of hyperinsulinemia, and weight loss is accompanied by a decrease in serum TNF-α concentration and an increase in insulin sensitivity [11-13]. It remains, however, unsolved whether the relationships between serum TNF-α and insulin resistance are caused by or are a result of obesity itself. Furthermore, it is suggested that glucose is proinflammatory and may potentially induce TNF-α. To address this, we recruited nonobese well-controlled Japanese type 2 diabetic patients carefully stratified by their resistance to insulin and explored the relationships between insulin resistance and the TNF-α system (serum TNF-α, serum-soluble TNF receptors). This is the first documented case where peripheral levels of TNF-α system activity (TNF-α, soluble TNF receptors) are not a major factor responsible for the evolution of insulin resistance, at least not in nonobese Japanese type 2 diabetic patients.

2. Subjects and methods

Eighty-eight nonobese Japanese type 2 diabetic patients who visited Kansai-Denryoku Hospital were enrolled for the present study. Type 2 diabetes mellitus was diagnosed based on the World Health Organization criteria [14]. The patients showed no evidence of acute infectious illness at the time of the study. The duration of diabetes was 11.0 ± 0.8 years (mean ± SEM) (range, 1-35 years). Seventy-six of 88 diabetic patients were taking sulfonylureas (gliclazide), and the rest were treated on a dietary regimen with no medication to alter blood glucose level. No patients have received insulin therapy. All subjects had ingested at least 150 g of carbohydrate for the 3 days preceding the study. None of the subjects had significant renal, hepatic, or cardiovascular disease. Patients did not consume alcohol or perform heavy exercise for at least 1 week before the study. Blood pressure was also measured.

Blood was drawn in the morning after a 12-hour fast. Plasma glucose was measured with glucose oxidase method. The triglycerides, total cholesterol, and high-density lipoprotein cholesterol (HDL-C) were also measured. Serum insulin was measured using a 2-site immunoradiometric assay (Insulin Riabead II, Dainabot, Osaka, Japan). Coefficients of variation were 4% for insulin greater than 25 μU/mL and 7% for insulin less than 25 μU/mL. Serum leptin and adiponectin concentrations were measured with a radioimmunoassay kit (Linco Research, St Charles, Mo) as described previously [7,8]. The intra-assay and interassay coefficients of variation were less than 5% for leptin and adiponectin. Serum TNF-α concentrations were measured by enzyme immunoassay kit (Quantikine HS Human TNF-α immunoassay kit, R&D Systems, Inc, Minneapolis, Minn), and serum concentrations of sTNF-R1 and sTNF-R2 were measured by enzyme-linked immunosorbent assay (BIOTRAK, Amersham Life Sciences, Uppsala, Sweden), as described previously [15]. The limits of sensitivity for TNF-α, sTNF-R1, and sTNF-R2 were 0.5, 25, and 50 pg/mL, respectively. Samples for insulin, leptin, adiponectin, and TNF-α system (TNF-α, sTNF-R1, and sTNF-R2) were prepared, frozen, and stored at −70°C until the assay.

The estimate of insulin resistance by homeostasis model assessment (HOMA-IR) was calculated with the following formula: fasting serum insulin (μU/mL) × fasting plasma glucose (mmol/L)/22.5 [16]. The HOMA-IR value of normal tolerant subjects was 1.6 ± 0.9 (mean ± SD), and we defined the values greater than 2.5 as an insulin-resistant state and the values less than 2.5 as an insulin-sensitive state [2,5,17]. The threshold value (2.5) for insulin resistance in our study is similar to that (2.77) in nonobese subjects with no metabolic disorders reported by Bonora et al [18]. It may be argued that the use of sulfonylureas in patients with diabetes might significantly affect the estimate of insulin resistance by HOMA, as these drugs are known to decrease fasting plasma glucose without substantially changing fasting plasma insulin [19]. It seems, however, unlikely because Bonora et al [20] and Emoto et al [21] showed that in the validation studies of HOMA, the correlation of insulin sensitivity measured by such method and that measured by the glucose clamp was not substantially different in diet-treated and sulfonylurea-treated type 2 diabetes. Another problem is that pancreatic B-cell function per se might affect HOMA-IR in Japanese type 2 diabetic patients because these patients are accompanied by mild impairments in pancreatic B-cell function [2]. In our present study, however, fasting C-peptide level was greater than 0.8 ng/mL, indicating that their pancreatic function is not severely impaired. Therefore, we used HOMA-IR in diet-treated and sulfonylurea-treated diabetic patients, taking into account pancreatic insulin secretion.

Table 1
Clinical characteristics in insulin-resistant and insulin-sensitive diabetic patients

	Insulin-resistant	Insulin-sensitive	*P*
No. of subjects	32	56	
Age (y)	61.9 ± 1.7	63.2 ± 1.1	.252
Men/women	25/7	38/18	.155
HOMA-IR	3.58 ± 0.22	1.58 ± 0.07	<.001
Diabetes duration (y)	10.7 ± 1.5	11.2 ± 0.8	.376
Smoking (no/yes)	25/7	42/14	.307
SU/diet	27/5	49/7	.343
BMI (kg/m^2)	23.7 ± 0.3	22.4 ± 0.3	.003
HbA1c (%)	7.4 ± 0.2	6.8 ± 0.1	.007
Triglycerides (mg/dL)	153 ± 12	104 ± 5	<.001
Total cholesterol (mg/dL)	214 ± 6	198 ± 5	.026
Leptin (ng/mL)	6.4 ± 0.8	4.7 ± 0.4	.018
HDL-C (mg/dL)	54 ± 2	61 ± 2	.012
Adiponectin (μg/mL)	10.7 ± 1.1	16.9 ± 1.6	.005
Fasting glucose (mg/dL)	150 ± 4	135 ± 3	.003
Fasting insulin (μU/mL)	9.8 ± 0.6	4.7 ± 0.2	<.001
Systolic blood pressure (mm Hg)	139 ± 3	135 ± 3	.107
Diastolic blood pressure (mm Hg)	86 ± 2	79 ± 1	.001
TNF-α (pg/mL)	3.70 ± 0.49	3.15 ± 0.19	.107
sTNF-R1 (pg/mL)	1132 ± 55	1208 ± 55	.185
sTNF-R2 (pg/mL)	2025 ± 88	2073 ± 67	.333

3. Statistical analysis

Data are presented as mean values ± SEM. Statistical analyses were conducted using the StatView 5 system (Statview, Berkeley, Calif). The mean values of the 2 groups were compared with Student t test. Spearman rank correlation coefficient analysis was also performed to calculate a correlation. $P < .05$ was considered as significant.

4. Results

The subjects studied were all Japanese type 2 diabetic patients (63 men and 25 women) with an age range of 43 to 84 years (62.8 ± 1.0 years) and a BMI of 17.1 to 26.7 kg/m^2 (21.0 ± 0.8 kg/m^2). They were all nonobese [22]. The fasting plasma glucose was 141 ± 3 mg/dL, and glycosylated hemoglobin (HbA1c) was 7.0% ± 0.1%. Fasting insulin level was 6.56 ± 0.39 μU/mL. Serum triglycerides, total cholesterol levels, and HDL-C levels were 121 ± 6, 204 ± 4, and 59 ± 2 mg/dL, respectively. Serum leptin and adiponectin concentrations were 5.3 ± 0.4 ng/mL and 14.6 ± 1.2 pg/mL, respectively. There was a wide variation in insulin resistance calculated by HOMA in our diabetic patients (range, 0.51-7.17; mean ± SD, 2.30 ± 0.15). Thirty-two (36%) of 88 patients had HOMA-IR greater than 2.5, indicating that they are insulin-resistant [4,5]. On the other hand, serum TNF-α, soluble TNF-R1(sTNF-R1), and soluble TNF-R2 (sTNF-R2) were 3.35 ± 0.22 (range, 1.6-15.7), 1180 ± 43 (range, 699-2920), and 2055 ± 56 pg/mL (range, 1250-3860 pg/mL), respectively.

Table 1 shows the clinical profile between insulin-resistant and insulin-sensitive type 2 diabetic patients. Compared with insulin-sensitive type 2 diabetic patients, insulin-resistant patients had significantly higher levels of BMI, HbA1c, triglycerides, total cholesterol, leptin, and diastolic blood pressure and lower concentrations of HDL-C and adiponectin. No significant difference was observed in age, sex, duration of diabetes, smoking, systolic blood pressure, and the 3 measures of TNF-α system (TNF-α, sTNF-R1, and sTNF-R2) between the 2 groups.

Table 2
Correlation of TNF-α, sTNF-R1, and sTNF-R2 to measures of variables in diabetic patients

	TNF-α		sTNF-R1		sTNF-R2	
	r	P	r	P	r	P
BMI	−0.062	.563	−0.013	.904	−0.159	.137
Systolic blood pressure	0.042	.712	0.208	.682	0.154	.177
Diastolic blood pressure	0.136	.233	0.006	.956	−0.009	.940
HbA1c	0.028	.790	−0.031	.769	−0.128	.233
Fasting glucose	−0.067	.948	−0.073	.496	−0.161	.133
Fasting insulin	0.048	.653	0.026	.811	−0.012	.908
HOMA-IR	0.026	.806	−0.008	.938	−0.061	.571
Triglycerides	0.082	.442	0.011	.920	−0.041	.705
Leptin	−0.204	.059	−0.004	.968	−0.093	.389
Adiponectin	−0.188	.089	0.111	.314	0.148	.180

The correlation between the 3 measures of TNF-α system (TNF-α, sTNF-R1, and sTNF-R2) and the factors associated with insulin resistance (BMI, systolic blood pressure, diastolic blood pressure, HbA1c, fasting glucose, fasting insulin, HOMA-IR, triglycerides, leptin, and adiponectin) was next investigated in our diabetic patients (Table 2). Peripheral levels of the 3 measures of the TNF-α system (TNF-α, sTNF-R1, and sTNF-R2) were not associated with these variables.

5. Discussion

Type 2 diabetes is a heterogenous syndrome characterized by insulin resistance and/or defective insulin secretion [1]. There seems to be racial difference in insulin resistance in type 2 diabetes. Haffner et al [23] surveyed the prevalence of white type 2 diabetic patients and found that 92% of type 2 diabetic patients were insulin-resistant. Chaiken et al [24] reported that 60% of type 2 diabetic patients with BMI less than 30 kg/m^2 were insulin-resistant in African-American populations. We recently demonstrated that 40% of type 2 diabetic patients are insulin-resistant in nonobese Japanese type 2 diabetic patients [4,5]. Whereas the patients with type 2 diabetes already manifest some elements of inflammation, the intriguing feature that nonobese Japanese type 2 diabetic patients are divided into 2 variants enables us to explore whether some inflammatory markers such as TNF-α participated in the worsening of insulin resistance. We therefore investigated TNF-α and sTNF-R in nonobese Japanese type 2 diabetic patients stratified into 2 different groups: one with insulin resistance and the other with normal insulin sensitivity.

The reason why Japanese type 2 diabetic patients are not always associated with insulin resistance is unclear, but it may be due to the fact that mean BMI in our type 2 diabetic patients is 21.0 kg/m^2 less than that in white populations (average BMI 30 kg/m^2). Chang et al [25] recently reported that only 23.6% of Korean type 2 diabetic patients are insulin-resistant. Their mean level of BMI was 22.6 kg/m^2.

Using HOMA-IR and/or minimal model analysis, we have investigated the factors underlying insulin resistance in nonobese Japanese type 2 diabetic patients [2-9]. Whereas BMI and triglycerides are considered to be the most important factors responsible for the evolution of insulin resistance, regional abdominal adipose tissue distribution per se contributes to insulin resistance in nonobese Japanese type 2 diabetic patients [19]. In contrast to white and African-American populations, subcutaneous and visceral fat areas are independently associated with insulin resistance in nonobese Japanese type 2 diabetic patients [26,27]. Not only serum triglycerides but also serum leptin and adiponectin levels are shown to be associated with insulin resistance in our populations [4,5,7,8]. Serum triglycerides level is positively correlated to visceral fat area [9]. Serum leptin level is positively correlated to subcutaneous fat areas, whereas serum adiponectin level is negatively correlated to

visceral fat areas [7,8]. Furthermore, we recently demonstrated that inflammation per se is independently associated with insulin resistance in nonobese Japanese type 2 diabetic patients [6]. We subsequently found that C-reactive protein, 1 of the inflammatory markers, is not only associated with insulin resistance but also with BMI and adipocytokine such as leptin and adiponectin (data not shown). Thus, the factors underlying insulin resistance in nonobese Japanese type 2 diabetic patients are hypothesized to be linked to adipose tissue–related insulin resistance.

