Loss-of-Function Mutation of the *GPR40* Gene Associates with Abnormal Stimulated Insulin Secretion by Acting on Intracellular Calcium Mobilization

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Background: Free fatty acids (FFAs) acutely stimulate but chronically impair glucose-stimulated insulin secretion from β-cells. The G protein-coupled transmembrane receptor 40 (GPR40) mediates both acute and chronic effects of FFAs on insulin secretion and plays a role in glucose homeostasis. Limited information is available on the effect of *GPR40* genetic abnormalities on insulin secretion and metabolic regulation in human subjects.

Study Design and Results: For *in vivo* studies, we screened 734 subjects for the coding region of *GPR40* and identified a new single-nucleotide mutation (Gly180Ser). The mean allele frequency was 0.75%, which progressively increased ($P < 0.05$) from nonobese subjects (0.42%) to moderately obese (body mass index = 30–39.9 kg/m^2, 1.07%) and severely obese patients (body mass index $\geq$ 40 kg/m^2, 2.60%). The relationship between the *GPR40* mutation, insulin secretion, and metabolic alterations was studied in 11 Gly/Ser mutation carriers. In these subjects, insulin secretion (insulinogenic index derived from oral glucose tolerance test) was significantly lower than in 692 Gly/Gly carriers (86.0 $\pm$ 48.2 *vs.* 183.7 $\pm$ 134.4, $P < 0.005$). Moreover, a case-control study indicated that plasma insulin and C-peptide responses to a lipid load were significantly ($P < 0.05$) lower in six Gly/Ser than in 12 Gly/Gly carriers. *In vitro* experiments in HeLa cells cotransfected with aequorin and the mutated Gly/Ser *GPR40* indicated that intracellular Ca^{2+} concentration increase after oleic acid was significantly lower than in Gly/Gly *GPR40*-transfected cells. This fact was confirmed using fura-2 acetoxymethyl ester.

Conclusions: This newly identified *GPR40* variant results in a loss of function that prevents the β-cell ability to adequately sense lipids as an insulin secretory stimulus because of impaired intracellular Ca^{2+} concentration increase. **(*J Clin Endocrinol Metab* 93: 3541–3550, 2008)**

Type 2 diabetes (T2D) is characterized by progressively increasing insulin resistance associated with inadequate insulin secretion after a period of compensatory hyperinsulinemia (1). The reasons for the β-cell secondary failure are not completely understood. Acquired pancreatic islet damage or exhaustion and/or genetic factors causing susceptibility to islet secretory insufficiency have been hypothesized (2).

Acute exposure to free fatty acids (FFAs) increases basal and glucose-stimulated insulin secretion (3–6). In contrast, chronic ex-

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Abbreviations: BMI, Body mass index; $[Ca^{2+}]_c$, cytosolic free Ca^{2+} concentration; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DI, disposition index; EGFP, enhanced green fluorescent protein; FFA, free fatty acid; fura-2/AM, fura-2 acetoxymethyl ester; GPR40, G protein-coupled transmembrane receptor 40; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; ISI, insulin sensitivity index; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test; OLTT, oral lipid tolerance test; T2D, type 2 diabetes.

posure to high levels of FFAs results in β-cell desensitization to glucose and fatty acids and blunted insulin secretion. This phenomenon is indicated as lipotoxicity (3, 7–9).

G protein-coupled transmembrane receptor 40 (GPR40), also known as FFA Receptor 1 (FFAR1), is a membrane-bound FFA receptor that is preferentially expressed in the brain and the pancreatic islets and specifically in β-cells (10, 11). The receptor is coupled to an intracellular G protein (G_q) that activates phospholipase C and the phosphatidylinositol (phosphatidylinositol 4,5-bisphosphate) signaling pathway.

At the pancreatic level, this receptor mediates both acute and chronic effects of FFAs on insulin secretion, including the amplification of glucose-stimulated insulin secretion (12–15). After FFA binding, GPR40 activates phospholipase-C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate, which mobilizes intracellular Ca^{2+} from the endoplasmic reticulum (16, 17).

GPR40-deficient mouse β-cells secrete less insulin in response to FFAs, and loss of *GPR40* prevents hyperinsulinemia due to obesity, hepatic steatosis, hypertriglyceridemia, increased hepatic glucose output, hyperglycemia, and glucose intolerance (13). Conversely, *GPR40* overexpression in mouse β-cells causes impaired β-cell function, hypoinsulinemia, and diabetes (13).

Abnormalities of *GPR40* expression and its consequences on metabolism have been poorly studied in human subjects (11). *GPR40* Arg211His gene polymorphism potentiated insulin secretion and predisposed to insulin resistance in healthy Japanese men (18). In a Danish Caucasian population, the association of this polymorphism with a rare mutation (Asp175Asn) was not associated with insulin secretion abnormalities (19).

At brain level, *GPR40* mRNA has been detected in both humans (10) and primates (20). Its functional role, however, has not been clarified. One hypothesis is that brain GPR40 acts as the target receptor for FFAs, which play the role of extracellular signaling molecules that regulate neural functions such as the lipid-sensing mechanism in the hypothalamus and the control of energy balance (21–23). Genetically determined functional modification of *GPR40*, therefore, could be associated with metabolic dysregulation, including obesity, abnormalities of insulin secretion, and susceptibility to progression toward T2D. We have therefore evaluated whether genetic abnormalities of *GPR40* are related with abnormalities of insulin response to an oral glucose or to a lipid load and whether carriers of *GPR40* variants are prone to obesity and/or glucose and lipid metabolism abnormalities.

By screening over 700 subjects, we have identified a new rare variant of the *GPR40* that causes a loss of function associated with impaired insulin secretion in response to oral glucose and lipid load. Moreover, the allele frequency of this *GPR40* variant progressively increases with increase of body mass index (BMI).

Subjects and Methods

Clinical studies

We studied 734 unrelated adults living in Sicily (district of Catania, approximately 1.1 million inhabitants). Two independent series of subjects were recruited: 495 obese (BMI ≥30 kg/m^2) patients, 144 males and 351 females (age 37.3 ± 12.2 yr; average BMI = 42.9 ± 7.6 kg/m^2, mean ± SD; median, 42.4 kg/m^2) were consecutively recruited from the outpatient obesity clinic of the Garibaldi Hospital (Catania, Italy). Nonobese (BMI <30 kg/m^2) subjects (n = 239, 111 males and 128 females; age 36.8 ± 12.6 yr; mean BMI = 24.5 ± 2.9 kg/m^2, mean ± SD; median 24.7 kg/m^2) were recruited from staff of four different cities and university hospitals volunteering to participate. Declaration of Helsinki guidelines were followed and informed consent obtained from all participants.

