The Gly^{972}→Arg amino acid polymorphism in IRS-1 impairs insulin secretion in pancreatic β cells

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Recent studies have identified several polymorphisms in the human insulin receptor substrate-1 (IRS-1) gene. The most prevalent IRS-1 variant, a Gly→Arg change at the codon 972, has been reported to be increased in prevalence among patients with type 2 diabetes. Carriers of the Arg^{972} substitution are characterized by lower fasting insulin and C-peptide levels compared with non-carriers, suggesting that the Arg^{972} IRS-1 variant may contribute to impairment of insulin secretion. In this study, we stably overexpressed both wild-type IRS-1 (RIN-WT) and Arg^{972} IRS-1 variant (RIN-Arg^{972}) in RIN β cells to investigate directly whether the polymorphism in codon 972 of IRS-1 impairs insulin secretion. The Arg^{972} IRS-1 variant did not affect expression or function of endogenous IRS-2. RIN-WT showed a marked increase in both glucose- and insulin-stimulated tyrosine phosphorylation of IRS-1 compared with control RIN cells. The Arg^{972} IRS-1 variant did not alter the extent of either glucose- or insulin-stimulated tyrosine phosphorylation of recombinant IRS-1. However, RIN-Arg^{972} showed a significant decrease in binding of the p85 subunit of phosphatidylinositol-3-kinase (PI 3-kinase) with IRS-1, compared with RIN-WT. Compared with control RIN cells, insulin content was reduced to the same extent in RIN-WT or RIN-Arg^{972} at both the protein and mRNA levels. Both glucose- and sulfonylurea-induced insulin secretion was increased in RIN-WT compared with control RIN cells. By contrast, RIN cells expressing Arg^{972} IRS-1 exhibited a marked decrease in both glucose- and sulfonylurea-stimulated insulin secretion compared with RIN-WT. These data suggest that the insulin signaling pathway involving the IRS-1/PI 3-kinase may play an important role in the insulin secretory process in pancreatic β cells. More importantly, the results suggest that the common Arg^{972} IRS-1 polymorphism may impair glucose-stimulated insulin secretion, thus contributing to the relative insulin deficiency observed in carriers of this variant.

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Introduction

Type 2 diabetes is a complex disease characterized by a combination of resistance to insulin action and inadequate compensatory insulin secretory response (1). Substantial evidence has accumulated to suggest that genetic factors contribute to the pathogenesis of type 2 diabetes, given the high concordance rate between monozygotic twins (2), the high prevalence in certain ethnic groups (3), and the increased prevalence in offspring of affected subjects (4). Susceptibility to both insulin resistance and insulin deficiency appears to be genetically determined (1). Longitudinal studies suggest that insulin resistance is the earliest detectable defect in the pathogenesis of type 2 diabetes (3, 5). Initially, increased insulin secretion by pancreatic β cells can compensate for insulin resistance, but hyperglycemia eventually develops as β-cell compensation fails. Mutations in the insulin receptor gene have been identified in rare syndromes of severe insulin resistance, such as leprechaunism and Rabson-Mendenhall (6). Similarly, defects in β-cell function caused by mutations in the genes encoding glucokinase (7, 8), transcription factors such as hepatic nuclear factor-1α, -1β, and -4α (9, 10), or insulin-promoting factor-1 (11) have been identified in subjects with maturity onset diabetes of the young (MODY), a rare monogenic form of type 2 diabetes. Despite intense investigations, genes responsible for the development of the common forms of type 2 diabetes remain unknown.

Insulin exerts its action by binding to its cell surface transmembrane receptor, stimulating receptor autophosphorylation and activation of the intrinsic tyrosine kinase activity, which results in tyrosine phosphorylation of several intracellular substrates (12). The

