Restoration of insulin secretion in pancreatic islets of protein-deficient rats by reduced expression of insulin receptor substrate (IRS)-1 and IRS-2

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Abstract

Autocrine and paracrine insulin signaling may participate in the fine control of insulin secretion. In the present study, tissue distribution and protein amounts of the insulin receptor and its major substrates, insulin receptor substrate (IRS)-1 and IRS-2, were evaluated in a model of impaired glucose-induced insulin secretion, the protein-deficient rat. Immunoblot and RT-PCR studies showed that the insulin receptor and IRS-2 expression are increased, whilst IRS-1 protein and mRNA contents are decreased in pancreatic islets of protein-deficient rats. Immunohistochemical studies revealed that the insulin receptor and IRS-1 and -2 are present in the great majority of islet cells; however, the greatest staining was localized at the periphery, suggesting a co-localization with non-insulin-secreting cells. Exogenous insulin stimulation of isolated islets promoted higher insulin receptor

and IRS-1 and -2 tyrosine phosphorylation in islets from protein-deficient rats, as compared with controls. Moreover, insulin-induced IRS-1- and IRS-2-associated phosphatidylinositol 3-kinase activity are increased in islets of protein-deficient rats. The reduction of IRS-1 and IRS-2 protein expression in islets isolated from protein-deficient rats by the use of antisense IRS-1 or IRS-2 phosphorothioate-modified oligonucleotides partially restored glucose-induced insulin secretion. Thus, the impairment of insulin cell signaling through members of the IRS family of proteins in isolated rat pancreatic islets improves glucose-induced insulin secretion. The present data reinforced the role of insulin paracrine and autocrine signaling in the control of its own secretion.

Journal of Endocrinology (2004) **181**, 25–38

Introduction

Type 2 diabetes is a multifactorial metabolic disease with rapidly increasing prevalence in most countries of the world (Zimmet *et al.* 2001). Genetic and environmental factors play decisive roles in the pathogenesis of the disease (Bell & Polonsky 2001, Zimmet *et al.* 2001). In several patients with type 2 diabetes, there is an initial peripheral resistance to insulin, which may be compensated for some time by β -cell hyperplasia and/or hyper-function (Bell & Polonsky 2001, Saltiel & Kahn 2001). As long as the pancreas is able to maintain a sufficient production of insulin, diabetes may not develop. However, upon failure of β -cell function, which may occur due to the cell's own incapacity to sustain high hormone production, or to a continuously increasing peripheral need for insulin, diabetes will ensue.

Following the demonstration of the presence of the insulin receptor (IR) and its main substrates, insulin receptor substrate (IRS)-1 and IRS-2 in pancreatic islets (Velloso *et al.* 1995), several reports have provided evidence for the participation of insulin and insulin-like growth factor-I (IGF-I) signaling in islet growth and insulin secretion (Aspinwall *et al.* 1999, Withers *et al.* 1999, Xu *et al.* 1999). IGF-I, acting through IRS-2, may co-ordinate islet growth during late embryogenesis and early postnatal life, and provide a *de novo* stimulus for growth in case of an extra demand for insulin during adult life, for example as a consequence of peripheral insulin resistance (Withers *et al.* 1999). In addition, IRS-2 may be operated by insulin inputs or metabolite-dependent signals, and influence the rate and amount of insulin secretion (Withers *et al.* 1998, 1999, Zhang *et al.* 1998, Lingohr *et al.* 2002). Conversely, IRS-1, acting mostly as a

substrate for the IR, may co-ordinate the islet response to autocrine insulin signaling and its linkage to the glucose-generated signal, which apparently provides a stimulus for enhanced insulin secretion, and depends on cytosolic Ca^{2+} accumulation (Xu *et al.* 1999, Aspinwall *et al.* 2000). Targeted disruption of the IRS-1 gene in the whole body of mice provokes growth retardation accompanied by pancreatic islet hyperplasia and increased insulin secretion (Araki *et al.* 1994, Kulkarni *et al.* 1999b). In contrast, IRS-2 whole-body knockout leads to severe insulin resistance, β -cell hypoplasia with decreased insulin secretion, and early onset diabetes (Kubota *et al.* 2000). It is possible that the same molecular defects in the insulin signaling pathway, which participate in the mechanisms of peripheral insulin resistance, produce functional modulation of pancreatic islets and, thus, play a role in defective compensation for the continuous demand for insulin, as occurs in incipient diabetes.

Protein malnutrition is associated with a defect in the secretion of insulin by pancreatic β -cells and with a variable degree of improved insulin sensitivity in peripheral tissues that are, in general, reciprocally counter-balanced to maintain glucose homeostasis (Reis *et al.* 1997). In humans exposed to low protein diets and in animal models of protein malnutrition, a subsequent prolonged period of hyper-feeding may provoke imbalance in the glucose equilibrium, leading to diabetes or glucose intolerance (Hales & Barker 2001). Since it has been observed that treatment of isolated pancreatic islets of Wistar rats, fed with standard chow, with a phosphorothioate-modified antisense oligonucleotide directed against IRS-1 promoted a significant increase in glucose-induced insulin secretion (Araujo *et al.* 2002), we decided to test if the same approach would lead to an improved functional response of the insulin secretion-defective pancreatic islets of protein-deficient rats. Moreover, we evaluated the participation of IRS-2 in the control of basal and glucose-induced insulin secretion of pancreatic islets of control and protein-deficient rats.