Glucose (glucose oxidase method, Beckman Glucose Analyzer 2; Beckman Coulter, Inc., Fullerton, CA), insulin (microparticle enzyme immunoassay; Abbott Laboratories, Abbott Park, IL), total cholesterol and triglycerides (enzymatic methods; Instrumentation Laboratory, Milan, Italy), and high-density lipoprotein cholesterol (separated by the use of Mg^{2+} and the dextran sulfate method; Sclavo Diagnostics, Siena, Italy) were measured in blood specimens obtained after an overnight fast and immediately frozen at −20 C. In 703 of 734 subjects without a previous diagnosis of diabetes, having fasting plasma glucose less than 8.3 mmol/liter, an oral glucose tolerance test (OGTT) was carried out. Glucose and insulin levels were measured before and 30, 60, 90, and 120 min after a 75-g oral glucose load. According to the American Diabetes Association criteria, 509 subjects had normal glucose tolerance (NGT), 24 impaired fasting glucose (IFG), 127 impaired glucose tolerance (IGT), and 74 T2D.

The insulinogenic index was calculated according to the following formula: [insulin (picomoles per liter) at 30 min − fasting insulin (picomoles per liter)]/[glucose (millimoles per liter) at 30 min − fasting glucose (millimoles per liter)].

The insulin sensitivity index (ISI) was calculated according to the following formula: $10{,}000/\sqrt{}$[fasting plasma glucose (milligrams per deciliter) × fasting plasma insulin (milliunits per liter)] × [mean OGTT glucose concentration (milligrams per deciliter) × mean OGTT insulin concentration (milliunits per liter)].

The disposition index (DI) was the product of the insulinogenic index by the ISI.

An oral lipid tolerance test (OLTT) was carried out to assess the fatty acid effect in regulating glucose-induced insulin secretion in 18 individuals (six subjects carrying the Gly/Ser mutation and 12 carrying the Gly/Gly matched for age, gender, BMI, and glycemic status), after an interval of 3–4 wk from OGTT. The OLTT is a modification of the standardized test proposed by Castro Cabezas *et al.* (24). Liquid fresh cream was used as the fat source; this is a 35% (wt/wt) fat emulsion with a P/S ratio of 0.06, enriched with 16% (wt/wt) carbohydrates. The total amount of nutrients ingested was 70.0 g fats, 32.9 g carbohydrates, and 4.6 g proteins. The corresponding caloric intake was 772 kcal with 82% fats, 16% carbohydrates, and 2% proteins. Plasma glucose, insulin, triglycerides, C-peptide (immunoradiometric assay; Adaltis Bologna, Italy) and FFAs (enzymatic colorimetric method; Wako Chemicals, Neuss, Germany) were measured at 0, 30, 60, 90, 120, 150, and 180 min.

Genetic studies

GPR40 gene screening in human subjects

The coding region of human *GPR40* gene was amplified starting from genomic DNA isolated from whole blood. Six nested primers were used to sequence the resulting PCR product on both strands using the BigDye terminator chemistry and an ABI 3100 automated DNA sequencing analyzer (Applied Biosystems, Foster City, CA).

DNA cloning and transfection

The PCR products (from a wild-type subject and from a mutant patient) were subcloned into the eukaryotic expression vector pIRES2-EGFP (Clontech, Palo Alto, CA), which allowed the expression of a bicistronic mRNA containing both *GPR40* and enhanced green fluorescent protein (EGFP).

For transient expression, subconfluent HeLa cells were cotransfected using either pGPR40wt or pGPR40mut with the calcium phosphate pre-

cipitation method. Control cells were transfected with a void vector (pIRES-EGFP). Ca^{2+} assays were performed 40 h after the beginning of transfection.

Immunofluorescence

HeLa cells were plated onto 24- × 24-mm coverslips at a density of 2.5×10^5 cells per coverslip. Transfected cells (pIRESEGFP, GPR40WT, and GPR40 Gly180Ser) were washed twice with PBS and fixed with a 4% formaldehyde solution for 15 min at room temperature. Cells were then washed twice with PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Unspecific binding was reduced by blocking with 2% fetal bovine serum for 30 min. Cells were incubated 2 h with a polyclonal rabbit anti-GPR40 antibody (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:50). After incubation, cells were extensively washed with PBS. A goat antirabbit, AlexaFluor546-conjugated antibody was used as secondary antibody for 1 h (Invitrogen Carlsbad, CA; dilution 1:500). Unbound antibody was removed by washing three times with PBS. Coverslips were then mounted with ProLong Gold antifade reagent (Invitrogen), and images were taken on a Zeiss LSM510 confocal microscope using a Plan-Apochromat ×63/1.4 oil immersion objective. GFP fluorescence was visualized using the 488-nm argon laser line as excitation and a 505–550 band pass filter in emission. Alexa546-conjugated antibody was excited with the 543-nm HeNe laser line, and emission was collected through a 585 long pass filter. Pinhole was set at 1 airy unit for each channel. Images were pseudocolored and exported in JPEG format using the ImageJ program (National Institutes of Health, Bethesda, MD).

Cell fractioning and Western blot

HeLa cells were plated in 10-cm petri dishes, and 36 h after transfection (pIRESEGFP, GPR40WT, and GPR40 Gly180Ser), cells were washed twice with PBS, scraped, centrifuged (1000 rpm for 5 min), and resuspended in lysis buffer (10 mM Tris-HCl, 2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, pH 8.0). Cells were then subjected to five freeze/thaw cycles and centrifuged at 1000 rpm for 5 min to remove gross cellular debris. The supernatant was then centrifuged at 12000 × *g* for 30 min at 4 C. The supernatant contained cytosolic proteins, whereas the pellet was resuspended in lysis buffer containing 10% sucrose.

The proteins were separated by SDS-PAGE on a 10% gel, and the amount of GPR40 and plasma membrane calcium ATPase was estimated by Western blotting using polyclonal rabbit anti-GPR40 (1:500; Santa Cruz Biotechnology) and monoclonal mouse anti-plasma membrane calcium ATPase (1:1000; AbCam) primary antibodies and antirabbit (1:3000; Santa Cruz Biotechnology) or antimouse (1:5000; Santa Cruz Biotechnology) IgG horseradish peroxidase-labeled secondary antibodies, according to standard protocols.

Biological studies on the *GPR40* (Gly180Ser) mutation

The functional properties of the newly identified *GPR40* (Gly180Ser) variant were investigated by transiently expressing the mutated *GPR40* in HeLa cells and exposing transfected cells to unsaturated fatty acid (C18:1) oleic acid. Changes in intracellular calcium mobilization in cells expressing either the Gly/Gly or the Gly/Ser GPR40 protein was then evaluated. Oleic acid and histamine were diluted to the final concentrations of 50 μM oleic acid and 1 μM histamine, respectively. Histamine was used as positive control because it binds to the H_1 receptor, constitutively expressed in HeLa cells. Oleate was preferred to palmitate because it possesses additive effects such as an anti-lipoapoptotic action on NIT-1 cells, effects that are mediated mainly via GPR40 (25).