Another candidate that is associated with adipose tissue–related insulin resistance is TNF-α, a potent proinflammatory cytokine [10]. Hotamisligil and Spiegelman [28] were the first workers who proposed that TNF-α represents a key mediator of obesity-linked insulin resistance. Overexpression of TNF-α from adipose tissue is shown in different rodent models of obesity. Dandona et al [12] showed that plasma concentration of TNF-α is increased among obese subjects, and it decreases with weight loss. In vitro studies have shown that TNF-α inhibits insulin-stimulated glucose uptake in adipocytes in vitro by decreasing phosphorylation of the insulin receptor [29].

In the present study, we used serum TNF-α, soluble TNF-R1, and soluble TNF-R2 as an index of TNF-α system activity since peripheral levels of TNF receptor remain elevated for a longer time than TNF-α itself and reflect the degree of TNF-α activation more accurately than the measurement of TNF-α itself. Using the 3 measures of TNF-α system activity, we first demonstrated that TNF-α system activity is not responsible for insulin resistance, at least not in nonobese Japanese type 2 diabetic patients. This is a surprising finding because TNF-α is suggested to have a key role in the assessment of insulin resistance of obese and type 2 diabetic patients [10,28]. Thus, the reason why we could not find the relationship between insulin resistance and peripheral levels of TNF-α system in our patients is not known, but it may be due to the difference in clinical characteristics studied. The previous studies supporting the relationship between insulin resistance and TNF-α are derived from the studies dealing with the obese diabetic patients [10-13]. Obese subjects are shown to have higher concentration of TNF-α than nonobese subjects. Moreover, adipose tissue TNF-R2 messenger RNA is shown to be correlated with BMI and hyperinsulinemia in obese diabetic patients. Weight loss is accompanied by a decrease in serum TNF-α concentration and an increase in insulin sensitivity.

On the other hand, there is some literature supporting our present finding that peripheral levels of TNF-α system activity are not associated with insulin resistance in human subjects. Kellerer et al [30] found no correlation between plasma TNF-α and insulin resistance in the offspring of type 2 diabetic patients. Two investigators [31,32] have shown that administration of antibodies or antagonists to TNF-α have not improved insulin sensitivity in insulin-resistant individuals. Zavarotoni et al [33] recently demonstrated that differences in TNF-α activity do not appear to contribute to the marked variation in insulin action that occurs in healthy individuals. Ghanim et al [34] very recently showed that TNF-α is not related to HOMA-IR in obese subjects. Thus, it may be speculated that adipose tissue–linked TNF-α system activity might function locally at the level of the adipocyte in a paracrine or autocrine fashion in our study's diabetic patients. Alternatively, adipose tissue may not play a major role in the determination of peripheral levels of TNF-α system activity in our nonobese, well-controlled, unique Japanese type 2 diabetic patients.

In summary, we demonstrated for the first time that although the number of patients with type 2 diabetes is limited, peripheral levels of TNF-α system activity do not appear to be a major explanation of the mechanisms underlying insulin resistance at least in nonobese well-controlled Japanese type 2 diabetic patients. This idea can be inferred from our present study that peripheral levels of TNF-α system activity are not associated with serum leptin and adiponectin which are another index of insulin resistance in human beings [10].

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Distinct Effects of Glucose-Dependent Insulinotropic Polypeptide and Glucagon-Like Peptide-1 on Insulin Secretion and Gut Motility

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Glucose-induced insulin secretion from pancreatic β-cells depends critically on ATP-sensitive K^+ channel (K_{ATP} channel) activity, but it is not known whether K_{ATP} channels are involved in the potentiation of insulin secretion by glucose-dependent insulinotropic polypeptide (GIP). In mice lacking K_{ATP} channels (Kir6.2$^{-/-}$ mice), we found that pretreatment with GIP in vivo failed to blunt the rise in blood glucose levels after oral glucose load. In Kir6.2$^{-/-}$ mice, potentiation of insulin secretion by GIP in vivo was markedly attenuated, indicating that K_{ATP} channels are essential in the insulinotropic effect of GIP. In contrast, pretreatment with glucagon-like peptide-1 (GLP-1) in Kir6.2$^{-/-}$ mice potentiated insulin secretion and blunted the rise in blood glucose levels. We also found that GLP-1 inhibited gut motility whereas GIP did not. Perfusion experiments of Kir6.2$^{-/-}$ mice revealed severely impaired potentiation of insulin secretion by 1 nmol/l GIP and substantial potentiation by 1 nmol/l GLP-1. Although both GIP and GLP-1 increase the intracellular cAMP concentration and potentiate insulin secretion, these results demonstrate that the GLP-1 and GIP signaling pathways involve the K_{ATP} channel differently. *Diabetes* 54: 1056–1063, 2005

Oral glucose load elicits larger insulin secretion and less increase in blood glucose levels than intravenous administration of the equivalent amount of glucose (1,2). This phenomenon is mostly due to incretins, gut-derived factors including glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) (3,4). GIP and GLP-1 are released from gastrointestinal endocrine K-cells and L-cells, respectively, into the blood stream in response to the ingestion of nutrients (5), which potentiates insulin secretion from pancreatic β-cells (6–9). GIP and GLP-1 exert their insulinotropic effects by binding to GIP receptors (10) and GLP-1 receptors on the β-cell surface (11), respectively, activating adenylyl cyclase (12,13), which leads to the rise in intracellular cAMP concentration that potentiates insulin secretion by activating protein kinase A– and/or cAMP–guanine nucleotide exchange factor (GEF)2–mediated signaling in normal pancreatic β-cells (14,15). Thus, GIP and GLP-1 share in part a common pathway of insulin secretion enhancement. However, many clinical findings suggest different mechanisms of GIP and GLP-1 action. In patients with type 2 diabetes, for example, the insulinotropic action of GLP-1 is well preserved whereas that of GIP is markedly reduced (16). The mechanism of the differing effects GLP-1 and GIP remains unknown.

Recent studies of GIP-receptor knockout (GIPR$^{-/-}$) mice have shown that potentiation of insulin secretion by GIP plays an important role in glucose metabolism (17). GIPR$^{-/-}$ mice have higher glucose levels in response to oral glucose load than in response to intraperitoneal load, showing that endogenous GIP plays an important role in preventing a rise in blood glucose levels after oral load. Unlike other secretagogues that stimulate insulin secretion, GIP exerts a potentiating effect on insulin secretion only in the presence of glucose (7,18,19). The glucose dependency of the insulinotropic action of GIP has been confirmed using stepwise glucose clamp in normal human subjects (9,20,21).

ATP-sensitive K^+ channel (K_{ATP} channel) null (Kir6.2$^{-/-}$ and SUR1$^{-/-}$) mice do not exhibit significant insulin secretion in response to oral glucose load (22–24). This raises the possibility that Kir6.2$^{-/-}$ mice have either a defect in glucose-induced GIP secretion from K-cells or a defect in potentiation by GIP of insulin secretion from β-cells. Because glucose-induced GIP secretion from K-cells has been shown to occur in a K_{ATP} channel–independent manner, we investigated the potentiating effect of GIP on insulin secretion from β-cells in Kir6.2$^{-/-}$ mice. We also examined the effects of GLP-1, the other important incretin hormone, on the potentiation of insulin secretion and

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AUC, area under the curve; GEF, guanine nucleotide exchange factor; GIP, glucose-dependent insulinotropic polypeptide; GIPR$^{-/-}$, GIP-receptor knockout; GLP-1, glucagon-like peptide-1; K_{ATP} channel, ATP-sensitive K^+ channel; KRBH, Krebs-Ringer bicarbonate HEPES; OGTT, oral glucose tolerance test; PKA, protein kinase A; PreTx, GIP pretreatment.

blood glucose levels after an oral glucose load in Kir6.2$^{-/-}$ mice.

RESEARCH DESIGN AND METHODS

Kir6.2$^{-/-}$ mice were generated as previously described (22). Because the Kir6.2$^{-/-}$ mice had been backcrossed to the C57BL/6 mouse strain over five generations, C57BL/6 mice were used as wild-type (Kir6.2$^{+/+}$) mice. All animal experiments were performed in accordance with the guidelines of the Animal Care Committee of Chiba and Kobe University.

GIP secretion assay in vivo. The secretion of GIP in response to oral glucose was examined in conscious male mice (18–20 weeks old, weighing 20–25 g) in vivo. After an overnight fast (16 h), Kir6.2$^{+/+}$ and Kir6.2$^{-/-}$ mice were administered D-glucose (150 mg/mouse in 0.5 ml) via gavage. A blood sample (~500 μl of whole blood) was taken 15 min after glucose load and separated by centrifugation at 12,000*g* for 15 min at 4°C and stored at −80°C until hormone radioimmunoassay. Blood samples for basal GIP and glucose level was taken independently 1 week before (n = 6 for both genotypes) and after (n = 6 for both genotypes) the glucose loading test. GIP concentrations and glucose levels were determined as previously described (25–27).

Oral glucose tolerance test and measurement of blood glucose and serum insulin levels. One-hundred micrograms of human GIP (in 0.1 ml), human GLP-1 (in 0.1 ml), or saline (0.1 ml) was given subcutaneously to overnight (16 h)-fasted male mice. Glucose (1.5 g/kg) was administered 5 min after GIP or GLP-1 pretreatment as a 15% solution via gavage. Blood glucose levels at 0, 10, 30, 60, 90, 120, and 180 min and serum insulin levels at 0, 10, and 30 min after the glucose load were measured as previously described (27). The areas under the curve (AUCs) were assessed for blood glucose levels ($AUC_{glucose}$) with the trapezoidal rule of suprabasal values.

Measurement of gastrointestinal transit. To evaluate gastrointestinal motility, male mice were fasted with free access to drinking water for 48 h. On the day of the experiment, the mice received an intragastric injection of 20 μl/g test solution (25% wt/vol barium sulfate suspended in water or 50% wt/vol D-glucose solution). The mice were killed 15 min later by cervical dislocation. After dissection, the length from the pylorus to the most distal point of migration of the barium (*A*) and from the pylorus to terminal ileum (*B*) was measured. Gastrointestinal transit was expressed as percentage of *A* to *B*. To determine the effects of GIP and GLP-1 on gastrointestinal motility, mice were pretreated 5 min before test solution ingestion with 100 μg human GIP or GLP-1.

Perfusion experiments of mouse pancreata. Overnight (16 h)-fasted male mice at 16–20 weeks of age were used in perfusion experiments as previously reported (28) with slight modifications. Briefly, after anesthesia with 80 mg/kg sodium pentobarbital, the superior mesenteric and renal arteries were ligated, and the aorta was tied off just below the diaphragm. The perfusate was infused from a catheter placed in the aorta and collected from the portal vein. The perfusate was Krebs-Ringer bicarbonate HEPES (KRBH) buffer supplemented with 4.6% dextran and 0.25% BSA and gassed with 95% O_2/5% CO_2. The flow rate of the perfusate was 1 ml/min. In experiments involving GIP and GLP-1, mouse pancreata were perfused with KRBH buffer containing 2.8 or 16.7 mmol/l glucose in the presence or absence of 1 nmol/l GIP or 1 nmol/l GLP-1. In experiments involving arginine and carbachol, pancreata were perfused with KRBH buffer containing 5.5 mmol/l glucose in the presence or absence of 20 mmol/l arginine or 50 μmol/l carbachol. The perfusion protocols began with a 10-min equilibration period with the same buffer used in the initial step (i.e., from 1 to 5 min) shown in the figures. The insulin levels in the perfusate were measured by an ELISA kit (Mesacup Insulin Test) from BML (Nagoya, Japan).

Measurement of insulin secretion in response to arginine and carbachol in vivo. To analyze arginine- and carbachol-induced insulin secretion, overnight (16 h)-fasted male mice were administered 250 mg/kg L(+)-arginine intravenously or 750 μg/kg carbachol intraperitoneally as previously described by Guenifi et al. (29) and Havel et al. (30). Blood samples were taken before and 2 min after load, and blood glucose and serum insulin levels were measured.

