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Putative role of polymorphisms in UCP1-3 genes for diabetic nephropathy

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Abstract

Increased production of reactive oxygen species (ROS) has been suggested as a cause of diabetic complications. Uncoupling proteins (UCPs) have been ascribed a role in reducing the formation of ROS, and genetic variation in genes encoding for UCPs could thus be putative candidate genes for diabetic nephropathy. To test this hypothesis we searched for association between the A → G (–3862) variant in UCP1, the insertion/deletion (I/D) polymorphism in exon 8 in UCP2, and the C → T (–55) polymorphism in UCP3 and diabetic nephropathy in 218 diabetic patients with normal urinary albumin excretion rate (AER), 216 with micro- or macroalbuminuria, and in 106 control subjects without a family history of diabetes. We did not find any association between the different polymorphisms and diabetic nephropathy, nor did we observe any difference in AER among carriers of different UCP1–3 genotypes. We could, however, confirm the reported association between BMI and the UCP3 –55 C → T polymorphism; patients carrying the T allele had higher BMI than patients homozygous for the C allele (26.4 ± 4.2 vs. 25.3 ± 4.3 kg/m²; $P = .01$). We conclude that studied polymorphisms in the UCP1–3 genes do not play a major role in the development of micro- or macroalbuminuria in Scandinavian diabetic patients.

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Keywords: Diabetes mellitus; Microalbuminuria; Uncoupling proteins; Polymorphisms

1. Introduction

Hitherto, studies on pathogenic mechanisms underlying the development of late diabetic complications have focused on three seemingly independent biochemical pathways, i.e., glucose-induced activation of protein kinase C (PKC) isoforms, increased formation of glucose-derived advanced glycation end (AGE) products, and increased flux through the aldose reductase pathway (RAAS). Hyperglycemia also increases production of reactive oxygen species (ROS) inside the cell. It has recently been shown that ROS may activate aldose reductase, induce diacylglycerol, activate PKC, induce AGE product formation, and activate the pleiotropic transcription factor nuclear factor B (NF-κB), suggesting that a unifying mechanism of induction, i.e., increased production of ROS, could serve as a link between elevated glucose and those three pathways. In addition,

normalizing mitochondria superoxide production in vitro can block these pathways (Nishikawa et al., 2000). Uncoupling of the oxidative phosphorylation with uncoupling proteins (UCPs) could also theoretically lead to inhibition of these pathways.

The UCPs represent a family of proteins that are able to dissipate the proton gradient in the inner mitochondrial membrane. The UCP1 is specific for brown adipose tissue, which is responsible for nonshivering thermogenesis in the newborn (Klingenberg, 1999). Human UCP2 is ubiquitous, expressed in white adipose tissue, kidney, heart, and pancreas (Fleury et al., 1997) whereas UCP3 is specific to skeletal muscle (Boss et al., 1997). The specific roles of UCP2 and UCP3 are still not clear (Dalgaard & Pedersen, 2001).

To address the question of whether UCP2, which is expressed in the kidney and has a capability of uncoupling oxidative phosphorylation and thereby reducing ROS formation, could have a role in diabetic nephropathy, we have studied the association between UCP2 insertion/deletion (I/D) polymorphism and diabetic nephropathy.

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Although not expressed in the kidney, UCP1 and UCP3 might have other indirect effects on albumin excretion rate (AER), e.g., through effects on insulin resistance (Forsblom et al., 1995) or dyslipidemia (Coonrod et al., 1993), both known risk factors for development of microalbuminuria. Therefore, we also included UCP1 and UCP3 in our study. Because little is known about the function of the known polymorphisms in UCP1–3 genes and no previous studies on association between these polymorphisms and diabetic nephropathy were available, we have chosen to also include polymorphisms that have previously been associated with increased BMI or weight gain.

