Physiological and genetic characterization of the Gly40Ser mutation in the glucagon receptor gene in the Sardinian population

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Summary A Gly40Ser amino acid substitution in the glucagon receptor gene has been associated with non-insulin-dependent diabetes mellitus (NIDDM), but the questions raised about its physiological implications have not been resolved. The aim of this study was to determine the frequency of the Gly40Ser mutation in different regions of Sardinia and to investigate the physiological implications of the mutation in glucose and insulin homeostasis. We studied a population of 691 subjects, selected on the basis of their Sardinian origin. Only heterozygous subjects were found, 21 of 574 (3.6 %) in NIDDM patients and 5 of 117 in non-diabetic subjects (4.2 %). In northern Sardinia 3.4 % of the NIDDM patients were carriers of the Gly40Ser substitution, 1.4 % in central Sardinia, while 7.6 % carried the substitution in the Southern part. No significant differences were found between hypertensive and normotensive subjects with respect to the presence of Gly40Ser. Ten subjects with Gly40Ser were carefully matched for diabetic state, BMI, age, sex, and geographical origin with 10 patients with Gly40, and a glucagon infusion test was performed using 1, 3, 9 and 27 ng glucagon $\text{kg}^{-1} \cdot \text{min}^{-1}$ for 30 min. Blood

for determination of glucose, glucagon, and insulin concentrations was drawn at 15-min intervals from the Controlateral arm. Plasma glucagon increased dose-dependently during the infusion with no significant difference between the two groups. Carriers of Gly40Ser had a significantly lower ($p < 0.02$) increase in plasma glucose concentration in response to glucagon infusion compared to Gly40 homozygous subjects at all times, while the plasma insulin increase was not significantly different at any time. In conclusion, our results indicate that the Gly40Ser variation is not associated with NIDDM in the Sardinian population, and that its frequency varies in different parts of Sardinia. Moreover *in vivo* Gly40Ser plays a physiological role in the glucose homeostasis under glucagon control both in NIDDM and non-diabetic subjects. This latter result suggests that this amino acid substitution in the glucagon receptor may lead to a decreased blood glucose concentration because of the reduced stimulation of liver glucose output via the glucagon receptor. [Diabetologia (1997) 40: 89–94]

Keywords NIDDM, genetics, hypertension.

Non-insulin-dependent diabetes mellitus (NIDDM) is a multifactorial disorder, displaying heterogeneity

both at the genotype and phenotype levels. It affects 3–8 % of the Caucasian population over 45 years [1, 2]. The genetic susceptibility to NIDDM is likely to involve separate but interrelated functions (for example insulin secretion, insulin resistance, obesity, abdominal fat patterning, intermediary metabolism, etc.) which have clinical consequences as a result of environmental influence. The increased understanding of the physiology and molecular biology of NIDDM has led to the identification of a number of genes. Among different candidate genes investigated so far, a possible association between NIDDM and a

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Abbreviations: NIDDM, Non-insulin-dependent diabetes mellitus.

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Gly40Ser amino acid substitution in the glucagon receptor has been reported in the French and British populations [3, 4]. No heterozygous carriers of this substitution were found in Japanese, Finnish, or a small sample of German NIDDM patients [5–8]. However, in Finland, non-diabetic control subjects had a carrier frequency of 1.3% (4/306). These data suggest that the Gly40Ser substitution is not widely associated with NIDDM and that there might be problems of population stratification when cases and control subjects are compared.

Moreover, the questions raised about the *in vivo* pathophysiological implications of this mutation have not been resolved. Glucagon may be an element in the pathogenesis of diabetes, and glucagon antagonists may be potentially useful in its treatment by alleviation of the characteristically high gluconeogenesis in NIDDM. Thus, the Gly40Ser amino acid variation in the glucagon receptor, generating a receptor less responsive to endogenous glucagon as clearly shown by *in vitro* studies [3, 9], could be protective against hyperglycaemia. On the other hand, glucagon may also stimulate insulin secretion since biologically active glucagon receptors have been found in the pancreatic beta-cells [10]. In any case, no reports so far have described the *in vivo* biological function of the Gly40Ser variation in exon 2 of the glucagon receptor gene in relation to glucagon-stimulated hyperglycaemia.

We have determined the frequency and geographical distribution of the Gly40Ser variation in the exon 2 of the glucagon receptor gene on chromosome 17 in a large number of NIDDM patients from families with multiple cases of diabetes and non-diabetic subjects of Sardinian origin, and investigated the physiological consequences of this amino acid variation *in vivo*.