Cell calcium measurements

Calcium was measured in HeLa transfected cells by two different methods.

Aequorin reporter assay. Cells were first incubated with 5 μM coelenterazine for 1–2 h in DMEM with 1% fetal bovine serum. A coverslip with transfected cells was then placed in a perfused thermostated chamber located in close proximity to a low-noise photomultiplier with a built-in amplifier/discriminator with or without 50 μM oleic acid. All aequorin measurements were carried out in Krebs-Ringer modified buffer (135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.4 mM K_2HPO_4, 5.5 mM glucose, 20 mM HEPES, pH 7.4 supplemented with 1 mM $CaCl_2$). Experiments were terminated by lysing cells with 100 μM digitonin in a hypotonic Ca^{2+}-containing solution (10 mM $CaCl_2$ in H_2O), thus discarding the remaining aequorin pool. The aequorin luminescence data were calibrated offline into $[Ca^{2+}]$ values using a computer algorithm based on the Ca^{2+} response curve of aequorin as previously described (14).

Fura-2 acetoxymethyl ester (fura-2/AM) assay. The cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) was also evaluated using the fluorescent Ca^{2+} indicator fura-2/AM (Molecular Probes, Inc., Eugene, OR). Briefly, cells incubated in medium supplemented with 2 μM fura-2/AM for 20 min were washed with Krebs-Ringer buffer to remove all extracellular probe, supplied with preheated Krebs-Ringer buffer (supplemented with 1 mM $CaCl_2$ or 100 μM EGTA when indicated) and placed in a thermostated (37 C) incubation chamber on the stage of an inverted fluorescence microscope (Zeiss Axiovert 200, Jena, Germany) equipped with a high-sensitivity cooled CCD camera (Photometrics Cascade 512B, Tucson, AZ). Fluorescence was measured every 80 msec with the excitation wavelength alternating between 340 and 380 nm and the emission fluorescence recorded at 510 nm. At the end of each experiment, a cell-free area was selected, and one average background frame was collected at each excitation wavelength for background correction. The $[Ca^{2+}]_c$ was calculated by the ratio method using the following equation: $[Ca^{2+}]_c = K_d(R - R_{min})/(R - R_{max}) \times Sf2/Sf1$, where K_d is the fura-2/AM dissociation constant at the two excitation wavelengths (F_{340}/F_{380}); R_{min} is the fluorescence ratio in the presence of minimal calcium, obtained by chelating Ca^{2+} with 50 mM EGTA; R_{max} is the fluorescence ratio in the presence of excess calcium, obtained by treating cells with 2 μM ionomycin; Sf2 is the fluorescence of the Ca^{2+}-free form and Sf1 is the fluorescence of the Ca^{2+}-bound form of fura-2/AM at excitation wavelengths of 380 and 340 nm, respectively.

Statistical analyses

Values are provided as mean ± SD (text and table) or as mean ± SEM (figures). Continuous variables were compared using an unpaired *t* test or one-way ANOVA or two-way ANOVA, followed by a *post hoc* analysis with a Fisher's protected least-significant difference test. Categorical variables were compared by the χ^2 test. Differences between groups, after adjusting for several covariates, were evaluated by covariance analysis test. The proportion of genotypes or alleles was compared by a χ^2 test.

To define the number of subjects to be recruited, power calculation was measured to obtain a two-sided test size of 5% and a power of 80% for differences in insulinogenic index (number of subjects to be examined by OGTT) and in glucose, insulin, and C-peptide responses to lipid load (number of subjects to be examined by OLTT). No normally distributed variables (serum triglycerides, insulinogenic index, and DI) were log-transformed for analyses. All analyses were performed by statistical package SPSS version 11.5 (Chicago, IL) and the StatView program (version 5.01; SAS Institute, Cary, NC).

Results

Genotyping

In the 734 subjects studied, two single-nucleotide substitutions were identified by sequencing the *GPR40* coding region (Fig. 1). One was the already described Arg211His polymorphism. Similar to previous data reported in the Danish population (19), in our cohort, the *GPR40* (Arg211His) variant prevalence was 86.5% for Arg/Arg, 13.0% for Arg/His, and

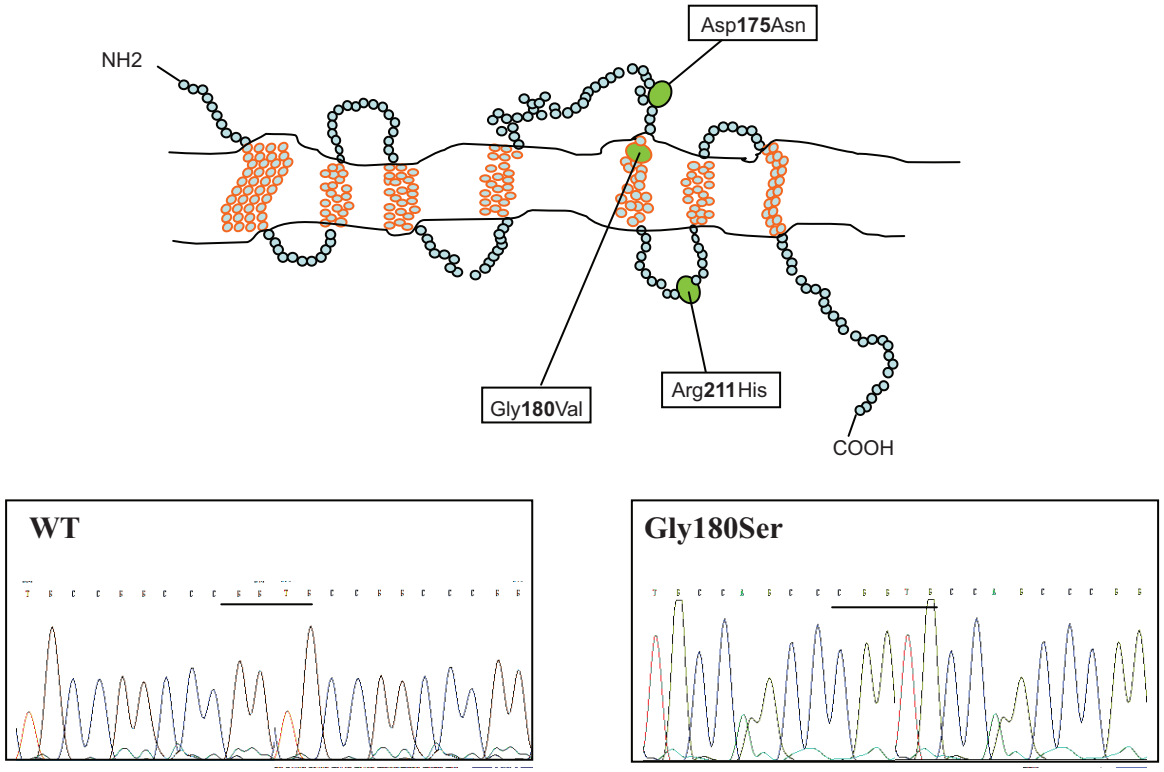

FIG. 1. Direct sequencing of the coding region of *GPR40* revealed a G/A substitution that replaced a glycine with a serine at codon 180, in the second extracellular loop region (between transmembrane 4 and 5 domains). WT, Wild-type.

