Brief Genetics Report

Insulin Secretory Function Is Impaired in Isolated Human Islets Carrying the $Gly^{972} \rightarrow Arg$ IRS-1 Polymorphism

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Type 2 (non–insulin-dependent) diabetes results from decreased insulin action in peripheral target tissues (insulin resistance) and impaired pancreatic β-cell function. These defects reflect both genetic components and environmental risk factors. Recently, the common $Gly^{972} \rightarrow Arg$ amino acid polymorphism of insulin receptor substrate 1 (Arg^{972} IRS-1) has been associated with human type 2 diabetes. In this study, we report on some functional and morphological properties of isolated human islets carrying the Arg^{972} IRS-1 polymorphism. Insulin content was lower in variant than control islets (94 ± 47 vs. 133 ± 56 μU/islet; $P < 0.05$). Stepwise glucose increase (1.7 to 16.7 mmol/l) significantly potentiated insulin secretion from control islets, but not Arg^{972} IRS-1 islets, with the latter also showing a relatively lower response to glyburide and a significantly higher response to arginine. Proinsulin release mirrored insulin secretion, and the insulin-to-proinsulin ratio in response to arginine was significantly lower from Arg^{972} IRS-1 islets than from control islets. Glucose utilization and oxidation did not differ in variant and wild-type islets at both low and high glucose levels. Electron microscopy showed that Arg^{972} IRS-1 β-cells had a severalfold greater number of immature secretory granules and a lower number of mature granules than control β-cells. In conclusion, Arg^{972} IRS-1 islets have reduced insulin content, impaired insulin secretion, and a lower amount of mature secretory granules. These alterations may account for the increased predisposition to type 2 diabetes in individuals carrying the $Gly^{972} \rightarrow Arg$ amino acid polymorphism of IRS-1. *Diabetes* 51:1419–1424, 2002

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HBSS, Hanks' balanced salt solution; IRS-1, insulin receptor substrate 1; PC 1/3, prohormone convertase 1/3; PDX-1, pancreatic duodenal homeobox 1; PI, phosphatidylinositol.

Type 2 (non–insulin-dependent) diabetes is the most frequent form of diabetes, and it affects ~5% of the population in Western countries (1). The association between type 2 diabetes and micro- and macrovascular complications, such as premature atherosclerosis, nephropathy, retinopathy, and neuropathy, has vast public implications. Consequently, intensive investigation into the causes of the disease to prevent its onset and optimize treatment after its diagnosis is required. Insulin resistance and impaired pancreatic β-cell function are pathogenetic hallmarks of type 2 diabetes (2–4). Insulin resistance is often believed to precede the development of glucose intolerance and in the early stages is associated with enhanced β-cell function that attempts to compensate for the reduced insulin sensitivity. As soon as insulin secretion becomes insufficient to meet the enhanced demand, diagnostic hyperglycemia develops. The progressive loss of insulin secretion is affected by a metabolic milieu (gluco- and lipotoxicity) acting upon a predisposing background. The predisposing condition is likely to result from the polygenic interactions of several simultaneously inherited gene mutations. To date, although some rare subtypes of type 2 diabetes have been described that are caused by mutations in genes encoding for crucial molecules involved in β-cell development and function (i.e., glucokinase, hepatocyte nuclear factor 1 and 4, insulin promoter factor 1, insulin, and mitochondrial DNA) (4), the genetic basis of the common forms of type 2 diabetes has not yet been identified.

