Decreased Tolbutamide-Stimulated Insulin Secretion in Healthy Subjects With Sequence Variants in the High-Affinity Sulfonylurea Receptor Gene

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The high-affinity sulfonylurea receptor (SUR1) is, as a subunit of the ATP-sensitive potassium channel, an important regulator of insulin secretion in the pancreatic β-cell. The aim of this study was to examine if genetic variability of the *SUR1* gene was associated with NIDDM or altered pancreatic β-cell function. Mutational analysis of all the 39 SUR1 exons, including intron-exon boundaries, in 63 NIDDM patients revealed two missense variants, five silent variants in the coding region, and four intron variants. The two missense variants (Asp673Asn and Ser1369Ala) and two sequence variants (ACC→ACT, Thr759Thr and a c→t intron variant in position –3 of the exon 16 splice acceptor site) were examined for association with NIDDM and for a possible influence on insulin and C-peptide secretion after intravenous glucose and tolbutamide loads in a random sample of unrelated, healthy, young Danish Caucasians. The Asp673Asn variant in exon 14 was only identified in one NIDDM patient, and the allelic frequency of the Ser1369Ala was similar among 247 control subjects (0.38 [95% CI 0.34–0.42]) and 406 NIDDM patients (0.40 [0.37–0.43]). The allelic frequency of the silent exon 18 Thr775Thr variant was 0.051 (0.035–0.067) in NIDDM patients (n = 392) and 0.027 (0.013–0.041) in control subjects (n = 246; χ^2 = 4.99, P = 0.03). The allelic frequency of the intron variant was similar among NIDDM patients (0.45 [0.42–0.48]) and control subjects (0.44 [0.40–0.48]). Of 386 NIDDM patients, 17 had the combined genotype exon 18 C/T and intron –3c/–3t (0.044 [0.024–0.064]), whereas 3 of 243 control subjects had the same combined genotype (0.012 [0–0.026]; χ^2= 4.87, P = 0.03; odds ratio: 3.69 [1.07–12.71]). Of 380 unrelated, healthy, young Danish Caucasians, 10 (0.026 [0.010–0.042]) had the combined at-risk genotype. These subjects had, on average, a 50% reduction in serum C-peptide and a 40% reduction in serum insulin responses upon tolbutamide injection (P = 0.002 and P = 0.05, respectively) but normal serum C-peptide and insulin responses upon glucose injection. In conclusion, a silent polymorphism in exon 18 of the *SUR1* gene is associated with NIDDM in a Danish Caucasian population. In combination with an intron variant, the association is higher, and young, healthy carriers of the intragenic combination have reduced serum C-peptide and insulin responses to a tolbutamide load. *Diabetes* 47:598–605, 1998

NIDDM, which is both a phenotypically and a genetically heterogeneous disorder (1), is characterized by hyperglycemia due to defects in insulin secretion and action (2). Despite the known inherited susceptibility to this disorder, only a small percentage of the late-onset NIDDM patients carries genetic variants shown to be associated with the disease (3–6).

A diminished acute insulin secretory response to intravenous glucose or sulfonylurea loads is a frequent feature of late-onset NIDDM. The mechanism of sulfonylurea action is through inhibition of an ATP-sensitive potassium channel (K_{ATP}), which controls the resting membrane potential of the pancreatic islet β-cell (7). A reduction of potassium outflow triggers β-cell depolarization with opening of voltage-gated calcium channels and influx of calcium and subsequently exocytosis of insulin. Recently, it was demonstrated that the K_{ATP} channel is a complex composed of at least two subunits, the high-affinity sulfonylurea receptor (SUR1) that appears to be the ATP/ADP sensor of the K_{ATP} (8) and a member of the inwardly rectifying ion channel family called BIR or Kir6.2 (9). The key role of SUR1 in the regulation of insulin secretion was shown in studies of subsets of patients with familial persistent hyperinsulinemic hypoglycemia of infancy, an autosomal recessive disorder characterized by unregulated hypersecretion of insulin (10). Also, a recent molecular scanning of selected exons of the *SUR1* gene identified sequence variants associated with late-onset NIDDM (11).

The present study was undertaken to examine the entire *SUR1* gene, including intron-exon boundaries, for sequence variants associated with NIDDM and for a possible influence

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K_{ATP}, ATP-sensitive potassium channel; PCR, polymerase chain reaction; QTL, quantitative trait locus; S_G, glucose effectiveness; S_I, insulin sensitivity index; SSCP, single-strand conformation polymorphism; SUR, sulfonylurea receptor.

of genetic variants on intravenous glucose and tolbutamide-stimulated insulin release in young healthy subjects.

RESEARCH DESIGN AND METHODS

Subjects. Forty-nine NIDDM patients recruited from the outpatient clinic at Steno Diabetes Center, Copenhagen, were initially screened by single-strand conformation polymorphism (SSCP) analysis. Subsequently, another group of 14 NIDDM patients with the exon 18/exon 16 (nt –3) SUR1 at-risk genotype were screened by SSCP. Association studies were performed in 449 unrelated Danish Caucasian NIDDM patients recruited from the outpatient clinic at Steno Diabetes Center, Copenhagen, and 250 age-matched, unrelated, and glucose-tolerant Danish Caucasian control subjects traced in the Danish Central Population Register and living in the same area of Copenhagen as the NIDDM patients. Diabetes was diagnosed by World Health Organization criteria, and all control subjects underwent a standard 75 g oral glucose tolerance test.

For studies of intravenous glucose- and tolbutamide-stimulated serum insulin and C-peptide release, 380 study participants were randomly recruited from a population of young individuals aged 18–32 years, who in 1979–1980 and again in 1984–1985 as children had participated in blood pressure surveys in a representative and specified part of Copenhagen city (12). All were Danish Caucasians by self-identification. Physiological characteristics of these subjects have been presented previously (12). Before participation in the study, informed consent was obtained from all subjects. The study was approved by the Ethical Committee of Copenhagen and was in accordance with the principles of the Declaration of Helsinki.