Materials and Methods

Antibodies, chemicals and buffers

Antibodies against IR β (SC-711), IRS-1 (SC-560), IRS-2 (SC-9299) and somatostatin (SC-7819) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against rat insulin (guinea pig raised) were kindly donated by Dr Leclercq-Meyer (Free University, Brussels, Belgium). Antibodies against phosphotyrosine (no. 05-321) were from UBI (Lake Placid, NY, USA). Somatostatin and glucagon RIA kits were from Phoenix Pharmaceuticals, Inc. (Belmont, CA, USA) and Linco Research Inc. (St Charles, MI, USA) respectively; insulin was determined by RIA. ^{125}I -Protein A Sepharose and ^{125}I -insulin were from Amersham International plc (Amersham, Bucks,

Table 1 Composition of control (17% protein) and low protein (6% protein) diets (Reeves *et al.* 1993)

Ingredient	Composition (g/kg)	
	Control	Low protein
Casein	202.0	71.5
Cornstarch	397.5	480.0
Sucrose	100.0	121.0
Fiber	50.0	50.0
Dextrinized cornstarch	130.0	159.0
L-cystein	3.0	1.0
Soybean oil	70.0	70.0
Mineral mix (AIN-93G-MX)	35.0	35.0
Vitamin mix (AIN-93-VX)	10.0	10.0
Choline chlorhydrate	2.5	2.5

UK). Protein A Sepharose 6 MB was from Pharmacia (Uppsala, Sweden). All the remaining chemicals used in the experiments were from Sigma (St Louis, MO, USA). Buffer A used in immunoblotting experiments consisted of 100 mM Tris, 10 g/l SDS, 50 mM HEPES (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride and 0.1 mg/ml aprotinin.

Phosphorothioate-modified oligonucleotides

Sense and antisense phosphorothioate oligonucleotides specific for IRS-1 (sense, 5' ACC CAC TCC TAT CCC G 3' and antisense, 5' CGG GAT AGG AGT GGG T 3') and IRS-2 (sense, 5' GGA GGC GGC TAC AAA G 3' and antisense, 5' CTT TGT AGC CGC CTC C 3') were produced by Invitrogen Corp. (Carlsbad, CA, USA). Both sequences were selected among three unrelated pairs of oligonucleotides on the basis of their ability to block IRS-1 and IRS-2 protein expression respectively, as evaluated by immunoblot of total protein extracts of isolated pancreatic islets utilizing specific anti-IRS-1 or anti-IRS-2 antibodies. The antisense oligonucleotide sequences were submitted to BLAST analyses (at www.ncbi.nlm.nih.gov) and matched only for the *Rattus norvegicus* IRS-1 or IRS-2 coding sequences respectively.

Experimental animals and islet isolation

All experiments were performed with male Wistar rats from the University of Campinas Breeding Center. At 28 days of age, the rats were randomly distributed into two groups, a control group that received standard rodent chow containing 17% protein and a protein-deficient group that received an isocaloric chow containing 6% protein (Table 1) for 12 weeks. For islet isolation, the rats were anesthetized by intraperitoneal injection of sodium amobarbital (15 mg/kg body weight) and, following the loss of corneal

and pedal reflexes, the animals were killed by decapitation. Abdominal cavities were rapidly opened and the pancreatic duct was cannulated for collagenase infusion. The pancreas was then removed and submitted to collagenase digestion. Islets were isolated by careful handpicking, following a method previously described (Bjork *et al.* 1994). The University of Campinas Ethical Committee approved all experiments.

Immunohistochemistry

Pancreata obtained from three rats of each experimental group were examined to determine the expression and tissue distribution of proteins that participate in insulin signaling. Hydrated, 5 µm sections of paraformaldehyde-fixed, paraffin-embedded tissue were stained by the avidin–peroxidase and single-staining fluorescence methods. Sections were incubated for 30 min with 2% normal rabbit or normal goat sera at room temperature, and then exposed for 12 h in a moisture chamber at 4 °C to the primary antibodies against IRβ (1/20), IRS-1 (1/50), IRS-2 (1/50) or somatostatin (1/20). For the avidin–peroxidase method, biotinylated secondary antibodies were used in incubations for 2 h at room temperature, followed by a 1-h incubation with ready-to-use avidin-coupled peroxidase from Vector (Burlingame, CA, USA). The resulting immunocomplexes were detected with 50 mg/100 ml diaminobenzidine–4 M HCl/0.01 ml/100 ml H₂O₂ dissolved in 5 mM Tris, pH 7.6. For single immunofluorescence staining, fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were employed. Analysis and photo-documentation were performed using an Olympus BX60 microscope and a Zeiss LSM 510 laser scanning confocal microscope.