Subjects and methods

Subjects. The subjects were recruited from our ongoing studies of genetic and environmental effects on diabetes in multiplex NIDDM families from all parts of Sardinia. This study was conducted as a collaboration between several Sardinian Antidiabetic Units (The Diabetes Genetic Study Group of Sardinia). A total of 691 subjects, 574 with NIDDM and 117 non-diabetic subjects were investigated for the Gly40Ser amino acid variation of the glucagon receptor. Of the subjects 468 were NIDDM siblings from a total of 189 families, while the remaining 223 (106 NIDDM and 117 non-diabetic subjects) were unrelated without documented family history for NIDDM. NIDDM was diagnosed according to the following criteria: basal plasma glucose concentration more than 7.8 mmol/l on two different occasions or glucose higher than 11.1 mmol/l at 120 min after a 75-g oral glucose tolerance test (OGTT). The age at diagnosis was between 29 and 61 years and insulin was not required during the first 3 years after diagnosis. Normal subjects, over 55 years of age, were selected on the basis of a plasma glucose concentration less than 7.6 mmol/l at 120 min

after an OGTT. All subjects had at least parents and grandparents born in Sardinia. Of the 574 patients with NIDDM, 148 (25.8%) originate from the Archaic area of Sardinia in the middle of the island, so defined due to the pre-Neolithic settlement of the population that has remained genetically conserved [11]. Hypertension was defined as either blood pressure higher than 140/90 mmHg on at least 3 different occasions over a period of three months without any antihypertensive treatment, or treatment with antihypertensive drugs.

The study was approved by the local ethics committee and all subjects gave informed oral consent to participation in the study.

Detection of the glucagon receptor mutation. Genomic DNA was isolated from leukocytes, and the GGT to AGT transition in exon 2 of the glucagon receptor gene, changing codon 40 from glycine to serine, was analysed after PCR amplification followed by restriction enzyme cleavage, using a previously described method [3] with modifications. PCR amplifications were performed with primers GCGR22, 5'-TCTGTGGCTCAG TTACCTCGTTA 3' and GCGR23, 5'-TCTGTCTGGTTGCTTGTCGA, 3' after initial denaturation for 3 min. at 94°C, 35 cycles consisting of 1 min at 94°C, 30 s at 55°C and 1 min at 72°C were performed, followed by a final extension of 5 min at 72°C using a Perkin Elmer DNA thermal cycler 4800 (Norwalk, CT, USA). Each 10 µl reaction contained 150 ng DNA, 10 mmol/l Tris-HCl, pH 8.8 at 25°C, 50 mmol/l KCl, 1.5 mmol/l MgCl₂, 0.1% Triton X-100, 0.2 mmol/l dNTP, 0.5 µmol/l of each primer, and 0.2 U of Dyna Zyme (Finnzymes Oy, Finland). After amplification, 10 µl of the PCR product was cleaved with 5 U BstEII at 60°C for 2 h, and the cleaved samples were separated by electrophoresis on a 3% agarose gel in Tris-Borate-Edta (TBE) buffer containing ethidium bromide (0.5 µg/ml) and visualized by ultraviolet Light. All BstEII resistant samples were re-analysed to verify the Gly40Ser coding change.

Glucagon infusion test. From our population we selected 20 normotensive subjects, 10 heterozygous Gly40Ser and 10 homozygous Gly40, for a glucagon infusion test. In each group six subjects were diabetic, while the other four were non-diabetic as defined by OGTT. The two groups were carefully matched for age, diabetes status and age at onset, body mass index. The NIDDM subjects were maintained in good metabolic control with oral antidiabetic agents ($HbA_{1c} < 7\%$) and were not on any other drug treatment. Oral antidiabetic agents were discontinued 3 days prior to the study. All subjects were on a isocaloric diet with 50% carbohydrate and body weights were steady for at least 3 months before the study. All experiments began between 07.00 and 08.00 hours after a 12-h fast. Indwelling catheters were placed into the antecubital veins in both arms for glucagon (Novo Nordisk Farmaceutici S.p.A., Roma, Italy) infusion and blood withdrawal. After a 30-min run-in period during which haemagel was infused at 3 ml/h, glucagon (dissolved in haemagel) was infused at doses progressively increasing from 13, 9 to 27 ng kg⁻¹ · min⁻¹, each concentration was infused for 30 min (study period). At the end of the glucagon infusions, haemagel was infused at 3 ml/h for an additional 60 min (recovery period). Blood was collected every 15 min in refrigerated (4°C) tubes, containing EDTA for glucose and insulin determinations. Plasma glucose was immediately assayed (Beckman Inc.) and the remaining plasma was stored at -35°C until insulin was assayed. Blood for glucagon determinations were collected in refrigerated glass tubes containing 500 kallekrein inhibitor units trasylol and 1 mg EDTA for each ml whole blood and immediately centrifuged at 3000 rev/min for 15 min at 4°C, and the plasma was collected and