2. Materials and methods

2.1. Patients

Data on 216 Scandinavian patients with diabetes mellitus and persistent micro- or macroalbuminuria were selected from a local diabetes registry (Diabetes 2000). Patients with known nondiabetic kidney disease were excluded. Inclusion criteria were at least two consecutive measurements with AER ≥ 20 $\mu\text{g}/\text{min}$ and the maximum AER value was selected. Thus, the patient was considered to have micro- or macroalbuminuria even if they had responded to the therapy with ACE inhibitors. Because Type 2 diabetic patients without retinopathy often suffer from nondiabetic kidney disease (Parving et al., 1992), only those Type 2 diabetic patients with micro- or macroalbuminuria and the presence of at least background retinopathy were included. A cohort of subjects with normal AER in at least three consecutive measurements and duration of diabetes for at least 10 years, matched for age, diabetes duration, and age at diagnosis of diabetes, glycated hemoglobin (HbA_{1c}), gender and type of diabetes, served as normoalbuminuric controls. In addition, a cohort of nondiabetic control subjects without a family history of diabetes ($n = 106$), matched for age and gender, was included (Table 1). All the genotypes were in Hardy–Weinberg equilibrium. All patients gave their informed consent and the Ethics Committee of Lund University approved the study.

2.2. Analytical techniques

Measuring the level of HbA_{1c} assessed metabolic control with HPLC (Mono S, Pharmacia LKB No. 17-0547-01). The reference range for healthy individuals with normal fasting blood glucose (fB-Glucose) was 3.6–5.0% for individuals < 50 years and 4.0–5.3% for individuals ≥ 50 years. One randomly selected HbA_{1c} value per year during the past 4 years was chosen, and the HbA_{1c} value that was used was calculated as the mean HbA_{1c} during these 4 years.

The albumin concentration in urine was determined by immunonephelometry (Beckman Instruments, CA, USA).

Table 1
Clinical characteristics of study patients

	Normoalbuminuria	Micro- or macroalbuminuria	Control subjects
Males/females	118/100	117/99	61/45
Types of diabetes ^a	128/88/2	127/87/2	–
Age (years)	54.3 \pm 14.7	55.9 \pm 14.6	55.0 \pm 14.1
Age at diagnosis (years)	30.4 \pm 18.3	32.6 \pm 18.6	–
Diabetes duration (years)	21.3 (14.5–29.5)	22.6 (14.8–29.5)	–
HbA _{1c} (%)	7.81 \pm 1.07	7.88 \pm 1.14	–
BMI (kg/m ²)*	25.1 \pm 3.9	26.6 \pm 4.3	26.2 \pm 4.6
AER ($\mu\text{g}/\text{min}$)***	5 (3–8)	243 (98–1041)	6 (4–9)
Antihypertensive medication***	89 (41.6%)	187 (87.0%)	43 (40.6%)
Myocardial infarction**	16 (8.4%)	30 (15.4%)	3 (2.9%)

Figures are mean \pm S.D. or median (interquartile range).

^a (Type 1/Type 2/Unknown).

* $P < .005$, normal AER vs. micro- or macroalbuminuria.

** $P < .05$, normal AER vs. micro- or macroalbuminuria.

*** $P < .001$, normal AER vs. micro- or macroalbuminuria.

The sensitivity of the method was 6 mg/l and intra- and interassay coefficient of variance (CV%) was < 5%. AER was calculated from timed overnight urinary specimens and reported as micrograms per minute.

2.3. UCP1–UCP3 polymorphisms

Genotyping of the UCP A \rightarrow G polymorphism (–3286) was performed with PCR using the following primers: 5'-CCAGTGGTGGCTAATGAGAGAA-3' and 5'-GCA-CAAAGAAGAAGCAGAGAGG-3'. PCR amplification was carried out in a volume of 20 μl containing 25 ng of genomic DNA, PCR buffer (Pharmacia Amersham), 5 $\mu\text{mol}/\text{l}$ of each primer, 4 mmol/l dNTP, 0.3 μl formamide, and 0.5 unit Amplitaq-polymerase (Pharmacia, Uppsala, Sweden). The cycling program was an initial denaturation at 94 $^{\circ}\text{C}$ for 3 min followed by 35 cycles of denaturation at 94 $^{\circ}\text{C}$ for 30 s, annealing at 55 $^{\circ}\text{C}$ for 30 s and elongation at 72 $^{\circ}\text{C}$ with a final elongation step at 72 $^{\circ}\text{C}$ for 5 min. The Bcl I endonuclease (New England Biolabs, Beverly, MA, USA) cut the A allele and the alleles were resolved on an agarose gel.