Measurements of intravenous glucose- and tolbutamide-stimulated serum insulin and C-peptide responses. Each subject underwent an intravenous glucose tolerance test after a 12-h overnight fast. Baseline values of serum insulin, serum C-peptide, and plasma glucose were taken in duplicate in 5-min intervals. Glucose was injected intravenously in the contralateral antecubital vein over a period of 1 min (0.3 g/kg body wt of 50% glucose). At 20 min following the end of the glucose injection, a bolus of 3 mg tolbutamide/kg body weight (Rastinon; Hoechst, Germany) was injected over 5 s to elicit a secondary pancreatic β-cell response. Venous blood was sampled at 2, 4, 8, 19, 22, 30, 40, 50, 70, 90, and 180 min (timed from the end of the glucose injection) for measurements of plasma glucose, serum insulin, and serum C-peptide. All intravenous glucose tolerance tests were done by the same investigator. Glucose-induced acute serum insulin and C-peptide responses (0–8 min) were calculated by means of the trapezoidal rule as the incremental values (area under the curve when expressed above basal values). Serum insulin and C-peptide responses upon tolbutamide injection (19–22 min) were calculated as the difference between measurements obtained at the two time points. The tolbutamide-stimulated serum insulin and C-peptide responses during 19–30 min were calculated by means of the trapezoidal rule as the incremental values (area under the curve when expressed above values obtained at 19 min). Insulin sensitivity index (S_I) and glucose effectiveness (S_G) were calculated using the Bergman MINMOD computer program developed specifically for the combined intravenous glucose and tolbutamide tolerance test (13,14). Serum insulin was determined by enzyme-linked immunosorbent assay with a narrow specificity excluding des(31,32)- and intact proinsulin (15). The serum concentration of C-peptide was determined by radioimmunoassay using the polyclonal antibody M1230 (16–18).

Preparation of genomic DNA from whole blood. Genomic DNA was isolated from human leukocyte nuclei isolated from whole blood by proteinase K digestion followed by phenol-chloroform extraction on an Applied Biosystems 341 Nucleic Acid Purification System (Foster City, CA), or proteins were precipitated by addition of a saturated NaCl solution. Subsequently, genomic DNA was ethanol precipitated (19).

SSCP-heteroduplex analysis. Polymerase chain reaction (PCR) amplification, SSCP analysis, and heteroduplex analysis were performed at Steno Diabetes Center as previously described (20). Oligonucleotide sequences for PCR amplification of the 39 SUR1 exons and intron-exon boundaries were designed from the genomic SUR1 sequence (G. Gonzales, L.A.-B., J.B., Genbank accession no. L78208–L78223, L78225–L78243, L78254, and L78255) to generate PCR products of 110–318 bp (Table 1). Exons are numbered in consecutive order from the 5′ end of the gene. In previous reports on polymorphisms in the *SUR1* gene, exons were numbered from the 3′ end of the gene (11). Variants identified 3 bp before the intron/exon 16 boundary, in exon 18, and in exon 33 were previously termed

TABLE 1
Nucleotide sequences of DNA primers, PCR product sizes, and annealing temperatures for SSCP analysis of the 39 SUR1 exons and intron-exon boundaries

Exon	Sequence	Product size (bp)	Annealing temperature (C°)
1a	5′-gcg-gag-cca-gcg-gag-cca-g-3′	232	65
1b	5′-ccc-ctc-ctc-cgc-ggc-tcg-3′		
2a	5′-atg-cac-cct-ctt-cct-cct-cca-ag-3′	255	60
2b	5′-agg-aag-tac-cct-gga-gca-gat-tca-3′		
3a	5′-ccc-cat-cct-gtc-tgt-ttc-tc-3′	186	65
3b	5′-gga-ctg-ccc-ctc-cgt-cct-a-3′		
4a	5′-gta-cac-aca-tga-tgc-aca-cac-g-3′	275	60
4b	5′-agg-aca-gag-cca-gag-cct-ct-3′		
5a	5′-gag-aag-ttg-act-tac-cca-tcc-3′	318	60
5b	5′-agt-gaa-tag-atg-gtg-tgc-tgt-3′		
6a	5′-gtg-aat-tag-ccc-tca-ggc-ac-3′	279	66
6b	5′-cca-tct-aga-ggg-tgc-ctt-ac-3′		
7a	5′-tca-ttt-tgc-tgg-tca-atg-ac-3′	253	66
7b	5′-ggt-tct-tat-ggc-aaa-gtg-aa-3′		
8a	5′-agc-ccc-tgg-cac-act-gta-aca-g-3′	281	60
8b	5′-tgt-cct-gct-gcc-ccc-ct-3′		
9a	5′-gca-ggg-caa-cca-tca-gg-3′	254	60
9b	5′-gag-gag-acc-tgc-tgc-tgt-cga-3′		
10a	5′-gaa-gcc-tct-ccc-ctc-tga-ct-3′	250	60
10b	5′-tct-cct-tgc-atg-tac-gca-gc-3′		
11a	5′-ctc-caa-gcc-atc-ctg-ggc-at-3′	240	60
11b	5′-ctg-gct-gtg-gag-cct-gtc-tt-3′		
12a	5′-ggg-acc-cac-acc-ctg-gcc-ac-3′	240	60
12b	5′-cag-acc-tgc-ctg-ccc-cag-tg-3′		
13a	5′-agc-tct-atc-agg-ctg-cgc-ct-3′	216	60
13b	5′-tgc-tgg-gag-tag-caa-ggg-ga-3′		

Continued on next page

as variants in intron 24, exon 22, and exon 7, respectively.

Direct sequencing. DNA was amplified as described above using biotinylated primers. Single-stranded DNA was generated using streptavidine-coated magnetic beads according to the manufacturer's recommendations (Dynal A/S, Oslo, Norway). Dideoxynucleotide sequencing using Version 2.0 DNA Sequencing Kit (United States Biochemical Corporation, Cleveland, OH) and [α-^{35}S]dATP (Amersham, Buckinghamshire, U.K.) was performed according to standard procedures.