Meal ingestion test. Glucose tolerance and insulin secretory response to mixed meal was evaluated using the enteral feeding formula Twinline, which is used clinically and which consists mainly of casein from milk protein, amino acids, maltodextrin, fat from safflower oil, and tricaprilin and contains 4.05 g/dl protein, 2.78 g/dl carbohydrate, and 2.78 g/dl fat (1 kcal/ml calorie in total). After overnight fasting (16 h), male mice were administered 20 μl/g Twinline (20 kcal/g energy and 3 g/kg carbohydrate), and blood glucose levels at 0, 30, 60, 120, and 180 min and serum insulin levels at 0, 30, and 60 min after the glucose load were measured.

Reagents. Synthetic human GIP and GLP-1 were purchased from Peptide Institute (Osaka, Japan). Arginine [L(+)-arginine monohydrochloride] was from Nacalai (033-23), and carbachol (carbamylcholine chloride, C-4382) was from Sigma. Twinline enteral formula was from Otsuka Pharmaceuticals (Tokushima, Japan).

Statistical calculations. All values are shown as means ± SE. *P* values were calculated with unpaired Student's *t* test. A value of $P < 0.05$ was considered statistically significant.

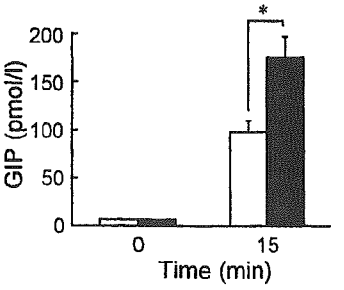

FIG. 1. GIP secretion in response to oral glucose. Plasma GIP levels in Kir6.2$^{+/+}$ (□; n = 12) and Kir6.2$^{-/-}$ (■; n = 12) mice. *$P < 0.0001$.

RESULTS

Glucose-induced GIP secretion. Oral glucose load elicited a significant increase in GIP secretion after 15 min in Kir6.2$^{+/+}$ mice (113.3 ± 2.6 pmol/l, n = 12) (Fig. 1). GIP secretion was significantly increased also in Kir6.2$^{-/-}$ mice (207.3 ± 14.7 pmol/l, n = 12), suggesting that glucose-induced GIP secretion is K_{ATP} channel independent. Interestingly, the increment in plasma GIP in Kir6.2$^{-/-}$ mice was enhanced in Kir6.2$^{-/-}$ mice ($P <$ 0.0001).

Glucose-lowering effect of GIP in vivo. Oral glucose tolerance test (OGTT) was performed on mice pretreated with or without GIP, as previously reported of GLP-1 (31). GIP pretreatment (PreTx) significantly increased glucose tolerance in Kir6.2$^{+/+}$ mice [$AUC_{glucose}$; PreTx(−), 2,146 ± 18 mmol/l in 180 min; PreTx(+), 1,649 ± 78 mmol/l in 180 min; $P < 0.005$] (Fig. 2*A*). However, GIP pretreatment failed to increase glucose tolerance in Kir6.2$^{-/-}$ mice [$AUC_{glucose}$; PreTx(−), 1,901 ± 75 mmol/l in 180 min; PreTx(+) 2,191 ± 375 mmol/l in 180 min; not significant] (Fig. 2*B*), indicating that the glucose-lowering effect of GIP is abolished completely in Kir6.2$^{-/-}$ mice.

Effect of GIP on gastrointestinal transit. We assessed gut motility by measuring gastrointestinal transit of orally ingested barium sulfate. GIP did not affect gastrointestinal transit [67.1 ± 6.7% in PreTx(−) and 74.9 ± 6.0% in PreTx(+)] in Kir6.2$^{+/+}$ mice, which shows that GIP does not inhibit gastrointestinal transit, at least in our protocol (Fig. 2*C*). However, there was a significant increase in gastrointestinal transit of oral glucose load. To evaluate involvement of the K_{ATP} channels in glucose-responsive enteric neurons in regulating gut motility of glucose load, we compared transit of oral glucose load in Kir6.2$^{+/+}$ and Kir6.2$^{-/-}$ mice. Gastrointestinal transit was similarly increased by oral glucose in Kir6.2$^{+/+}$ [glucose (−), 41.9 ± 2.1%; glucose (+), 57.5 ± 4.0%] and Kir6.2$^{-/-}$ [glucose (−), 34.1 ± 1.7%; glucose (+), 61.4 ± 1.7%] mice (Fig. 2*D*), indicating that gut motility is not regulated by K_{ATP} channel–mediated glucose sensing in enteric neurons.

Glucose-lowering effect of GLP-1 in vivo. We then performed OGTTs with and without GLP-1 pretreatment, as was done with GIP. GLP-1 pretreatment reduced the elevation in blood glucose significantly in Kir6.2$^{+/+}$ mice

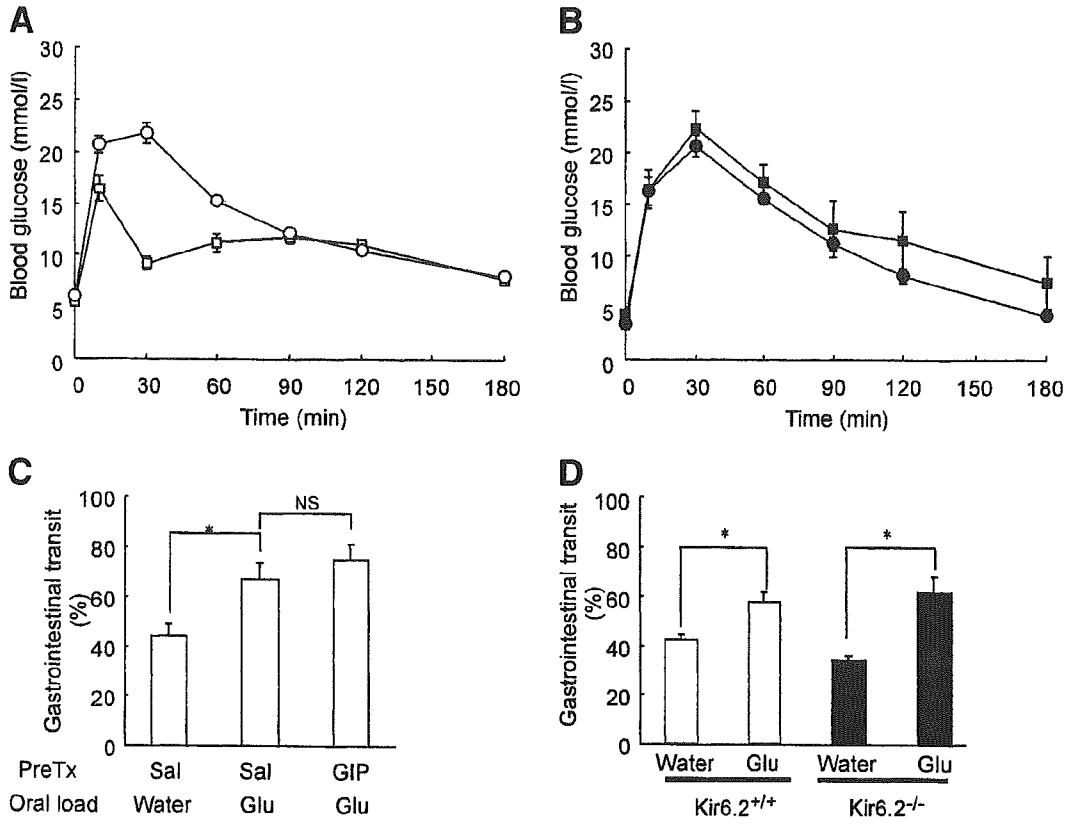

FIG. 2. Effect of GIP on blood glucose and gastrointestinal transit. *A* and *B*: Effect of GIP on blood glucose levels after oral glucose challenge in Kir6.2$^{+/+}$ and Kir6.2$^{-/-}$ mice. Changes in blood glucose levels of Kir6.2$^{+/+}$ (*A*) and Kir6.2$^{-/-}$ (*B*) mice (n = 5–9, for each group) during OGTT are shown. Kir6.2$^{+/+}$ (*A*) and Kir6.2$^{-/-}$ (*B*) mice were pretreated subcutaneously with saline (●) or GIP (■), and glucose was administered orally. *C*: Effect of GIP on gut motility in Kir6.2$^{+/+}$ mice. Gastrointestinal transit was measured in the three groups (n = 4 for each group). The mice were pretreated subcutaneously with saline (Sal) or GIP and then administered barium sulfate orally suspended in glucose (Glu) or water. *P < 0.05; NS, not significant. *D*: Effect of glucose on gut motility in Kir6.2$^{+/+}$ (□) and Kir6.2$^{-/-}$ (■) mice. Gastrointestinal transit was measured in Kir6.2$^{+/+}$ (n = 9) and Kir6.2$^{-/-}$ (n = 9) mice in response to orally administered barium sulfate suspended in glucose or water. *P < 0.005.

[$AUC_{glucose}$; PreTx(−), 2,057 ± 86 mmol/l in 180 min; PreTx(+), 1,347 ± 127 mmol/l in 180 min; $P < 0.005$] (Fig. 3*A*) as well as Kir6.2$^{-/-}$ mice [$AUC_{glucose}$; PreTx(−), 2,513 ± 156 mmol/l in 180 min; PreTx(+) 1,403 ± 155 mmol/l in 180 min; $P < 0.0001$] (Fig. 3*B*).

Effect of GLP-1 on gastrointestinal transit in vivo. We also examined the effect of GLP-1 on gut motility. Gastrointestinal transit was significantly suppressed by GLP-1 pretreatment (Fig. 3*C*). GLP-1 was similarly effective on gut motility in Kir6.2$^{+/+}$ and Kir6.2$^{-/-}$ mice, suggesting that the effect of GLP-1 is independent of K_{ATP} channel activity. In addition, GLP-1 similarly suppressed gastrointestinal transit in glucose-loaded and water-loaded mice, showing that GLP-1 and glucose regulate gut motility through independent mechanism.

Potentiation of insulin secretion by GIP and GLP-1 in vivo. We examined insulin secretion during OGTT with and without GIP or GLP-1 pretreatment (Fig. 4). Ten minutes after glucose loading, serum insulin levels were already elevated in Kir6.2$^{+/+}$ mice (77.2 ± 11.5 pmol/l at 0 min; 275.5 ± 42.5 pmol/l at 10 min) (Fig. 4). Insulin secretion at 10 min was significantly enhanced by GIP pretreatment (440.8 ± 53.8 pmol/l, $P < 0.05$) or by GLP-1 pretreatment (474.7 ± 49.2 pmol/l, $P < 0.05$). Secretion in Kir6.2$^{+/+}$ mice was no longer enhanced by GIP or GLP-1 pretreatment at 30 min, when the blood glucose levels are lower (Figs. 2*A* and 3*A*).

Interestingly, the insulinotropic effect of GIP was completely absent in Kir6.2$^{-/-}$ mice at 10 min (83.1 ± 9.0 pmol/l at 0 min; 66.2 ± 17.0 pmol/l at 10 min; not significant); however, there was significant potentiation of insulin secretion at 10 min by GLP-1 pretreatment (242.9 ± 13.0 pmol/l, $P < 0.0005$) (Fig. 4). In contrast, at 30 min after glucose load, there was significant potentiation of insulin secretion in 30 min in Kir6.2$^{-/-}$ mice both by GIP pretreatment (245.5 ± 8.51 pmol/l) and by GLP-1 pretreatment (240.4 ± 21.1 pmol/l), even though glucose-induced insulin secretion was not observed (112.8 ± 12.5 pmol/l) (Fig. 4).