major substrates of the insulin receptor are insulin receptor substrates 1 and 2 (IRS-1 and IRS-2) (13–15). IRS molecules are rapidly tyrosine phosphorylated after insulin stimulation and serve as multisite docking proteins for various effector molecules possessing src homology 2 (SH2) domains, including phosphatidylinositol-3-kinase (PI 3-kinase) (15). IRS-1 was the first characterized substrate. IRS-1 is ubiquitously expressed in tissues that are responsible for glucose production, glucose uptake, and insulin production — such as liver, skeletal muscle, and pancreatic β cells (15–17) — and is thought to play a major role in mediating both metabolic and mitogenic effects of insulin (18, 19). Molecular scanning of the IRS-1 gene in normal individuals and patients with type 2 diabetes has revealed several polymorphisms resulting in amino acid substitutions (20–24), the most common of which is a Gly→Arg substitution at codon 972 (Arg[972]). According to available data, the Arg[972] polymorphism is twice as prevalent in patients with type 2 diabetes as in control subjects. Transfection studies have shown that the Arg[972] polymorphism impairs insulin-stimulated signaling via the PI 3-kinase pathway (25, 26). These data suggest that the Arg[972] IRS-1 variant may contribute to the development of type 2 diabetes. Carriers of the Arg[972] IRS-1 variant are characterized by low fasting plasma concentrations of insulin and C-peptide (20). A young, healthy, lean male homozygous for the Arg[972] variant had low fasting plasma insulin levels and a low acute insulin response (22). In addition, it has been reported that glucose-tolerant offspring of patients with type 2 diabetes who carry the Arg[972] IRS-1 variant are characterized by lower fasting plasma insulin levels and a lower insulin response to an oral glucose load compared with non-carriers (27). It has been shown that glucose-stimulated insulin secretion may be modulated by autocrine activation of the insulin signal-transduction pathway involving insulin receptor phosphorylation, tyrosine phosphorylation of IRS-1, and activation of PI 3-kinase (17, 28). Taken together, these data raise the intriguing hypothesis that a single molecular defect in insulin signaling, involving the defective interaction between IRS-1 and PI 3-kinase, might result in both peripheral insulin resistance and impaired insulin secretion. To address this issue, we overexpressed both wild-type IRS-1 and Arg[972] IRS-1 variant in RIN β cells, and measured glucose-stimulated insulin secretion. Data obtained showed that in β cells expressing the Arg[972] IRS-1 variant, insulin secretion stimulated by glucose is impaired compared with β cells expressing wild-type IRS-1, thus suggesting that this polymorphism may compromise the ability of β cells to compensate for insulin resistance.

Methods

Reagents. Hybond-P PVDF transfer membranes, protein A-Sepharose, and all radiochemicals were from Amersham Pharmacia Biotech (Milan, Italy). Geneticin G-418 sulphate, Lipofectamine transfection reagent, media, and FCS for cell culture were from GIBCO BRL (Grand Island, New York, USA). Glybenclamide was from Guidotti Laboratory (Pisa, Italy). SDS-PAGE and Western blot reagents were from Bio-Rad Laboratories Inc. (Richmond, California, USA). Anti–IRS-1, anti–IRS-2, and anti-rat PI 3-kinase polyclonal antibodies were purchased from Upstate Biotechnology Inc. (Lake Placid, New York, USA). Anti-phosphotyrosine polyclonal antibody was from Transduction Laboratories (Lexington, Kentucky, USA). Enhanced chemiluminescence reagent detection system (SuperSignal CL-HRP Substrate System) was from Pierce Chemical Co. (Rockford, Illinois, USA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA).

Cell cultures. RIN 1046-38 cells were subcultured weekly in M199 medium supplemented with 10% FCS, and were used at passages 16–25, at which they maintain a glucose responsivity in the range of 0.1–2.8 mM (29).

Generation of human IRS-1 cDNAs. A fragment of human genomic DNA containing the entire coding sequence of IRS-1 was cloned and ligated into pcDNA3 expression vector (pcDNA3-WT-IRS-1) as previously described (18). The cDNA construct containing the Arg[972] substitution was generated by site-directed mutagenesis by overlap extension using PCR. Wild-type IRS-1 was used as template for PCR-mediated oligonucleotide-directed mutagenesis. The PCR fragment containing the codon 972 variant of IRS-1 was digested with *Bam*HI and *Nhe*I restriction endonucleases and inserted into pcDNA3-WT-IRS-1 that had been previously digested with the same enzymes. The presence of the substitution and the entire sequence of the fragment inserted was confirmed by nucleotide sequencing.

Transfection of IRS-1 cDNAs. RIN cells were seeded at a density of 2×10^5 cells/mL, treated for 4 hours with transfection medium containing 2 μg pcDNA3 alone, pcDNA3-WT-IRS-1, or mutant IRS-1 (pcDNA3-Arg[972]-IRS-1), and suspended with 15 μL Lipofectamine in 1 mL serum-free M199. One milliliter of M199 supplemented with 10% FCS was then added to the transfection mixture, according to the manufacturer's instructions.(GIBCO BRL). After 24 hours, the medium was replaced with M199 supplemented with 10% FCS, and the cells were incubated for a further 72 hours. Thereafter, the cells were incubated with selective medium containing 0.4 mg/mL geneticin G-418 sulfate. Individual G-418–resistant clones were collected and lysed for 30 minutes in buffer containing 50 mM Tris-HCl (pH 7.8), 150 mM NaCl, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonylfluoride, and 1% Triton X-100. Insoluble material was removed by centrifugation in microfuge for 10 minutes, and the supernatant was stored for Western blot analysis. Protein content of each sample was determined by Bradford assay using BSA as standard.