Table 1. Clinical and biochemical characteristics of the NIDDM patients and the non-diabetic subjects according to the presence of the Gly40Ser amino acid substitution

	NIDDM		Non-diabetic subjects	
	Gly40	Gly40Ser	Gly40	Gly40Ser
<i>n</i>	503	21	112	5
Age (years)	60.6 ± 9.6	62.4 ± 8.8	60.8 ± 5.4	59 ± 6
Sex (female/male)	245/258	10/11	67/45	3/2
Body mass index	29.2 ± 4.5	29.1 ± 4.7	28.3 ± 4.9	24.6 ± 6.3
Hypertension (yes/no)	256/247	10/11	51/61	3/2
Diabetes onset (years)	49.5 ± 9.6	48.6 ± 8.7	–	–
HbA _{1c} (%)	7.6 ± 2.1	8.1 ± 2.5	–	–
Treatment (D/T/T + I/I)	137/264/46/56	7/8/6/0	–	–
Total cholesterol (mmol/l)	5.66 ± 1.06	5.74 ± 0.85	5.82 ± 1.21	6.05 ± 1.00
HDL-cholesterol (mmol/l)	1.34 ± 0.40	1.24 ± 0.31	1.34 ± 0.36	1.55 ± 0.38
LDL-cholesterol (mmol/l)	3.67 ± 1.03	3.67 ± 0.64	3.67 ± 1.00	3.90 ± 0.67
Triglycerides (mmol/l)	1.55 ± 1.04	1.39 ± 0.62	1.42 ± 0.74	1.22 ± 0.14

Data are given only for subjects in whom all data were available, data are expressed as mean ± SD, the only significant difference was detected for HDL-cholesterol between heterozy-

gous subjects with the Gly40Ser variation, NIDDM and non-diabetic subjects ($p < 0.05$)

stored in glass tubes. All the samples from each patient were run in the same assay. Blood pressure was measured three times at the end of each glucagon infusion step with a mercury sphygmomanometer, at the same time radial pulse rate was determined. Plasma glucagon was measured by radioimmunoassay, using a double antibody kit (Diagnostic Products Corporation, Los Angeles, Calif., USA; intra- and inter-assay coefficients of variation were less than 6%), plasma insulin was measured by radioimmunoassay (INCSTAR, Stillwater, Minn., USA, intra- and inter-assays coefficients of variation were less than 9%).

Statistical analysis

Values are mean ± SD except for the glucagon infusion test where SEM was used. Differences between groups were tested using the paired or unpaired Student's *t*-test and ANOVA with repeated measurements design was used to analyse the results of the glucagon infusion test. Differences in frequency were tested with the chi-square analysis. The statistical calculations were performed using the SIGMA STAT statistical package.

Results

Glucagon receptor gene screening. Of our population of 691 subjects, a total of 26 (13 males and 13 females) were heterozygous carriers of the Gly40Ser amino acid substitution. No homozygous Ser40 subjects were found. In particular 21 of 574 NIDDM patients and 5 of 117 non-diabetic subjects were heterozygous carriers of the Gly40Ser amino acid variation. Thus, the prevalence of this mutation was 3.6% in NIDDM patients and 4.2% in non-diabetic subjects (non significant, $\chi^2 = 0.003$, $p = 0.96$). In detail, among the 514 NIDDM patients from whom

ancestral data were available: in north Sardinia 9 of 261 (3.4%) were heterozygous carriers of the Gly40Ser, while in the center 2 of 148 (1.4%) were heterozygous, and in the south 8 of 105 (7.6%). The prevalence was significantly different between south and central ($\chi^2 = 4.81$, $p = 0.028$) and between south and the combined central and north regions ($\chi^2 = 4.4$, $p = 0.036$), while there was no statistical significance between the north and the central regions ($\chi^2 = 0.89$, $p = 0.346$) and between the south and the north regions ($\chi^2 = 0.89$, $p = 0.346$). No significant differences for sex or for the presence of hypertension were present in relation to the glucagon amino acid variation. None of the variables measured in NIDDM patients were significantly different in the heterozygous carriers as compared to the patients homozygous for Gly40. A slight difference was observed for plasma HDL-cholesterol between diabetic and non-diabetic carriers (Table 1). All the measured variables were not significantly different as shown Table 1.

