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Rapid communication

Sequence variations in the human Kir6.2 gene, a subunit of the beta-cell ATP-sensitive K-channel: no association with NIDDM in white Caucasian subjects or evidence of abnormal function when expressed in vitro

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Summary The ATP-sensitive K-channel plays a central role in insulin release from pancreatic beta cells. This channel consists of two subunits: a sulphonylurea receptor, SUR1, and an inwardly rectifying K-channel subunit, Kir6.2. We screened 135 white Caucasian patients with non-insulin-dependent diabetes mellitus (NIDDM) and 90 non-diabetic subjects for mutations in the Kir6.2 gene by single-stranded conformational polymorphism (SSCP) analysis. We identified one silent mutation (A190A) and four missense mutations (E23K, L270V, I337V and S385C) in normal and diabetic individuals. In a single diabetic subject, we identified a two-amino acid insertion (380KP). We also screened 39 Afro-Caribbean diabetic subjects and identified one additional missense (L355P) and one more silent (S363S) mutation. The E23K and I337V variants were completely linked. The common variants (E23K, I337V and L270V) were found with similar frequency in diabetic and normal subjects. Diabetic subjects with the variants responded normally to sulphonylurea therapy. When mutant Kir6.2 subunits were coexpressed with SUR1 in *Xenopus* oocytes, there was no difference in the sensitivity of the whole-cell currents to metabolic inhibition or to the sulphonylurea tolbutamide. We therefore conclude that mutations in Kir6.2 are unlikely to be a major cause of NIDDM. [Diabetologia (1996) 39: 1233–1236]

Keywords ATP-sensitive K-channel, Kir6.2, pancreatic beta cell, insulin secretion, non-insulin-dependent diabetes mellitus.

Non-insulin-dependent diabetes mellitus (NIDDM) is a common metabolic disorder characterised by both impaired insulin secretion and insulin resistance. Although there is good evidence for a genetic component associated with disease susceptibility, the genes associated with the common forms of NIDDM have not yet been identified. Several beta-cell genes involved in the regulation of insulin secretion have been shown to be associated with maturity-onset diabetes of the young, and certain unusual forms of NIDDM but these mutations account for only a small percentage of cases. This suggests that other genes involved in beta-cell stimulus-secretion coupling may be good candidates for investigation as disease susceptibility genes.

ATP-sensitive K-channels (K_{ATP} channels) play a key role in insulin secretion elicited both by glucose and by sulphonylureas [1]. The beta-cell K_{ATP} channel is a complex of (at least) two proteins: an inwardly rectifying K_{ATP} channel subunit, Kir6.2, and a sulphonylurea receptor, SUR1 [2, 3]. The latter acts as a drug-binding receptor, while it is believed that Kir6.2 forms at least part of the channel pore. Although no evidence for linkage between SUR1 and NIDDM was reported in a recent study of Mexican-American sibpairs [4], or between NIDDM and microsatellite markers close to the beta-cell K_{ATP} channel genes in Japanese subjects [5], we and others have identified sequence variants in SUR1 associated with NIDDM in Caucasians [6]. Given the central role of this channel in insulin secretion, we have now

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Abbreviations: NIDDM, Non-insulin-dependent diabetes mellitus; PCR, polymerase chain reaction; SSPC, single-stranded conformational polymorphism; bp, base pair; wt, wild type; SUR, sulphonylurea receptor; K_{ATP} ATP sensitive K-channel.