Effects of GIP and GLP-1 on insulin secretion in perfused pancreas. To examine the time course of the insulin secretory response to GIP and GLP-1 in Kir6.2$^{-/-}$ mice, perfusion experiments were performed in the absence (Fig. 5*A*) or presence of GIP (Fig. 5*B* and *C*) or GLP-1 (Fig. 5*D*). In Kir6.2$^{+/+}$ mice, 16.7 mmol/l glucose elicited insulin secretion [the amount of secreted insulin ($AUC_{insulin}$) after glucose stimulation (from 5 to 25 min); 61.4 ± 5.5 ng in 20 min, n = 3] (Fig. 5*A*), which was further potentiated by 1 nmol/l [$AUC_{insulin}$; 217.9 ± 12.3 ng, n = 3, $P < 0.005$ vs. GIP(−)] or 10 nmol/l GIP [$AUC_{insulin}$; 278.8 ± 25.4 ng, n = 3, $P < 0.05$ vs. GIP(−)] (Fig. 5*B* and *C*). In contrast, in Kir6.2$^{-/-}$ mice, 16.7 mmol/l glucose barely elicited a rise in insulin secretion ($AUC_{insulin}$; 23.3 ± 2.7 ng, n = 3) (Fig. 5*A*), and there was only slight potentiation in insulin secretion by 1 nmol/l ($AUC_{insulin}$; 37.1 ± 4.2 ng, n =

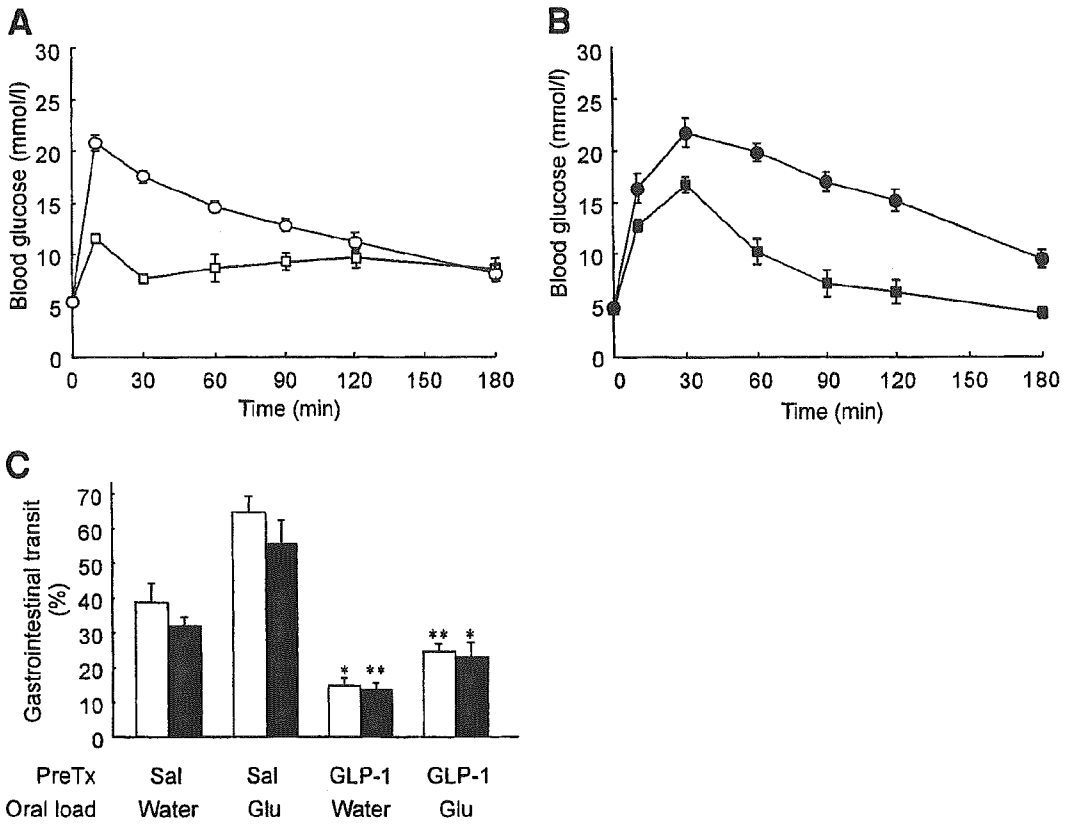

FIG. 3. Effect of GLP-1 on blood glucose after oral glucose challenge and gastrointestinal transit in Kir6.2$^{+/+}$ and Kir6.2$^{-/-}$ mice. *A* and *B*: Changes in blood glucose levels in Kir6.2$^{+/+}$ (*A*) and Kir6.2$^{-/-}$ (*B*) mice (n = 10–14, for each group) during OGTT are shown. Mice were pretreated subcutaneously with saline (●) or GLP-1 (■) or and were administered orally ingested glucose. *C*: Effect of glucose on gut motility in Kir6.2$^{+/+}$ (□) and Kir6.2$^{-/-}$ (■) mice. Gastrointestinal transit was measured (n = 7–9 for each group) as in Fig. 2*C*. The mice were pretreated subcutaneously with saline (Sal) or GLP-1 and then administered barium sulfate orally suspended in glucose (Glu) or water. *P < 0.005 and **P < 0.0001 for comparison between GLP-1–pretreated (GLP-1) versus GLP-1–untreated (Sal) mice.

3) or 10 nmol/l GIP ($AUC_{insulin}$; 55.8 ± 12.7 ng, n = 3) (Fig. 5*B* and *C*). The potentiation of insulin secretion by 1 nmol/l GLP-1 also was attenuated in Kir6.2$^{-/-}$ mice ($AUC_{insulin}$; 103.8 ± 40.6 ng, n = 3) compared with that of Kir6.2$^{+/+}$ mice ($AUC_{insulin}$; 329.1 ± 20.1 ng, n = 3), but the secretion was nevertheless more potent than that by 1 nmol/l GIP (Fig. 5*D*). When insulin secretion was assessed

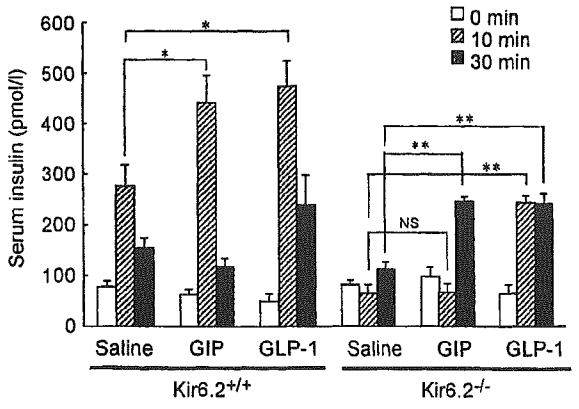

FIG. 4. Effect of GIP and GLP-1 pretreatment on insulin secretion after oral glucose challenge. Serum insulin levels in Kir6.2$^{+/+}$ and Kir6.2$^{-/-}$ mice before (□, n = 5–9) and 10 min (▨, n = 5–9) or 30 min (■, n = 5–9) after glucose load. *P < 0.05; **P < 0.0005.

by the $AUC_{insulin}$, 1, 10, and 1 nmol/l GLP-1 potentiated insulin secretion in Kir6.2$^{+/+}$ mice 3.5-, 3.5-, and 5.4-fold, respectively (Fig. 5*E*). In contrast, in Kir6.2$^{-/-}$ mice, 1 and 10 nmol/l GIP increased insulin secretion only by 1.6- and 2.4-fold, whereas 1 nmol/l GLP-1 increased insulin secretion by 4.5-fold (Fig. 5*E*). In addition, glucose-induced insulin secretion in Kir6.2$^{-/-}$ mice became apparent in the presence of 1 nmol/l GLP-1 [fold increase in the insulin secretory rate before and after stimulation with 16.7 mmol/l glucose; 1.52 ± 0.10-fold in the absence of GLP-1 (n = 3) (Fig. 5*A*), 4.14 ± 0.06-fold in 1 nmol/l GLP-1 (n = 3) (Fig. 5*D*); P < 0.05], indicating that Kir6.2$^{-/-}$ mice were endued with glucose responsiveness by stimulation with 1 nmol/l GLP-1.

Insulin secretory response to arginine, cholinergic stimuli, and mixed meal. Insulin secretion by arginine in vivo was significantly impaired in Kir6.2$^{-/-}$ mice (Fig. 6*A*), but marked secretion was observed 2 min after administration of carbachol in vivo in both Kir6.2$^{+/+}$ and Kir6.2$^{-/-}$ mice (Fig. 6*B*). The insulin secretory response was also examined in perfusion experiments. Similar to the findings in vivo, insulin secretion from Kir6.2$^{-/-}$ pancreata was markedly impaired in response to 20 mmol/l arginine [$AUC_{insulin}$ during stimulation (from 5 to 10 min); 39.1 ± 4.2 ng in Kir6.2$^{+/+}$ mice (n = 3), 8.4 ± 2.8 ng in Kir6.2$^{-/-}$ mice (n = 3)] but remained unaffected in response to 50

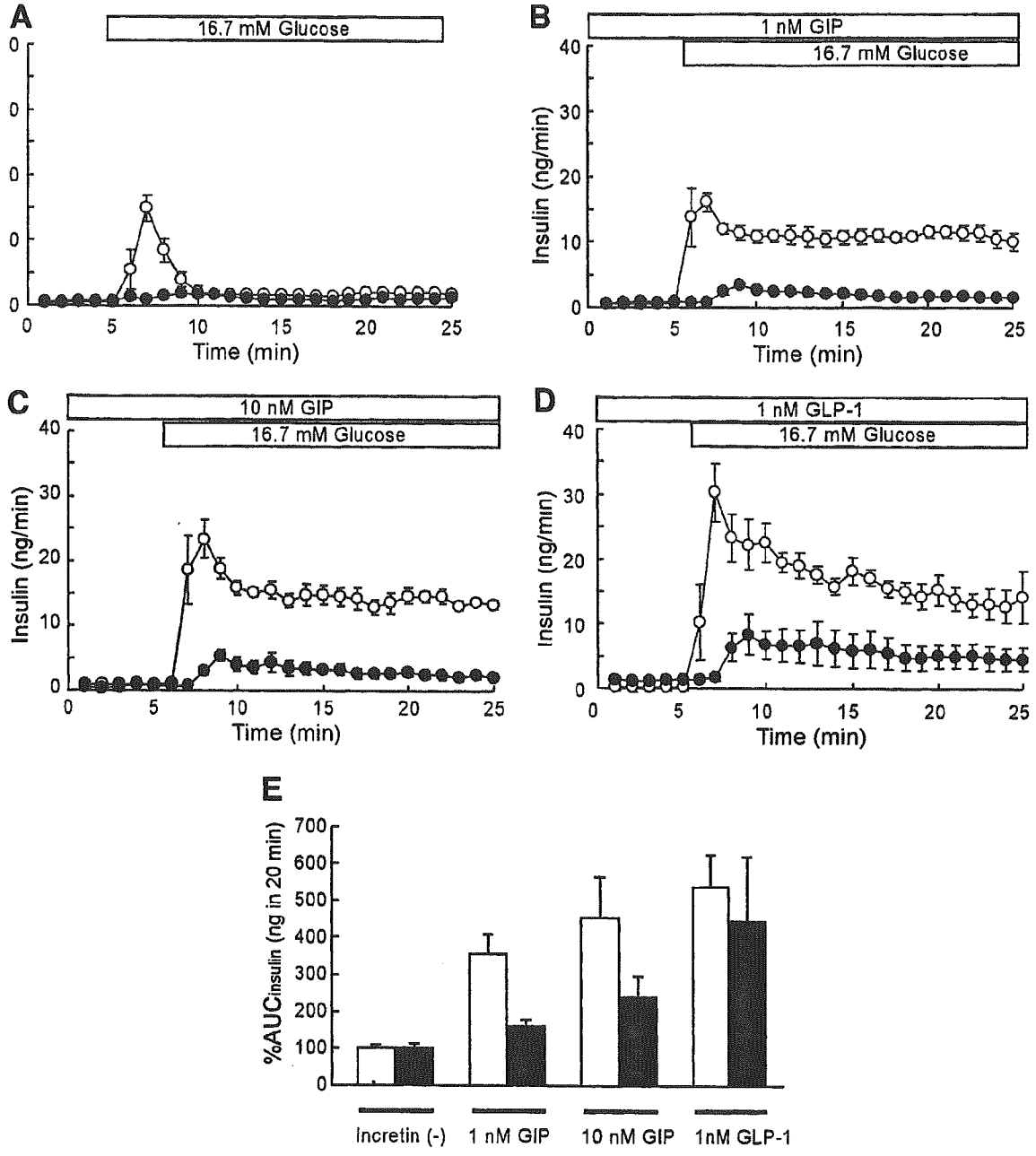

FIG. 5. Insulin secretion in perfused pancreata in response to high glucose, GIP, and GLP-1. *A–D*: Insulin secretion from perfused pancreata of Kir6.2$^{+/+}$ (○) and Kir6.2$^{-/-}$ (●) mice is shown. Glucose concentration was shifted from 2.8 to 16.7 mmol/l at 5 min in the absence of incretin (*A*) or in the presence of 1 nmol/l GIP (*B*), 10 nmol/l GIP (*C*), or 1 nmol/l GLP-1 (*D*). The data represent the mean ± SE of three mice. *E*: The amounts of secreted insulin of Kir6.2$^{+/+}$ (□) and Kir6.2$^{-/-}$ (■) mice after glucose stimulation are expressed as the $AUC_{insulin}$ from 5 to 25 min in *A–D*. Data are percent relative to that in the absence of incretin.