0.5% for His/His genotypes. Clinical features and fasting metabolic parameters of subjects subdivided according to the Arg211His genotype are shown in Table 1. The presence of this variant was not associated with insulin secretion alterations (insulinogenic index, 179.7 ± 129.9 in 605 subject carrying Arg/Arg genotype and 197.1 ± 157.5 in 98 Arg/His or His/His carriers, $P = 0.2$), as well as with differences in glucose and insulin profiles during OGTT ($P = 0.8$ and $P = 0.5$, respectively; Fig. 2). No difference ($P = 0.6$) in the mutation (Arg/His or His/His genotypes) prevalence was observed among patients having glucose metabolism abnormalities (IFG, IGT, or T2D, 28 of 225, 12.4%) and NGT subjects (71 of 509, 13.9%).

The second variant was a newly identified single-nucleotide mutation (Gly180Ser) replacing the apolar amino acid glycine with the polar amino acid serine at residue 180 of the *GPR40* gene (Fig. 1). As the previous one, this mutation also lies within the second extracellular loop that links transmembrane domains 4 and 5 of the GPR40 protein (Fig. 1). The overall prevalence of this mutation was 1.5% (11 of 734), corresponding to an allele frequency of 0.75%. Interestingly, a significantly ($P < 0.05$) increasing mutation frequency was observed among nonobese

TABLE 1. Subject clinical features and fasting metabolic parameters according to the *GPR40* variants

	Gly180Ser		Arg211His	
	Gly/Gly (n = 723)	Gly/Ser (n = 11)	Arg/Arg (n = 635)	Arg or His/His (n = 99)
Gender (male/female)	250/473	5/6	221/414	34/65
Age (yr)	37.1 ± 12.3	38.7 ± 12.5	37.3 ± 12.1	35.5 ± 13.6
BMI (kg/m^2)	36.8 ± 10.7	43.4 ± 10.9[a]	36.9 ± 10.9	37.4 ± 10.1
Waist circumference (cm)	112.0 ± 24.5	125.2 ± 23.9	111.8 ± 24.7	114.7 ± 23.2
SBP (mm Hg)	121.2 ± 14.7	120.7 ± 12.6	121.1 ± 14.7	121.7 ± 14.4
DBP (mm Hg)	78.0 ± 10.5	80.2 ± 7.9	78.0 ± 10.5	78.1 ± 10.1
FPG (mmol/liter)	5.5 ± 1.7	5.6 ± 1.0	5.5 ± 1.7	5.3 ± 0.9
IRI (pmol/liter)	88.5 ± 56.1	109.4 ± 75.0	88.1 ± 55.4	93.3 ± 62.7
TC (mmol/liter)	5.1 ± 1.1	5.2 ± 0.9	5.1 ± 1.0	4.9 ± 1.2
HDL-C (mmol/liter)	1.2 ± 0.3	1.1 ± 0.2	1.2 ± 0.3	1.1 ± 0.4
TG (mmol/liter)	1.3 ± 0.8	1.4 ± 0.5	1.3 ± 0.8	1.2 ± 0.9

Data are mean ± SD. DBP, Diastolic blood pressure; FPG, fasting plasma glucose; HDL-C, high-density lipoprotein cholesterol; IRI, fasting plasma insulin; SBP, systolic blood pressure; TC, fasting total cholesterol; TG, fasting triglycerides.

[a] $P < 0.05$ *vs.* Gly/Gly carriers.

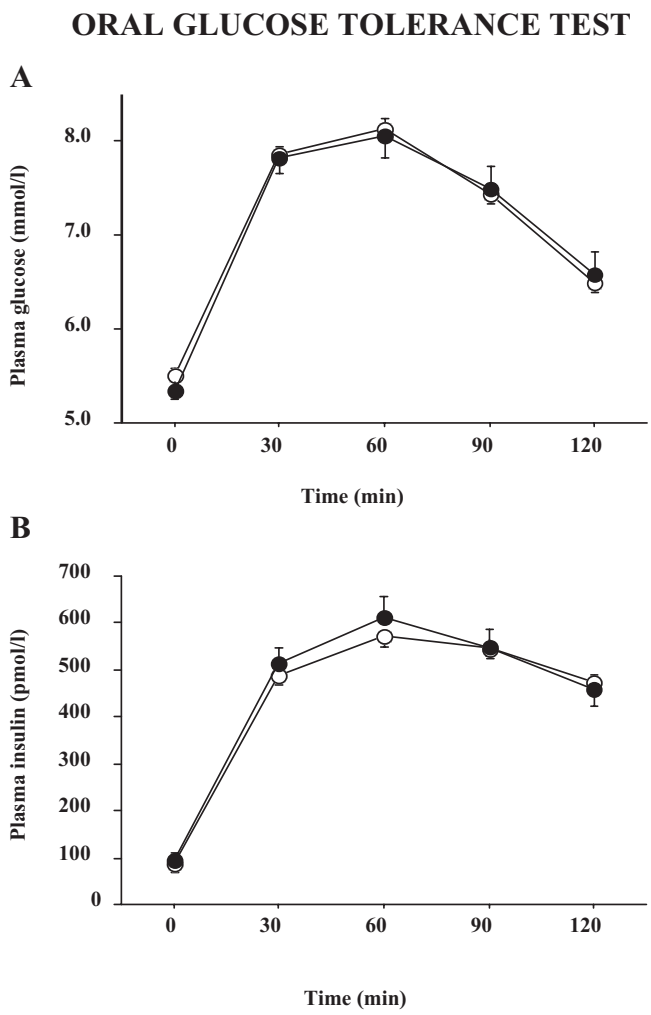

FIG. 2. OGTT in the 703 subjects without a previous diagnosis of diabetes, grouped according to the Arg211His polymorphism (605 Arg/Arg and 98 Arg/His or His/His carriers). Plasma glucose (A) and insulin (B) levels were not different among subjects carrying Arg/Arg (○) and Arg/His or His/His (●) genotypes.

(n = 1 of 239, 0.42%), obese patients with a BMI ranging from 30–39.9 kg/m^2 (n = 2 of 187, 1.07%), and patients with severe obesity (BMI ≥ 40 kg/m^2, n = 8 of 308, 2.60%). No difference ($P = 0.8$) in the mutation prevalence was observed between patients having glucose metabolism abnormalities (IFG, IGT, or T2D, 3 of 225, 1.33%) and NGT subjects (8 of 509, 1.57%).