Static secretion studies

One hundred groups of freshly isolated islets were initially incubated for 6 h at 37 °C, in RPMI (Life Technologies, Gaithersburg, MD, USA) containing 2.8 mM glucose with no oligonucleotide or in the presence of either 4 nmol (in 10 ml RPMI) sense or 4 nmol antisense IRS-1 or IRS-2 oligonucleotides. RPMI was then replaced and islets were maintained for 3 h (for insulin measurement) or 90 min (for glucagon and somatostatin measurement) under the experimental conditions (RPMI containing 2.8 mM or 16.7 mM glucose, with either no oligonucleotide addition or in the presence of sense or antisense oligonucleotides). At the end of the incubation time, samples of the supernatants of the incubation medium were collected for hormone (insulin, glucagon and somatostatin) measurement.

Dynamic insulin secretion studies

Groups of 100 freshly isolated islets were preincubated for 6 h at 37 °C in RPMI containing 2.8 mM glucose with no

oligonucleotide or in the presence of either 4 nmol (in 10 ml RPMI) sense or 4 nmol antisense IRS-1 or IRS-2 oligonucleotides. Thereafter, the islets were placed on a Millipore SW 1300 filter (8 µm pore) in a perfusion chamber. Islets were continuously perfused at a flow rate of 1 ml/min. During the initial 20 min of perfusion, the buffer consisted of Krebs–bicarbonate solution containing 2.8 mM glucose (when required, oligonucleotides at the concentration of 0.4 mM were maintained in the perfusate). Finally, perfusion buffer containing 16.7 mM glucose was introduced. Samples of perfusate for quantification of insulin were collected every second minute starting at the tenth minute after the onset of perfusion.

Immunoblot, immunoprecipitation and phosphatidylinositol 3-kinase (PI3-kinase) activity analysis

For specific protein determination, groups of 300 freshly isolated islets were incubated for 6 h in RPMI containing 2.8 mM glucose in the presence of 4 nmol (in 10 ml RPMI) sense or antisense IRS-1 or IRS-2 oligonucleotides or with no addition of oligonucleotide. Following incubation, islets were lysed in 0.3 ml boiling buffer A and insoluble material was removed by centrifugation for 20 min at 8000 g. Protein quantification in the supernatants was determined by the Bradford (1976) method. Samples containing 0.2 mg total proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with specific antibodies. For immunoprecipitation followed by immunoblot analysis and for IRS-1- and IRS-2-associated PI3-kinase activity assays, groups of 800 pancreatic islets were exposed to experimental conditions and technical procedures were performed as previously described (Velloso *et al.* 1996, Luciano *et al.* 2002). Visualization of specific protein bands was performed by incubating membranes with ¹²⁵I-protein A followed by exposure to RX films (Kodak). For phosphoinositol phosphate (PIP) evaluation in PI3-kinase assays, thin layer chromatography (TLC) plates were exposed to RX films.

Semi-quantitative analysis of IRS-1, IRS-2 and somatostatin mRNA

Groups of islets were cultured for 12 h at 37 °C in RPMI 1640 medium containing 5.6 mM glucose and 5% fetal calf serum. Sense or antisense IRS-1 phosphorothioate-modified oligonucleotide were added as required following the same doses and protocol as described. To determine the expression level of IRS-1, IRS-2 and somatostatin mRNA, the relative amount of respective products obtained by RT-PCR was compared with external control potato virus X (PVX) or β-actin mRNA. Total cellular RNA was extracted from groups of 500 islets using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed on 1 µg total RNA using

Moloney murine leukemia virus-reverse transcriptase (Superscript II) and random hexamers according to the manufacturer's instructions (Invitrogen). RT-PCR assays were performed using Taq DNA polymerase, recombinant (Invitrogen) containing 10 pmol of each primer in a master mix of 50 µl. PCR primers for rat somatostatin (forward, 5' ACC GGG AAA CAG GAA CTG GC 3'; reverse, 5' TGG GAT TTG GAG GAG AGG GAT C 3') amplify a 277 bp cDNA fragment, and for rat β -actin (forward, 5' ATG AAG ATC CTG ACC GAG CGT G 3'; reverse, 5' CTT GCT GAT CCA CAT CTG CTG G 3') amplify a 510 bp cDNA fragment. PCR amplification conditions were 2 min at 95 °C followed by cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. The number of cycles was 26 for β -actin and 32 for somatostatin. PCR primers for rat IRS-1 (forward, 5' ACC CAC TCC TAT CCC G 3'; reverse, 5' CCC TAC TCC GTT TGT CC 3') amplify a 375 bp cDNA fragment, for rat IRS-2 (forward, 5' AGC TGG TGG TAG TCA TAC CC 3'; reverse, 5' CAG GTT CAT ATA GTC AGA 3') amplify a 390 bp cDNA fragment, and for PVX (forward, 5' CCT GCT CTA CGG ACT TAT C 3'; reverse, 5' GTA GTT GAG GTA GTT GAC CC 3') amplify a 125 bp cDNA fragment. PCR amplification conditions were 2 min at 94 °C followed by cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s. The number of cycles was 37 for PVX, 32 for IRS-1 and 34 for IRS-2. Cycle numbers were defined after titration between 20 and 42 cycles and were within the logarithmic phase of amplification. PCR samples were submitted to electrophoresis on a 2% agarose gel, and DNA was visualized by ethidium bromide staining. The band intensities were determined by digital scanning, followed by quantification using Scion Image analysis software.