Tyrosine phosphorylation of IRS-1 and IRS-2, and association of IRS-1 and IRS-2 with the p85 subunit of PI 3-kinase. RIN cells cultured in 100-mm-diameter dishes were

washed 3 times with glucose-free buffer A containing 114 mM NaCl, 25.5 mM NaHCO$_3$, 10 mM HEPES, 2.5 mM CaCl$_2$, 4.7 mM KCl, 1.21 mM KH$_2$PO$_4$, 1.16 mM MgSO$_4$, and 0.1% BSA (pH 7.4). Thereafter, the cells were incubated in the presence or absence of maximally stimulating concentrations of glucose (2.8 mM) or insulin (100 nM) for the indicated period of time. At the end of incubation, cells were washed with ice-cold PBS and lysed for 45 minutes at 4°C in lysis buffer containing 20 mM Tris-HCL (pH 7.6), 137 mM NaCl, 2 mM EDTA, 10 mM NaPP, 2 mM Na$_3$VO$_4$, 10 mM NaF, 8 µg/mL leupeptin, 2 mM phenylmethylsulfonylfluoride, 10% glycerol, and 1.5% NP-40. Insoluble material was removed by centrifugation in microfuge for 5 minutes, and the supernatant was saved for analysis by immunoprecipitation and Western blotting.

Immunoprecipitation and Western blotting. Cell lysates were incubated for 16 hours at 4°C with anti–IRS-1 antibody (4 µg for 1 mg of cell lysate) or anti–IRS-2 antibody (1:1,000). Immunocomplexes were collected by incubation with protein A-Sepharose for 2 hours at 4°C. Immunoprecipitates proteins were washed 3 times with wash buffer containing 150 mM NaCl, 10 mM Tris (pH 7.8), 1.5% NP-40, 1 mM Na$_3$VO$_4$, and 1 mM phenylmethylsulfonylfluoride; they were then resuspended in Laemmli buffer. Equal amounts of cell lysates or immunoprecipitated proteins were subjected to SDS-PAGE under reducing conditions. Proteins resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose membrane. The filters were then incubated for 16 hours at 4°C with the indicated primary antibodies, followed by incubation with peroxidase-conjugated goat anti-rabbit antibodies. Proteins were detected by using enhanced chemiluminescence, and band densities were quantified by densitometry.

Insulin secretion. RIN cells were seeded in 24-multiwell plates at a density of 10^5 cells/mL. After 48 hours, the medium was removed, and the cells were washed twice for 30 minutes at 37°C with glucose-free buffer A. The cells were incubated in the presence or absence of increasing concentrations of glucose for different periods of time. To study sulfonylurea-induced insulin secretion, RIN cells were incubated for 30 minutes at 37°C in glucose-free buffer A in the presence or absence of different concentrations of Glybenclamide, a second-generation sulfonylurea. Aliquots of the supernatant were collected and stored at –20°C for subsequent insulin RIA. Cells were extracted overnight at 4°C with a solution of acidified ethanol for measurement of the intracellular insulin content. Insulin amount was determined by RIA.

Northern blot analysis of insulin mRNA expression. Total RNA was extracted from RIN cells using the acid-guanidinium-thiocyanate-phenol-chloroform procedure (30). Twenty micrograms of total RNA was heated at 65°C and applied to a 1% agarose gel containing 5% formaldehyde. RNAs were then blotted onto nylon membrane using a PosiBlot apparatus (Stratagene, La Jolla, California, USA) and cross-linked. The blots were hybridized with a 450-kb mouse insulin cDNA (kindly provided by A.L. Notkins, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland, USA) labeled with [α-^{32}P]dCTP by random priming procedure using 2 U of Klenow polymerase. Band densities were quantified by densitometry.

Statistics. Statistical analysis was carried out by 1-way ANOVA followed by nonpaired Student's t test. Data in figures are expressed as mean ± SE. P values less than 0.05 were considered significant.