Almost a decade ago, Almind et al. (5) reported an association between the insulin receptor substrate 1 (IRS-1) polymorphism causing a Gly-to-Arg substitution at codon 972 (Arg^{972} IRS-1) and type 2 diabetes. IRS-1 is a major substrate for the insulin receptor and is present in tissues that are involved in glucose production, glucose clearance, and insulin secretion (6,7). It acts as a multisite docking protein that binds signal proteins and links the receptor kinase to a number of cellular functions that are regulated by insulin. For these reasons, the IRS-1 gene has been considered a candidate gene for common forms of type 2 diabetes. Indeed, after the above-mentioned study

TABLE 1
Clinical characteristics of pancreas donors

	Age (years)	Sex	Weight (kg)	Height (cm)	Fasting plasma glucose (mg/dl)	Cause of death
Control #1	30	M	87	188	123	Trauma
Control #2	60	F	67	164	151	Cardiac arrest
Gly^{972}→Arg IRS-1 #1	15	F	58	156	117	Trauma
Gly^{972}→Arg IRS-1 #2	64	F	65	160	121	Cardiac arrest

(5), several investigations have been performed, usually suggesting that the Arg^{972} IRS-1 polymorphism could predispose individuals to type 2 diabetes (8–12). A greater degree of insulin resistance in type 2 diabetic patients carrying the Arg^{972} IRS-1 polymorphism has been observed in some (13,14), but not all, investigations (15,16). Interestingly, type 2 diabetic patients as well as their nondiabetic nonobese first-degree relatives carrying the Arg^{972} IRS-1 polymorphism have lower fasting plasma insulin levels and lower insulin responses to glucose than noncarriers (17,18). Lately, it has been reported that normal glucose-tolerant subjects with the polymorphism have decreased insulin secretion and normal insulin sensitivity (19), thus suggesting that the Arg^{972} IRS modification can indeed play a role in β-cell dysfunction.

Defects of insulin signaling at the β-cell level have been shown to contribute to impaired insulin secretion (20–22). An impairment of glucose-mediated insulin secretion has been reported with disruption of the insulin receptor gene of the β-cell (21), a knockout for islet IRS-1 (22), and overexpression of the Arg^{972} IRS-1 polymorphism in a rat insulinoma cell line (22). More recently, we have demonstrated that the Arg^{972} IRS-1 polymorphism impairs human pancreatic β-cell survival and causes resistance to the anti-apoptotic effects of insulin (23).

Our laboratory processes pancreata of large mammals and humans on a regular basis for the preparation of isolated islets (24–27). We received pancreata from two nondiabetic donors (Table 1) in whom the Arg^{972} IRS-1 polymorphism was demonstrated; this gave us the unique opportunity to study a number of morphological and functional properties of the isolated islets and to compare the results with those obtained with the islets from two control donors (Table 1).

When islet insulin content was measured, it was significantly lower in islets carrying the Arg^{972} IRS-1 polymorphism (94 ± 47 μU/islet; n = 33) than in wild-type islets (133 ± 56 μU/islet; n = 35; $P < 0.05$). Insulin mRNA levels analyzed by semiquantitative RT-PCR were slightly, but not significantly, decreased (by 13%) in variant islets compared with wild-type islets (data not shown). Insulin secretion results (expressed as percent of insulin content) in response to glucose, arginine, and glibenclamide are reported in Table 2. Control islets showed a progressive and significant potentiation of insulin release at increasing glucose concentrations ($P < 0.05$ by ANOVA). Conversely, the Arg^{972} IRS-1 islets had lower insulin secretion, with no significant increase in response to the stepwise increase of glucose levels in the medium. The Arg^{972} IRS-1 islets also showed a relatively lower insulin release in response to glibenclamide and a higher secretion of the hormone in response to arginine than control islets (Table 2).

Table 3 illustrates proinsulin secretion. Similar to insulin release, proinsulin secretion from Arg^{972} IRS-1 islets showed no significant increase in response to glucose. When the insulin-to-proinsulin molar ratio was calculated, no significant difference was found between wild-type and polymorphic islets when the cells were challenged with glucose or glibenclamide (data not shown). However, a lower ratio was apparent for Arg^{972} IRS-1 than for control islets in response to arginine (4.4 ± 2.1 vs. 7.9 ± 2.9; $P <$ 0.05).

Glucose utilization and oxidation increased as a function of the increasing glucose concentration, both in control and Arg^{972} IRS-1 islets, with no difference between wild-type and variant islets (Table 4).