Detection of sequence variants in the *SUR1* gene. PCR amplification of the DNA segments containing the SUR1 exon 18 C/T variant and the exon 16 (nt –3) c/t variant was carried out in a volume of 25 µl, containing 100 ng of DNA, 200 mmol/l of each dNTP, 1 mmol/l $MgCl_2$ (exon 18 variant), 1.5 mmol/l $MgCl_2$ (exon 16 (nt –3) variant), 5 pmol of each primer (exon 18: forward primer 5′-aga-gct-cag-

TABLE 1
(Continued)

Exon	Sequence	Product size (bp)	Annealing temperature (C°)
14a	5′-cca-ggg-aag-agg-ggc-tcc-ct-3′	240	60
14b	5′-gct-ccc-tct-ggg-gat-tgg-tg-3′		
15a	5′-gtg-tct-ttt-ggc-ttt-cat-gg-3′	264	60
15b	5′-caa-taa-atg-cag-ctt-tgt-ct-3′		
16a	5′-tct-ggg-taa-tgg-ttg-ttc-ag-3′	257	60
16b	5′-tcc-aat-aaa-tgt-gtg-tgc-at-3′		
17a	5′-ggg-gtg-gga-act-aac-tgg-tg-3′	110	58
17b	5′-ctt-ctg-acc-cca-gtc-cca-ag-3′		
18a	5′-tct-tcc-tgg-agg-ata-tgg-tt-3′	224	60
18b	5′-cct-ggt-cct-ccc-cca-aca-ct-3′		
19a	5′-tct-gag-aac-aag-ccc-tga-ga-3′	255	60
19b	5′-ggg-tgt-ggg-tga-tcg-atg-ga-3′		
20a	5′-act-aat-gac-cct-ctg-caa-gt-3′	260	60
20b	5′-cct-att-tcc-tag-tta-ccc-at-3′		
21a	5′-tag-tcc-agt-agg-gta-cag-ga-3′	180	60
21b	5′-tgg-tgg-ggt-tgt-cgg-ggg-ag-3′		
22a	5′-cta-ccc-tct-act-ctc-ttc-ct-3′	250	66
22b	5′-gga-ttg-gtt-cct-gcc-aag-at-3′		
23a	5′-att-tga-gtg-agg-aaa-ggg-atg-t-3′	256	63
23b	5′-ctt-ggc-cat-ccc-tgg-ata-t-3′		
24a	5′-cca-tat-atc-cta-aga-cat-tgc-c-3′	179	60
24b	5′-agg-cca-taa-ttt-cac-tcc-ca-3′		
25a	5′-aca-gag-tgg-ttc-ttg-cgg-cc-3′	284	60
25b	5′-tag-ggc-gcc-agt-cac-ac-3′		
26a	5′-cac-cct-gga-act-cca-aat-ca-3′	255	60
26b	5′-aga-gaa-cag-tgg-act-tgc-tc-3′		
27a	5′-tga-atg-act-cca-gag-aca-ctt-a-3′	245	55
27b	5′-aga-cag-gag-aag-ccc-cca-g-3′		
28a	5′-caa-aat-tca-cct-cac-tct-ct-3′	243	68
28b	5′-ctg-cac-att-gca-aag-cac-ct-3′		
29a	5′-att-cac-cca-cac-agc-ggt-a-3′	304	60
29b	5′-gag-aga-gca-acg-tgt-cct-tg-3′		
30a	5′- gct-atg-tgg-acc-aac-act-gt-3′	248	60
30b	5′-gta-tcc-tat-cct-ctc-ttt-ca-3′		
31a	5′-ggt-aga-aca-ggg-tcc-tgt-gg-3′	240	61
31b	5′-gac-gaa-ggt-gct-ccg-gga-gt-3′		
32a	5′-aga-aga-gcc-att-ctg-gtg-gt-3′	248	53
32b	5′-cga-cac-act-cct-cct-tgg-ac-3′		
33a	5′-cca-caa-gga-ggc-tgg-gga-tg-3′	266	60
33b	5′-cag-cat-tgg-gtt-ggg-ccc-gt-3′		
34a	5′-cac-ctg-cac-aca-cac-cca-ga-3′	239	60
34b	5′-tga-cca-cgt-gcc-agg-gct-ga-3′		
35a	5′-gac-agg-agg-ccc-agc-agt-ca-3′	240	60
35b	5′-cct-ctt-tgt-gct-cca-gat-ct-3′		
36a	5′-cac-tgc-cct-ctt-tgc-cgc-ct-3′	264	68
36b	5′-aac-ctg-aga-cac-ggg-ctt-ct-3′		
37a	5′-atc-cca-tct-gct-cca-ctc-ac-3′	254	60
37b	5′-atc-cca-cta-aac-cct-ttc-cag-3′		
38a	5′- cca-ggc-cag-acc-cca-tcc-ac-3′	153	60
38b	5′-tcc-ctc-tgc-acc-cca-tca-at-3′		
39a	5′-cac-agt-gac-agg-aca-ttc-t-3′	312	66
39b	5′-gta-tgg-gca-ggg-tcc-gaa-t-3′		

For each exon, forward primers are indicated by a, reverse primers by b.

TABLE 2
Genetic variants identified in the *SUR1* gene in 63 NIDDM patients

Location	Codon	Nucleotide change	Amino acid change
Exon			
2	69	CCT → CCC	Pro → Pro
12	562	CAT → CAC	His → His
14	649	AAG → AAA	Lys → Lys
14	673	GAT → AAT*	Asp → Asn
18	759	ACC → ACT*	Thr → Thr
31	1273	AGG → AGA	Arg → Arg
33	1369	TCC → GCC*	Ser → Ala
16 (nt –3)		c → t*	
26 (nt +6)		c → t	
34 (nt –25)		t → c	
39 (nt –100)		+ c (insertion)	

Codon positions are according to the short alternatively spliced SUR1 cDNA sequence (GenBank accession no. L78207; L78223). Intron variants are located according to the number of nucleotides before (nt –) or after (nt +) an exon. *Nucleotide variants examined in associations studies.