Results

Expression of recombinant IRS-1 in RIN cells. RIN cells were stably transfected with expression vectors for the wild-type human IRS-1 (RIN-WT) and the Arg^{972} IRS-1 variant (RIN-Arg^{972}). Expression of wild-type IRS-1 and Arg^{972} IRS-1 was 2.5- to 3-fold higher compared with that of endogenous IRS-1, as determined by immunoblotting (Figure 1). Similar amounts of recombinant IRS-1 were observed in cells expressing the wild-type IRS-1 or Arg^{972} IRS-1 (Figure 1), thus confirming that the Gly^{972}→Arg substitution does not affect the level of expression of IRS-1 (25, 26). Expression of IRS-2 did not change in RIN cells expressing either wild-type IRS-1 or Arg^{972} IRS-1, compared with control cells (Figure 1). Two independent clones of RIN cells expressing comparable amounts of either wild-type or mutant IRS-1 were used for further experiments. No significant differences in the DNA content or total protein content per cell were observed in cells expressing either wild-type IRS-1 or Arg^{972} IRS-1, compared with control cells (data not shown).

Figure 1

Quantitation of IRS-1 and IRS-2 expressed in RIN β cells. RIN cells were stably transfected with expression vectors for wild-type IRS-1 or Arg^{972} IRS-1. The cells were lysed, and equal amount of proteins were immunoprecipitated (IP) with anti–IRS-1 or anti–IRS-2 antibody, separated by SDS-PAGE, transferred to PVDF membrane, and Western immunoblotted (WB) with either anti–IRS-1 (top) or anti–IRS-2 (bottom) antibody. Proteins were detected by using enhanced chemiluminescence, and band densities were quantified by densitometry. The autoradiographs shown are representative of 4 independent experiments.
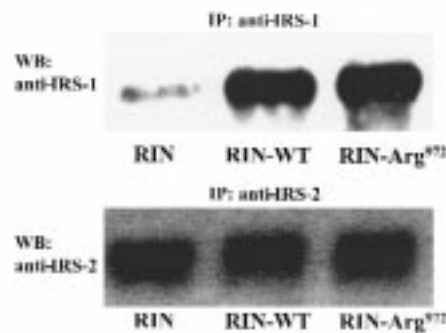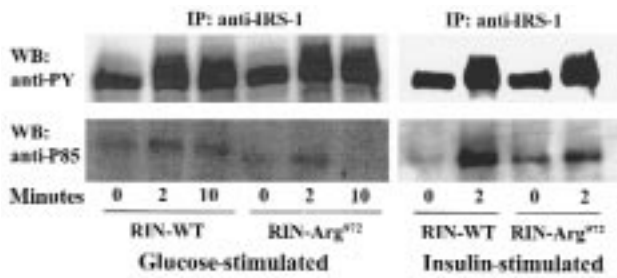
Figure 2
Glucose- and insulin-stimulated tyrosine phosphorylation of IRS-1, and association with p85 subunit of PI 3-kinase in RIN β cells expressing wild-type IRS-1 and Arg[972] IRS-1. RIN-WT and RIN-Arg[972] cells were incubated in the presence or absence of 2.8 mM glucose for 0, 2, and 10 minutes, or in the presence or absence of 100 nM insulin for 2 minutes, at 37°C. The cells were then lysed, and equal amounts of total proteins were immunoprecipitated (IP) with anti–IRS-1 antibody, subjected to SDS-PAGE, and Western immunoblotted (WB) with either anti-phosphotyrosine antibody (top) or anti-p85 antibody (bottom). Proteins were detected by using enhanced chemiluminescence, and band densities were quantified by densitometry. The autoradiographs shown are representative of 3 independent experiments.

Tyrosine phosphorylation of IRS-1 and IRS-2, and association with p85 subunit of PI 3-kinase. To determine whether the Arg[972] IRS-1 variant alters phosphorylation of IRS-1, lysates from glucose- or insulin-treated RIN cells were immunoprecipitated with anti–IRS-1 antibody and immunoblotted with anti-phosphotyrosine. Expression of the Arg[972] IRS-1 variant did not alter the extent of glucose-stimulated tyrosine phosphorylation of recombinant IRS-1 (Figure 2). The extent of tyrosine phosphorylation of IRS-1 was also similar in both RIN-WT and RIN-Arg[972] after insulin stimulation (Figure 2). To determine whether the Arg[972] IRS-1 variant had any influence on glucose- and insulin-stimulated association of p85 subunit of PI 3-kinase with IRS-1, lysates from glucose- or insulin-treated RIN cells were immunoprecipitated with anti–IRS-1 antibody and immunoblotted with anti-p85 antibody. In RIN-WT, binding of p85 subunit to IRS-1 was increased 3.2-fold after glucose stimulation. In RIN-Arg[972], binding of p85 subunit to IRS-1 was reduced by 57% compared with RIN-WT ($P < 0.001$) (Figure 2). After insulin stimulation in RIN-WT, binding of p85 subunit to IRS-1 was increased 4-fold. In RIN-Arg[972], binding of p85 subunit to IRS-1 was reduced by 59% compared with RIN-WT ($P < 0.003$) (Figure 2).