Glucagon infusion test. The recovery of glucagon dissolved in haemagel was $91 \pm 3\%$ (mean ± SD). Plasma glucose changes during the test are expressed as incremental variations calculated after subtraction of the basal value (time 0). Plasma glucose increased in a dose-dependent manner in both carriers of Gly40Ser and non-carriers during glucagon infusion. The glucose incremental values were significantly higher at all time points during the glucagon infusion test in the Gly40 subjects as compared to the Gly40Ser heterozygotes ($p = 0.02$, Fig. 1). A dose as small as $1 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, which increased the basal glucagon concentration only by 50% significantly increased

Fig 1. The incremental plasma glucose concentrations above basal glucose are shown for heterozygous Gly40Ser (●) and Gly40 homozygous (■) mean $\pm$ SEM. Glucagon was infused at stepwise increased concentrations $1 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ (30–120 min), $3 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ (30–60 min), $9 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ (60–90 min) and $27 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ (90–120 min)

Table 2. Plasma glucagon and insulin levels before (–15 and 0 min) during (30–120 min) and after (135–180 min) glucagon infusion at the different doses in the two groups of patients, heterozygous carriers of the Gly40Ser variation and homozygous Gly40

Time (min)	Glucagon (pmol/l)		Insulin (pmol/l)	
	Gly40	Gly40Ser	Gly40	Gly40Ser
–15	40 ± 1.7	43 ± 1	56 ± 12	44 ± 7
0	40 ± 2.3	42 ± 1	62 ± 12	38 ± 5
30	65 ± 2.3	59 ± 4	67 ± 6	52 ± 5
60	78 ± 17	77 ± 12	72 ± 11	55 ± 5
90	118 ± 20	142 ± 18	114 ± 21	109 ± 22
120	258 ± 27	303 ± 26	194 ± 59	158 ± 42
135	109 ± 33	89 ± 18	142 ± 23	105 ± 20
150	47 ± 8	54 ± 7	85 ± 12	62 ± 14
180	47 ± 8	42 ± 1	43 ± 9	31 ± 12

Data are mean $\pm$ SEM. No significant differences were evident between groups at any time for both plasma glucagon and insulin. A significant increase within groups was evident for both plasma glucagon and insulin levels ($p < 0.001$) during exogenous glucagon infusion

the plasma glucose in both groups. However, these physiological glucagon levels increased the glucose concentration significantly more in Gly40 subjects as compared to the heterozygous carriers ($p = 0.014$). The area under the curve (AUC) of the incremental plasma glucose levels during the glucagon infusion (from 0 min to 120 min) was significantly larger ($p = 0.011$) in Gly40 homozygotes 184 ± 33 (mmol/l) $\cdot$ min vs Gly40Ser heterozygotes 96 ± 23 (mmol/l) $\cdot$ min. Plasma insulin and glucagon were not significantly different in the two groups before glucagon infusion. During the glucagon infusion, both insulin and glucagon increased in a dose-dependent manner to similar levels (Table 2). After the end of glucagon infusion, plasma glucose, insulin, and glucagon decreased in the two groups, returning to basal values after 60 min. Systolic and diastolic blood pressure and heart rate were stable during the test with no differences between the groups (data not shown).

Discussion

The results of our study demonstrate that the Gly40Ser variation in the glucagon receptor gene is not associated with NIDDM in the Sardinian population, suggesting that this amino acid substitution alone is not a significant factor in the development of familiar NIDDM in this population. Previously Hager et al. [3] showed a non-significant difference in the prevalence of the Gly40Ser mutation in a small samples of Sardinian NIDDM patients (8.3%) when compared with non-diabetic subjects (3.1%), although significant association was found in the French population. Our data do not differ from those obtained by Hager et al. with respect to the Sardinian population, and when the two data sets from Sardinia are combined the difference between NIDDM and control subjects is still not significant (4.3% in NIDDM and 3.8% in control subjects). Indeed, in our subgroup of NIDDM patients who originated from the south of Sardinia, we found a prevalence of heterozygotes for the Gly40Ser substitution of about 8%. An interesting result from our study is the major difference in distribution of the Gly40Ser variation across the different Sardinian regions. In fact, in the south the frequency of Gly40Ser was significantly higher than in the central region (the most archaic and genetically homogeneous area), and almost twice as high, although not significantly, than in the north. Genetically, Sardinians are very different from most European populations since the colonization dates back to pre-Neolithic times, and cultural, linguistic and geographical factors have promoted isolation of the Sardinians [11, 12]. The Gly40Ser amino acid substitution has been observed at different prevalences in different populations [3–6], and also within the same population [7, 8]. These data fit well with our findings in the different regions of Sardinia.