μmol/l carbachol [$AUC_{insulin}$ during stimulation (from 20 to 25 min); 8.7 ± 1.5 ng in Kir6.2$^{+/+}$ mice (n = 3), 10.6 ± 1.2 ng in Kir6.2$^{-/-}$ mice (n = 3)] (Fig. 6*C*). We also compared glucose tolerance and insulin secretion in response to orally ingested meal in Kir6.2$^{+/+}$ and Kir6.2$^{-/-}$ mice (Fig. 6*D* and *E*). In Kir6.2$^{-/-}$ mice, glucose tolerance was significantly impaired in response to mixed meal (Fig. 6*D*), and early-phase insulin secretion was significantly diminished (serum insulin levels 30 min after meal ingestion; Kir6.2$^{+/+}$, 322.2 ± 47.5 pmol/l; Kir6.2$^{-/-}$, 134.0 ± 14.9 pmol/l, $P < 0.005$) (Fig. 6*E*).

DISCUSSION

GIP is released from gastrointestinal endocrine K-cells (32) in a glucose-dependent manner (7,33). Although glucose-induced insulin secretion from pancreatic β-cells is critically dependent on K_{ATP} channel function (22–24), our present study indicated that glucose-induced GIP secretion occurs independently of K_{ATP} channel function (Fig. 1).

Because GIP pretreatment did not reduce the elevation of blood glucose in Kir6.2$^{-/-}$ mice after oral glucose load

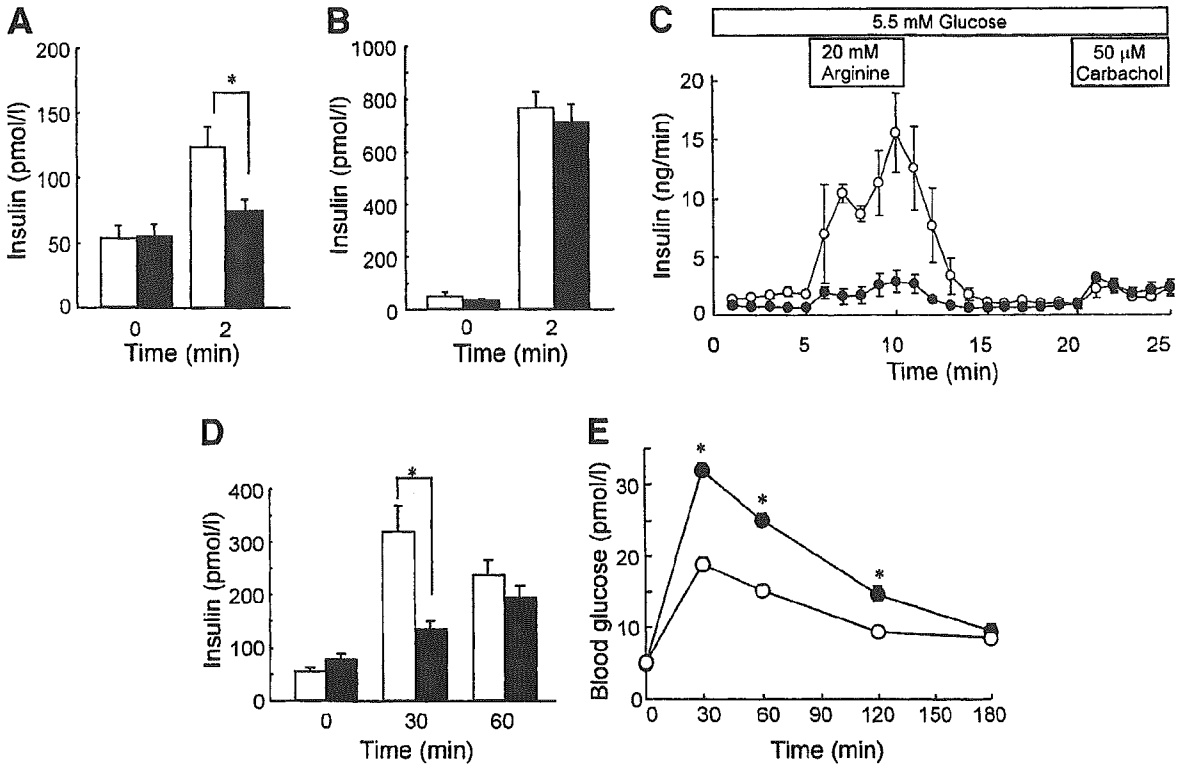

FIG. 6. Insulin secretion and glucose tolerance in response to arginine, carbachol, and a mixed meal. *A*: Early phase of insulin secretion in response to arginine is shown. Serum insulin levels in Kir6.2$^{+/+}$ (□, n = 8) and Kir6.2$^{-/-}$ (■, n = 9) mice 2 min after intravenous arginine administration. *P < 0.005. *B*: The insulin secretion in response to carbachol is shown. Serum insulin levels in Kir6.2$^{+/+}$ (□, n = 9) and Kir6.2$^{-/-}$ (■, n = 10) mice 2 min after intraperitoneal carbachol administration. There is no significant difference in serum insulin levels between Kir6.2$^{+/+}$ and Kir6.2$^{-/-}$ mice. *C*: Insulin secretion from perfused pancreata of Kir6.2$^{+/+}$ (○) and Kir6.2$^{-/-}$ (●) mice in response to 20 mmol/l arginine and 50 μmol/l carbachol is shown. *D*: Changes in serum insulin levels in response to mixed meal ingestion. Serum insulin levels in Kir6.2$^{+/+}$ (□, n = 15) and Kir6.2$^{-/-}$ (■, n = 15) mice at indicated time points are shown. *P < 0.005. *E*: Changes in blood glucose levels in response to mixed meal ingestion. Blood glucose levels in Kir6.2$^{+/+}$ (○, n = 15) and Kir6.2$^{-/-}$ (●, n = 15) mice are shown. *P < 0.0001.

(Fig. 2*B*), we considered the possibility that GIP inhibits gut motility in a K_{ATP} channel–dependent manner, but this apparently is not the case (Fig. 2*C*). This is compatible with a recent study of GIP action on gut motility in humans (34). We also found a significant increase in gastrointestinal transit by oral glucose load. We established previously that K_{ATP} channels comprising Kir6.2 and SUR1 are found in glucose-responsive neurons in the hypothalamus (35) and in the ileum (36), and we proposed that the K_{ATP} channel in gut cholinergic neurons plays a role in glucose-evoked reflexes (36). Ingestion of carbohydrate is known to stimulate gastrointestinal motility (37), but it was unclear whether the K_{ATP} channel in glucose-responsive enteric neurons is involved in regulating glucose-induced gut motility. Our present findings on gastrointestinal transit in Kir6.2$^{-/-}$ mice clearly show that gut motility is not regulated by K_{ATP} channel–mediated glucose sensing in the enteric neurons (Fig. 2*D*).

Measurement of serum insulin at 10 min after oral glucose load revealed that GIP pretreatment in vivo failed to potentiate the early-phase (38) insulin secretion during OGTT in Kir6.2$^{-/-}$ mice (Fig. 3), indicating that the K_{ATP} channel in β-cells is essential in the insulinotropic effect of GIP. It would be likely, therefore, that the glucose-dependent effects of GIP depend on the activity of the K_{ATP} channel. In contrast, there was significant potentiation of late-phase insulin secretion (2.17-fold increase) in Kir6.2$^{-/-}$ mice by GIP pretreatment. However, the physiological significance of this late-phase insulin secretion remains uncertain, because there was no significant reduction in blood glucose levels even after 30 min in GIP-pretreated Kir6.2$^{-/-}$ mice compared with GIP-untreated Kir6.2$^{-/-}$ mice. These results also suggest that rapid enhancement of early-phase insulin secretion by GIP is required for its glucose-lowering effect after oral glucose load.

In contrast to GIP, GLP-1 did potentiate the insulin secretion (3.7-fold increase in 10 min) and had an obvious antihyperglycemic effect in Kir6.2$^{-/-}$ mice (Fig. 4*A*, *B*, and *D*). Perfusion experiments of mouse pancreata are applicable only for a short period (less than 45 min of sampling) of secretion study of insulin. Thus, it is difficult to perform multiple stimuli in the same mouse pancreas, and a number of experiments are required to compare the secretory differences among different stimuli. However, when compared with the study of isolated islets, this method has an advantage because we can neglect cellular damage during islet isolation or unexpected effects by culturing the islets.

We performed perfusion experiments in Kir6.2$^{-/-}$ mice and found that differences in the insulinotropic effects between GIP and GLP-1 in Kir6.2$^{-/-}$ mice were also shown in the perfusion experiments (Fig. 5). Accordingly, the mechanism of potentiation of insulin secretion differs for GIP and GLP-1: insulin secretion by GIP depends critically on the K_{ATP} channel, whereas that by GLP-1 does not. Both

GIP and GLP-1 increase the intracellular cAMP concentration and potentiate insulin secretion by activating protein kinase A (PKA)– and/or cAMP-GEF2–mediated signaling in normal pancreatic β-cells (39). We previously reported that GIP-potentiated insulin secretion is almost completely suppressed in islets treated both with PKA blocker H-89 and antisense oligodeoxynucleotides against cAMP-GEF2, whereas GLP-1–potentiated insulin secretion remains nearly normal (15). Apparently, the insulinotropic action of GLP-1 is mediated by a pathway other than that involving PKA and cAMP-GEF2. In addition, we found that whereas GIP had almost no effect on gut motility, GLP-1 significantly suppressed gastrointestinal transit (Fig. 3*C*). Because the effect of GLP-1 is independent of the K_{ATP} channels, GLP-1 may well delay glucose absorption and prevent a rise in blood glucose levels after glucose load in Kir6.2$^{-/-}$ mice. Thus, GLP-1 is suggested to participate in the postprandial glycemic control in K_{ATP} channel–independent manners by potentiating insulin secretion and by delaying gastric emptying. Although the importance of the K_{ATP} channel in the potentiation of insulin secretion by cAMP has been shown in SUR1 knockout mice (24,40), we clarify here the involvement of the channel in the potentiation of insulin secretion by GIP and GLP-1.

Although arginine treatment elicited impaired insulin secretion, the insulin secretion of Kir6.2$^{-/-}$ mice in response to carbachol was intact, indicating that the exocytotic machinery of Kir6.2$^{-/-}$ β-cells is intact and that the cause of impaired insulin secretion differs according to the stimulus (Fig. 6*A–C*). Insulin secretion is stimulated by multiple signals in pancreatic β-cells, including nutrients (carbohydrate, proteins, and fat), incretins (GIP and GLP-1), and neuronal input (mainly cholinergic). Our results indicate that mice lacking the Kir6.2 pore-forming subunit of K_{ATP} channels have an impaired insulin secretory response to glucose, arginine, and GIP, whereas the insulin secretion elicited by carbachol is comparable with that in Kir6.2$^{+/+}$ mice. Kir6.2$^{-/-}$ mice were also shown to exhibit glucose intolerance and delayed insulin secretion in response to mixed meal (Fig. 6*C* and *D*). The K_{ATP} channel thus plays an important role in regulating blood glucose levels both after glucose load and after ingestion of a mixed meal. The present study shows that the K_{ATP} channel in pancreatic β-cells is required for the insulinotropic effects of GIP through the potentiation of glucose-induced insulin secretion. In contrast, the potentiation of insulin secretion by GLP-1 depends on K_{ATP} channel–independent and –dependent mechanisms. The differing pathways of the action of GLP-1 and GIP on both the potentiation of insulin and gut motility might well account for the differences seen in their therapeutic efficacy in type 2 diabetes.