To determine whether overexpression of the Arg[972] IRS-1 variant alters phosphorylation of endogenous IRS-2, lysates from glucose- or insulin-treated RIN cells were immunoprecipitated with anti–IRS-2 antibody and immunoblotted with anti-phosphotyrosine. Expression of the Arg[972] IRS-1 variant did not alter the extent of glucose-stimulated tyrosine phosphorylation of endogenous IRS-2 (Figure 3). The extent of tyrosine phosphorylation of endogenous IRS-2 was also similar in both RIN-WT and RIN-Arg[972] after insulin stimulation (Figure 3). To determine whether overexpression of the Arg[972] IRS-1 variant had any influence on glucose- and insulin-stimulated association of p85 subunit of PI 3-kinase with endogenous IRS-2, lysates from glucose- or insulin-treated RIN cells were immunoprecipitated with anti–IRS-2 antibody and immunoblotted with anti-p85 antibody. The extent of binding of p85 subunit to endogenous IRS-2 was similar in both RIN-WT and RIN-Arg[972] after either glucose or insulin stimulation (Figure 3). Thus, expression of the Arg[972] IRS-1 variant did not alter function of endogenous IRS-2.

Intracellular insulin content and insulin mRNA level. In the basal glucose-free state, total intracellular insulin content measured by RIA was similar in RIN cells expressing both wild-type IRS-1 and Arg[972] IRS-1 variant. However, insulin content was significantly decreased in both RIN-WT and RIN-Arg[972] compared with control RIN cells (4.84 ± 0.46, 4.91 ± 0.40, and 6.58 ± 0.50 ng/10[5] cells for RIN-WT, RIN-Arg[972], and RIN control cells, respectively; $P < 0.05$). To determine whether the decrease in insulin content in RIN cells expressing both wild-type IRS-1 and Arg[972] IRS-1 variant was due to changes in their insulin mRNA levels, total RNA was extracted from each cell line, and insulin mRNA expression was analyzed by Northern blot. A 450-kb mouse insulin cDNA labeled with [α-^{32}P]dCTP was used as probe, and a GAPDH cDNA probe was used as control to ensure equal RNA loading. As shown in Figure 4, overexpression of both wild-type IRS-1 and Arg[972] IRS-1 in RIN cells results in a similar decrease in insulin mRNA levels compared with control RIN cells.

Glucose-stimulated insulin secretion. Time course of glucose-stimulated insulin secretion revealed that maximal increase was reached after 30 minutes of incuba-
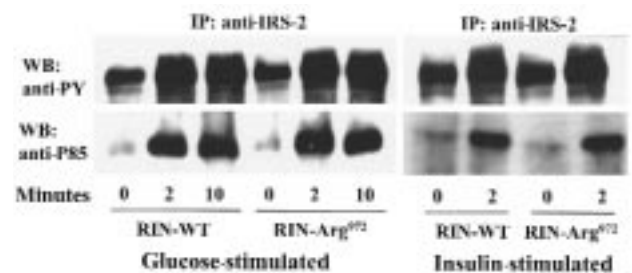

Figure 3
Glucose- and insulin-stimulated tyrosine phosphorylation of endogenous IRS-2, and association with p85 subunit of PI 3-kinase in RIN β cells expressing wild-type IRS-1 and Arg[972] IRS-1. RIN-WT and RIN-Arg[972] cells were incubated in the presence or absence of 2.8 mM glucose for 0, 2, and 10 minutes, or in the presence or absence of 100 nM insulin for 2 minutes, at 37°C. The cells were then lysed, and equal amounts of total proteins were immunoprecipitated (IP) with anti–IRS-2 antibody, subjected to SDS-PAGE, and Western immunoblotted (WB) with either anti-phosphotyrosine antibody (top) or anti-p85 antibody (bottom). Proteins were detected by using enhanced chemiluminescence, and band densities were quantified by densitometry. The autoradiographs shown are representative of 3 independent experiments.
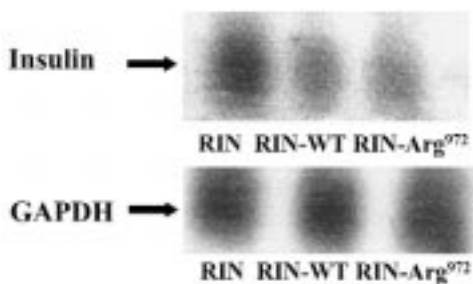
Figure 4
Insulin mRNA analysis. Total RNA was extracted from control RIN, RIN-WT, and RIN-Arg[972] and blotted onto nylon membranes. The blots were hybridized with a 450-kb mouse insulin cDNA labeled with [α-^{32}P]dCTP and were analyzed by an image-analyzer. The autoradiograph shown is representative of 3 independent experiments.