Metabolic characteristics of *GPR40* Gly180Ser mutation carriers

Clinical characteristics of the 734 studied subjects subdivided according to the *GPR40* Gly180Ser genotype are shown in Table 1. BMI was significantly higher ($P < 0.05$) in Gly/Ser mutation carriers in respect to Gly/Gly *GPR40* individuals (43.4 ± 10.9 and 36.8 ± 10.7 kg/m^2, respectively), also after adjusting data for age and gender. No other significant difference was observed in biochemical parameters under fasting conditions (Table 1). However, during OGTT, plasma glucose was significantly ($P < 0.005$, power 80%) higher in the Gly/Ser *GPR40* mutation carriers than in Gly/Gly *GPR40* carriers (Fig. 3A), also after adjusting for age and gender, but not when data were further adjusted for BMI. Despite the higher glucose levels, in the 11 Gly/Ser mutation carriers, insulin levels during OGTT were lower than in the 692 Gly/Gly *GPR40* individuals, although the difference did not reach statistical significance ($P = 0.1$) (Fig. 3B). Insulin secretion, measured by the insulinogenic index derived from OGTT, was significantly lower in *GPR40* Gly/Ser in respect to Gly/Gly *GPR40* carriers (86.0 ± 48.2 *vs.* 183.7 ± 134.4, respectively; $P < 0.005$, power 83%, also after adjusting data for age, gender, and BMI; Fig. 3C). Although insulin sensitivity (as measured by ISI) was not significantly different between Gly/Ser and Gly/Gly *GPR40* carriers (3.8 ± 3.2 *vs.* 4.6 ± 3.9, $P = 0.5$), the interplay between early insulin secretion (insulinogenic index) and insulin sensitivity (ISI), as indicated by the DI, was significantly ($P = 0.01$, power 70%) lower in subjects carrying the Gly/Ser *GPR40* mutation (311.1 ± 229.7) compared with Gly/Gly (905.0 ± 875.3) individuals. This difference remained significant also after adjusting for age and gender, but not after further adjustment for BMI.

In six subjects carrying the Gly/Ser *GPR40* mutation (three males and three females; age, 43.5 ± 14.2 yr; BMI, 41.0 ± 12.5 kg/m^2; NGT to IGT, 5:1) and in 12 Gly/Gly subjects (six males and six females; age, 44.2 ± 13.2 yr; BMI, 40.2 ± 11.7 kg/m^2; NGT to IGT, 10:2) matched for gender, age, BMI, and glucose tolerance status, insulin secretion in response to an oral fat load was evaluated by OLTT. During OLTT, triglyceride levels significantly ($P < 0.001$) increased from baseline both in Gly/Ser *GPR40* carriers (baseline level, 1.02 ± 0.43 mmol/liter; peak level, 1.67 ± 0.79 mmol/liter) and in Gly/Gly subjects (baseline level, 0.89 ± 0.33 mmol/liter; peak level, 1.45 ± 0.69 mmol/liter), with no significant difference between the two groups at the different times. Interestingly, the plasma glucose profile after OLTT was significantly higher ($P < 0.001$, power 98%, Fig. 3D) and plasma insulin and C-peptide levels significantly lower ($P < 0.05$ and $P < 0.001$, power 70 and 98%; Fig. 3, E and F, respectively) in Gly/Ser than in Gly/Gly *GPR40* carriers. As a consequence of this difference in insulin levels, a significantly ($P < 0.05$) more pronounced FFA suppression was observed in Gly/Ser in respect to Gly/Gly *GPR40* carriers (peak levels, 0.59 ± 0.06 *vs.* 0.48 ± 0.11 mmol/liter, respectively).

In vitro studies

To investigate the mechanism of the reduced insulin secretion in subjects with the Gly/Ser *GPR40* mutation, HeLa cells were transfected with either wild-type or Gly180Ser mutated *GPR40*, and cytosolic calcium changes were measured after lipid stimulation. In HeLa cells, cotransfected with either the mutated or the wild-type *GPR40* and with the photoprotein aequorin trapped in the cytosol, no difference in the cytosolic Ca^{2+} concentration was observed under basal conditions (data not shown). However, after cell exposure to 50 μM oleic acid, a significantly higher intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) rise was observed in Gly/Gly *GPR40* cells in respect to cells carrying the Gly/Ser *GPR40* (n = 24; 0.58 ± 0.06 *vs.* 0.37 ± 0.01 μM; $P < 0.01$) (Fig. 4A).

To investigate the specificity of the HeLa cell $[Ca^{2+}]_i$ response to oleic acid, transfected HeLa cells that constitutively express the H_1-receptor for histamine (26), whose activation induces $[Ca^{2+}]_i$ increase (27), were exposed to histamine (1 μM). After