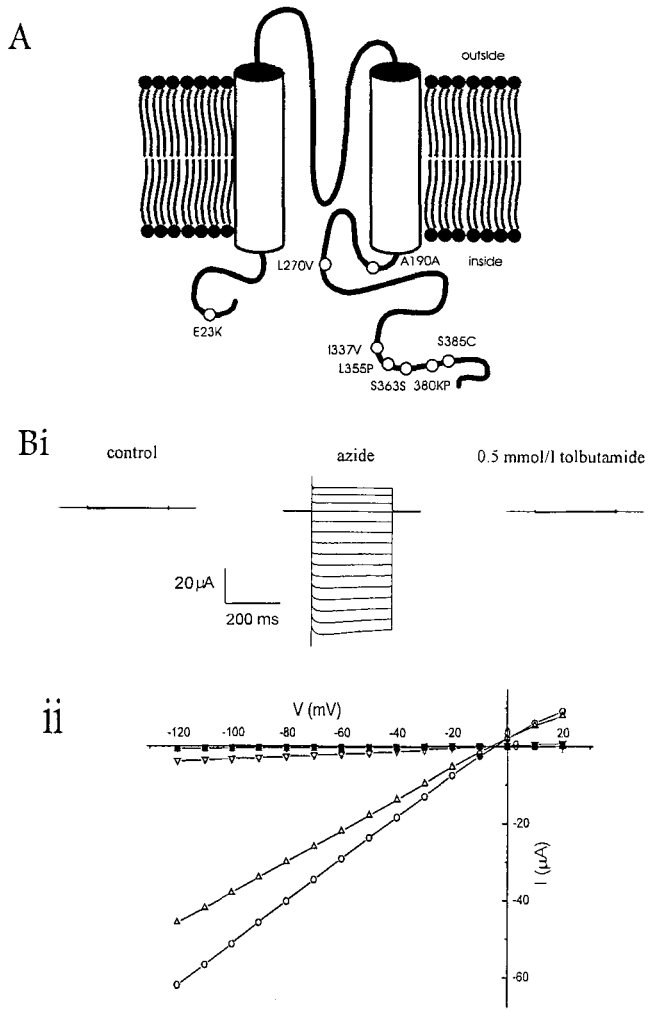

Fig. 1. **A** Putative topology of Kir6.2 showing the location of the mutations and polymorphisms identified. **B.i.** Whole-cell currents recorded from an oocyte injected with mRNA encoding wtKir6.2 and SUR1 in control solution (left), 10 min after exposure to 3 mmol/l azide (middle) and in azide plus 0.5 mmol/l tolbutamide (right). Currents were elicited by a series of voltage steps from −120 to +20 mV from a holding potential of −10 mV. **Bii.** Corresponding current-voltage (I-V) relationships recorded in the absence (•) and in presence (open symbols) of 3 mmol/l azide in the absence (○) and presence of 5 μmol/l (△) or 50 μmol/l (▽) tolbutamide

screened the Kir6.2 gene in Caucasians for mutations associated with NIDDM.

Materials and methods

Polymerase chain reaction (PCR) – single-stranded conformational polymorphism (SSCP) analysis. Genomic DNA was obtained from peripheral leukocytes from 90 normal humans and from 135 consecutive white Caucasian patients with NIDDM chosen from one centre of the UK Prospective Diabetes Study [7]. At diagnosis, these individuals had mean (± SD) age 52 ± 8 years, body mass index (BMI), 28.9 ± 5.5 kg · m^{-2}, fasting plasma glucose 12.3 ± 3.7 mmol/l and haemoglobin A_{1c} 9.5 ± 2.7 %.

The coding region of the Kir6.2 gene is contained within a single exon [2] and PCR primers were chosen to amplify this exon and the adjacent flanking regions: Sense (antisense) strand, Segment 1, 5'-GGTGCCTCCGATGGGGGAAG-3' (AAGGACATGGTGAAGATGAG); 2, GACCTCAAGTGGCCACACAC (TCCTCAGTCACCATGCGCCC); 3, CTTCCTTTTCTCCATTGAGG (ATCATGCTCTTGCGGAGGTC); 4, CCGCCTCTGCTTCATGCTAC (ACCACGCCTTCCAGGATGAC); 5, CCTGCACCACCACCAGGACC (GTGTGGGCACTTTGATGGTG); 6, GTGGACTACTCCAAGTTTGG (GGCTACATACCACATGGTCC). In order to maximize the possibility of detecting genetic variations by SSCP, the amplified DNA fragments were designed to overlap one another and to be smaller than 300 base pairs (bp). SSCP was performed using the method of Sakura et al. [8] except for annealing temperatures of 55 °C (segments 2, 3, 4 and 5) or 60 °C (segments 1 and 6). When a polymorphism was identified, the PCR fragment was subcloned and sequenced. More than two independent clones were sequenced for each polymorphism.

Electrophysiology. Mutant Kir6.2 DNA was amplified from genomic DNA using the sense and antisense primers for segments 1 and 6, respectively, cloned into the PCRII expression vector, and sequenced to confirm accurate amplification. In vitro synthesis of mRNA and oocyte injection and handling were carried out as previously described [10]. Rat SUR1 mRNA (25 ng) and Kir6.2 (or mutant Kir6.2; 100 ng) were mixed and approximately 50 nl of solution injected per oocyte. Whole-cell currents were measured 2 days after injection using a 2-electrode voltage-clamp (Geneclamp 500; Axon Instruments, Foster City, Calif., USA) at 18–24 °C. The external (bath) solution contained (mmol/l): 90 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (pH 7.4 with KOH). Tolbutamide was dissolved in dimethyl sulphoxide (DMSO), and Na-azide in water. Recorded currents were filtered at 1 kHz, digitised at 4 kHz and measured 280–295 ms after the start of the voltage pulse.

