calculated from combined case and control population data by using the expectation maximization algorithm [2LD program (30)]. Two statistics were calculated, D' (29, 30) and r^2, which is a better measure of how well the results from two SNPs will correlate (8). Three locus haplotype frequencies were estimated in the population sample by using the EM algorithm, and differences in the frequency of the most common haplotype were compared between cases and controls with the Fisher Exact test. We tested the impact of each variant on S_I and insulin secretion by use of mixed-effects regression models (2, 18). S_I was calculated from the MinMod analysis of FSIGT data (5), and insulin secretion was determined as the mean 2- to 10-min AIR_G (15). Previous studies have shown that AIR_G varies according to S_I and that the relationship between AIR_G and S_I may be described as a hyperbolic function (25). To account for the ability of the pancreatic β-cell to compensate for prevailing S_I, we calculated the disposition index ($S_I \cdot AIR_G$), which describes this hyperbolic relationship. The model tested S_I, AIR_G, and disposition index as dependent variables, with BMI and age as covariates; sex, genotype, and diagnosis (impaired vs. normal glucose tolerance) as fixed factors, and pedigree membership as a random factor. Interaction between genotype and pedigree membership was included for all analyses. Three dependent variables were assessed in models that included the genotypes for each of the three variants. Because individual haplotype combinations could not be established with certainty, they were not tested in this analysis. Similarly, analysis of gene expression treated the UCP2 mRNA value as the dependent variable, genotype and sex as fixed factors, and age and natural logarithm (ln)-BMI as covariates. All skewed variables (BMI, S_I, UCP2 mRNA levels) were ln-transformed to normality before analysis. Because ethnicity was not found to have any influence on UCP2 mRNA levels on exploratory analyses (*F* statistic 0.14), data were included from all ethnic groups and ethnicity was not included in the model. Correlations of adipocyte UCP2 mRNA and S_I, BMI, and percent body fat were tested by Pearson correlation after ln-transformation of all values. Statistical tests were performed in SPSS for Windows, v. 11.5 (SPSS, Chicago, IL).

Family-based analyses were conducted in the Pedigree Analysis Package (PAP) (22). Transmission Disequilibrium Tests were performed for T2DM and obesity by means of a maximum likelihood method (12), which examines the transmission likelihood of each allele from parents to affected offspring compared with the expectation of 0.5. Obesity was defined as BMI >27.2 kg/m^2 for men and >26.9 kg/m^2 for women, according to Burton et al. (7). Results were not significantly altered when obesity was defined as BMI >30 kg/m^2. T2DM was defined according to World Health Organization criteria (16). Haplotypes were established from the pedigree data by using maximum likelihood methods implemented in PAP (36). The effect of UCP2 variation on quantitative traits was tested primarily in nondiabetic family members (glucose, insulin) or all family members (BMI, lipids). All skewed measures, including insulin, glucose, BMI, and lipid measures were natural logarithm-transformed to normality and adjusted for age and sex before analysis. An effect of each SNP on the quantitative traits was tested using measured genotype analysis (13, 36). The likelihood was maximized by assuming genotype-specific means for each trait. This likelihood was compared with the likelihood maximized from a model assuming a single mean (no genotype effects). Significance was assessed from a χ^2 statistic calculated as twice the natural logarithm of the ratio between the two likelihoods (genotypic means estimated/single mean), with the degrees of freedom (9 df) determined by the difference in the number of estimated parameters between the two models. Estimated parameters for each genotype were the mean, standard deviation, and heritability, yielding 12 df for the restricted model (3 genotypes and a 4th genotype encompassing all other effects) and 3 df for the general model (a single mean representing non-UCP2 genes).

RESULTS

Characteristics of the case-control study population are shown in Table 1. All three variants showed a trend toward an association with T2DM (Table 2), with a higher frequency of the common alleles for both the −866 G and 3′UTR D alleles in subjects with T2DM but a lower frequency of the A55 allele. To explore the relationship among these three variants, we estimated haplotypes among 127 case and 115 control individuals who were successfully typed for all three variants. Only three haplotypes were observed at frequencies >5%. The frequency of the haplotype −866 G, A55, 3′D was estimated to be 0.626 among case alleles and 0.534 among control alleles ($P = 0.043$), consistent with the trends observed for individual variants. However, global tests comparing the frequency of all observed haplotypes did not reach significance.