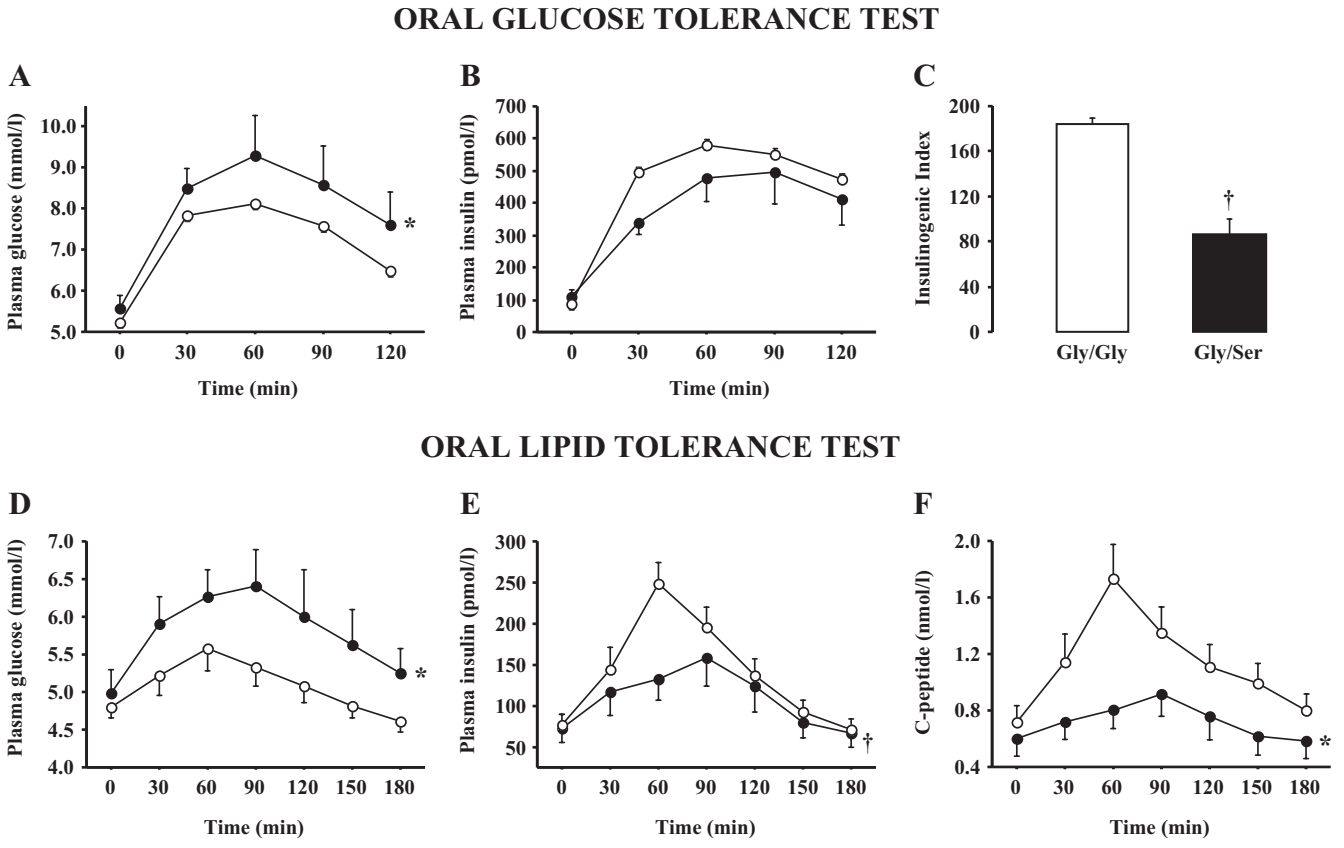

FIG. 3. OGTT (A–C) in the 703 subjects without a previous diagnosis of diabetes and OLTT (D–F) in the subgroup of 18 subjects (six Gly/Ser and 12 Gly/Gly carriers, matched for glucose tolerance status, gender, age, and BMI). Glucose plasma levels (A) were significantly higher in the 11 subjects carrying the mutation (●) when compared with the 692 Gly/Gly individuals (○). *, $P < 0.005$, also after adjusting data for age and gender. Plasma insulin profiles (B), in contrast, were lower in the 11 mutation carriers, although the difference did not reach the statistical significance ($P = 0.1$). Insulin secretion, measured by the insulinogenic index (C), was significantly lower in Gly/Ser (*black bar*) than in Gly/Gly carriers (*white bar*). †, $P < 0.005$, also after adjusting data for age, gender, and BMI. After OLTT, six subjects carrying the *GPR40* (Gly180Ser) mutation showed significantly higher glucose plasma levels (D) (*, $P < 0.001$) and lower insulin (E) (†, $P < 0.05$) and C-peptide (F) (*, $P < 0.001$) levels in respect to 12 Gly/Gly individuals.

histamine, a rapid $[Ca^{2+}]_i$ increase was observed in both Gly/Gly and Gly/Ser *GPR40* transfected cells, with no difference in the amplitude or the kinetics of the response (Fig. 4B). However, pIRES-EGFP transfected cells had a more robust $[Ca^{2+}]$ increase upon histamine stimulation, probably due to the toxicity of the exogenous expression of the FFA receptor in HeLa cells.

Experiments with the fluorescent Ca^{2+} indicator fura-2/AM confirmed that oleic acid induced a higher $[Ca^{2+}]_i$ response in Gly/Gly *GPR40*-expressing cells in respect to cells carrying the mutated *GPR40* (Gly180Ser) ($n = 12$; 0.8 ± 0.01 *vs.* 0.5 ± 0.02; $P < 0.01$) (Fig. 4C). Cells transfected with the empty vector (pIRES-EGFP) were unresponsive.

To identify whether the oleic acid-induced $[Ca^{2+}]_i$ increase was due to either Ca^{2+} release from intracellular stores or Ca^{2+} influx from the incubation medium (Krebs Ringer modified buffer), transfected HeLa cells were exposed to oleic acid in a Ca^{2+}-free medium added with 100 μM EGTA. In the absence of extracellular calcium, oleic acid-induced $[Ca^{2+}]_i$ increase was similar to that observed in cells incubated in Ca^{2+}-containing medium for both amplitude and kinetics of $[Ca^{2+}]_i$ increase, with no difference between wile-type or mutated *GPR40*-transfected cells in respect to the correspondent cells incubated in the presence of Ca^{2+} in the medium ($n = 5$; 0.70 ± 0.1 *vs.* 0.32 ± 0.1; $P < 0.05$; Fig. 4D). These data indicate that the GPR40-stimulated $[Ca^{2+}]_i$ increase is due to the $[Ca^{2+}]_i$ release from the intracellular stores.

Finally, we investigate whether these differences in Ca^{2+} mobilization from the intracellular stores could be due to a different localization or processing of the Gly/Gly and Gly/Ser GPR40 proteins. Immunofluorescence staining of the exogenous expressed GPR40 demonstrates that Gly/Ser substitution has no effect on intracellular localization, showing a clear signal from both the endoplasmic reticulum and the plasma membrane (Fig. 5A). Moreover, we verified that expression levels of Gly/Gly and Gly/Ser GPR40 protein at the cell surface are comparable by isolating a plasma membrane-enriched fraction. Western blot analysis shows no appreciable differences in expression levels between Gly/Gly and Gly/Ser GPR40 (Fig. 5B).

Discussion

Several pieces of *in vitro* and *in vivo* evidence suggest that *GPR40* can be considered a new candidate gene for T2D (11–13, 28–30). However, up to now, in different ethnic populations, *GPR40* genotyping has not reached unequivocal conclusions on the possible link between a specific gene polymorphism or mutation and the presence of T2D (18, 19). Besides, the genomic locus of