Genotyping of the UCP2 I/D polymorphism was performed with PCR using the following primers: 5'-CAGT-GAGGGAAGTGGAGG-3' and 5'-GGGCAGGACGAA GATTC-3' at annealing temperature of 56 $^{\circ}\text{C}$ and resolved on a 4% agarose gel.

Genotyping of the UCP3 C \rightarrow T polymorphism was performed with PCR as above using the following primers: 5'-CCTCCCCTCTCACCTCACTG-3' and 5'-GGCAC TGGTCTTATACCCAC-3' at annealing temperature of 56 $^{\circ}\text{C}$. The BsaJ I endonuclease (New England Biolabs) cut the C allele and the alleles were resolved on an agarose gel.

Table 2
UCP1-3 genotype and frequencies

	UCP1		UCP2		UCP3	
	G/G or A/G	A/A	I/I or I/D	D/D	T/T or C/T	C/C
Control subjects	38 (35.8%)	68 (64.2%)	59 (55.7%)	47 (44.3%)	55 (51.9%)	51 (48.1%)
Diabetic subjects						
Normoal-bumuria	97 (44.5%)	121 (55.5%)	115 (52.8%)	103 (47.2%)	104 (45.7%)	114 (52.3%)
Micro- or macroalbuminuria	84 (38.9%)	132 (61.1%)	105 (48.6%)	111 (51.4%)	110 (50.9%)	106 (49.1%)

Data are number of subjects (percent). The UCP2 I/D and UCP3 C → T (−55) polymorphisms were in linkage disequilibrium (93% of the subjects homozygous for the D/D genotype were homozygous for the UCP3 C allele; $P < .0005$).

2.4. Statistical analysis

The significance of differences between groups was tested by *t* test or Mann–Whitney nonparametric test using NCSS Statistical Software (UT, USA). *P* values were adjusted for multiple comparisons by multiplying the *P* value with the number of comparisons. *P* values of less than .05 were considered statistically significant. Allele frequency distribution was tested by χ^2 analysis.

3. Results

There were no significant differences in allele and genotype frequencies in the UCP1–3 polymorphisms between healthy control subjects, diabetic subjects with normal AER, and diabetic subjects with micro- or macroalbuminuria (Table 2). Stratifying the group with albuminuria in micro- and macroalbuminuria did not change the picture (data not shown). Neither was there any association between AER, plasma cholesterol, HDL-cholesterol, and plasma triglycerides with the exemption for the finding of lower HDL-cholesterol levels in patients carrying the G allele of UCP1 (Table 3). The G/G genotype has previously been reported to be associated with lower HDL-cholesterol level (Kiec-Wilk et al., 2002). We do not know whether this represents an effect of the G allele or linkage disequilibrium with the nearby located intestinal fatty acid binding protein 2 (FABP2), which has been associated with cholesterol and triglyceride levels (Carlsson, Orho-Melander, Hedenbro, Almgren, & Groop, 2000).

The UCP3 T allele was associated with higher BMI (Table 3); patients carrying the T allele (C/T or T/T) had higher BMI than patients homozygous for the C allele (C/C). There was no association between UCP1–3 polymorphisms and macrovascular disease using myocardial infarction as end point (data not shown).