Although case-control studies offer the greatest power to detect small gene effects, they can be subject to spurious associations. To further test the role of UCP2 polymorphisms and haplotypes, we typed the three variants in up to 768 members of families ascertained for at least two siblings with T2DM. Because of missing phenotype and genotype data, the

Table 3. *Sample size by variable in family association study*

Variable	No. of Individuals
Obese men, T2DM	75
Obese women, T2DM	109
Nonobese men, T2DM	47
Nonobese women, T2DM	40
BMI (all)	661
Triglycerides (all)	616
Fasting insulin (nondiabetic)	341
Fasting glucose (nondiabetic)	355
Insulin sensitivity (HOMA)	338
β-Cell compensation (OCI60)	325

HOMA, homeostasis model assessment.

Table 4. *Standardized genotypic means by haplotype combination for familial T2DM*

Trait	*P*	Genotype Combinations								
		GAD/GAD	GAD/GVI	GAD/AVD	GVI/AVD	AVD/AVD	GAD/AVI	GVI/AVI	AVD/AVI	AVI/AVI
Number range		113–238	13–29	42–101	4–11	8–13	92–159	7–17	25–50	17–33
BMI	0.028	26.29	24.52	26.71	30.34	25.90	26.32	28.15	24.97	26.96
Triglycerides	0.026	175.9	144.1	178.1	254.6	201.2	155.2	234.0	168.5	157.6
Fast Ins	0.029	14.13	8.54	11.98	26.63	12.66	13.11	20.21	11.89	13.16
Fast Glc	0.089	89.38	83.98	92.45	91.30	88.40	91.22	94.36	90.87	87.07
HOMA	0.031	0.122	−0.748	−0.061	1.228	−0.072	0.053	0.726	−0.113	−0.040
β-Cell Compen.	0.088	−0.106	0.413	−0.055	−1.455	−0.128	0.107	−0.153	0.009	−0.115

Genotype means are shown for each haplotype combination. Means are adjusted to male and age 30 yr. Genotype combinations are in the order: −866 (G/A), exon 4 (A, A55; V, V55), 3′ UTR (45-bp insertion, I; 45-bp deletion, D). Fast Ins, fasting insulin; Fast Glc, fasting glucose; β-Cell Compen, oral measure off the disposition index based on fasting and 60-min glucose and insulin levels during an oral glucose tolerance test. Total no. of individuals studied is shown in Table 3; the no. with each genotype varies with the variable.

actual number of individuals tested for each trait was lower than the total number of subjects typed (Table 3). In contrast to the case-control study, no individual variant was transmitted from parents to obese offspring, obese diabetic offspring, or obese nondiabetic offspring more often than expected by chance ($P > 0.5$ for all comparisons). Likewise, no haplotype was overtransmitted to either obese offspring or diabetic offspring. Thus family association studies did not support the trends toward an association seen in the population case-control study.

Our large family study allowed us to assess the impact of the UCP2 variants on quantitative measures of insulin sensitivity, insulin secretion, and obesity. Among full families and considering individual polymorphisms, the only statistically significant association was with the −866 promoter variant on fasting glucose ($P = 0.029$), but differences in adjusted fasting glucose levels between genotypes was small (3 mg/dl). If each of the individual variants were to impact UCP2 expression or function, the full impact of UCP2 variation would be evident only by testing haplotype combinations. We identified nine predicted genotype combinations (Table 4). We found nominally significant associations for BMI ($P = 0.028$), triglyceride levels ($P = 0.026$), fasting insulin levels ($P = 0.029$), and S_I by homeostasis model assessment (HOMA; $P = 0.031$). Notably, neither fasting glucose nor indexes of β-cell compensation (indexes based on S_I derived from fasting insulin and AIR_G from the 30-min and 60-min rises in insulin after a glucose challenge) were associated with individual UCP2 polymorphisms or with genotype combinations (Table 4).

Although the family study involved a large number of participants, the larger study only provided data from the OGTT. These available data lacked the precision of measurements of S_I and insulin secretion derived from the FSIGT, which was available only in 125 subjects from 26 families that were part of the larger family study (15). FSIGT-based estimates of S_I, AIR_G, and disposition index were corrected for age, sex, BMI, glucose tolerance status, and family membership, and interaction between each genotype and family membership was tested in addition to main effects. We found a highly significant interaction of each genotype with family membership in the determination of disposition index ($P < 10^{-6}$ for all 3 genotypes). We observed less significant interactions in the determination of AIR_G for both the −866 promoter ($P = 0.012$) and A55V ($P = 0.028$) variants, but not for the 3′UTR I/D variant. Disposition index was significantly different among genotypes when marginal means (corrected for other model parameters) were compared for the −866 ($P = 0.014$), A55V ($P = 0.0003$), and 3′UTR I/D variants ($P = 0.0006$). In each case, heterozygous individuals showed the lowest disposition index, whereas differences between the two homozygous genotypes were not significant (Table 5). Differences in insulin sensitivity, fasting plasma glucose, and serum insulin were not significantly different (Table 5).