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Soluble E-selectin, leptin, triglycerides, and insulin resistance in nonobese Japanese type 2 diabetic patients

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Abstract

The aim of the present study was to investigate the relationships between insulin resistance and soluble E-selectin, body mass index (BMI), leptin, and serum lipid profile including triglycerides in nonobese Japanese type 2 diabetic patients.

A total of 97 nonobese Japanese type 2 diabetic patients aged 43 to 84 years were examined. The duration of diabetes was 11.2 ± 0.8 years. In conjunction with BMI and fasting concentrations of plasma glucose, serum lipids (triglycerides, total cholesterol, and high-density lipoprotein cholesterol) and serum insulin, soluble E-selectin, and leptin were also measured. The low-density lipoprotein (LDL) cholesterol level was calculated using the Friedewald formula. Insulin resistance was estimated by the homeostasis model assessment. The subjects were divided into 2 groups according to the value of insulin resistance estimated by the homeostasis model assessment. Values greater than 2.5 were indicative of the insulin-resistant state, and values less than 2.5 were indicative of the insulin-sensitive state.

The insulin-resistant group had significantly higher levels of E-selectin, leptin, triglycerides, total and LDL cholesterol, and diastolic blood pressure as compared with the insulin-sensitive group. There was, however, no significant difference in age, sex, diabetes duration, BMI, systolic blood pressure, HbA1c, and high-density lipoprotein cholesterol between the 2 groups. Univariate regression analysis showed that insulin resistance was positively correlated to E-selectin ($r = 0.305$, $P = .003$), BMI ($r = 0.283$, $P = .006$), leptin ($r = 0.296$, $P = .004$), HbA1c ($r = 0.241$, $P = .018$), serum triglycerides ($r = 0.385$, $P < .001$), serum total ($r = 0.240$, $P = .019$) and LDL cholesterol ($r = 0.254$, $P = .013$) levels, and systolic ($r = 0.247$, $P = .024$) and diastolic ($r = 0.305$, $P = .006$) blood pressure. Multiple regression analyses showed that insulin resistance was independently predicted by serum E-selectin ($F = 18.4$), serum leptin ($F = 14.0$) and serum triglycerides ($F = 20.0$) levels, which explained 45.0% of the variability of insulin resistance.

From these results, it can be concluded that in conjunction with serum triglycerides and serum leptin, serum E-selectin is another important independent factor associated with insulin resistance in nonobese Japanese type 2 diabetic patients.

1. Introduction

Type 2 diabetes is a heterogeneous syndrome characterized by insulin resistance and/or defective insulin secretion [1,2]. There seem to be ethnic differences in insulin resistance in type 2 diabetes. Nonobese Japanese type 2 diabetic patients are unique in that they are divided into 2 variants: one with insulin resistance and the other with normal insulin sensitivity [3,4].

The mechanisms underlying insulin resistance in nonobese Japanese type 2 diabetes are not fully understood. We recently demonstrated that insulin resistance in nonobese Japanese type 2 diabetic patients is mostly associated with triglycerides but not with body mass index (BMI) [5,6]. The reduction in triglycerides level by bezafibrate [7] or exercise [8] leads to an enhancement in insulin action without affecting BMI in nonobese Japanese type 2 diabetic patients. Abassi et al [9] are the first to show that plasma insulin concentration is more tightly linked to plasma leptin concentration than is the BMI in human beings. Thus, in conjunction with serum triglycerides, leptin is suggested to

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be another factor that is linked to insulin resistance in nonobese Japanese type 2 diabetic patients.

Furthermore, there are some literatures suggesting that insulin resistance is closely associated with the pathogenesis of atherosclerosis. The earliest morphological evidence of atherosclerosis is the attachment of monocytes to the cell surface of the endothelium. Monocytes attach at the cell surface of adhesion molecules such as E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1). In contrast to ICAM-1 and VCAM-1, E-selectin is expressed only on activated endothelium [10]. Thus, E-selectin is hypothesized to be one of the most important adhesion molecules for the evolution of atherosclerosis. Whereas serum E-selectin level is reported to be high in type 2 diabetic patients, the relationship between serum concentration of E-selectin and insulin resistance is very limited [11-13]. Furthermore, the relationship has not yet been fully investigated in nonobese Japanese type 2 diabetic patients without confounding the effects of serum triglycerides and serum leptin levels. In this respect, a major problem is that the degree of overweight or of hyperglycemia, insulin therapy, or the medications known to improve insulin resistance is shown to affect serum soluble E-selectin level. Thus, the aim of the present study is to investigate the relationship between insulin resistance andÂserum E-selectin in nonobese unique Japanese type 2 diabetic patients taking into account of the effects of leptin, triglycerides, BMI, and hemoglobin (Hb) A1c. This is the first description that in conjunction with serum triglycerides and serum leptin, serum E-selectin is another independent factor closely associated with insulin resistance in nonobese Japanese type 2 diabetic patients who had no insulin therapy and no evidence of diabetic vascular complications.

2. Subjects and methods

Ninety-seven Japanese type 2 diabetic patients who visited our clinic were enrolled for the present study. Type 2 diabetes mellitus was diagnosed based on the criteria of the World Health Organization [14]. The patients who had chronic heart or renal failure, symptomatic coronary heart disease, symptomatic stroke, and symptomatic peripheral artery disease were excluded. They had no evidence of current acute illness including clinically significant infectious disease. Their age and BMI levels were 62.9 ± 0.9 years (mean ± SEM) and 23.0 ± 0.2 (range, 19.1 to 26.7 kg/m^2), respectively. They all were nonobese [15]. The duration of diabetes was 11.2 ± 0.8 (range, 1 to 35 years). HbA1c level was 7.0% ± 0.1% (range, 5.2% to 10.4%). Systolic and diastolic blood pressure was 137 ± 2 and 83 ± 1 mm Hg, respectively. Forty-two of 97 patients had hypertension that was treated with angiotensin-converting enzyme inhibitors (21/42), calcium-channel blockers (20/42), or both (1/42). Eighty-three patients were taking sulfonylureas (gliclazide) and the rest with diet alone. Seventeen and 14 of 97 patients were treated with bezafibrate and 3-hydroxy-3 methylglutaryl coenzyme A (HMG CoA) reductase inhibitors, respectively. They all were not treated with insulin, biguanides, or pioglitazone. All subjects had ingested at least 150 g of carbohydrate for the 3 days preceding the study. They did not consume alcohol or perform heavy exercise for at least 1 week before the study.

Blood was drawn at the morning after a 12-hour fast. Plasma glucose was measured with glucose oxidase method. The triglycerides, total cholesterol, and high-density lipoprotein (HDL) cholesterol were also measured. Serum insulin was measured using a 2-site immunoradiometric assay (Insulin Riabead II, Dainabot, Japan). Coefficients of variation were 4% for insulin greater than 25 μU/mL and 7% for insulin less than 25 μU/mL, respectively. There was no detectable cross-reactivity of proinsulin in the insulin assay. Soluble E-selectin was measured by commercially available enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, Minn) using baseline samples. Serum leptin concentration was measured with a radioimmunoassay kit (Linco Research, St Charles, Mo) using specific human leptin antibody. The intra-assay and interassay CVs were less than 6% for E-selectin and leptin. Samples for insulin, E-selectin, and leptin were prepared, frozen, and stored at −70°C until the assay. The estimate of insulin resistance by homeostasis model assessment (HOMA-IR) was calculated with the formula: fasting serum insulin (μU/mL) × fasting plasma glucose (mmol/L)/22.5 [16]. The HOMA-IR value of normal glucose tolerant subjects was 1.6 ± 0.9 (mean ± SD), and we defined the value greater than 2.5 (mean ± SD of normal glucose-tolerant subjects) as an insulin-resistant state and the value less than 2.5 as an insulin-sensitive state [5,6]. The threshold value for insulin resistance in our study (ie, 2.5) is similar to that (2.77) reported in nonobese subjects with no metabolic disorders reported by Borona et al [17].

3. Statistical analysis

Data are presented as mean ± SEM. Statistical analyses were conducted using the StatView 5 system (StatView, Berkeley, Calif). Means of 2 groups were compared with Student t test. Simple (Spearman rank) correlation coefficients between HOMA-IR and measures of variables were calculated, and a stepwise multiple regression analysis was then used to evaluate the independent association of these variables with HOMA-IR. P value less than .05 was considered as significant. In multivariate analysis, F value ≥4 was considered significant.

4. Results

Table 1 illustrates the mean ± SEM of the clinical characteristics and clinical profile in insulin-resistant and insulin-sensitive nonobese Japanese type 2 diabetic patients. HOMA-IR values in the patients with insulin resistance and normal insulin sensitivity were 3.68 ± 0.25 and 1.63 ±

0.06, respectively. Thirty-two (30%) of 97 type 2 diabetic patients had HOMA-IR of greater than 2.5, indicating that they are insulin-resistant. There was no significant difference in age, duration of diabetes, BMI, HbA1c, and HDL cholesterol levels between the 2 subpopulations. Fasting glucose and insulin concentrations were significantly higher in insulin-resistant group than in insulin-sensitive group. In contrast, the patients with insulin resistance had significantly higher concentrations of E-selectin (58.2 ± 4.2 vs 47.2 ± 2.3 mg/dL, $P = .008$), leptin (6.18 ± 0.73 vs 4.47 ± 0.34 mg/dL, $P = .009$), triglycerides (148 ± 12 vs 109 ± 5 mg/dL, $P < .001$), total (213 ± 6 vs 196 ± 4 mg/dL, $P = .018$), and low-density lipoprotein (LDL) cholesterol (134 ± 6 vs 120 ± 4 mg/dL, $P = .018$) as compared with those with normal insulin sensitivity. Whereas no significant difference was observed in systolic blood pressure, diastolic blood pressure was significantly higher in insulin-resistant group than in insulin-sensitive group. There was no significant difference in the mode of therapy for hypertension or lipidemia between the 2 groups (data not shown).

Spearman rank correlations of insulin resistance with measures of variables were calculated for all our diabetic patients (Table 2). Insulin resistance was positively correlated with E-selectin, leptin, triglycerides, BMI, HbA1c, and total and LDL cholesterol levels. Other variables including age, sex, duration of diabetes, and HDL cholesterol were not associated with insulin resistance.

Multiple regression analyses were carried out using the stepwise procedure. The analysis included insulin resistance as a dependent variable and candidate risk factors (E-selectin, leptin, triglycerides, BMI, HbA1c, total cholesterol, and LDL cholesterol) as independent variables. Insulin resistance was independently predicted by serum concentrations of E-selectin, leptin, and triglycerides, which explained 45.0% of the variability of insulin resistance in our patients. Other variables including BMI, HbA1c, and total and LDL cholesterol were not independently associated with insulin resistance in our nonobese Japanese type 2 diabetic patients.

Table 1
Clinical characteristics and clinical profile in insulin-resistant and insulin-sensitive diabetic patients

	Insulin-resistant	Insulin-sensitive	P
HOMA-IR	3.68 ± 0.25	1.63 ± 0.06	<.001
Number of subjects	32	65	
M/F	30/9	42/5	.384
Age (y)	62.4 ± 1.7	63.1 ± 1.0	.360
BMI (kg/m^2)	23.4 ± 0.3	22.8 ± 0.2	.064
Duration of diabetes (y)	11.3 ± 1.6	11.1 ± 0.8	.459
HbA1c (%)	7.2 ± 0.2	6.9 ± 0.1	.071
HDL cholesterol (mg/dL)	56 ± 2	60 ± 2	.135
Fasting glucose (mg/dL)	151 ± 4	137 ± 3	.005
Fasting insulin (μU/mL)	10.0 ± 0.7	4.8 ± 0.2	<.001
E-selectin (mg/dL)	58.2 ± 4.2	47.2 ± 2.3	.008
Leptin (mg/dL)	6.18 ± 0.73	4.47 ± 0.34	.009
Triglycerides (mg/dL)	148 ± 12	109 ± 5	<.001
Total cholesterol (mg/dL)	213 ± 6	196 ± 4	.018
LDL cholesterol (mg/dL)	134 ± 6	120 ± 4	.018
Systolic blood pressure (mm Hg)	141 ± 3	135 ± 2	.081
Diastolic blood pressure (mm Hg)	88±2	81±1	.001

Table 2
Correlation of insulin resistance to measures of variables in diabetic patients

	r	P
E-selectin	0.305	.003
Leptin	0.296	.004
Triglycerides	0.385	<.001
BMI	0.283	.006
HbA1c	0.241	.018
Total cholesterol	0.240	.019
LDL cholesterol	0.254	.013
Systolic blood pressure	0.247	.024
Diastolic blood pressure	0.305	.006
Age	−0.065	.522
Sex	0.007	.946
HDL cholesterol	−0.178	.804
Duration of diabetes	−0.018	.860

Finally, the relationships between soluble E-selectin and serum leptin, BMI, or serum triglycerides level were investigated. There were no significant relationships between serum soluble E-selectin level and serum leptin, BMI, or serum triglycerides level in our patients (data not shown).