TABLE 3
Genotype and allele frequencies of the SUR1 exon 18, codon 759, ACC (Thr) → ACT (Thr) polymorphism

	Control subjects	NIDDM subjects	χ^2	*P* value
n	246 of 250	392 of 449	—	—
Genotype				
C/C	233 (0.95)	352 (0.90)	—	—
C/T	13 (0.05)	39 (0.10)	—	—
T/T	0 (0.00)	1 (0.00)	4.80, 1 df*	0.03
Allele				
C	479 (0.97)	743 (0.95)	—	—
T	13 (0.03)	41 (0.05)	4.99, 1 df	0.03

*C/T and T/T genotypes are combined in the χ^2 analysis.

tat-gcc-ttt-cc-3′, reverse primer 5′-ggt-gat-gtg-gct-ccc-ttg-g-3′; exon 16 (nt –3): forward primer 5′-ccc-ggc-ccc-act-cac-atc-tg-3′, reverse primer 5′-gga-gga-tgg-tta-aaa-gga-gat-t-3′), 0.3 units of Taq DNA polymerase (Promega), 50 mmol/l KCl, 10 mmol/l Tris-HCl, and 0.1% Triton X-100. The exon 16 (nt –3) reverse primer was fluorescently labeled at the 5′ end. The PCR (Perkin Elmer 9600) started with denaturation at 95°C for 3 min, followed by 36 cycles of denaturation (95°C, 30 s), annealing (58°C, 30 s), and extension (72°C, 30 s), with a final extension at 72°C for 9 min. The amplified exon 18 and exon 16 (nt –3) products, 25 µl and 1 µl, respectively, were digested by restriction enzymes in a total volume of 30 µl and 10 µl for at least 2 h with 2 units of *Bsa*O1 (Promega) and 5 units of *Pst* I (New England Biolabs), respectively. The exon 18 fragments (wild-type: 91 and 54 bp; heterozygous: 145, 91, and 54 bp; homozygous: 145 bp) were resolved on a 4% agarose gel (2% GTG Nusieve, 2% Seakem Agarose) and visualized under ultraviolet illumination after being stained with ethidium bromide. The exon 16 (nt –3) fragments (wild-type: 164 and 38 bp; heterozygous: 202, 164, and 38 bp; homozygous: 202 bp) were separated by acrylamide electrophoresis and analyzed on an ABI 373 automated sequencer. Fluorescently labeled fragments were seen as wild-type: 164 bp; heterozygous: 202 and 164 bp; and homozygous: 202 bp. SSCP was used for screening for the exon 14 G/A variant. The exon 33 variant was screened for using forward primer 5′-agg-gag-agg-ggt-ggg-aag-agt-cca-a-3′ and reverse primer 5′-att-ggg-ttg-ggc-ccg-tgc-act-gac-3′. The PCR used an annealing temperature of 66°C and 1.0 mmol/l $MgCl_2$. The PCR product was digested with *Mwo* I, and products (wild-type: 206 and 82 bp; heterozygous: 206, 82, and 41 bp; homozygous: 206 and 41 bp, respectively) were visualized on an agarose gel.

Statistics. χ^2 analysis with Yates correction when appropriate was applied to test for significance of differences in allele and genotype frequencies. A nonparametric Kruskall-Wallis test or a Mann-Whitney test was used for comparison between groups. To control for possible confounders on the tolbutamide-induced serum insulin and C-peptide responses, multiple regression analyses were performed. Fasting plasma glucose or plasma glucose at 19 min, sex, BMI, S_I, S_G, and the combined genotypes exon 18 C/T and exon 16 (nt –3) c/t were entered as explanatory variables. Serum insulin and C-peptide responses (19–22 min and 19–30 min, respectively) were response variables. If necessary, the variables included in the analysis were logarithmically transformed. Data are means ± SE. A *P* value < 0.05 (two-tailed) was considered significant. Statistical Package of Social Science (SPSS) for Windows, version 6.01, was used for statistical analysis.

RESULTS

SSCP-heteroduplex scanning in 49 NIDDM patients of the coding region, including intron-exon boundaries of the *SUR1* gene, and direct sequencing revealed 11 sequence variants (Table 2). Additional SSCP-heteroduplex scanning of 14 NIDDM patients carrying the combined SUR1 at-risk genotype did not reveal further sequence variants. Two missense variants, Asp673Asn and Ser1369Ala, and two silent variants that previously have been shown to be associated with NIDDM were all examined for association with NIDDM and for a possible influence on glucose- and tolbutamide-induced C-peptide and insulin secretion in a cohort of 380 young white Danes.

Neither of the two missense variants appeared to be associated with NIDDM. The Asp673Asn variant was found in only one NIDDM patient. The phenotype of this patient did not differ from the average NIDDM patient of this study. A normal glucose-tolerant brother of the mutation-carrying patient was heterozygous for the mutation, whereas another brother with NIDDM had the wild-type genotype. The allele frequency of the Ser1369Ala polymorphism was similar among 247 (of 250) control subjects (0.38 [0.34–0.42]) and among 406 (of 449) NIDDM patients (0.40 [0.37–0.43]).

The silent exon 18 variant was more prevalent among NIDDM patients (allelic frequency, 0.051 [0.035–0.067]) compared with control subjects (0.027 [0.013–0.041]; $\chi^2 = 4.99$, $P = 0.025$; Table 3). In contrast, the frequency of the exon 16 (nt –3) variant did not differ between the two groups (control subjects 0.44 [0.40–0.48]; NIDDM patients 0.45 [0.42–0.48]; $\chi^2 = 0.11$, $P = 0.742$; Table 4). However, the combined genotype exon 18 C/T and exon 16 (nt –3) –3c/–3t was even more frequently associated with NIDDM (Table 5). This genotype combination was found with a frequency of 0.012 (0.000–0.026) among control subjects and 0.044 (0.024–0.064) among NIDDM patients ($\chi^2 = 4.87$, $P = 0.03$; odds ratio 3.69 [1.07–12.71]).

No differences in glucose-induced or tolbutamide-induced serum insulin and C-peptide responses were seen between groups of young healthy carriers of either the exon 18 variant or the exon 16 (nt –3) variant (data not shown). In contrast, 10 carriers of the combined genotype exon 18/exon 16 (nt –3) at-risk genotype had reduced serum C-peptide (19–22 min, 266 ± 102 vs. 683 ± 23 min × pmol/l, $P = 0.002$; Fig. 1*A*; 19–30 min, 3,792 ± 624 vs. 6,462 ± 167 min × pmol/l, $P = 0.004$) and insulin responses (19–22 min, 124 ± 27 vs. 231 ± 10 min × pmol/l, $P = 0.045$; Fig. 1*B*; 19–30 min, 1,154 ± 238 vs. 1,838 ± 72 min × pmol/l, $P = 0.077$) upon tolbutamide injection. But the carriers had normal acute serum C-peptide (0–8 min, 6,632 ± 962 vs. 7,111 ± 177 min × pmol/l, $P = 0.567$; Fig. 1*A*) and acute serum insulin responses (0–8 min, 2,242 ± 415 vs.