Statistical analysis

Specific protein or cDNA bands present on the blots or on the agarose gels respectively, or PIP dots on TLC plates were quantified by densitometry. Mean values $\pm$ S.E.M. obtained from densitometric scans, and values for insulin and glucagon during static secretion studies were compared utilizing the Turkey–Kramer test (ANOVA). Insulin quantification during dynamic secretion studies was compared after log transformation in order to correct for variance heterogeneity or non-normality utilizing the Turkey–Kramer test (ANOVA). A $P < 0.05$ was accepted as statistically significant.

Results

Characteristics of the protein-deficient rat

After 12 weeks of treatment, there were significant differences between the control and protein-deficient rats

in total body weight (274 ± 38 vs 152 ± 22 g respectively, $n=12$, $P < 0.05$), liver glycogen concentration (0.68 ± 0.21 vs 1.38 ± 0.37 mg/100 g respectively, $n=6$, $P < 0.05$), total serum albumin (35.2 ± 0.5 vs 32.1 ± 0.05 g/l respectively, $n=6$, $P < 0.05$) and fasting insulin level (175 ± 31 vs 119 ± 18 pmol/l respectively, $n=6$, $P < 0.05$). No significant difference was detected in daily food ingestion, fasting glucose concentration and plasma free fatty acids.

Immunohistochemical characterization of IR, IRS-1 and IRS-2 expression in pancreatic islets of protein-deficient rats

Using specific polyclonal antisera against IR, IRS-1 and IRS-2, it was demonstrated that all three proteins that participate in insulin signal transduction are expressed in most cells of the pancreatic islets, in control and in protein-deficient rats (Fig. 1). The highest expression of IR, IRS-1 and IRS-2 occurred in cells at the periphery of the islets, according to the pattern of staining obtained in three independent experiments, and based on the evaluation of at least ten islets per histological section. Comparative evaluation of the pattern of staining suggested that pancreatic islets of protein-deficient rats expressed higher protein levels of IR and IRS-2, and lower protein levels of IRS-1 than control rats.

Immunoblot evaluation of IR, IRS-1 and IRS-2 protein expression and RT-PCR evaluation of IRS-1 and IRS-2 mRNA levels in pancreatic islets of protein-deficient rats

As depicted in Fig. 2, pancreatic islets of protein-deficient rats expressed higher quantities of IR ($\sim 150\%$, $P < 0.05$, Fig. 2a) and IRS-2 ($\sim 160\%$, $P < 0.05$, Fig. 2c) and a lower quantity of IRS-1 ($\sim 60\%$, $P < 0.05$, Fig. 2b) than pancreatic islets of control rats. The results for IRS-1 and IRS-2 were further confirmed by evaluation of mRNA levels by RT-PCR, which showed a significantly lower level of IRS-1 ($\sim 50\%$, $P < 0.05$, Fig. 2d) and a significantly higher level of IRS-2 ($\sim 170\%$, $P < 0.05$, Fig. 2e) in pancreatic islets of protein-deficient rats.

Insulin-induced IR, IRS-1 and IRS-2 tyrosine phosphorylation, and IRS-1/IRS-2-associated PI3-kinase activity in pancreatic islets of protein-deficient rats

To evaluate the ability of exogenous insulin to induce molecular activation of IR, IRS-1 and IRS-2, and the IRS-1/IRS-2-associated PI3-kinase activity, groups of 800 islets were incubated in the presence of 2.8 mM glucose and challenged, or not, for 2 min with exogenous insulin (10^{-7} M). As shown in Fig. 3a, b and c respectively, insulin induced significantly higher tyrosine phosphorylation levels of IR ($\sim 140\%$, $P < 0.05$), IRS-1 ($\sim 140\%$, $P < 0.05$) and IRS-2 ($\sim 140\%$, $P < 0.05$) in

Figure 1 Immunohistochemical characterization of IR (a–d), IRS-1 (e–h) and IRS-2 (i–l) in pancreatic islets of Wistar rats fed regular chow (a, c, e, g, i and k) and protein-deficient Wistar rats (b, d, f, h, j and l). Sections (5 µm) of paraformaldehyde-fixed, paraffin-embedded pancreata of rats were stained using IR (c–d), IRS-1 (g–h) or IRS-2 (k–l) primary antibodies and FITC-coupled secondary antibody (c–d), or biotinylated secondary antibody followed by treatment with avidin coupled to peroxidase (g–h and k–l). Signal detection and image acquisition were performed in fluorescence (a–d) or optical (e–l) microscopes. Methodological controls were exposed to species-specific pre-immune sera (a–b, e–f and i–j). Photographs are representative of three distinct experiments.