By electron microscopy, it was shown that the percent of β-cells was similar in control islets (61 ± 3%) as in Arg^{972} IRS-1 islets (63 ± 2%). In addition, the amount of α-cells did not differ significantly between wild-type (22 ± 4%) and variant (16 ± 3%) islets. Quantification of β-cell secretory granules indicated a greater number of immature and a severalfold lower amount of mature secretory granules in the Arg^{972} IRS-1 islets compared to control islets (Fig. 1 and Table 5).

Type 2 diabetes is a dual-disorder condition, characterized by insulin resistance and impaired insulin secretion. Although type 2 diabetes is likely to reflect a polygenic background to account for the defect of peripheral insulin action and reduced β-cell response to glucose, recent studies have pointed out that a common defect may be shared by the β-cell and the insulin-target cell. This hypothesis is supported by the observation that alteration of the insulin receptor or disruption of the insulin signaling pathway may result in both insulin resistance and reduced β-cell function (3–5).

TABLE 2
Insulin release from control and Gly^{972} →Arg IRS-1 islets in response to different stimuli

	Glucose			Glibenclamide	Arginine
	1.8 mmol/l	5.5 mmol/l	16.7 mmol/l	100 μmol/l	20 mmol/l
Control	1.4 ± 0.3	2.6 ± 1.6	4.3 ± 1.4	4.5 ± 1.6	2.6 ± 0.6
Gly^{972}→Arg	1.6 ± 0.4	1.9 ± 0.4	2.9 ± 1.1*	3.5 ± 2.4	4.7 ± 1.3†

Data are percent of insulin content and are means ± SD. ANOVA was performed to test differences in the response to stepwise increase of glucose concentrations, which was significantly higher ($P < 0.05$) in control islets. *P = 0.05, †$P < 0.05$ vs. control.

TABLE 3
Proinsulin secretion results from control and Gly^{972}→Arg islets in response to different stimuli

	Glucose			Glibenclamide	Arginine
	1.8 mmol/l	5.5 mmol/l	16.7 mmol/l	100 μmol/l	20 mmol/l
Control	1.46 ± 0.4	1.96 ± 0.3	2.64 ± 0.7	2.7 ± 0.9	2.5 ± 0.4
Gly^{972}→Arg	1.08 ± 0.7	0.90 ± 0.2*	1.28 ± 0.8*	1.5 ± 0.5*	2.6 ± 0.5

Data are pmol/islet and are means ± SD. ANOVA was performed to test differences in the response to stepwise increase of glucose concentrations, which was significantly higher ($P < 0.05$) in control islets. *$P < 0.05$ vs. control.

In the present study, we showed for the first time that human pancreatic islets from carriers of the Gly^{972}→Arg amino acid polymorphism in IRS-1 have reduced insulin content, altered insulin release, and greater number of immature secretory granules. Because no major difference was seen in the percent of β-cells in control and variant islets, it is suggested that these alterations are mainly attributable to functional abnormalities of the insulin-secreting cells. This might be caused by several factors, but the present experimental evidence supports the primary role of the Gly^{972}→Arg amino acid polymorphism in IRS-1. In fact, the insulin signaling involving the IRS-1/phosphatidylinositol (PI) 3-kinase pathway has been shown to play an important role in insulin secretion by regulating the mobilization of intracellular Ca^{2+} stores (28). Moreover, in experimental rodent models, the IRS-1/PI 3-kinase system is altered in β-cells carrying the Arg^{972} IRS-1 polymorphism (22,23).

The integrity of the insulin-signaling pathways is conceived as essential for normal insulin action and in particular for insulin-mediated glucose transport and metabolism. In our study, however, the Gly^{972}→Arg amino acid polymorphism was not associated with any detectable defect in glucose utilization and/or oxidation. It should be recalled that the IRS-1/PI 3-kinase system is more involved in GLUT4 translocation in insulin-target tissues, whereas β-cells express the GLUT2 transporter.