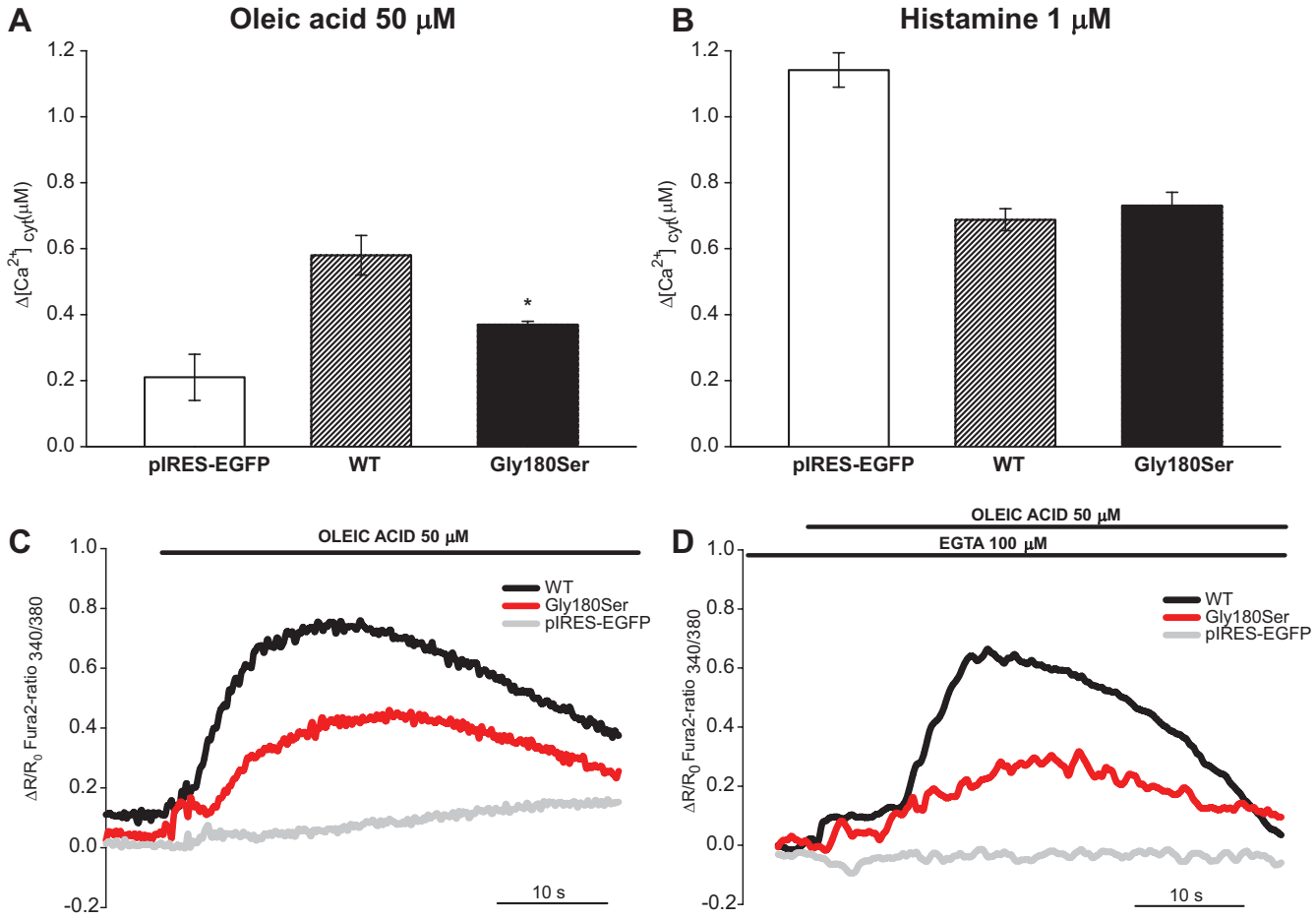

FIG. 4. A, Effect of oleic acid (50 μM) on $[Ca^{2+}]_i$ changes in transfected HeLa cells. HeLa cells were transiently cotransfected with the cDNA coding for cyt-Aeq and either wild-type or Gly180Ser mutant form of *GPR40*. Cells transfected with the void vector (pIRES-EGFP) were used as controls. *Bars* represent mean ± SD values. B, Effect of histamine (1 μM) on $[Ca^{2+}]_i$ changes in transfected HeLa cells. *Bars* represent mean ± SD values. C, Representative traces of the effect of oleic acid addition to transfected HeLa cells loaded with fura-2 on $[Ca^{2+}]_i$ changes expressed as the ratio F340/F380. D, Representative traces of the effect of oleic acid addition to transfected HeLa cells loaded with fura-2 in Ca^{2+}-free medium containing 100 μM EGTA. Changes in $[Ca^{2+}]_i$, expressed as the ratio F340/F380 were normalized to the average value obtained within the first minute of the experiment. WT, Wild-type.

GPR40 (19q13.1) was outside the 19q region associated to T2D by a recent genome-wide study (31). In the present study, we examined the entire coding region of the human *GPR40* gene in a population that included a wide range of individual BMI, to evaluate whether the link of *GPR40* mutations or polymorphisms and T2D was present in subjects prone to diabetes because of insulin resistance due to obesity.

We found the well known polymorphism Arg211His in almost 13% of the subjects examined, with no correlation with metabolic abnormalities. We also identified a new rare mutation, Gly180Ser, characterized by the replacement of the polar amino acid serine by the apolar amino acid glycine at residue 180 in the second extracellular loop of the GPR40. This extracellular portion seems crucial for the ligand activation of the GPR40 protein, as already described for other members of the same family. GPR42 (Chr 19q13.1) differs from GPR41 because it is unable to respond to short-chain fatty acids. Binding studies with GPR41 identified in position 174 of the second extracellular loop an arginine (tryptophan in GPR42), which is essential for ligand activation, presumably because it forms a salt bridge with the carboxylate ligand (32, 33). Mutations or changes of the amino acid sequence in the second extracellular loop of GPR40, therefore, may possibly lead to a reduced capacity to bind the ligand and to transfer the ligand signal inside the cell (32, 33).

This new mutation was found in 10 obese patients and in one normal-weight subject. Interestingly, subdividing subjects according to BMI (nonobese, obese, and severely obese), the greater the BMI the higher the mutation frequency, suggesting a possible link between the presence of *GPR40* (Gly180Ser) mutation and the propensity to develop obesity. As far as the possible mechanisms why *GPR40* (Gly180Ser) carriers could be more susceptible to obesity, one possibility is related to the up to now unclear role of GPR40 expression in monkey and human brain (10, 20). In the hypothalamic neurons, GPR40 could be involved in the lipid-sensing mechanism that controls energy balance and food intake (21–23). An adequate sensing of increased fatty acid availability may initiate a negative feedback on energy homeostasis that includes restraint of food intake, stimulation of energy expenditure, and decreased output of nutrients from endogenous sources (predominantly the liver) (22). It is possible, therefore, that a GPR40 loss-of function mutation could impair this negative feedback mechanism at the hypo-