TABLE 4
Genotype and allele frequencies of the SUR1 exon 16 (nt –3), c → t polymorphism

	Control subjects	NIDDM subjects	χ^2	*P* value
n	244 of 250	419 of 449	—	—
Genotype				
–3c/–3c	79 (0.32)	118 (0.28)	—	—
–3c/–3t	115 (0.47)	225 (0.54)	—	—
–3t/–3t	50 (0.20)	76 (0.18)	2.67, 2 df	0.26
Allele				
–3c	273 (0.56)	461 (0.55)	—	—
–3t	215 (0.44)	377 (0.45)	0.11, 1 df	0.74

2,249 ± 84 min × pmol/l, $P = 0.781$; Fig. 1*B*) upon glucose injection compared with 370 subjects with other genotype combinations. The two groups did not differ in sex, age, BMI, body fat, waist-to-hip ratio, or S_I (Table 6). The S_G of the at-risk genotype carriers tended to be increased ($P = 0.06$, Table 6).

When adjusting for sex, BMI, S_I, S_G, fasting plasma glucose level, or plasma glucose level at 19 min (the last sample point before the tolbutamide load), and the combined SUR1 genotypes in a multiple regression analysis, the associations between the combined exon 18/exon 16 (nt –3) and serum insulin (19–22 min, $P = 0.016$; 19–30 min, $P = 0.024$) and serum C-peptide responses (19–22 min, $P = 0.005$; 19–30 min, $P = 0.004$) were still significant.

DISCUSSION

Recent studies in Mexican-Americans with highly polymorphic DNA markers near the *SUR1* gene located at chromosome 11p15.1 failed to show evidence for a linkage of the SUR1 locus with NIDDM (21). Also, sib pair linkage studies with NIDDM in French Caucasian families gave no evidence for linkage to the SUR1 locus (22). In contrast, two studies of the Goto-Kakizaki (GK) rat, a well-characterized animal model for NIDDM, have revealed a quantitative trait locus (QTL) linked to the diabetic phenotype in a genomic region in which SUR1 is located (23,24). In addition, evidence for linkage of the plasma glucose concentration 2 h after oral glucose administration was demonstrated with markers near the SUR1 locus in Mexican-American families, ascertained via an NIDDM proband (25). Also, a silent polymorphism in exon 18 of the high-affinity *SUR1* gene has been reported to be significantly associated with NIDDM in Utah Caucasians, U.K. Caucasians (11), and French Caucasians (22). These data, together with the above-mentioned QTLs near the SUR1 locus, indicate that *SUR1* or a gene near the SUR1 locus may contribute to the genetic susceptibility to subsets of NIDDM.

In the present study, we have scanned the entire coding region of the *SUR1* gene, including intron-exon boundaries, for sequence variants associated with NIDDM. Of 11 identified sequence variants, only two predicted an amino acid change, but none of these appear to be associated with NIDDM. However, we did confirm that the silent Thr759Thr exon 18 variant is associated with NIDDM being present in about 10% of patients with NIDDM compared with about 5% in normal control subjects. This silent variant has now been shown to be associated with NIDDM in four independent studies (11,22, the present study). Pooling the data obtained in these studies, the exon 18 T allele is found in 2.2% of 1,176 control alleles and in 5.6% of 1,786 NIDDM alleles ($P < 0.001$), emphasizing that a genetic variant either at or near the SUR1 locus may contribute to the inherited basis of NIDDM. Combining this genetic variant with an intron variant at position –3 of the intron/exon 16 boundary resulted in a doubling of the odds ratio for NIDDM. About 4% of NIDDM patients had the combined SUR1 variants; only 1% of control subjects had them. No difference in mode of treatment, age of diagnosis, or BMI was observed between carriers and noncarriers of the combined genotype.

The present combined at-risk genotype is difficult to explain. The combined at-risk genotype found by Inoue et al. (11) was exon 18 C/T and exon 16 (nt –3) c/c; whereas the combined at-risk genotype found in this study was the double heterozygote, exon 18 C/T and exon 16 (nt –3) c/t. Because the phase is unknown for this genotype, we do not know how many of this group have exon 18/16 haplotypes C-c/T-t and how many have C-t/T-c. In contrast, all individuals in the combined at-risk genotype in Inoue's study are obligate carriers of T-c. Therefore, a hypothesis to be investigated in further studies is that the T-c haplotype is a common element of the combined at-risk genotypes identified in both studies. As none of the variants predicts an amino-acid change, the T-c haplotype may be in linkage disequilibrium with a nearby functional mutation that has not been identified. Apart from the *SUR1* gene itself, one obvious candidate gene for such a mutation is Kir6.2, located just downstream of the *SUR1* gene at chromosome 11. However, three recent molec-

TABLE 5
Frequencies of the combined exon 18 and exon 16 (nt –3) genotypes

Exon 18 genotype	Exon 16 (nt –3) genotype	Control subjects (n = 243 of 250)	NIDDM subjects (n = 386 of 449)	χ^2	*P* value
C/C	–3c/–3c	69	87	—	—
C/C	–3c/–3t	111	191	—	—
C/C	–3t/–3t	50	70	—	—
C/T	–3c/–3c	10	21*	—	—
C/T	–3c/–3t	3	17	4.87, 1 df	0.03

No subject was found to carry the combined genotype exon 18 C/T and exon 16 –3t/–3t, exon 18 T/T and exon 16 –3c/–3t, or exon 18 T/T and exon 16 –3t/–3t. These groups are therefore not included in the table. *One NIDDM subject with the exon 18 T/T exon 16 –3c/–3c genotype is included in this group.