pancreatic islets of protein-deficient rats as compared with control. In subsequent experiments, 2 mg protein from samples prepared as above were used in immuno-precipitation protocols with anti-IRS-1 or anti-IRS-2 antibodies. Immunocomplexes were recovered with protein A Sepharose and evaluated for associated PI3-

kinase activity. As shown in Fig. 3d and e respectively, higher insulin-induced IRS-1-associated PI3-kinase activity ($\sim 140\%$, $P < 0.05$) and higher IRS-2-associated PI3-kinase activity ($\sim 140\%$, $P < 0.05$) were encountered in pancreatic islets of protein-deficient rats, as compared with control.

Figure 2 IR, IRS-1 and IRS-2 expression in pancreatic islets of Wistar rats fed regular (Ctr) or protein-deficient (LP) chow. Protein extracts (0.2 mg total protein) obtained from control and protein-deficient rats were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-IR (a), -IRS-1 (b) or -IRS-2 antibodies (c). IRS-1 and IRS-2 mRNAs expression were determined by RT-PCR (d and e respectively) in pancreatic islets of control and protein-deficient rats. mRNA quantifications were performed in parallel with internal control PVX. In all experiments, $n=6$ and $*P<0.05$ vs Ctr. Values are means $\pm$ S.E.M.

Figure 3 Insulin-induced tyrosine phosphorylation of IR (a), IRS-1 (b) and IRS-2 (c), and IRS-1- (d) or IRS-2 (e)-associated PI3-kinase activity in pancreatic islets of control (Ctr) and protein-deficient Wistar rats (LP). Samples containing 0.5 mg (a–c) or 2.0 mg (d–e) total protein obtained from pancreatic islets of control or protein-deficient rats and treated (+) or not (–) with 10^{-7} M insulin were submitted to immunoprecipitation (IP) with anti-IR (a), anti-IRS-1 (b and d) or IRS-2 (c and e) antibodies. Immunoprecipitated proteins were separated by SDS-PAGE (a–c), transferred to nitrocellulose membranes and blotted (IB) with anti-phosphotyrosine (pY) antibodies, or utilized for determination of PI3-kinase activity (d–e), according to the method described in Materials and Methods, and resolved by TLC. In all experiments $n=6$ and $*P<0.05$ vs Ctr+. Values are means $\pm$ S.E.M.

Effect of the reduction of pancreatic islet IRS-1 and IRS-2 expression upon insulin secretion

To determine the role of IRS-1 and IRS-2 signaling upon insulin secretion, pancreatic islets were incubated in the presence of phosphorothioate-modified IRS-1 or IRS-2 antisense oligonucleotide (and respective controls, sense and with no addition of oligonucleotide) and evaluated for basal and glucose-stimulated insulin secretion in static and dynamic secretion protocols. Initially the effect of IRS-1 antisense oligonucleotide treatment upon IRS-1 protein expression was evaluated by immunoblot. As shown in Fig. 4a, oligonucleotide antisense treatment led to a significant reduction in IRS-1 expression in pancreatic islets of control ($\sim 35\%$, $P < 0.05$) and protein-deficient rats ($\sim 45\%$, $P < 0.05$). No effect was detected with sense oligonucleotide treatment. During static insulin secretion studies (Fig. 4b and c), the reduction of IRS-1 expression provoked an increase in basal ($\sim 170\%$, $P < 0.05$) and glucose-stimulated ($\sim 180\%$, $P < 0.05$) insulin secretion in islets from protein-deficient rats. The same treatment led to an increase in glucose-induced ($\sim 210\%$, $P < 0.05$), but not basal, insulin secretion from pancreatic islets of control rats. In addition, a significant increase in the insulin-secretion rate was observed during the high glucose perfusion phase, both in islets of control ($P < 0.05$) and protein-deficient ($P < 0.05$) rats (Fig. 4d and e). Interestingly, the greatest difference in insulin secretion rates between islets treated with IRS-1 antisense oligonucleotide and islets incubated with no oligonucleotide occurred in the final samples of the perfusate, suggesting that reduction of IRS-1 expression modulates mostly second phase insulin secretion.

With respect to IRS-2, significant reductions in control ($\sim 40\%$, $P < 0.05$) and protein-deficient ($\sim 40\%$, $P < 0.05$) IRS-2 expression were obtained in pancreatic islets treated with IRS-2 antisense oligonucleotide (Fig. 5a). This approach did not produce any significant modulation of basal and glucose-stimulated insulin secretion in islets of control rats (Fig. 5b, c and d). However, the reduction of IRS-2 expression led to a significant increase in glucose-induced insulin secretion from islets from protein-deficient rats, as evaluated in static (Fig. 5c) and dynamic (Fig. 5e) secretion studies.