tion in all cell lines (Figure 5). Under basal glucose-free conditions, insulin release was similar in the 3 cell lines (25.29 ± 2.43, 27.89 ± 3.02, and 32.52 ± 4.02 pg/10[5] cells for RIN-WT, RIN-Arg[972], and control RIN cells, respectively). However, maximal glucose-stimulated insulin secretion was decreased by 27% in RIN-Arg[972] compared with RIN-WT (*P* < 0.05). Basal fractional insulin secretion, expressed as the amount of secreted insulin divided by the total insulin content, did not differ between control RIN cells, RIN-WT, and RIN-Arg[972] (5.21 ± 0.61, 5.27 ± 0.5 and 5.11 ± 0.8%, respectively). Expression of wild-type IRS-1 in RIN cells resulted in a significant increase in fractional insulin secretion compared with control RIN cells, at all concentrations of glucose tested (Figure 6). By contrast, expression of the Arg[972] IRS-1 variant in RIN cells caused a significant reduction in glucose-dependent insulin secretion compared with RIN cells expressing wild-type IRS-1 (Figure 6a). At 0.1 mM, glucose induced a 75 ± 12 and 33 ± 2% increase in insulin release above the basal values (*P* < 0.005), and at 2.8 mM, glucose induced a 120 ± 14 and 50 ± 5% increase (*P* < 0.01), in RIN cells expressing wild-type IRS-1 and Arg[972] IRS-1, respectively (Figure 6b).

Sulfonylurea-induced insulin secretion. To determine whether the Arg[972] IRS-1 variant impairs the response to other secretagogues, we evaluated the effects of Glybenclamide, a second-generation sulfonylurea. Sulfonylureas stimulate insulin release from pancreatic β cells through the interaction with a specific plasma membrane receptor coupled to the ATP-dependent K^{+} channel, leading to its closure. As a consequence of the inhibition of K^{+} efflux, β cells depolarize, inducing Ca^{2+} entry, which in turn stimulates extrusion of insulin granules. Expression of wild-type IRS-1 in RIN cells resulted in a significant increase in Glybenclamide-induced insulin secretion compared with control RIN cells, at any concentrations of drug tested (Figure 7). By contrast, expression of the Arg[972] IRS-1 variant in RIN cells caused a significant reduction in Glybenclamide-dependent insulin secretion compared with RIN cells expressing wild-type IRS-1 (Figure 7).

Discussion

As a consequence of its extreme complexity, the regulation of insulin secretion from pancreatic β cells is still not completely clarified. Glucose is the principal stimulus for insulin secretion. Through its metabolism, glucose determines the closure of the ATP-dependent K^{+} channels, resulting in plasma membrane depolarization and influx of extracellular Ca^{2+} into cytosol, which, in turn, triggers exocytosis of insulin granules. It has been shown that glucose-induced insulin secretion activates, via an autocrine loop, the β-cell surface insulin receptor tyrosine kinase and its downstream signal-transduction pathway involving tyrosine phosphorylation of IRS-1 and activation of PI 3-kinase (17, 28). IRS-1 is a major intracellular substrate of the insulin receptor in most insulin-sensitive tissues, and has been considered a plausible candidate gene for type 2 diabetes. The IRS-1 gene is highly polymorphic, with coding sequence variations in about 5% of normal subjects and in 10–20% of patients with type 2 diabetes. The most prevalent polymorphism is a Gly→Arg substitution at codon 972 (Arg[972]) of the IRS-1 gene, and its overall frequency is increased in type 2 diabetic patients compared with normal individuals (10.7 vs. 5.8%, respectively; *P* < 0.02). Normal and diabetic subjects heterozygous for the Arg[972] variant exhibited both low fasting plasma insulin and C-peptide levels (20). In addition, nondiabetic first-degree relatives of patients with type 2 diabetes who are heterozygous for the Arg[972] variant exhibited both a lower fasting plasma insulin level and a lower insulin response to an oral glucose load when compared with non-carriers (27). These observations have led us to hypothesize that the Arg[972] IRS-1 variant may affect pancre-
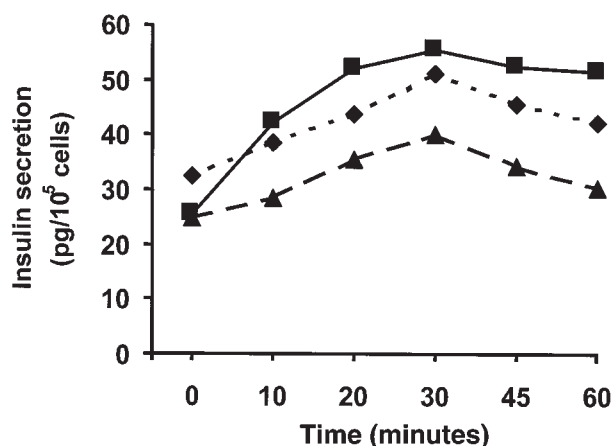