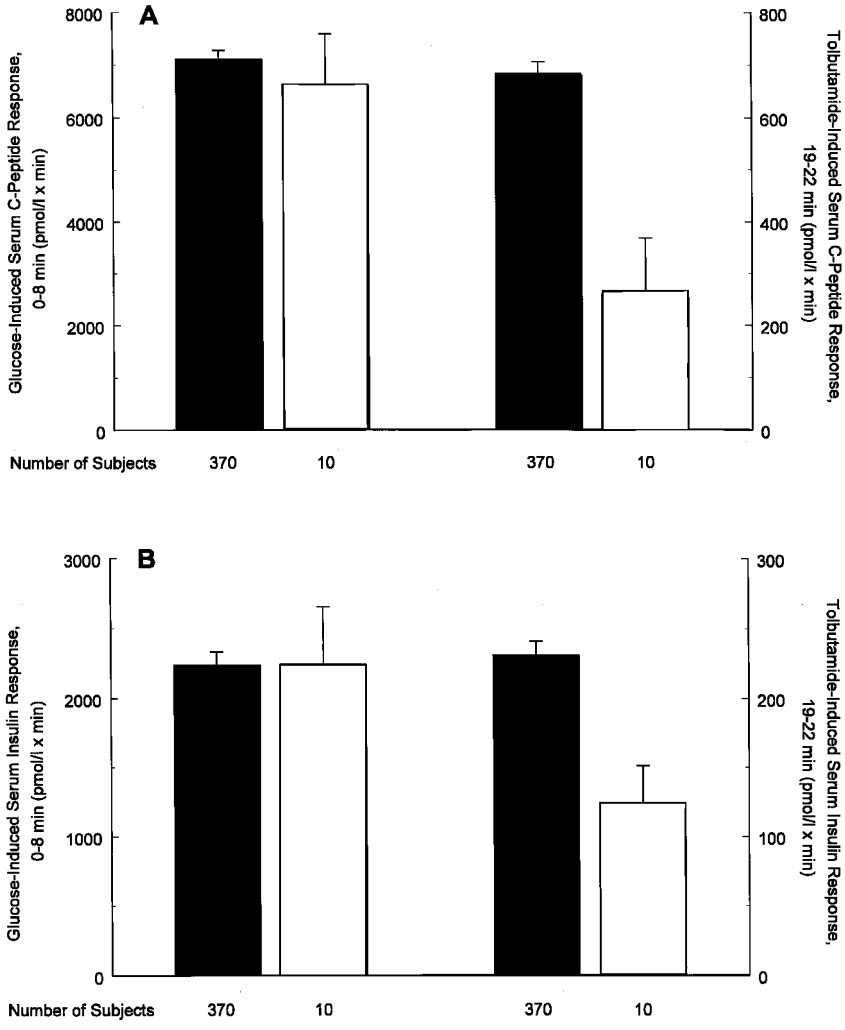

FIG. 1. Incremental serum C-peptide responses (*A*) and serum insulin responses (*B*) in 380 young, healthy Caucasians during a 3-h intravenous glucose tolerance test (0.3 g/kg body wt of 50% glucose at t = 0 min) with an intravenous tolbutamide load (3 mg tolbutamide/kg body wt) after 20 min. ■, individuals with all other genotype combinations (n = 370); □, individuals (n = 10) with the exon 18 C/T or T/T and exon 16 (nt –3) c/t or t/t SUR1 at-risk genotype. Glucose-induced acute serum C-peptide and insulin responses (0–8 min) were calculated by means of the trapezoidal rule as the incremental values (area under the curve when expressed above basal values). Serum C-peptide and insulin responses upon tolbutamide injection (19–22 min) were calculated as the difference between measurements obtained at the two time points. Data are means ± SE.

ular scannings of this gene have not revealed genetic sequence variants associated with NIDDM (26–28). In addition, we failed in the present study to demonstrate any functional mutations within the *SUR1* gene in either a primary SSCP-heteroduplex scanning of 49 NIDDM patients or a second scanning of 14 NIDDM patients carrying the combined SUR1 at-risk genotype.

To address the potential physiological implications of the combined exon 18 and exon 16 (nt –3) at-risk genotype on pancreatic β-cell function, we studied insulin and C-peptide secretion during the 3 h after an intravenous glucose tolerance test in combination with intravenous tolbutamide injection in

TABLE 6
Clinical and biochemical characteristics of carriers of nucleotide variants in the *SUR1* gene in a population sample of 380 young healthy Caucasians

	Combined genotype exon 18 (C/T or T/T) and exon 16 (nt –3) (–3c/–3t or –3t/–3t)	All other genotypes	*P* value
n (F/M)	10 (8/2)	370 (186/184)	0.12
Age years	27 (23–30	25 (18–32)	0.17
BMI (kg/m^2)	25.5 ± 2.0	23.5 ± 0.2	0.47
Body fat (%)	29.6 ± 4.0	22.9 ± 0.4	0.11
Waist-to-hip ratio	0.80 ± 0.02	0.82 ± 0.01	0.61
S_I [$10^{-5} \cdot$ (min $\cdot$ pmol/l)$^{-1}$]	20.8 ± 5.5	15.2 ± 0.5	0.69
S_G ($10^{-2} \cdot$ min^{-1})	2.69 ± 0.37	2.13 ± 0.03	0.06

Data are means ± SE or means (ranges).

a random sample of 380 young, healthy Danish Caucasians. The prevalence of the combined at-risk genotype was slightly higher in the young cohort compared with the prevalence in the middle-aged control cohort used for the association study to NIDDM. This is probably due to the age difference, as an excess number of the young at-risk genotype carriers may develop NIDDM later in life. When 10 subjects with the combined nucleotide variants in the *SUR* gene were compared with the remaining 370 subjects, they were not different from the remaining cohort regarding the acute glucose-triggered serum insulin and C-peptide release. However, these subjects had, on average, a 50% decrease in C-peptide secretion and a 40% decrease in insulin secretion after tolbutamide stimulation. The 10 normal subjects with the at-risk genotype had, in addition, a 30% increase in glucose effectiveness (P = 0.06), which may indicate that they somehow compensate for an impaired insulin secretion by increasing glucose effectiveness. Interestingly, studies of nondiabetic, first-degree relatives of NIDDM patients have shown that individuals at high risk of later development of NIDDM may have increased glucose effectiveness (29).