4. Discussion

Several association studies have been published on the association of different gene polymorphisms in UCP1–3 genes and obesity. The UCP1 A → G (−3862) polymorphism in the promoter of the UCP1 gene has been associated with increased BMI (Hayakawa et al., 1999; Heilbronn et al., 2000) whereas other studies have failed to demonstrate any association between obesity and this UCP1 polymorphism (Gagnon et al., 1998; Urhammer et al., 1997). The I/D variant of UCP2 in the 3' untranslated region of exon 8 of UCP2 was found to be associated with increased sleeping metabolic rate and 24-h energy expenditure and with lower BMI in Pima Indians (Walder et al., 1998), and homozygous carriers of the insertion had increased BMI in a South Indian population (Cassell et al., 1999). The T allele in UCP3 C → T (−55) polymorphism was associated with increased waist to hip ratio in women (Cassell et al., 2000), and the T/T genotype with higher BMI (Otabe et al., 2000). The −55 T/T genotype was also found to be associated with an atherogenic lipid profile in French Caucasians, and the T/T genotype conferred decreased risk of Type 2 diabetes in French but not in Danish subjects

Table 3
BMI and urinary AER and plasma lipid concentrations in different UCP1-3 genotypes

	UCP1 A → G (−3286)		UCP2 I/D (exon 8)		UCP3 C → T (−55)	
	G/G or A/G	A/A	I/I or I/D	D/D	T/T or C/T	C/C
AER ($\mu\text{g}/\text{min}$)	11 (5–168)	13 (5–180)	11 (5–171)	14 (5–175)	12 (5–169)	12 (5–220)
BMI (kg/m^2)	26.0 ± 4.4	25.8 ± 3.9	25.6 ± 4.5	26.2 ± 4.0	26.4 ± 4.2	25.4 ± 4.0*
P-cholesterol (mmol/l)	4.97 ± 0.97	5.15 ± 1.07	5.03 ± 1.03	5.13 ± 1.04	5.13 ± 1.04	5.02 ± 1.02
P-HDL-cholesterol (mmol/l)	1.39 ± 0.43	1.52 ± 0.54*	1.45 ± 0.51	1.49 ± 0.49	1.47 ± 0.47	1.47 ± 0.53
P-triglycerides (mmol/l)	1.14 (0.79–1.74)	1.06 (0.72–1.51)	1.07 (0.77–1.59)	1.11 (0.73–1.67)	1.11 (0.79–1.72)	1.06 (0.72–1.55)

Figures are mean ± S.D. or median (interquartile range).

* $P = .01$ vs. carriers of the UCP3 T allele (T/T or C/T) and UCP1 G allele (GG or AG), respectively, after adjustment for multiple comparisons.

(Dalgaard et al., 2001; Meirhaeghe et al., 2000). However, studies on the UCP2–3 knockout mice do not support a major role for these genes in energy metabolism, e.g., the UCP2 null mice showed no changes in basal or cold-induced maintenance of body temperature, and the UCP null mice had normal body weight, fatty acid oxidation, exercise tolerance, and cold-induced thermogenesis. The results from these mice also suggest that UCP2–3 may be important for the regulation of ROS. The UCP2 null mice seem to produce less ROS than the wild-type mice (Arsenijevic et al., 2000), and the UCP3 null mice have increased production of ROS (Vidal-Puig et al., 2000). Recently, Eghtay et al. (2002) showed that superoxide activates UCP1–3 proteins and the uncoupling is induced by fatty acids.

The present study, to our knowledge, is the first to study a putative association between diabetic nephropathy and polymorphisms in the UCP1–3 genes. Chronic hyperglycemia not only generates AGE, but also ROS and attenuates antioxidative mechanisms through glycation of the scavenging enzymes. The UCPs are able to reduce the electrochemical gradient inside cells thereby reducing the production of ROS. In fact, the recently published studies of the UCP2 and UCP3 gene knockout mice suggest that a major role of these proteins would be to limit ROS formation in cells. The UCP2 in turn is widely expressed in all tissues including the kidney (Fleury et al., 1997; Gimeno et al., 1997) and could therefore play a role in the development of diabetic nephropathy.