At variance with the impaired insulin response to glucose, islets expressing the Gly^{972}→Arg amino acid polymorphism for IRS-1 exhibited a larger insulin release when challenged with arginine as compared to wild-type β-cells. Arginine can stimulate insulin through various mechanisms, including depolarization of the β-cell membrane because of its transport in a positively charged form (29), by production of nitric oxide (30), and, indirectly, by stimulation of glucagon secretion from α-cells (31). Although drawing conclusions in this regard is beyond the purpose of our study, it may be of interest that we did not find any evident change in the percent of α-cells between control and Arg^{972} IRS-1 islets. In any event, the effect of arginine on insulin secretion from Arg^{972} IRS-1 islets was accompanied by a relative hypersecretion of proinsulin, as suggested by the decreased insulin-to-proinsulin molar ratio, which is a feature of β-cell dysfunction (32).

Human pancreatic β-cells expressing the Gly^{972}→Arg amino acid IRS-1 polymorphism showed a much larger number of immature secretory granules than control islets. In this regard, there is evidence that insulin stimulates pancreatic duodenal homeobox 1 (PDX-1) DNA-binding activity and insulin promoter activity via a pathway involving PI 3-kinase (33). More recently, it has been demonstrated that expression of PDX-1 in liver by recombinant adenovirus–mediated gene transfer results in the induction of expression of prohormone convertase 1/3 (PC 1/3), a protease that converts proinsulin to mature insulin (34). Although we have not directly addressed this issue, it seems possible that in the Arg972 IRS-1 β-cells, the reduced insulin action might decrease PDX-1 activity, which, in turn, might reduce the expression of PC 1/3.

If the glucose and arginine challenges had divergent responses in terms of insulin release in Gly^{972}→Arg islets, only a slight alteration of insulin secretion was detected in response to glibenclamide. This finding may well be in agreement with different and alternative pathways regulating insulin secretion, but it may also provide some clinical information. From these results it could be argued that sulfonylureas can be initially used for the treatment of type 2 diabetic patients carrying the Gly^{972}→Arg IRS-1 polymorphism. Nonetheless, these individuals can be expected to fail soon, as suggested by the higher prevalence of the Gly^{972}→Arg IRS-1 polymorphism in insulin-requiring type 2 diabetic patients as compared with patients who don't require insulin (S.G., M.F., unpublished observations). By and large, these findings point out how, in the near future, it will be of importance to investigate the molecular characterization of the pathogenetic mechanisms responsible for hyperglycemia in order to identify the most appropriate therapeutic approach for a given patient.

Therefore, the presence of the Gly^{972}→Arg IRS-1 polymorphism appears to be strongly associated with a primary defect of β-cell function characterized by decreased glucose-stimulated insulin secretion, lower insulin content, and a reduced number of mature secretory granules.

TABLE 4
Glucose utilization and oxidation in islets isolated from subjects carrying the Gly^{972}→Arg IRS-1 polymorphism and in control islets

	Glucose (3.3 mmol/l)		Glucose (16.7 mmol/l)	
	Utilization	Oxidation	Utilization	Oxidation
Control	37.6 ± 5.9	18.3 ± 3.5	118.2 ± 23.6*	33.6 ± 6.8*
Gly^{972}→Arg IRS-1	33.7 ± 3.6	15.3 ± 2.7	105.4 ± 11.2*	36.3 ± 4.4*

Data represent pmol · islet^{-1} · 120 min^{-1} and are means ± SD. *$P < 0.05$ vs. low glucose.