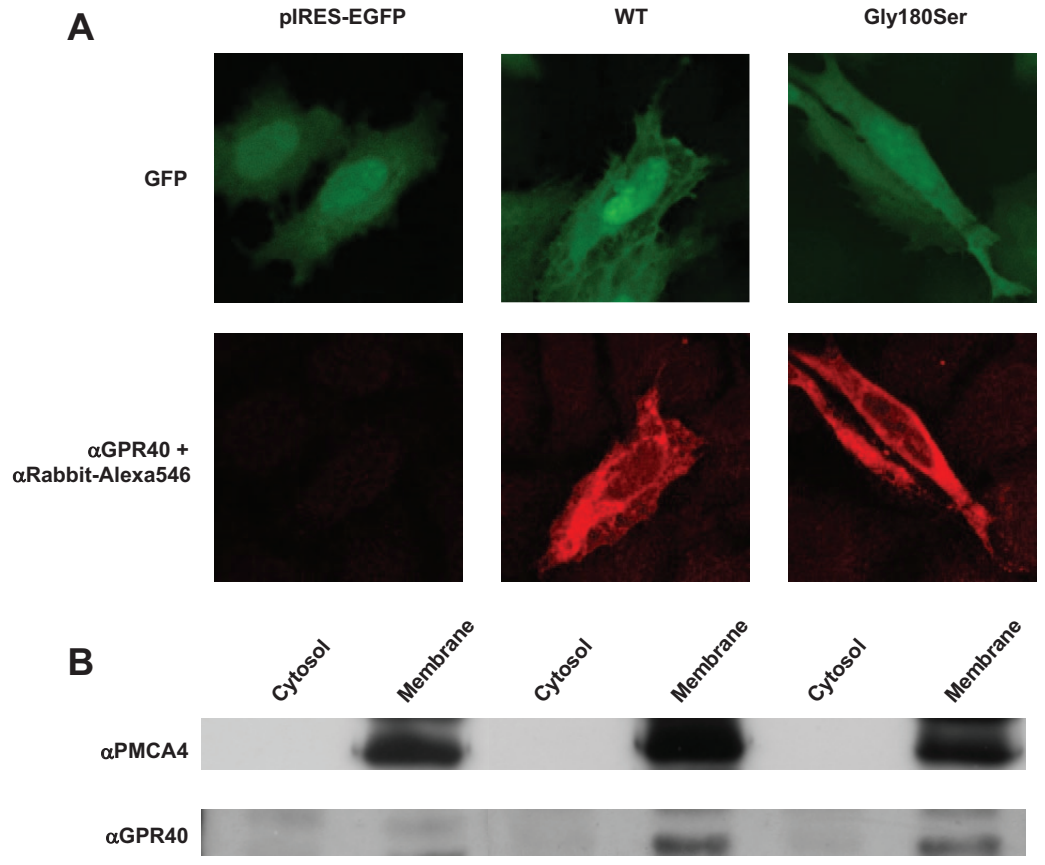

FIG. 5. A, Immunofluorescence staining of GPR40 in pIRES-EGFP, Gly/Gly and Gly/Ser *GPR40*-transfected HeLa cells. Gly/Gly and Gly/Ser *GPR40*-expressing cells show a characteristic signal from both endoplasmic reticulum and plasma membrane, whereas pIRES-EGFP cells show only nonspecific binding. B, Subcellular fractioning and Western blot of HeLa cells transfected with pIRES-EGFP, Gly/Gly, or Gly/Ser *GPR40*. Expression levels of *GPR40* at plasma membrane were comparable in Gly/Gly and Gly/Ser *GPR40*, whereas no specific bands were detected in pIRES-EGFP-expressing cells. GFP, Green fluorescence protein; WT, wild-type.

thalamic level and favor obesity. The observation that $GPR40^{-/-}$ mice are lean on a standard diet and gain weight and become as obese as their $GPR40^{+/+}$ littermates on a high-fat diet does not necessarily disprove this hypothesis because *GPR40* expression was not documented in mouse or rat brain (10, 13, 14, 28).

The present studies confirm the important role of GPR40 for the β-cell function in human subjects. Fatty acids can function as signaling molecules at the β-cell level, regulating insulin secretion both acutely and chronically acting on the GPR40 receptor (12–15). We investigated, therefore, the effect of the *GPR40* (Gly180Ser) mutation with both *in vivo* and *in vitro* studies.

In vivo, the biological profile of mutation carriers was investigated by evaluating insulin secretion after oral glucose or a lipid-rich meal test. Data indicate that insulin secretion, as assessed by the insulinogenic index after OGTT, and the insulin response to OLTT, were significantly lower in individuals carrying the Gly/Ser *GPR40* mutation in respect to subjects with the Gly/Gly genotype. These results parallel those recently described in *GPR40* knockout mice, where insulin secretion in response to Intralipid was markedly reduced (30) and suggest that subjects with this mutation are characterized by reduced glucose tolerance and reduced insulin response to both glucose and lipids.

In vitro studies were carried out in transfected cells and indicate that the mutated Gly/Ser *GPR40* receptor is unable to adequately sense the oleic acid and consequently activate G protein signaling and induce intracellular calcium mobilization. It is already known that inhibition of Ca^{2+} influx suppresses FFA stimulation of insulin release (34).

Therefore, this type of GPR40 mutation alters β-cell function, reducing its ability to respond to changes of metabolic substrates. Because most carriers of mutated *GPR40* alleles identified in the studied population were obese, we cannot exclude that the insulin resistance associated with this condition may be an independent factor, other than the *GPR40* mutation. In any case, insulin resistance coupled to the insufficient β-cell secretory response to both fatty acids and glucose is a condition clearly predisposing to T2D. Our study does not demonstrate a direct association between *GPR40* (Gly180Ser) mutation and T2D, probably because of the low allele frequency (statistical power <50% in our cohort). Consequently, false-negative results cannot be excluded. However, our data demonstrate a significant association between *GPR40 (Gly180ser)* mutation and DI, an important predictor of T2D (35). Therefore, genetic studies in larger series of T2D patients and longitudinal prospective study with an accurate follow-up of *GPR40* (Gly180Ser) mutation carriers will be required to confirm the link between the insulin secretion abnormalities observed in Gly/Ser *GPR40* carriers and the susceptibility to develop T2D. Other factors, besides *GPR40* gene mutation and loss-of-function, could affect β-cell secretion pattern. Chronic exposure to high FFA concentrations and/or

hyperglycemia itself could differently influence β-cell function (2). In addition to glucotoxicity and lipotoxicity, there is evidence (both *in vivo* and *in vitro*) indicating that elevated FFAs, in the absence of hyperglycemia, are major signals that permit β-cell adaptation to insulin resistance (36). In this case, a genetic defect in the β-cell lipid-sensing mechanisms, due to the reduced *GPR40* function, will not allow the β-cell to adapt to insulin resistance.

In a situation of chronic lipid (FFA) overflow, as it occurs in obesity and T2D, the impact of GPR40 (Gly180Ser) mutation is unclear. The reduced GPR40 function may be potentially beneficial or detrimental in modulating β-cell function under long-term exposure to FFA excess. Paradoxically, β-cells from *GPR40*-deficient mice under chronic exposure to FFA excess are protected from lipotoxicity. In contrast, mice expressing *GPR40* under the control of PDX-1 (pancreatic duodenal homeobox-1) promoter had impaired β-cell function and developed diabetes (13). Hence, in this model, GPR40 loss-of-function mutation would protect from progression to diabetes. If this is the case also in human subjects, development of a GPR40 antagonist could be an attractive approach for the treatment of diabetes.

In summary, we found that at least two nonsynonymous variants of GPR40 exist in the Sicilian population and that the rare GPR40 (Gly180Ser) variant results in a loss of the FFA receptor function that prevents its ability to adequately sense lipids as a stimulus for intracellular $[Ca^{2+}]_i$ increase and pancreatic β-cell secretion. Data in carriers of this mutation indicate a possible relationship with obesity, insulin resistance, and reduced insulin response to metabolic stimuli (both glucose and lipids). However, the relationship of such a *GPR40* variant to obesity and T2D remains to be established.

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