Statistical analysis

Frequencies of genotypes between normal and diabetic subjects were assessed by chi-square using SAS. Differences in phenotypic variables at diagnosis of diabetes, between subjects with different genotypes, were assessed by analysis of variance adjusting for age, gender and BMI. Insulin sensitivity was assessed by homeostasis model assessment [9] from fasting plasma glucose and insulin concentrations and is expressed in terms of insulin sensitivity (% S), with 100 % being the mean of normal subjects, aged 18–25 years with BMI 18–25 kg · m^{-2}.

Results

Identification of genetic variations detected by SSCP. Normal and NIDDM white Caucasian subjects were screened for polymorphisms in the Kir6.2 gene by SSCP under two different conditions (the presence or absence of 10 % glycerol). Six differences were detected (Fig. 1 A). Direct sequence mucleotide analysis demonstrated that one of these polymorphisms was a silent mutation, A190A (GCT to GCC), but that four resulted in amino acid changes, these being E23K

Table 1A. Genotypic frequencies of the missense variants in normal and NIDDM subjects

	Genotype	Caucasian normal *n*	Caucasian NIDDM *n*	Afro-Caribbean NIDDM *n*
E23K	E/E	44 (0.54)	38 (0.38)	31 (0.94)
	E/K	27 (0.33)	45 (0.45)	2 (0.06)
	K/K	11 (0.13)	17 (0.17)	0
L270V	L/L	73 (0.88)	89 (0.88)	32 (0.97)
	L/V	9 (0.11)	11 (0.11)	1 (0.03)
	V/V	1 (0.01)	1 (0.01)	0
I337V	I/I	44 (0.54)	46 (0.39)	32 (0.91)
	I/V	27 (0.33)	56 (0.47)	3 (0.09)
	V/V	10 (0.12)	16 (0.14)	0
L355P	L/L	81	118	34
	L/P	0	0	1
380KP	–KP	81	117	35
	+KP	0	1	0
S385C	S/S	80	117	34
	S/C	1	1	1

Numbers in parentheses give frequency of the missense variant as a proportion of the total number of subjects

Table 1B. Electrophysiological properties of Kir6.2 variants

Clone	Control I (μA)	Azide I (μA)	5 μmol/l Tolbutamide % block	0.5 mmol/l Tolbutamide % block
Water	0.33 ± 0.08	0.25 ± 0.03	NT	100 ± 0
Wild type	0.95 ± 0.23	42.5 ± 5.1	31.8 ± 2.4	98.7 ± 0.3
23K/337V	1.12 ± 0.23	53.1 ± 7.1	35.3 ± 6.5	98.8 ± 0.3
23K/337V/270V	0.65 ± 0.14	50.8 ± 8.1	30.9 ± 9.5	98.3 ± 0.5
L355P	0.57 ± 0.06	34.8 ± 5.0	51.6 ± 2.2[a]	98.5 ± 0.5
23K/337V/380KP	0.88 ± 0.24	41.5 ± 7.5	31.4 ± 2.2	98.1 ± 0.7
S385C	0.87 ± 0.15	52.9 ± 6.1	39.1 ± 3.9	98.8 ± 0.5
n	6	7	4	4

Wild type = E23, I270 and I337. Each clone was coinjected with SUR1. Data are mean ± SEM. NT, not tested. [a] Significantly different at $p = 0.05$ with respect to wild type. All other data were not significantly different from wild type (Student's *t*-test). Currents were measured at –100 mV. The degree of tolbutamide block was determined by measuring the current in presence of 3 mmol/l azide, then in 3 mmol/l azide plus 5 μmol/l tolbutamide and finally in 3 mmol/l azide plus 0.5 mmol/l tolbutamide

(GAG to AAG), L270V (CTG to GTG), I337V (ATC to GTC) and S385C (TCT to TGT). In addition, we detected an insertion of two amino acids, KP (AAGCCC), after position 380 in one diabetic individual. In all cases where the full length Kir6.2 DNA was sequenced (> 10 individuals), we found that the residue at position 148 was I (ATC) not S (AGC) as previously reported [2]. The E23K, I377V and L270V variants were highly polymorphic, and found with similar frequency in normal and NIDDM subjects (Table 1).