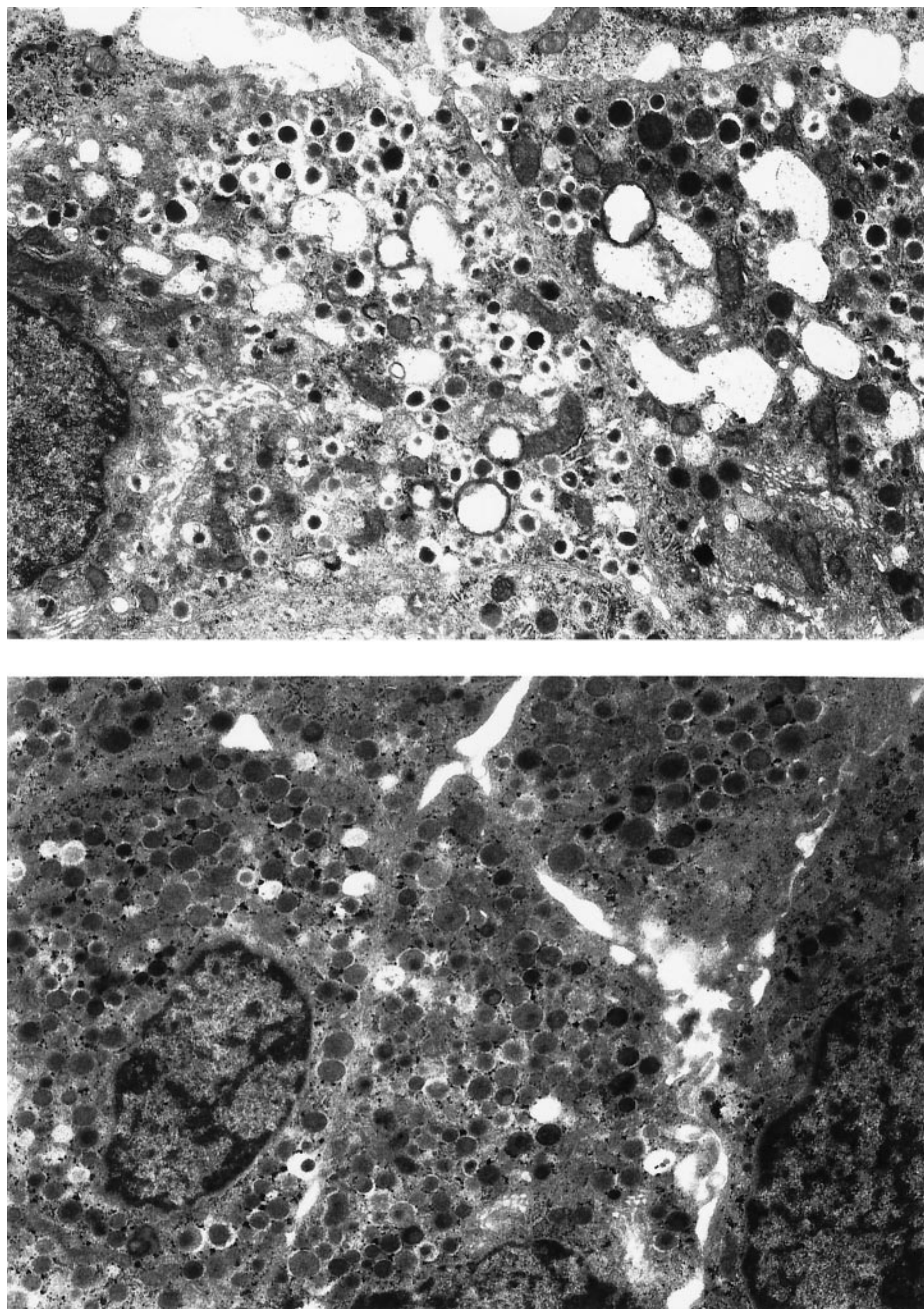

FIG. 1. Electron microscopy of control (upper) and Gly^{972}→Arg IRS-1 (lower) islets, showing a greater number of more immature granules in the latter.

As such, this condition may represent a typical predisposing condition for future development of type 2 diabetes, since it seems unlikely that these β-cells will be able to cope with any severe and/or long-lasting increased demand of insulin secretion as it occurs in the presence of a condition of insulin resistance.