Although genetic factors have been proposed to be responsible for the development and progression of diabetic nephropathy, duration of diabetes and glycemic control are still the most important factors for development of microvascular complications in diabetes mellitus (The Diabetes Control and Complications Trial Research Group, 1993; UK Prospective Diabetes Study (UKPDS) Group, 1998). Therefore, to be able to detect possible genetic differences, the study groups were carefully matched for duration of diabetes and glycemic control. Further, the long duration of diabetes in the normoalbuminuric group reduces the possibility of later development of microalbuminuria in patients with normal AER. Another concern is whether the elevated AER really reflects development of diabetic nephropathy, or if other factors such as hypertension, urinary infections, or heart disease can contribute to this elevation. Hypertension was common as well in patients with diabetes mellitus as in the control group; 87% of the patients with micro- or macroalbuminuria were treated with antihypertensive drugs (73.3% with ACE inhibitors) as well as 42% of the normoalbuminuric group and 41% of the controls. Therefore, we cannot rule out the possibility that hypertension could have contributed to the elevation in AER, especially in Type 2 diabetes. However, all the Type 2 diabetic patients with elevated AER had also at least background retinopathy, which would indicate that these patients had

also developed diabetic nephropathy (Parving et al., 1992). Of the patients with normal AER, 45 were treated with ACE inhibitors. This could of course be a possible source of misclassification because it is not known whether these patients would have developed micro- or macroalbuminuria without treatment. Therefore, we made a subanalysis excluding all the patients with treatment with ACE inhibitors or angiotensin 2 blockers. This subanalysis did not change the results (data not shown). We have also made a regression analysis with hypertension, treatment with ACE inhibitors, systolic and diastolic blood pressure, and the respective genotype as independent variables. This analysis did not show any significant association with micro- or macroalbuminuria and the polymorphism studied (data not shown). To minimize the risk of including patients with only temporary rise in AER we chose only patients with at least two consequent measurements over 20 $\mu\text{g}/\text{min}$. Furthermore, the AER was rather high, over 50% of the patients had macroalbuminuria and 75% had AER > 100 $\mu\text{g}/\text{min}$.

There was also a difference in the prevalence of macrovascular complications between the two groups with diabetes using myocardial infarction as end point. Out of the patients with micro- or macroalbuminuria, 15.3% and 8.4% of the patients with normal AER had had myocardial infarction. This finding is quite expected keeping in mind that microalbuminuria is also a marker for damage not only in the kidney but also to the cardiovascular system (Bigazzi, Bianchi, Baldari, & Campese, 1998). There was, however, no significant difference in the prevalence of myocardial infarction in Type 2 diabetic patients with and without micro- or macroalbuminuria.

The UCP2 protein is the most likely candidate to contribute to diabetic nephropathy as it is expressed in the kidney. We did not observe any significant association between the UCP2 exon 8 I/D variant and signs of nephropathy. Neither was there any association between UCP1 or UCP3 gene polymorphisms and micro- or macroalbuminuria. Although there might be some differences in the pathogenic mechanisms in the development of diabetic nephropathy, the course of renal abnormalities is similar in Type 1 and Type 2 diabetes (Mogensen, 1999). We have therefore chosen to pool the data between Type 1 and Type 2 diabetes. We also made a subanalysis separately in Type 1 and Type 2 diabetes, and no significant differences were observed in the genotype frequencies of the studied polymorphisms between the normoalbuminuric and micro- or macroalbuminuric groups (data not shown). Although the size of the study group was rather small, it was large enough to confirm the previously reported association between BMI and UCP3 C \rightarrow T polymorphism. We could also confirm a previously reported association between HDL-cholesterol and UCP1 A \rightarrow G polymorphism (Kiec-Wilk et al., 2002). Our conclusion is that the studied polymorphisms in UCP1–3 genes do not play a major role in the development of diabetic nephropathy in Scandinavian patients.

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