The −866 variant was previously reported to alter intraperitoneal adipose UCP2 mRNA levels, and the G allele showed reduced expression in cultured preadipocytes (19). We tested the association of both the −866 variant and the 3′UTR I/D variant with UCP2 mRNA from subcutaneous fat in individu-

Table 5. *Marginal population means for metabolic parameters in nondiabetic family members*

	−866 Promoter			A55V			3′ UTR I/D		
	G/G	G/A	A/A	A/A	A/V	V/V	D/D	D/I	I/I
S_I, 10^{-5} pmol·min^{-1}·l^{-1}	6.86 (5.15, 9.12)	5.70 (4.37, 7.44)	6.11 (3.81, 9.64)	6.34 (4.12, 9.74)	5.62 (4.30, 7.34)	6.88 (5.13, 9.24)	6.97 (5.37, 9.04)	5.33 (4.00, 7.11)	4.75 (2.87, 7.88)
AIR_G, pmol/l	154 (124, 191)	173 (142, 211)	160 (114, 225)	154 (112, 213)	175 (143, 213)	167 (133, 208)	159 (129, 197)	195 (155, 245)	163 (109, 243)
Disposition index, min^{-1}	0.124[a] (0.090, 0.160)	0.063[a,b] (0.057, 0.097)	0.146[b] (0.079, 0.202)	0.124[c] (0.077, 0.199)	0.063[c,d] (0.085, 0.146)	0.146[d] (0.106, 0.201)	0.143[e] (0.107, 0.191)	0.066[e] (0.048, 0.090)	0.116 (0.066, 0.204)

S_I, insulin sensitivity; AIR_G, acute insulin response to initial glucose bolus; disposition index, S_I·AIR_G. Marginal means are shown from general linear model regression for each trait, each of the 3 markers, and each genotype. Means were converted back to linear units from natural logarithms used in the analysis. Confidence intervals (95%) are shown in parentheses. Overall significance is stated in the text. Significant comparisons among genotypes are denoted with superscripts; [a]$P = 0.007$; [b]$P = 0.040$; [c]$P = 0.012$; [d]$P = 0.00007$; [e]$P = 0.0001$. Comparisons not marked were not significant ($P > 0.05$) in pairwise comparisons, although differences may have been significant in overall comparisons (see text). *P* values were calculated by least significant difference and were not corrected for multiple testing.

als without diabetes. Only one individual was homozygous for the 3′UTR I allele. No differences were observed with mRNA levels between individuals homozygous for the D allele and heterozygous individuals, when either nonparametric tests (Mann-Whitney *U*-test) or ln-transformed mRNA levels and a regression analysis were used. Although individuals with the G/G genotype at the −866 polymorphism tended to have higher mRNA levels than individuals carrying the G/A and A/A genotypes (Fig. 1), these differences were not significant by ANOVA without covariates. A mixed-effects regression model that included BMI, age, sex, and genotype likewise found no term that significantly determined ln-transformed UCP2 mRNA levels, but when UCP2 mRNA levels for 12 Caucasian individuals with the G/G genotype were compared with two individuals with the A/A genotype in exploratory analyses, the differences were significant ($P = 0.009$, 9.58 ± 1.86 vs. 2.25 ± 1.43, both $\times 10^6$ copies/μg RNA). UCP2 mRNA levels were not correlated with BMI or S_I when only Caucasian individuals were examined, but we found a trend toward a negative correlation of adipocyte UCP2 mRNA and percent body fat ($r = -0.38$, $P = 0.05$).