5. Discussion

Type 2 diabetes is a heterogenous syndrome characterized by insulin resistance and/or defective insulin secretion [1,2]. There seems to be ethnic difference in insulin resistance in type 2 diabetes. Haffner et al [18] recently disclosed that 92% of type 2 diabetic patients are insulin-resistant in white populations. In contrast, Chaiken et al [19] previously showed that 60% of type 2 diabetic patients are insulin-resistant in black Americans with a BMI less than 30 kg/m^2. Using minimal model approach shown by Bergman et al [20], our team previously demonstrated that Japanese type 2 diabetic patients are divided into 2 variants: one with primary insulin resistance and the other with normal insulin sensitivity [3,4]. Thereafter, we have shown that 40% of type 2 diabetic patients are insulin-resistant in Japanese populations [5]. In conjunction with the present study that 30% of type 2 diabetic patients are insulin-resistant in nonobese Japanese type 2 diabetic patients, Japanese type 2 diabetic patients are assumed to be unique in terms of clinical profiles.

There are some factors associated with insulin resistance in nonobese Japanese type 2 diabetic patients. We recently demonstrated that serum triglycerides but not BMI are mostly associated with insulin resistance in nonobese Japanese type 2 diabetic patients [5,6]. Thereafter, our group clarified that not only serum leptin but also adiponectin levels are linked to insulin resistance in nonobese Japanese type 2 diabetic patients [21,22]. Serum triglyceride level is positively correlated to visceral fat areas in nonobese Japanese type 2 diabetic patients [23]. Serum

leptin level is positively correlated to subcutaneous fat areas, whereas serum adiponectin level is negatively correlated to visceral fat areas in nonobese Japanese type 2 diabetic patients [21,22]. Thus, the factors associated with insulin resistance in nonobese Japanese type 2 diabetic patients are hypothesized to be linked to adipose tissue–related insulin resistance.

Another factor that is associated with insulin resistance is adhesion molecules such as E-selectin, ICAM-1, and VCAM-1. These adhesion molecules are associated with the evolution of atherosclerosis. Atherosclerosis is assumed to be linked to insulin resistance in human beings. Thus, these adhesion molecules are suggested to be associated with insulin resistance in nonobese Japanese type 2 diabetic patients. In contrast to ICAM-1 and VCAM-1, E-selectin is expressed only on activated endothelium [10]. We therefore investigated the relationship between insulin resistance and serum soluble E-selectin in our nonobese unique Japanese type 2 diabetic patients.

In the present study, we disclosed that not only serum triglyceride but also serum soluble E-selectin and serum leptin levels are higher in insulin-resistant group than in insulin-sensitive groups in nonobese Japanese type 2 diabetic patients matched for sex. Furthermore, in conjunction with serum triglyceride and leptin, E-selectin is independently associated with insulin resistance in nonobese Japanese type 2 diabetic patients. It may be argued that our results are influenced by sex because leptin concentration is influenced by sex. However, not only insulin resistance but also serum triglycerides and E-selectin were not affected by sex in our present study.

Interestingly, serum leptin and triglycerides levels were independently associated with insulin resistance, whereas BMI was not, in our nonobese Japanese type 2 diabetic patients. Serum leptin level is shown to be associated with subcutaneous fat area in nonobese Japanese type 2 diabetic patients [21]. In contrast, serum triglyceride level is shown to be reflective of visceral abdominal fat area in nonobese Japanese type 2 diabetic patients [23]. Thus, body fat distribution but not the degree of BMI seems to affect insulin resistance in nonobese unique Japanese type 2 diabetic patients. This idea is supported from the recent study shown by Abassi et al [9] that plasma insulin concentration is more tightly linked to plasma leptin concentration than is the BMI. We recently demonstrated that both subcutaneous and visceral abdominal fat areas are independently associated with insulin resistance in nonobese Japanese type 2 diabetic patients [23].

E-selectin level was not associated with adipose tissue–related insulin resistance such as leptin, triglycerides, and BMI in our present study. Thus, E-selectin is considered to be another most important factor associated with insulin resistance in nonobese Japanese type 2 diabetic patients.

The mechanisms underlying the relationship between insulin resistance and soluble E-selectin are not known at present. One possible explanation for the relationship is nitric oxide released from endothelium. Steinberg et al [24] showed that insulin resistance is associated with blunted endothelium-dependent vasodilation and that this phenomenon is related to low nitric oxide release from endothelium. Elevated E-selectin level is known to be reflective of endothelial damage [25]. Another possible explanation for the finding is the mode of therapy. Intensive insulin therapy is reported to reduce serum E-selectin level in diabetic patients [26]. Agents that improve insulin resistance are shown to reduce serum E-selectin concentration. Cominacini et al [27] reported that troglitazone decreased serum E-selectin level in patients with type 2 diabetes. In the present study, our patients were not treated with insulin therapy or the medications known to improve insulin resistance such as biguanide or pioglitazone. Finally, the role of oxidative stress should not be overlooked in type 2 diabetic patients. Cominacini et al [11] showed that serum E-selectin concentration is related to plasma hydroperoxides and to susceptibility to LDL to oxidation in type 2 diabetic patients.

Irrespective of this, it can be concluded that in conjunction with serum triglycerides, serum E-selectin and leptin are another independent factors associated with insulin resistance in nonobese unique Japanese type 2 diabetic patients.

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The C42R Mutation in the Kir6.2 (KCNJ11) Gene as a Cause of Transient Neonatal Diabetes, Childhood Diabetes, or Later-Onset, Apparently Type 2 Diabetes Mellitus

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Context: Known genes in maturity-onset diabetes of the young account for only a fraction of families with dominantly inherited diabetes in Japan. There should be as-yet-unidentified genes that account for the rest of the patients.

Objective: To identify and characterize the mutation responsible for a Japanese family with dominantly inherited diabetes mellitus.

Subjects: Members of a four-generation family with dominantly inherited diabetes mellitus observed in three generations. None of the patients in this family had permanent neonatal diabetes. One had transient neonatal diabetes, one had childhood diabetes, and the others had adult-onset diabetes without autoantibodies or insulin resistance.

Methods: Screening of the chromosomal location of the gene by a genome-wide linkage analysis followed by candidate gene sequencing. Confirmation of the functional significance of the identified mutation by the population survey and the physiological analysis.

Results: We identified a novel mutation (C42R) in the KCNJ11 gene coding for the Kir6.2 subunit of the pancreatic ATP-sensitive potassium channel. The patch-clamp experiments using the mutated KCNJ11 showed that the mutation causes increased spontaneous open probability and reduced ATP sensitivity. The effect, however, was partially compensated by the reduction of functional ATP-sensitive potassium channel expression at the cell surface, which could account for the milder phenotype of our patients.

Conclusions: These results broaden the spectrum of diabetes phenotypes caused by mutations of KCNJ11 and suggest that mutations in this gene should be taken into consideration for not only permanent neonatal diabetes but also other forms of diabetes with milder phenotypes and later onset. (*J Clin Endocrinol Metab* **90: 3174–3178, 2005**)

Kir6.2, TOGETHER WITH SUR1, forms an inwardly rectifying ATP-sensitive potassium channel (K_{ATP}) channel, which plays a key role in ATP-dependent insulin secretion from pancreatic β-cells (1). After entry of glucose into the β-cells, elevated intracellular ATP levels cause closure of the K_{ATP} channels on the cell surface, which then depolarize the cell membrane leading to opening of the voltage-dependent calcium channels. The resultant influx of calcium leads to secretion of insulin from the β-cells.

Inactivating mutations in the KCNJ11 gene coding for Kir6.2 have been known to cause persistent hyperinsulinemic hypoglycemia in infancy through constitutive closure of the channel (2). On the other hand, the role of the variations of KCNJ11 in the pathogenesis of diabetes mellitus has remained less definitive only with some of the polymorphisms, such as E23K, reported to be associated with the risk of developing type 2 diabetes (3–8). Recently, however, Gloyn *et al.* (9) showed that activating mutations in KCNJ11 are the leading cause of permanent neonatal diabetes mellitus (PND). Other studies (10, 11) have also established the role of activating Kir6.2 mutations in the pathogenesis of PND.

Through a genome-wide linkage analysis and candidate gene sequencing conducted on a four-generation family with dominantly inherited diabetes mellitus observed in three generations, we identified a novel mutation (C42R) of the KCNJ11 gene. Unlike the cases discussed in the previous reports, the onset and severity of diabetes were variable: transient neonatal diabetes, childhood-onset diabetes, gestational diabetes, or adult-onset diabetes. Functional analysis through patch-clamp experiments revealed the biochemical basis of these milder phenotypes.

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Abbreviations: GAD, Glutamic acid decarboxylase; HbA_{1c}, hemoglobin A_{1c}; K_{ATP}, ATP-sensitive potassium channel; MODY, maturity-onset diabetes of the young; PND, permanent neonatal diabetes mellitus; Po, open probability; SNP, single-nucleotide polymorphism.

Patients and Methods

Patients' profiles

Figure 1 shows the pedigree of the family. The proband (IV-2) came to our clinic at 6 wk of age because of hyperglycemia [626 mg/dl (34.8 mmol/liter)]. He was born after 41 wk of uneventful pregnancy, with a birth weight of 2866 g. The neonatal period was uneventful. On admission, laboratory data revealed elevated hemoglobin A_{1c} (HbA_{1c}) (7.7%: normal range: 4.3–5.6%) and diminished blood C-peptide (0.35 ng/ml

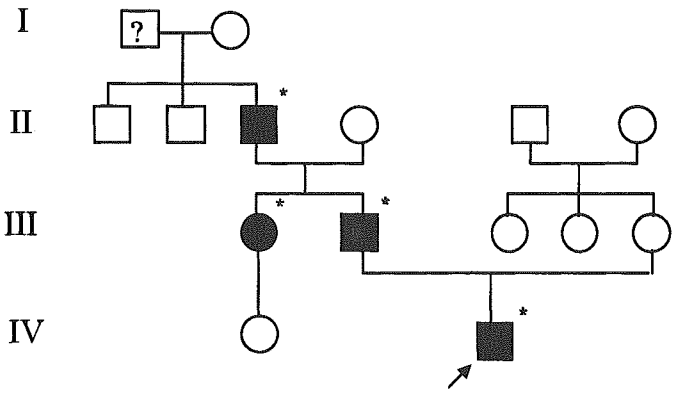

FIG. 1. Pedigree of the family. *Open symbols* show the unaffected family members, whereas *closed symbols* show the affected. The morbidity status of I-1 was uncertain. *Asterisks* show individuals with the C42R mutation.

(0.12 nmol/liter), normal range 1.0–2.5 ng/ml). Antiglutamic acid decarboxylase (GAD) or antiislet antibodies were negative. Frequent insulin injections at 2.2 U/kg·d gradually normalized his blood glucose levels, and he was discharged at 10 wk of age. After discharge, his insulin requirements gradually decreased and insulin could be stopped at 12 months of age. Currently, the patient is 1 yr 9 months old and maintains normal HbA_{1c} without any treatment.

The proband's father (III-2) developed diabetes at 22 yr of age. His neonatal and infantile periods were uneventful, and yearly urinalyses during school age were all negative for glucosuria. At the initial visit, he was not obese, with a body mass index of 17.8. His HbA_{1c} was slightly elevated, at 5.9%. He showed diminished insulin secretion, with postprandial C-peptide at 0.7 ng/ml (0.23 nmol/liter). Anti-GAD antibody was negative. Diet therapy was instituted, which kept his HbA_{1c} at less than 6.5% for 2 yr, after which his blood glucose levels gradually elevated, with HbA_{1c} at 6.8–7.3% at 25 yr of age. Currently he is on sc insulin injections at 14 U/d.