Effects of reduction of IRS-1 protein expression upon glucagon secretion and somatostatin expression in pancreatic islets of protein-deficient rats

In a previous study, we observed that the reduction of IRS-1 expression in pancreatic islets isolated from Wistar rats fed with regular chow induced modulation of glucagon secretion and somatostatin expression (Araujo *et al.* 2002). Since the effect of reduction of IRS-1 protein expression upon insulin secretion was more pronounced than the effect of reduction of IRS-2 protein expression

we evaluated the role of IRS-1 in the secretion of glucagon and expression of somatostatin in pancreatic islets of protein-deficient rats. For this, groups of 100 islets were incubated in the presence of antisense IRS-1 oligonucleotide (or respective controls, sense and with no addition of oligonucleotide) and exposed to low (2.8 mM) or high (16.7 mM) glucose concentration. Similarly to the control, the reduction of IRS-1 in islets of protein-deficient rats led to a reduction in glucagon secretion from islets exposed to low glucose. However, in contrast to islets not exposed to antisense oligonucleotide, the reduction of IRS-1 expression induced a reversal of the pattern of glucagon secretion in such a way that instead of reducing its secretion in high glucose concentrations, as compared with the low glucose concentrations, an increased glucagon secretion occurred in islets exposed to high glucose (Fig. 6a). Since the amounts of somatostatin secreted by isolated pancreatic islets are below the detectability limit of all RIA kits available, islets of control and protein-deficient rats were incubated in the presence of antisense IRS-1 oligonucleotide (and respective controls) and, following incubation, total RNA was obtained, reverse transcribed and cDNA was utilized in a semi-quantitative PCR protocol in parallel with an internal control, β -actin. As shown in Fig. 6b, islets from protein-deficient rats expressed higher levels of somatostatin mRNA than control rats ($\sim 220\%$, $P < 0.05$). This finding was further supported by immunostaining of pancreatic islets of control and protein-deficient rats with anti-somatostatin primary antibody (Fig. 6c–e), which clearly showed, in all islets evaluated (three pancreata from each condition, ten islets evaluated per section), that higher levels of somatostatin are present in islets of protein-deficient rats. Finally, the reduction of IRS-1 expression in pancreatic islets of protein-deficient rats leads to a significant reduction in somatostatin mRNA expression ($\sim 60\%$, $P < 0.05$ vs low protein-treated with no oligonucleotide) (Fig. 6b), similar to the effect of IRS-1 expression impairment in islets of control rats (Araujo *et al.* 2002).

Discussion

There are several reasons for studying insulin secretion in animal models of protein deficiency. Initially, protein deficiency has been implicated as one of the pathogenic factors involved in malnutrition-related diabetes, a rare type of diabetes that may occur in malnourished patients from tropical, developing countries (Abu-Bakare *et al.* 1986, Rao 1988). Secondly, according to the theory of the thrifty phenotype, malnutrition during the intra-uterine period, or during early postnatal life, may predispose to development of type 2 diabetes and obesity during adulthood (Hales 1997, Hales & Barker 2001). In both cases, defective insulin secretion by the pancreatic β -cell may play a central role in the pathogenesis of these diseases.

Figure 4 Effects of pancreatic islet IRS-1 protein expression reduction by IRS-1 phosphothioate antisense oligonucleotide on static and dynamic insulin secretion by isolated pancreatic islets of control (Ctr) and protein-deficient (LP) rats. Total protein (200 μ g) from pancreatic islets of control and protein-deficient rats, treated with IRS-1 phosphothioate antisense (as) or sense (s) oligonucleotide, or not exposed to oligonucleotide (wo), were separated by SDS-PAGE, transferred to nitrocellulose membrane and blotted with anti-IRS-1 antibody (a). Groups of six (b–c) or 100 (d–e) islets isolated from control or protein-deficient rats and treated with IRS-1 phosphothioate antisense, sense oligonucleotide, or not exposed to oligonucleotide, were submitted to a protocol for evaluation of static insulin secretion in low (2.8 mM) (b) or high (16.7 mM) (c) glucose concentration, or for evaluation of dynamic insulin secretion (d, control; e, protein-deficient rats), according to the methods described in Materials and Methods. In all experiments $n=6$ and $*P<0.05$ vs wo within the experimental group. Values are means $\pm$ S.E.M.

Figure 5 Effects of pancreatic islet IRS-2 protein expression reduction by IRS-2 phosphorthioate antisense oligonucleotide on static and dynamic insulin secretion by isolated pancreatic islets of control (Ctr) and protein-deficient (LP) rats. Total protein (200 µg) from pancreatic islets of control and protein-deficient rats, treated with IRS-2 phosphorthioate antisense (as) or sense (s) oligonucleotide, or not exposed to oligonucleotide (wo), were separated by SDS-PAGE, transferred to nitrocellulose membrane and blotted with anti-IRS-2 antibody (a). Groups of six (b–c) or 100 (d–e) islets isolated from control or protein-deficient rats and treated with IRS-2 phosphorthioate antisense, sense oligonucleotide, or not exposed to oligonucleotide, were submitted to a protocol for evaluation of static insulin secretion in low (2.8 mM) (b) or high (16.7 mM) (c) glucose concentration, or for evaluation of dynamic insulin secretion (d, control; e, protein-deficient rats), according to the methods described in Materials and Methods. In all experiments $n=6$ and $*P<0.05$ vs wo within the experimental group. Values are means $\pm$ S.E.M.