Figure 5
Time course of glucose-stimulated insulin secretion. Control RIN (diamonds), RIN-WT (squares), and RIN-Arg[972] (triangles) cells were incubated in the presence or absence of 2.8 mM glucose for the indicated periods of time. Aliquots of the supernatant were collected, and insulin concentration was determined by RIA. Results shown are representative of 3 independent experiments.
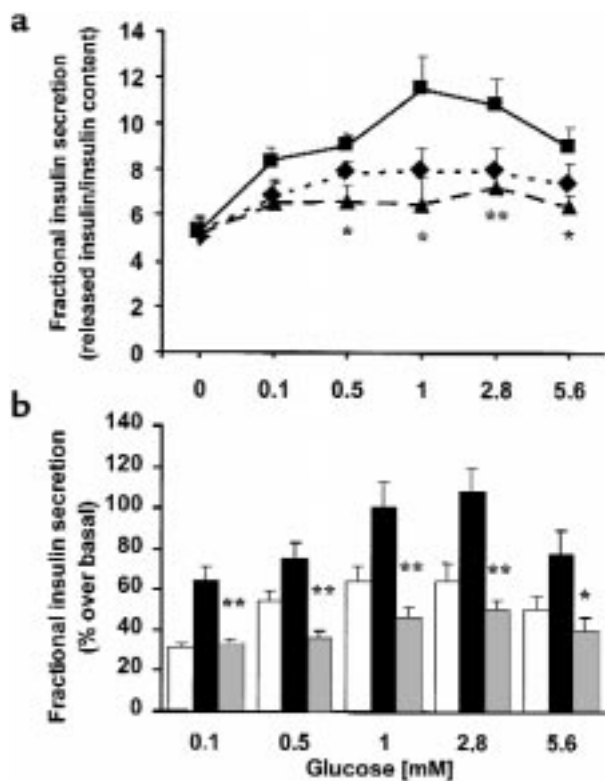
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atic β-cell insulin secretion, thus contributing to the development of type 2 diabetes. In this study, we stably transfected RIN 1046-38 β cells with either wild-type IRS-1 or Arg[972] IRS-1 variant to investigate directly whether this amino acid substitution impairs β-cell function. As noted above, it has been shown that glucose induces tyrosine phosphorylation of the insulin receptor and IRS-1, via an autocrine loop of the exocytosed insulin. We therefore determined both glucose- and insulin-induced tyrosine phosphorylation of the insulin receptor and IRS-1. Both glucose and insulin stimulated tyrosine phosphorylation of the insulin receptor and IRS-1 in control RIN cells, consistent with previous studies performed in a different insulinoma cell line (17) and in rat pancreatic islets (28). RIN cells overexpressing wild-type IRS-1 showed a marked increase in both glucose- and insulin-stimulated tyrosine phosphorylation of IRS-1. However, expression of Arg[972] IRS-1 variant in RIN cells did not alter the extent of either glucose- or insulin-stimulated tyrosine phosphorylation of IRS-1. These results indicate that the Arg[972] IRS-1 variant does not impact the interaction of the insulin receptor with IRS-1, which results in tyrosine phosphorylation of IRS-1. Furthermore, expression of either wild-type IRS-1 or Arg[972] IRS-1 did not affect expression of, glucose- and insulin-stimulated tyrosine phosphorylation of, or PI 3-kinase binding of endogenous IRS-2, thus ruling out the possibility that changes in endogenous IRS-2 content or function would complicate interpretation of the present data. There is evidence indicating that insulin might