Glucagon is a 29 amino acid peptide hormone that is secreted from the pancreatic alpha-cells in response to hypoglycaemia. It is a key regulator of hepatic glucose production through hepatic gluconeogenesis and glycogenolysis [13]. Glucagon receptors have been identified in a restricted number of tissues besides liver including heart, adipose tissue, pancreatic beta-cells – where glucagon may potentiate glucose-induced insulin secretion from beta cells during fasting – kidney, and brain, but the role of glucagon in these tissues is not fully understood [14–17]. In cultured rat hepatocytes, glucagon receptor mRNA levels are up-regulated in the presence of high glucose concentrations in the culture medium, and glucagon is able to down-regulate the expression of its own receptor [18]. Increased endogenous glucose production through gluconeogenesis and glycogenolysis contribute to the pathogenesis of hyperglycaemia in NIDDM. Additionally, it has been shown that the regulation of glucagon secretion is impaired in

NIDDM and can contribute to the hyperglycaemic state [19]. Thus, glucagon might be an additional contributing factor in the pathogenesis of diabetes and glucagon receptor antagonists have been proposed as being potentially useful in the treatment of diabetes, by alleviating the characteristically high liver glucose output. From these data we can hypothesize that an amino acid variant in the glucagon receptor gene, which reduces the effects of glucagon would not play a major role in the development of NIDDM. The fact that the Gly40Ser amino acid substitution results in a reversion to the sequence of the rat glucagon receptor sequence, suggests a role of this region in the receptor activity, as has been described for the glucagon like peptide-1 receptor [20]. In any case, although *in vitro* binding studies suggest a reduced activity of the mutated receptor [3, 9], the exact *in vivo* physiological role of the Gly40Ser amino acid substitution in the glucagon receptor gene has not been addressed until now. The present study was therefore designed to determine the *in vivo* role of the variant in terms of glucose production under glucagon control. To explore the biological effects of the Gly40Ser variant we evaluated the response of plasma glucose and insulin to a stepwise increase in the glucagon concentration. Even though insulin plasma concentration increased during glucagon infusion, the infusion of only 1 and 3 ng · kg⁻¹ · min⁻¹, which raised the endogenous glucagon plasma levels within the physiological range, only 20 and 35 pmol/l, respectively, resulted in a statistically significant rise in glucose in both groups. Subjects who were heterozygous carriers of the Gly40Ser variant of the glucagon receptor showed similar glucagon and insulin increases, but a significantly lower increase in plasma glucose, irrespective of diabetes status. This indicates that the Gly40Ser variant reduces the activity of the hepatic receptor *in vivo*. Thus, from our data, it appears that this variant of the glucagon receptor is responsible for a significant reduction of glucose production in response to physiological or slightly over-physiological increase in plasma glucagon, while insulin secretion appeared unaffected by the presence of the variant glucagon receptor. Recently, it was shown that BHK cells transfected with the Gly40Ser glucagon receptor had a right shift in the dose-response curve for glucagon stimulated cAMP production at glucagon concentrations of 1–10 pmol/l [9], compatible with our concentrations during *in vivo* infusion.

It has long been known that partial glucagon suppression could be useful in the management of diabetic patients [21]. Since glucagon receptors are present on the beta cell and are supposed to stimulate insulin secretion [10, 22], it may be that a reduction in the activity of this receptor may contribute to a failure of insulin secretion in NIDDM patients with inadequate insulin secretion. Indeed a right shift has been reported in the dose response curve of

glucagon stimulated insulin secretion in RIN cells transfected with the mutant receptor, as compared to the same cell line transfected with the wild type receptor [9]. In that experiment the effects were seen at concentrations of glucagon 10³ to 10⁴ higher than those obtained during our *in vivo* experiment. Our data indicate that *in vivo* insulin secretion is not different in heterozygous carriers of the Gly40Ser as compared to normal subjects during increase in plasma glucagon concentration in the physiological range. This observation suggests that the effects of glucagon on insulin secretion are minimal under these conditions.

In summary our data demonstrate that 1), in the Sardinian population the Gly40Ser variant in the glucagon receptor gene is not preferentially associated with NIDDM; 2), there are significant differences in Gly40Ser carrier frequency in different parts of Sardinia; 3), heterozygous carriers of the Gly40Ser glucagon receptor variant display significantly decreased glucose levels during glucagon stimulation within the physiological range.

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