RESEARCH DESIGN AND METHODS

The pancreata were procured through the local organ procurement agency, within the framework of a project aimed at clinical islet allotransplantation into diabetic patients and approved by the ethics committee of the University of Pisa. The presence of the polymorphism was detected by nested RT-PCR, as previously described (23). Procedures for islet preparation have also been reported in detail previously (24–27). Briefly, the enzyme collagenase (collagenase P; Roche Diagnostics) was used for digestion of the gland. The pancreatic duct was cannulated, and the digestion solution (collagenase, 1.5–2 mg/ml, dissolved in 200 ml Hanks' balanced salt solution [HBSS]) was slowly injected to distend the tissue. After distension, the gland was placed into a 500-ml glass beaker, and the solution not used for distension was added into the beaker. This was

TABLE 5
Volume density and number of immature and mature secretory granules in beta-cells from control and Gly^{972}→Arg IRS-1 islets

	Volume density (% ml)		Number (per microscopy field)	
	Immature	Mature	Immature	Mature
Control	0.5 ± 0.2	3.5 ± 0.8	5.5 ± 0.8	62 ± 5
Gly^{972}→Arg IRS-1	3.3 ± 0.7*	0.9 ± 0.2*	49 ± 6.5*	11 ± 1.1*

Data are means ± SD. *$P < 0.02$ vs. control.

loaded into a shaking water bath at 37°C, activated at 120 rpm. After ~10 min, the pancreas was shaken with forceps for 60 s; then the digestate was filtered through 300- and 90-μm mesh stainless steel filters, in sequence. The solution passed through the filter, and the tissue entrapped on the 300-μm mesh filter was placed back into the water bath for further digestion. The tissue remaining in the 90-μm filter was washed with HBSS and 2% human albumin. The same procedure of filtration, washing, and settling in HBSS solution was repeated at 8–10 intervals for up to 40–50 min. For purification, 3 ml of tissue was loaded into 220-ml plastic conicals and resuspended in 60 ml of 80% histopaque 1.077 (Sigma) in HBSS, topped with 40 ml of HBSS. After centrifugation at 800*g* for 5 min at 4°C, the islets were recovered at the interface between the two layers. The islets were then washed with HBSS and 2% human albumin, centrifuged at 800*g* for 2 min at 4°C, resuspended in M199 culture medium, and cultured at 37°C in a CO_2 incubator for 5–6 days to perform the experiments described below.

Islet secretory function was evaluated as previously described (24–27). After a 30-min preincubation period at 37°C in Krebs-Ringer bicarbonate solution with 0.5% albumin (pH 7.4) containing 3.3 mmol/l glucose, the islets were incubated for 45 min in the Krebs-Ringer solution with 1.8, 5.5, or 16.7 mmol/l glucose or 3.3 mmol/l glucose plus 20 mmol/l arginine or 100 μmol/l glibenclamide. At the end of the incubation time, aliquots of the incubation medium were taken for insulin (Medgenix Diagnostics, Fleurs, Belgium; no cross-reactivity with human proinsulin) and proinsulin (Tecnogenetics, Turin, Italy; cross-reactivity with human insulin of $<0.1\%$) immunoassay measurement. Islet insulin extraction was performed at the end of the incubation by overnight acid-alcohol incubation.

Glucose utilization and oxidation were determined at low (3.3 mmol/l) and high (16.7 mmol/l) glucose by measuring the formation of ^3H_2O from [5-^{3}H]glucose and $^{14}CO_2$ from [U- ^{14}C]glucose, respectively, according to methods previously reported in detail (35).

Electron microscopy evaluation and quantification of β- and α-cells, as well as insulin secretory granules, were performed according to standard procedures (36,37).

RNA extraction from human islets and semiquantitative RT-PCR analysis of insulin and insulin housekeeping control genes was performed as previously described (38).

Results are given as means ± SD. Comparison of data were performed by ANOVA or the two-tailed Student's *t* test.

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