Figure 6 Effects of IRS-1 protein expression reduction by IRS-1 phosphorthioate antisense oligonucleotide on glucagon (a) secretion, and somatostatin (b–e) expression in pancreatic islets of control (Ctr) and protein-deficient (LP) rats. Groups of 100 islets isolated from protein-deficient rats and treated with IRS-1 phosphorthioate antisense (as) or sense (s) oligonucleotide, or not exposed to oligonucleotide (wo) were submitted to a protocol for evaluation of static glucagon secretion under low (2.8 mM) or high (16.7 mM) glucose concentration (a). Somatostatin (Somatost) mRNA expression was determined by RT-PCR (b) in pancreatic islets of control and protein-deficient rats treated with IRS-1 phosphorthioate antisense or sense oligonucleotide, or not exposed to oligonucleotide. mRNA quantification was performed in parallel with β -actin internal control. Immunohistochemical characterization of somatostatin expression in pancreatic islets of rats fed regular chow (d) and protein-deficient rats (e). Sections (5 μ m) of paraformaldehyde-fixed, paraffin-embedded pancreata of rats were stained using somatostatin primary antibodies and biotinylated secondary antibody followed by treatment with avidin coupled to peroxidase. Signal detection and image acquisition were performed with an optical microscope. In (a) $n=6$ and $*P<0.05$ vs wo2.8. In (b) $n=6$, $*P<0.05$ vs Ctr and $#P<0.05$ vs LP/wo. A methodological control for immunohistochemical characterization of somatostatin expression was obtained by exposing sections of pancreas from protein-deficient (c) and control (not shown) rats to species-specific pre-immune sera. In (c–e) photographs are representative of three distinct experiments.

Thus, another reason for studying insulin secretion in protein-deficient states is the fact that a full characterization of the mechanisms that lead to defective functioning of pancreatic β -cells may provide clues for understanding the pathways that contribute to impaired insulin secretion in the highly prevalent type 2 diabetes.

Several studies have attempted to characterize the molecular mechanisms underlying the impaired response of the pancreatic islets of protein-deficient animals to glucose and other insulinotrophic agents (Rao & Menon

1993, Reis *et al.* 1997, Latorraca *et al.* 1998, Barbosa *et al.* 2002, Ferreira *et al.* 2003). Caloric and protein deficiency produce phenotypic changes in pancreatic islets, which are smaller and composed of a lower number of β -cells, than islets of eutrophic animals (Petrik *et al.* 2001). In addition, the remaining β -cells are less responsive to glucose, presenting a higher K_m and a lower maximum insulin secretion rate (Reis *et al.* 1997). In a recent study, Pende *et al.* (2000) showed that mice made deficient for S6 kinase 1 (S6K1) are hypoinsulinemic and glucose intolerant.

Their pancreatic islets are considerably smaller than those of wild-type mice and, according to the authors, their islets resemble and function like pancreatic islets of protein-deficient mice. S6K1 is an effector of PI3-kinase and may integrate signals generated by hormones, growth factors and metabolites (Pende *et al.* 2000). This same intracellular signaling pathway is employed by insulin to exert autocrine control of its own transcription, acting through IR subtype A and engaging IRS proteins (Leibiger *et al.* 2001). Experiments with β -cell-specific IR knockout mice (Kulkarni *et al.* 1999a) and with IRS-1 (Araki *et al.* 1994) and IRS-2 (Withers *et al.* 1999) whole-body knockouts suggest that autocrine insulin signaling favors islet mass growth and stimulates insulin synthesis and secretion. However, previous physiological studies (Malaisse & Malaisse-Lagae 1969) and recently published data (Araujo *et al.* 2002, Persaud *et al.* 2002) demonstrate that by acting upon undisrupted and non-genetically manipulated islets, insulin exerts a negative regulatory effect upon its own secretion, which apparently occurs through intra-islet paracrine signaling (Araujo *et al.* 2002). By partially and transiently inhibiting IRS-1 expression in pancreatic islet of Wistar rats, we were able to promote an increase in the amount and rate of insulin secretion (Araujo *et al.* 2002). Since pancreatic islets of protein-deficient rats present a defective insulin secretion, and since abrogation of expression of elements that participate in insulin signaling may produce islets that are phenotypically similar to islets from protein-deficient rodents (Pende *et al.* 2000), we decided to evaluate initial and intermediary steps of the insulin-signaling pathway in undisrupted pancreatic islets from protein-deficient rats.