modulate its own synthesis and/or secretion in pancreatic β cells (31). We therefore determined insulin mRNA expression and intracellular insulin content in parental and transfected cells. Insulin content was similar in RIN cells overexpressing either wild-type IRS-1 or Arg[972] IRS-1 at both the mRNA and protein levels, thus suggesting that the polymorphism in codon 972 of IRS-1 does not affect the synthesis of insulin. However, insulin content and insulin mRNA levels were reduced in RIN cells expressing wild-type IRS-1 or Arg[972] IRS-1, compared with control RIN cells. These findings suggest that the insulin signaling pathway involving IRS-1 may play an important role in regulating insulin synthesis by decreasing insulin gene transcription and/or increasing insulin mRNA degradation, and that the Arg[972] IRS-1 variant does not alter this regulatory mechanism. By contrast, overexpression of wild-type IRS-1 in RIN cells increased glucose-induced insulin secretion compared with control RIN cells. RIN cells expressing Arg[972] IRS-1 exhibited a marked decrease in glucose-
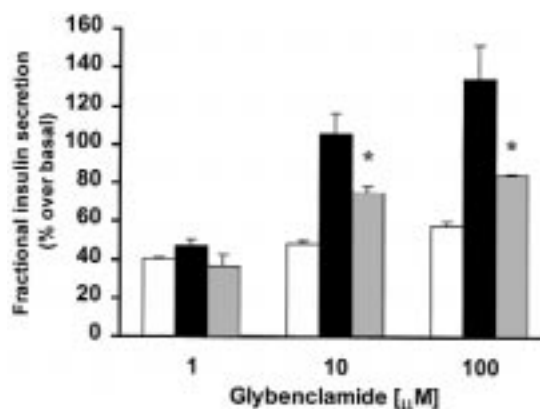
stimulated insulin secretion compared with RIN cells expressing wild-type IRS-1. These data suggest that the mutant Arg[972] IRS-1 may differentially impair insulin synthesis and glucose-stimulated insulin secretion. After ligand stimulation, IRS-1 is tyrosine phosphorylated, and binds and activates a variety of signaling proteins containing the SH domain, including the PI 3-kinase. PI 3-kinase activation has been implicated in histamine and leukotriene secretion by cultured basophilic leukemic cells (32) and in the regulation of vesicular trafficking via its association with dynamin (33). Furthermore, different experimental approaches have suggested that PI 3-kinase may be involved in insulin-stimulated translocation of glucose transporters from an intracellular pool to the plasma membrane (34, 35). We therefore determine the insulin-stimulated association of both wild-type IRS-1 and Arg[972] IRS-1 with PI 3-kinase. RIN cells expressing the mutant Arg[972] showed a significant decrease (59%) in binding of the p85 subunit of PI 3-kinase with IRS-1, compared with RIN cells expressing wild-type IRS-1. Taken together, these data suggest that the insulin signaling pathway involving the IRS-1/PI 3-kinase might participate in the insulin secretory process in pancreatic β cells.

In an attempt to elucidate whether the Arg[972] IRS-1 variant impairs insulin secretion induced by secretagogues other than glucose, we examined the effect of Glybenclamide, a second-generation sulfonylurea. RIN cells expressing wild-type IRS-1 showed a marked increase in sulfonylurea-stimulated insulin secretion compared with control RIN cells. By contrast, RIN cells expressing Arg[972] IRS-1 exhibited a significant decrease in sulfonylurea-stimulated insulin secretion compared with RIN cells expressing wild-type IRS-1, similar to data obtained using glucose. These data raise the intriguing hypothesis that subjects carrying the Arg[972] IRS-1 variant may have an altered response to these drugs. Further in vivo studies are necessary to elucidate this issue.

To our knowledge, this is the first study to demonstrate the functional impact of a naturally occurring IRS-1 polymorphism on insulin secretion by pancreatic β cell. The Arg[972] IRS-1 variant is more prevalent in subjects with type 2 diabetes who have insulin resistance associated or not with dyslipidemia (24). Equilibrium glucose infusion rates during a euglycemic clamp in normal and type 2 diabetic subjects with the Arg[972] polymorphism are decreased compared with those in comparable groups without polymorphism (23). In obese nondiabetic subjects, the Arg[972] polymorphism of IRS-1 in its heterozygous form is associated with a 50% reduction in insulin sensitivity compared with obese subjects without polymorphism (22). We have recently observed that expression of the Arg[972] IRS-1 variant in L6 skeletal muscle cells causes an impairment in glucose transport due to a defect in translocation of glucose transporters GLUT1 and GLUT4 to the plasma membrane (ref. 36; and M.L.

Hribal et al, manuscript submitted for publication). These observations suggest that the Arg[972] polymorphism of IRS-1 might contribute to insulin resistance as well. Taken together with the results of the present investigation, we suggest that amino acid substitution at codon 972 of IRS-1 may represent the first identified defect of a single gene involved in insulin signaling, resulting in both peripheral insulin resistance and impaired insulin secretion, the 2 key components in the pathogenesis of type 2 diabetes.

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