In addition to being associated with NIDDM, the exon 18 Thr759Thr variant was shown to be associated with morbid obesity in French Caucasians (22). In the present study of young, healthy Danes, we did not find an association with BMI, percentage body fat, or waist-to-hip ratio either in carriers of the Thr759Thr T allele (data not shown) or in carriers of the combined at-risk genotype (Table 6). However, further genetic studies of the SUR1 locus in morbidly obese patients are necessary to evaluate whether modulation of insulin secretion may have any impact on BMI in individuals at high risk for obesity.

Some mutations in the *SUR1* gene have been shown to cause familial persistent hyperinsulinemic hypoglycemia of infancy (10,30) due to dysfunction of the K_{ATP} channel, leading to closure of the potassium channel with a subsequent increase in insulin secretion. The present findings do not point to a similar mechanism in the at-risk genotype carriers. Carriers of the SUR1 at-risk genotype have a normal pancreatic β-cell response to an intravenous glucose load but a reduced β-cell response after intravenous tolbutamide, suggesting that the ligand-induced activation of SUR1 may be impaired. Recently, both bovine and porcine endosulfines, putative endogenous ligands for the SUR1, have been isolated and partially characterized (31,32). If, as suggested (31), one or more endosulfines represent the natural regulators of K_{ATP} channels, their biological function in the pancreatic β-cells could be impaired in carriers of SUR1 variants, conferring increased susceptibility to NIDDM.

In summary, although several studies showed no linkage between SUR1 and NIDDM, quantitative traits in one human and in two animal studies were linked to this locus. Now the current study reports an association between SUR1 and NIDDM; this is the fourth population in which such an association was found. How do we explain these discrepant results? It is well recognized that the ability to detect linkage in a complex disorder such as NIDDM is determined by the risk contributed by a particular locus and the number of family members studied (33). In fact, Cox and Bell (34) suggested that many susceptibility genes involved in complex traits, neither necessary nor sufficient for the disease, might be detected only by association studies. Therefore, a careful screen for mutations in coding regions of an important candidate gene such as the SUR1/K_{ATP} channel, followed by assessment of the potential significance of the variants by association and phenotype studies, appears warranted. In this case, variants were found that were associated both with the disease and in young, healthy subjects with an impaired stimulation of pancreatic β-cells. These variants might therefore be linked to mutations that alter the function of the gene. Yet nothing was found in a careful screen of all exons. We know, however, that the control of gene function is not limited to the coding regions. We have assessed only the exons in genomic DNA. It is conceivable that intronic mutations alter RNA splicing, creating variant exons and thus variant SUR1 protein. It is also possible that promoter variants, or even imprinted regions as far away as 100 kb, may alter SUR1 expression, as for example the control of the insulin/IGF2 locus by the H19 gene (35). These hypotheses need to be tested by additional studies.

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REFERENCES

1. Turner RC, Hattersley AT, Shaw JTE, Levy JC: Type II diabetes: clinical aspects of molecular biological studies. *Diabetes* 44:1–10, 1995
2. DeFronzo RA, Bonadonna RC, Ferrannini E: Pathogenesis of NIDDM: a balanced overview. *Diabetes Care* 15:318–368, 1992
3. Accili D, Cama A, Barbetti F, Kadowaki H, Kadowaki T, Taylor SI: Insulin resistance due to mutations of the insulin-receptor gene: an overview. *J Endocrinol Invest* 15:857–864, 1992
4. Ouweland JMW, Lemkes HHPJ, Ruitenbeek W, Sandkuijl LA, Vijlder MF, Struyvenberg PAA, Kamp JJP, Maassen JA: Mutation in mitochondrial $tRNA^{Leu(UUR)}$ gene in a large pedigree with maternally transmitted type II diabetes mellitus and deafness. *Nature Genet* 1:368–371, 1992
5. Almind K, Bjørbæk C, Vestergaard H, Hansen T, Echwald S, Pedersen O: Aminoacid polymorphisms of insulin receptor substrate-1 in non-insulin-dependent diabetes mellitus. *Lancet* 342:828–832, 1993
6. Hager J, Hansen L, Vaisse C, Vionnet N, Philippi A, Poller W, Velho G, Carcassi C, Contu L, Julier C, Cambien F, Passa P, Lathrop M, Kindsvogel W, Demenais F, Nishimura E, Froguel P: A missense mutation in the glucagon receptor gene is associated with non-insulin-dependent diabetes mellitus. *Nature Genet* 9:299–304, 1995
7. Ashcroft SJ, Ashcroft FM: Properties and functions of ATP-sensitive K-channels. *Cell Signals* 2:197–214, 1990
8. Aguilar-Bryan L, Nichols CG, Wechsler SW, Clement JP, Boyd AE, Gonzales G, Herrerasosa H, Nguy K, Bryan J, Nelson DA: Cloning of the b cell high-affinity sulfonylurea receptor: a regulator of insulin secretion. *Science* 268:423–426, 1995
9. Inagaki N, Gonoi T, Clement IV JP, Namba N, Inazawa J, Gonzales G, Aguilar-Bryan L, Seino S, Bryan J: Reconstitution of I_{KATP}: an inward rectifier subunit plus the sulfonylurea receptor. *Science* 270:1166–1170, 1995
10. Thomas PM, Cote GJ, Wohllk N, Haddad B, Mathew PM, Rabl W, Aguilar-Bryan L, Gagel RF, Bryan J: Mutations in the sulfonylurea receptor gene in familial persistent hyperinsulinemic hypoglycemia of infancy. *Science* 268:426–429, 1995
11. Inoue H, Ferrer J, Wellin CM, Elbein SC, Hoffman M, Mayorga R, Warren-Perry M, Zhang Y, Millns H, Turner R, Province M, Bryan J, Permutt MA, Aguilar-Bryan L: Sequence variants in the sulfonylurea receptor (SUR) gene are associated with NIDDM in Caucasians. *Diabetes* 45:825–831, 1996
12. Clausen JO, Borch-Johnsen K, Ibsen H, Bergman RN, Hougaard P, Winther K, Pedersen O: Insulin sensitivity index, acute insulin response, and glucose effec-