The proband's paternal grandfather (II-3) was first diagnosed with diabetes at 3 yr of age. Data on the autoantibodies at diagnosis are not available. He had been on insulin (dose unknown) since diagnosis until 21 yr of age, when sulfonylurea therapy was introduced. At 23 yr of age, insulin was stopped, and he was entirely on sulfonylurea. Currently he is 58 yr of age. He is still taking sulfonylurea (chlorpropamide 500 mg/d). His diabetes is well controlled (HbA_{1c} ranging between 5.0 and 5.8%), and he does not have any diabetic complications. He has never been obese, which suggests that the etiology of his diabetes was not insulin resistance. Actually, his current homeostasis model assessment index is low, at 0.58.

The proband's paternal aunt (III-1) had been well until 26 yr of age, when she was diagnosed with gestational diabetes. Her yearly urinalyses during school age were negative for glucosuria. Although she initially required insulin injections, the episode was transient, and her blood glucose returned to normal (HbA_{1c} 4%) after delivery. At the age of 28 yr, she was found to have elevated postprandial blood glucose [228 mg/dl (12.7 mmol/liter)] again and was diagnosed with diabetes. At diagnosis, she was not obese (height 160 cm, weight 44 kg). Blood HbA_{1c} was elevated, at 9.6%, and insulin secretion was diminished, at 3.3 μU/ml (19.8 pmol/liter), when fasting blood glucose was 200 mg/dl (11.0 mmol/liter). Anti-GAD autoantibody was negative. Insulin injections (16 U/d) were initiated. However, after 4 months, insulin therapy could be stopped, and she remains in fair control of her diabetes (HbA_{1c} 5.1–6.4%) with oral sulfonylurea alone (glimepiride, 1 mg/d).

The proband's mother, paternal grandmother (II-5), and great grandmother (I-2) have never been diabetic. The morbidity status of his paternal great grandfather (I-1) is uncertain, although he is currently not overtly diabetic. Although the proband's cousin (IV-1) is still at younger age, her HbA_{1c} and postprandial plasma glucose measurements gave normal results.

DNA isolation

After obtaining written informed consent, genomic DNA was isolated from peripheral blood leukocytes by using the QIAmp DNA maxikit (QIAGEN, Hilden, Germany). The study protocol was approved by the institutional review board.

Genome-wide linkage analysis

Polymorphic markers covering the whole genome at approximately 10-cM intervals were typed with the Linkage Mapping Set (version 2.5; Applied Biosystems, Foster City, CA) in accordance with the manufacturer's recommendations. Multipoint linkage analysis was performed with the Genehunter 2 program (12) under an assumption of dominant inheritance and full penetrance. Although the morbidity status of I-1 was uncertain because I-2 was clearly unaffected, the initial analysis was performed assuming that he was affected.

Sequencing analysis

The whole coding region of the KCNJ11 gene was amplified as two overlapping fragments using primer pairs 5′-CGAGAGGACT CTGCAGTGAG-3′ (Kir1)/5′-GCTTGCTGAAGATGAGGGTC-3′ (Kir2) and 5′-CATCGTGCAGAACATCGTG-3′ (Kir3)/5′-TAACACCCTGGATGAGCAG-3′ (Kir4) in 25-μl reactions containing 1 × GC buffer I, 200 μM each of the deoxynucleotide triphosphates, 30 ng of template DNA, and 0.5 U of LA-Taq DNA polymerase (Takara, Shiga, Japan). The initial denaturation at 94 C for 2 min was followed by 30 cycles of denaturation at 94 C for 30 sec, annealing at 60 C for 30 sec, and extension at 72 C for 60 sec. The amplified products were purified with the Wizard PCR Preps DNA purification system (Promega, Madison, WI) and directly sequenced with the BigDye Terminator cycle sequencing kit (version 3.1; Applied Biosystems). Similarly, the whole coding region of the NEUROG3 gene and the SUR1 (ABCC8) gene was directly sequenced (the sequences of the primers are available from the authors).

Population survey

A reverse mismatched primer, 5′-GATGTTCTTGTGGGCCACGCTGC-3′, which would generate a novel *PstI* site in the wild-type allele, and a forward primer, 5′-CGCTTTGTGTCCAAGAAAG-3′, were used to amplify a 48-bp fragment spanning the cysteine at position 42 in 5-μl reactions containing 20 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 12.5 pmol of each primer, and 0.1 U AmpliTaq Gold DNA polymerase (Applied Biosystems). The initial denaturation at 94 C for 10 min was followed by 35 cycles of denaturation at 94 C for 30 sec, annealing at 45 C for 15 sec, and extension at 72 C for 30 sec. Two microliters of the PCR products were then digested with *PstI* in 5-μl reactions and analyzed by PAGE and staining with ethidium-bromide.

Plasmids

The mammalian expression plasmids containing the whole coding region of the human Kir6.2 and SUR1 have been described previously (1, 13). To generate the expression plasmid containing C42R, the whole coding region of the KCNJ11 gene was amplified from IV-2's DNA by using the primers Kir1 and Kir4 under the same conditions as for the sequencing. Then the amplification products were doubly digested with *KpnI*/*XhoI* and ligated to the Kir6.2 expression plasmid, generating pKir6.2C42R. The presence of C42R and the absence of other mutations were confirmed by sequencing the whole insert.

Cell culture and DNA transfection

COS-1 cells were plated on 35-mm dishes containing cover slips. The cells were then transiently transfected with wild-type or mutated human Kir6.2 cDNA (1.5 μg/dish) plus human SUR1 (1.5 μg/dish) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). pEGFP-N1 (Clontech, Palo Alto, CA) coding for the green fluorescent protein was cotransfected as a reporter gene.

Patch-clamp experiments

Recordings were made 24–72 h after transfection. The ATP sensitivity of the wild-type and mutant channels were determined basically as described previously (1, 13) with a patch-clamp amplifier, Axopatch 200B (Axon Instruments, Foster City, CA). Sulfonylurea sensitivity was assessed as the ratio between the amplitudes of the K_{ATP} channel currents before and after tolbutamide application. To determine the open probability, the channel density at the cell surface, and the detectable rate of the K_{ATP} channels, single-channel recordings were performed by using an ATP-free bath solution (1, 13). The detectable rate of the channels was determined by the ratio between the patches expressing K_{ATP} channels and the total number of examined patches. The number of channels in a patch was estimated by dividing the maximum current amplitude by the K_{ATP} channel unitary current. Single-channel currents were analyzed by a combination of pCLAMP (version 9.0, Axon Instruments) and in-house software. Mann-Whitney *U* tests (detectable rate of the channels) or unpaired Student's *t* tests (others) were used to test for statistical significances, and the results were expressed as mean ± SE.

Results

By the genome-wide linkage analysis, nine chromosomal regions of interest with LOD scores higher than 1.2 were identified (chromosomes 2p, 3p, 9p, 10q, 11p, 14q, 18q, 19p, 22q). These regions were then compared with a list of the known chromosomal locations of 32 candidate genes involved in pancreatogenesis or insulin secretion (list not shown). All known genes in maturity-onset diabetes of the young (MODY) were readily excluded and three final candidate genes, KCNJ11, ABCC8, and NUUROG3, were further analyzed for sequence alterations, leading to the identification of a novel mutation, C42R (124T>C) in the KCNJ11 gene. No other mutations were identified in the other two genes. Sequencing analysis of all family members for C42R showed that the initial assumption that I-1 was affected was incorrect, and that the mutation arose *de novo* in II-3. The presence of diabetes was in complete concordance with the presence of the C42R mutation (Fig. 1). PSI-BLAST analysis (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) showed that the cysteine at position 42 was evolutionally well conserved and identical in the human, mouse, rat, chicken, and even *Drosophila melanogaster*, suggesting the functional importance of this amino acid residue. In addition, by the population survey, the C42R mutation was absent in 100 normal volunteers without diabetes, further suggesting that the mutation is not a simple polymorphism.

The electrophysiological studies have shown reduced ATP sensitivity of the mutant channel (Fig. 2). A response to tolbutamide was detected in the mutant channel, but the sensitivity was significantly reduced (Fig. 3). Spontaneous open probability (Po) was also increased in the mutant channel (Table 1), suggesting a shift toward the open state even in the absence of ATP. On the other hand, the functional expression at the cell surface, as measured by the channel density (the rate of detectable channels and the number of channels in a patch), was markedly reduced in cells expressing mutant K_{ATP} channels (Table 1). Although assessed in the homozygous state, these results suggest that the increase of Po and the reduction of ATP sensitivity account for the decrease in insulin secretion but that this is partially compensated by the reduction of functional K_{ATP} channel expression at the cell surface.

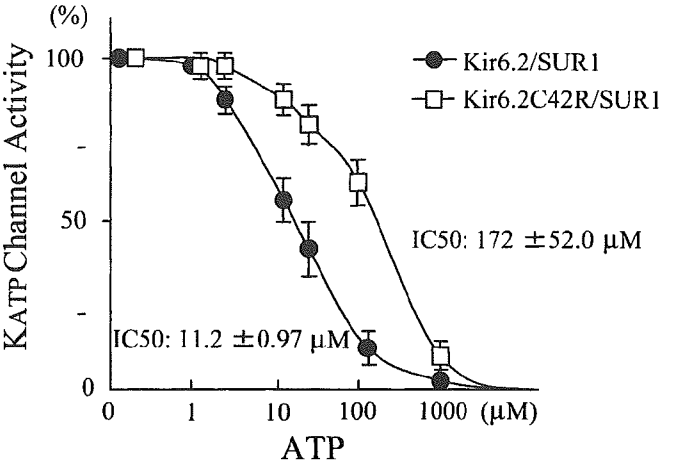

FIG. 2. ATP sensitivity of wild-type and mutant K_{ATP} channels. The dose-dependent effects of ATP concentration on the activities of the wild-type (Kir6.2/SUR1) and mutant (Kir6.2 C42R/SUR1) K_{ATP} channels are shown. Five experiments were conducted for each type of channel. IC_{50} shows the concentration of ATP at which the inhibition is half of the maximal.

Discussion

In this study we initially tried to identify a novel gene involved in the pathogenesis of dominantly inherited diabetes mellitus in Japanese patients because, unlike Caucasians, known MODY genes account for only 10–20% of Japanese patients with MODY, and there should be as-yet-unidentified genes that account for the rest of the Japanese patients.

Taking into account the characteristics of diabetes in this family, *i.e.* diminished insulin secretion without autoantibodies or insulin resistance, we identified a novel mutation (C42R) in the KCNJ11 gene.

The results of the patch-clamp experiments showed that the degree of ATP insensitivity and the increase in Po was

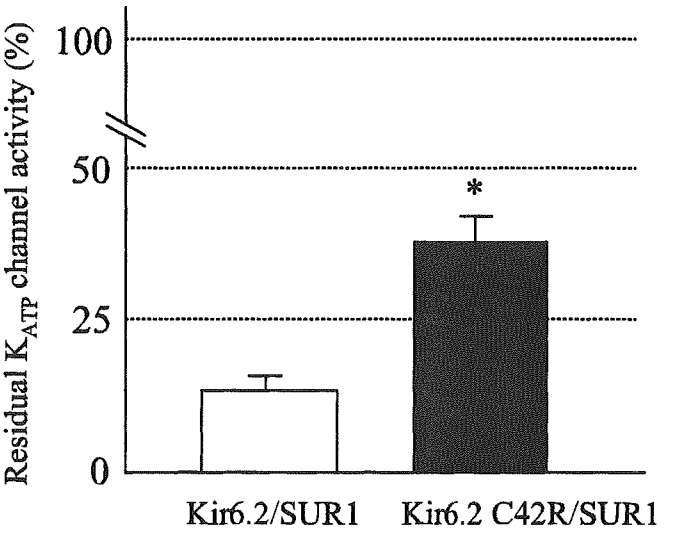

FIG. 3. Sulfonylurea sensitivity of the wild-type (Kir6.2/SUR1) and mutant (Kir6.2 C42R/SUR1) K_{ATP} channels. Residual K_{ATP} channel activities were determined by the ratio between the amplitudes of K_{ATP} channel currents before and after 100 mM tolbutamide application. *Asterisk*, $P < 0.01$.