As noted previously by Esterbauer et al. (19), the three variants were in strong linkage disequilibrium, with standardized linkage disequilibrium coefficients (D') of 0.97 between the −866 variant and the A55V variant and between the A55V variant and the 3′UTR I/D variant, whereas linkage disequilibrium was incomplete ($D' = 0.75$) between the −866 variant and the 3′UTR I/D variant. Similarly, the correlation coefficient (r^2) value, which is a better measure of whether the same disease association would be observed with the two variants, was highest between the −866 and A55V variants (0.82) but showed a lower correlation between these two polymorphisms and the 3′UTR I/D ($r^2 < 0.55$). Of the eight predicted haplotypes, only six were observed among family members. Two haplotypes (−866 G-A55–3′UTR D; −866 A-V55–3′UTR I) accounted for 78% of all observed haplotypes. Of the remaining haplotypes, only one (−866 A-A55–3′UTR I) accounted for 15%, and the other haplotypes were observed at a frequency of <5%. Thus only three of the eight predicted haplotypes accounted for 93% of all observed haplotypes. Notably, the most common haplotypes placed the −866 G allele, which has been reported to decrease UCP2 mRNA expression (19), with the 3′UTR D allele, which has been reported to increase mRNA stability; similarly, the −866 A allele, which was reported to increase transcription (19), was present most often on the haplotype with the 3′UTR I allele, which has been proposed to reduce UCP2 mRNA stability. These haplotypes, in view of data from Esterbauer et al., suggest that the promoter and 3′UTR variants might have counterbalancing effects. Although we could not establish haplotype combinations in individuals tested for mRNA expression, the lack of haplotype diversity suggests that the mRNA levels that we observed were from the combined effects of all three variants.

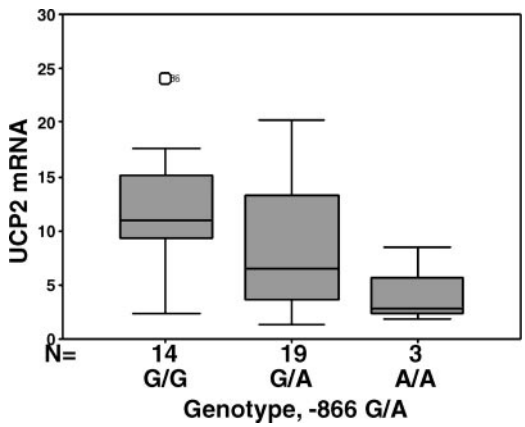

Fig. 1. Gene expression by genotype at −866 promoter (G/A) polymorphism. Boxplot shows the number of copies of UCP2 mRNA × 10^6 for each genotype at the −866 locus. Number of subjects is shown below each box. Data are shown without logarithmic transformation. Median value is shown by horizontal line; interquartile range is shown by length of box; lines show outliers (between 1.5 and 3 box lengths); points show extreme values. Differences among the 3 genotypes are not significant, but GG vs. AA genotypes are significant at $P = 0.009$.

DISCUSSION

By dissipating proton gradients, uncoupling respiration from oxidative phosphorylation, and converting fuel to heat, UCP may play an important role in the regulation of human energy metabolism. One linkage study mapped the trait of resting metabolic rate to chromosome 11q in the vicinity of UCP2 (6). Additionally, UCP2 is widely expressed in adult humans, including in white adipose, skeletal muscle, and, as demonstrated more recently, the pancreatic β-cell (20). Several findings recently strengthened the hypothesis that sequence variation in UCP2 might contribute to the pathogenesis of T2DM. In transgenic mice overexpressing UCP2 in the pancreatic β-cell, glucose-stimulated insulin secretion was decreased (9), whereas mice deficient in UCP2 showed increased glucose-stimulated insulin secretion (38). Furthermore, UCP2 levels may be modulated by free fatty acids, and thus UCP2 may also be involved in fat and skeletal muscle lipid metabolism (33). These findings, along with the recent report by Esterbauer et al. (19) that the −866 promoter variant and the 3′UTR I/D variant altered mRNA levels in vitro and in intraperitoneal fat, suggested that known UCP2 variants might predispose to diabetes through multiple pathways affecting both insulin secretion and insulin action. On the other hand, whereas decreased UCP2 message might result in decreased energy expenditure and obesity, this same defect would increase insulin secretion and thus protect against T2DM.

Our findings support some, but not all, of the previous hypotheses. In the case-control study, we found some support for an association of the more common −866 G allele with diabetes, with a trend for the A55V and 3′UTR I/D variants that did reach nominal statistical significance. We could not replicate the diabetes association in the family study, but the transmission disequilibrium test lacks the power of the case-control study. Had we performed a Bonferroni correction, the observed associations would no longer be statistically significant, but in a test of a previous hypothesis, such a correction was overly conservative. The G allele was also associated with a higher prevalence of obesity in the study of Esterbauer et al. (19), and in their study resulted in decreased expression of UCP2. If anything, our data suggested higher levels of mRNA in individuals with the G/G genotype. The differences in effect on mRNA levels might suggest that other factors, either independently or in interaction with the −866 variant, influence the mRNA levels and account for the different conclusions.