We found the E23K and I337V variants to be completely linked in individuals in which we were able to examine both polymorphisms. Thus, all individuals possessing E at residue 23 also had I at 337, while those having K at position 23 also had V at 337. Furthermore, V at position 270 appeared to be linked to V at position 337 (and thus also to K at 23). This was confirmed by full-length sequencing of Kir6.2 in more than ten patients.

No significant differences were found in the clinical characteristics of diabetic subjects (at diagnosis of diabetes) with different E23K or I270V genotypes. However, for L270V, 11 heterozygous LV individuals had a higher fasting plasma insulin concentration than 89 homozygous LL individuals (geometric mean (SD range)) 151 (92–248) vs 88 (46–166) pmol/l respectively). No differences were observed in BMI, waist-to-hip ratio, fasting plasma glucose, HbA_{1c}, triglyceride, total cholesterol, HDL cholesterol or LDL cholesterol levels. The plasma insulin difference for LL/VV vs. LL was significant at $p = 0.0063$ taking into account age (LV = 60 ± 4 years, LL = 51 ± 8 years), BMI (LV = 30 ± 5, LL = 28 ± 6 kg/m^2) and gender (LV = 36 % male, LL = 70 % male). Insulin sensitivity showed a similar difference (% S, 26 (17–39) vs 51 (26–101), $p = 0.0035$ adjusting for age, gender and BMI) but neither insulin nor %S were significant with a Bonferroni correction. Only one patient, aged 52 years, BMI 27 kg/m^2, was homozygous for the V allele (plasma insulin 161 pmol/l, matched that of the heterozygotes). The systolic blood pressures in those with LV and LL were 147 ± 26 and 136 ± 16 mm Hg, respectively (NS) and

the diastolic blood pressures were 92 ± 15 and 84 ± 9 mm Hg, respectively ($p = 0.02$ adjusting for age, gender and BMI, but NS with a Bonferroni correction). Four diabetic subjects with the L270V polymorphism were treated with sulphonylurea therapy, two with chlorpropamide and two with glibenclamide, and responded normally.

We also examined 39 Afro-Caribbean NIDDM subjects. One subject had new mutations, L355P (CTA to CCA) and S363S (TCA to TCG; which is silent), but otherwise the same polymorphisms were identified as in white Caucasian subjects (Table 1 A).

To determine if the mutations and polymorphisms we identified produced functional changes in K_{ATP} channel activity, or markedly altered the level of channel expression, we examined the effect of metabolic inhibition, and of tolbutamide, on K_{ATP} currents heterologously expressed in *Xenopus* oocytes. A marked increase in whole-cell K-currents was observed in oocytes injected with wild-type (wt) Kir6.2 and SUR1 following 10 min incubation with 3 mmol/l azide, an inhibitor of mitochondrial metabolism (Fig. 1 B, Table 1 B), and these currents were fully blocked by 0.5 mmol/l tolbutamide. This indicates that wtKir6.2 and SUR1 form functional K_{ATP} channels in *Xenopus* oocytes, which are normally suppressed by resting ATP levels but which can be activated by metabolic inhibition. There was no significant difference in the magnitude of the azide-activated currents when whole-cell K_{ATP} currents were recorded from oocytes injected with SUR1 and mutant Kir6.2 containing the polymorphisms/mutations observed in man (Table 1 B). Likewise, there was no significant difference in tolbutamide sensitivity, with the exception of L335P which appeared to be slightly more sensitive.

Discussion

We conclude that mutations in Kir6.2 are unlikely to be a major cause of NIDDM, since we observed no difference in the genotypic frequency of the mutations we identified between normal and NIDDM individuals. In addition, when mutant Kir6.2 co-expressed with SUR1 in *Xenopus* oocytes, the K_{ATP} currents appeared to be grossly normal, being activated by metabolic inhibition and blocked by tolbutamide.

All of the mutations we identified were found in the cytosolic domains of the channel, with E23K being located in the N-terminal tail and the others in the C-terminal tail (Fig. 1 A). Most polymorphisms were conservative (e.g. L270V; I337V) and unlikely to result in changes in protein function. Although the mutations we identified were not associated with NIDDM, and do not produce marked changes in the functional properties of heterologously expressed K_{ATP} currents, it is possible that they may alter channel function in more subtle ways. The association of L270V with impaired insulin sensitivity raises the possibility that this mutation may affect insulin action in muscle, in which Kir6.2 is expressed [2, 3]. However, this association was not significant after a Bonferroni correction and needs cautious interpretation until further studies have been done.

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