tiveness in a population-based sample of 380 young healthy caucasians: analysis of the impact of gender, body fat, physical fitness, and life-style factors. *J Clin Invest* 98:1195–1205, 1996
13. Pacini G, Bergman RN: MINMOD: a computer program to calculate insulin sensitivity and pancreatic responsivity from the frequently sampled intravenous glucose tolerance test. *Comput Methods Progr Biomed* 23:113–122, 1986
14. Steil GM, Vølund A, Kahn SE, Bergman RN: Reduced sample number for calculation of insulin sensitivity and glucose effectiveness from the minimal model: suitability for use in population studies. *Diabetes* 42:250–256, 1993
15. Andersen L, Dinesen B, Jørgensen PN, Poulsen F, Røder ME: Enzyme immunoassay for intact human insulin in serum or plasma. *Clin Chem* 39:578–582, 1993
16. Heding LG: Radioimmunological determination of human C-peptide in serum. *Diabetologia* 11:541–548, 1975
17. Faber OK, Marcussen J, Naithani VK, Binder C: Production of antisera to synthetic benzyloxycarbonyl-C-peptide. *Hoppe-Seylor's Z Physiol Chem* 357:751–757, 1976
18. Faber OK, Binder C, Marcussen J, Heding LG, Naithani VK, Kuzuya H, Blix P, Horwitz DL, Rubenstein AH: Characterization of seven C-peptide antisera. *Diabetes* 27 (Suppl. 1):170–177, 1978
19. Miller SA, Dykes DD, Polesky HF: A simple procedure for extracting DNA from human nucleated cells. *Nucleic Acid Res* 16:1215–1216, 1988
20. Hansen L, Hansen T, Vestergaard H, Bjørbæk C, Echwald SM, Clausen JO, Chen YH, Chen MX, Cohen PTW, Pedersen O: A widespread amino acid polymorphism at codon 905 of the glycogen-associated regulatory subunit of protein phosphatase-1 is associated with insulin resistance and hypersecretion of insulin. *Hum Mol Genet* 4:1313–1320, 1995
21. Stirling B, Cox NJ, Bell GI, Hanis CL, Spielman RS, Concannon P: Linkage studies in NIDDM patients with markers near the sulphonylurea receptor gene. *Diabetologia* 38:1479–1481, 1995
22. Hani EH, Clément K, Velho G, Vionnet N, Hager J, Philippi A, Dina C, Inoue H, Permutt MA, Basdevant A, North M, Demenais F, Guy-Grand B, Froguel P: Genetic studies of the sulfonylurea receptor gene locus in NIDDM and in morbid obesity among French Caucasians. *Diabetes* 46:688–694, 1997
23. Galli J, Li L, Glaser A, Östenson CG, Jiao H, Fakhrai-Rad H, Jacob HJ, Lander ES, Luthman H: Genetic analysis of non-insulin dependent diabetes mellitus in the GK rat. *Nature Genet* 12:31–37, 1996
24. Gauguier D, Froguel P, Parent V, Bernard C, Bihoreau MT, Portha B, James MR, Penicaud L, Lathrop M, Ktorza A: Genetic analysis of non-insulin dependent diabetes mellitus in the GK rat. *Nature Genet* 12:38–43, 1996
25. Stern MP, Ravindranath D, Braxton DM, Reinhart LJ, Shivakumar S, Shipman PA, Uresandi OC, Benavides E, Lanero J, O'Connell P: Evidence for linkage of regions on chromosomes 6 and 11 to plasma glucose concentrations in Mexican Americans. *Genome Res* 6:724–734, 1996
26. Sakura H, Wat N, Horton V, Milns H, Turner RC, Ashcroft FM: 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. *Diabetologia* 39:1233–1236, 1996
27. Hansen L, Echwald SM, Hansen T, Urhammer SA, Clausen JO, Pedersen O: Amino acid polymorphisms in the ATP-regulatable inward rectifier Kir6.2 and their relationships to glucose- and tolbutamide-induced insulin secretion, the insulin sensitivity index, and NIDDM. *Diabetes* 46:508–512, 1997
28. Inoue H, Ferrer J, Warren-Perry M, Zhang Y, Millns H, Turner R, Elbein SC, Hampe CL, Suarez BK, Inagaki N, Seino S, Permutt MA: Sequence variants in the pancreatic islet β-cell inwardly rectifying K^+ channel Kir6.2 (Bir) gene: identification and lack of role in Caucasian patients with NIDDM. *Diabetes* 46:502–507, 1997
29. Henriksen JE, Alford F, Handberg A, Vaag A, Ward GM, Kalfas A, Beck-Nielsen H: Increased glucose effectiveness in normoglycemic but insulin-resistant relatives of patients with non-insulin-dependent diabetes mellitus: a novel compensatory mechanism. *J Clin Invest* 94:1196–1204, 1994
30. Nestorowicz A, Wilson BA, Schoor KP, Inoue H, Glaser B, Landau H, Stanley CA, Thornton S, Clement IV JP, Bryan J, Aguilar-Bryan L, Permutt MA: Mutations in the sulfonylurea receptor gene are associated with familial hyperinsulinism in Ashkenazi Jews. *Hum Mol Genet* 5:1813–1822, 1996
31. Virsolvy-Vergine A, Leray H, Kuroki S, Lupo B, Dufour M, Bataille D: Endosulphine, an endogenous peptidic ligand for the sulfonylurea receptor: purification and partial characterization from bovine brain. *Proc Natl Acad Sci USA* 89:6629–6633, 1992
32. Virsolvy-Vergine A, Salazar G, Sillard R, Denoroy L, Mutt V, Bataille D: Endosulfine, endogenous ligand for the sulphonylurea receptor: isolation from porcine brain and partial structural determination of the a form. *Diabetologia* 39:135–141, 1996
33. Risch N, Merikangas K: The future of genetic studies of complex human diseases. *Science* 273:1516–1517, 1996
34. Cox N, Bell GI: Disease associations: chance, artefact, or susceptibility genes? *Diabetes* 38:947–950, 1989
35. Zemel S, Bartolomei MS, Tilghman SM: Physical linkage of two mammalian imprinted genes, H19 and insulin-like growth factor 2. *Nature Genet* 2:61–65, 1992

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Q2: what do wt, he, and ho stand for?>
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Q4: Please spell out EEC.
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Ref 11: Please add the first initial(s) for Province in ref. 11.>
Ref 25: Please add first initial(s) for O’Connell in ref. 25.>