One unique aspect of our study was our ability to use extended families to establish haplotypes and haplotype combinations and to test these combinations in a large number of carefully phenotyped individuals. Indeed, among family members, we found evidence that combinations of the haplotypes of the three variants altered BMI, triglyceride levels, fasting insulin, and insulin resistance, as measured by HOMA (28), although we did not find an association of any individual variant with S_I. Because of the large number of genotype combinations and because the variables are somewhat correlated, the true significance of these findings is difficult to assess. The highest BMI, triglyceride levels, and fasting insulin levels were observed in individuals heterozygous for the combinations GVI/AAD and GVI/AVI (−866 G/A, A55V, 3′ I/D). Similarly, the greatest effect of individual variants on insulin secretion (disposition index) was in heterozygous individuals relative to both homozygous genotypes. Greatest risk among heterozygous individuals was also observed for calpain 10 (24) and is well documented for HLA region genes and type 1 diabetes. In contrast, lowest trait values were observed with the GAD/GVI genotype. These observations could represent chance effects, but four of six traits tested were significant at $P < 0.05$ for an effect of haplotype combinations. We could find no other explanation for the observations. These heterozygous effects might explain the discrepancies between our studies and those previous studies that tested only single polymorphisms.

Several of our findings failed to confirm earlier studies. We could not demonstrate lower UCP2 mRNA levels in individuals carrying either the −866 G allele or the deletion variant; our findings (Fig. 1) were in the opposite direction: higher mRNA in individuals homozygous for the −866 G allele. These differences might result from different expression patterns in intra-abdominal vs. subcutaneous fat or other interacting factors that influence mRNA levels and differed between the studies. However, a limitation of our gene expression study was the relatively small sample size and the diverse nature of our population, which included both sexes and spanned a broad range of ages and BMIs.

As with earlier reports (27, 34), we found an effect of all three UCP2 variants on insulin secretion measured by the disposition index and to a lesser degree on AIR_G. However, the most striking effects were in interaction with family membership and not with the main effects of the −866 genotype that were observed by Krempler et al. (27). Furthermore, analysis of disposition index by genotype suggested that the most significant differences were between individuals homozygous for the common allele (G/G) and heterozygous individuals (A/G), rather than the additive effects observed by Krempler et al. and, more recently, using OGTT-derived indexes, by Sesti et al. (34). These differences may derive from the study of a larger number of subjects, such that individuals homozygous for the rare allele were better represented in our study. However, all of our subjects were drawn from 26 high-risk families. We have previously shown that disposition index in these families is highly heritable (15), and we identified the sulfonylurea receptor as one gene contributing to that heritability (18). The highly significant interaction between UCP2 variants and family membership suggests that family-specific factors such as sulfonylurea receptor variants and other interacting genes influenced the effect of UCP2 polymorphisms on insulin secretion. Other differences in our studies, including the younger age of our subjects or lower BMI, might also contribute to the differences in our observations and those of previous investigations. We examined the AIR_G from 2 to 10 min following glucose, rather than 3 to 5 min as in the study by Krempler et al. or the response to oral glucose in the recent study of Sesti et al.

Krempler et al. (27) found a lower frequency of the −866 G allele among their diabetic subjects than among controls, whereas our association was in the opposite direction. Different ascertainment might account for these discrepancies. Our diabetic group was ascertained from individuals with a strong family history of T2DM, and our control population was not particularly obese. In contrast, both the diabetic and control groups studied by Krempler et al. were obese, high-risk subjects. Additional studies are needed to define and resolve these discrepancies.

In summary, we find evidence that sequence variants, particularly combinations of the three variants previously described, alter BMI, insulin secretion, triglyceride levels, and diabetes risk. In our study, the effects on insulin secretion, although highly significant, were observed only in interaction with other family-specific factors. Furthermore, as in recent studies of calpain 10, we observed the greatest reduction of insulin secretion in individuals heterozygous for all three UCP2 polymorphisms. We find trends in UCP2 gene expression in subcutaneous adipose tissue that were opposite to those of a previous report in intraperitoneal fat. Our findings suggest that the three UCP2 variants interact and together are likely to have different impacts in different tissues and in the presence of different gene-gene or gene-environment interactions. Notably, earlier studies of the 3′UTR I/D variant and the A55V variant have yielded inconsistent results (11). These inconsistencies and the failure of many linkage studies to map either obesity or diabetes to this region (17, 35) are most consistent with a small effect of these polymorphisms on phenotypes related to diabetes and obesity. Very large studies that permit stratification by other genetic and environmental factors will be required to resolve these discrepancies.

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