The present animal model matches all hormonal, biochemical and phenotypic characteristics of those reported in previously published studies (Latorraca *et al.* 1998, Prada *et al.* 2001). The pancreatic islets of protein-deficient rats are smaller and possess fewer insulin granules than islets from eutrophic rats (not shown). Nevertheless, IR, IRS-1 and IRS-2 are expressed in pancreatic islets with the same pattern and histological distribution to those observed in control rats, i.e. more strongly stained in cells of the periphery of the islets, suggesting higher expression in non-insulin-secreting cells. Although not an appropriate method for quantitative analysis, the immunostainings suggested that pancreatic islets from protein-deficient rats express higher levels of IR and IRS-2, and a lower level of IRS-1 than islets of control rats. These findings were further confirmed by immunoblot and RT-PCR.

The treatment of isolated pancreatic islets with exogenous insulin promoted higher IR, IRS-1 and IRS-2 tyrosine phosphorylation and increased IRS-1/IRS-2-associated PI3-kinase activity in pancreatic islets from protein-deficient rats compared with control rats. Thus, there is a direct relationship between the amounts of IR and IRS-2 and their molecular response to insulin. On the other hand, although IRS-1 expression is considerably

lower in islets from protein-deficient rats, it responds with higher insulin-induced molecular signaling than islets of control rats. A similar finding was described in some animal models of insulin resistance and, apparently, the induction of tyrosine phosphorylation of substrates of the IR by insulin reflects more appropriately the activation of this particular pathway than the simple measurement of specific protein expression (Kahn & Saad 1992, Bezerra *et al.* 2000, Bertelli *et al.* 2003). In exercise-trained rats, which present a better response to insulin action, a similar phenomenon was described and, according to the authors, the net functional result in this particular situation is the same, i.e. tyrosine phosphorylation of the IR and its substrates is a more appropriate parameter for evaluating the cellular action of insulin than the amount of proteins engaged by the signaling pathway (Chibalin *et al.* 2000). In conclusion, islets of protein-deficient rats express proteins that participate in insulin signaling with a similar tissue distribution to that observed in islets of eutrophic rats. Upon insulin stimulation, however, islets of protein-deficient rats respond with higher molecular activation of the IR/IRS-1, -2/PI3-kinase signaling pathway than islets of control rats.

The treatment of pancreatic islets of protein-deficient rats with IRS-1 antisense oligonucleotide promoted an ~60% reduction in IRS-1 expression compared with islets of protein-deficient rats not treated with antisense oligonucleotide, and ~85% reduction compared with islets of control rats not treated with antisense oligonucleotide. The treatment of pancreatic islets of control rats with IRS-1 antisense oligonucleotide also caused a considerable reduction in IRS-1 expression. In both cases (protein-deficient and control islets), the partial inhibition of IRS-1 expression led to a significant and sustained increase in glucose-induced insulin secretion. These phenomena were accompanied by a reversal of the pattern of glucose-regulated glucagon secretion and to an increase in somatostatin mRNA expression.

Since insulin is known to induce an increase in its own secretion in isolated β -cells (Kulkarni *et al.* 1999b, Leibiger *et al.* 2001), we believe that the effect of the inhibition of IRS-1 signaling, leading to an improvement, instead of impairment, of insulin secretion, is due to indirect mechanisms. In the present and a previous study (Araujo *et al.* 2002), we have demonstrated that the highest expression of proteins that participate in insulin signaling occurs in non-insulin-secreting cells, most specifically δ -cells, but also in α -cells. Moreover, islets from control and protein-deficient rats present an increased somatostatin expression accompanied by a reversal of the glucagon secretion pattern when treated with IRS-1 antisense oligonucleotide. This pattern of hormone secretion may participate in paracrine signaling and favor insulin secretion.

Finally, the possible reasons why protein deprivation promoted a reduction of IRS-1 accompanied by an increase of IRS-2 expression deserves some considerations.

Since IRS-2 signaling in pancreatic islets seems to be predominantly related to growth promotion (Withers *et al.* 1999), we suspect that its induction may occur as an effort to stimulate pancreatic islet mass growth, which is impaired by the lack of nutrients. On the other hand, the pancreatic islet IRS-1 protein participates mostly in the control of insulin secretion, and its activation in undisrupted islets leads to inhibition of insulin secretion (Araki *et al.* 1994, Kulkarni *et al.* 1999b). Therefore, the reduction of IRS-1 expression in islets of protein-deficient rats may reflect an adaptation for improving insulin secretion in this insulinopenic animal model. The highest effect of IRS-1 protein synthesis reduction by oligonucleotide antisense treatment upon insulin secretion as compared with IRS-2 protein synthesis reduction provides further support for the specialized roles of each of these substrates for insulin (and possibly IGF-I) signaling in pancreatic islets.

In conclusion, a higher molecular response to exogenous insulin is detected in pancreatic islets of protein-deficient rats. The impairment of IRS-1 expression in isolated islets of protein-deficient rats partially restores the insulin secretion defect and modulates glucagon secretion and somatostatin expression. These data provide further support for a more robust role of paracrine, rather than autocrine, insulin signaling in pancreatic islets.

Acknowledgements

This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Pesquisa (PRONEX).

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Received in final form 4 December 2003

Accepted 11 December 2003