Blockade of IRS1 in isolated rat pancreatic islets improves glucose-induced insulin secretion

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Abstract Several neural, hormonal and biochemical inputs actively participate in the balance of insulin secretion induced by blood glucose fluctuations. The exact role of insulin as an autocrine and paracrine participant in the control of its own secretion remains to be determined, mostly due to insufficient knowledge about the molecular phenomena that govern insulin signaling in pancreatic islets. In the present experiments we demonstrate that higher insulin receptor and insulin receptor substrates-1 and -2 (IRS1 and IRS2) concentrations are predominantly encountered in cells of the periphery of rat pancreatic islets, as compared to centrally located cells, and that partial blockade of IRS1 protein expression by antisense oligonucleotide treatment leads to improved insulin secretion induced by glucose overload, which is accompanied by lower steady-state glucagon secretion and blunted glucose-induced glucagon fall. These data reinforce the inhibitory role of insulin upon its own secretion in isolated, undisrupted pancreatic islets.

Key words: Insulin; Insulin receptor; Insulin receptor substrate-1; Glucagon; Somatostatin

1. Introduction

Following the demonstration of the presence of insulin receptor (IR) and its main substrates, insulin receptor substrate-1 and -2 (IRS1 and IRS2, respectively) in pancreatic islets [1–3], a series of studies have attempted to characterize the molecular events that link insulin to the autocrine control of its own secretion. In that respect, the generation of mice carrying targeted disruption of the genes encoding for proteins participating in insulin signaling has been rather elucidating. Spontaneous mutations leading to whole body ablation of the IR leads to severe insulin resistance and neonatal death in both humans and animals [4]. Homozygous targeted disruption of the IR in the whole body promotes hyperinsulinemia and insulin resistance, leading to severe ketosis and death within few days [5]. However, heterozygous IR disruption produces a mild phenotype with only 10% of animals developing diabetes at later stages of life [6]. The disruption of IR specifically in pancreatic β cells using the Cre-lox system under the control of the insulin promoter leads to a progressive loss in glucose-induced first-phase insulin secretion, decreased islet volume and insulin content, and age-dependent glucose intolerance [7]. Genetic manipulation of the main substrates of the IR offered further information on the role of insulin upon the control of β cell function. Thus, IRS1 whole body knockout produces growth retardation accompanied by islet hyperplasia and increased insulin secretion [8,9], while IRS2 whole body knockout leads to severe insulin resistance, β cell hypoplasia with decreased insulin secretion and early-onset diabetes [10].

In isolated β cells IRS1 participates in the control of insulin secretion through a mechanism that involves the lipid metabolizing enzyme phosphatidylinositol 3-kinase (PI3-kinase) and the mobilization of Ca^{+2} [11–14]. On the other hand, IRS2 expression in β cells seems to be involved in the control of cell growth and mitogenesis [15,16].

According to the current concepts a paradigm has been generated proposing that, once secreted by pancreatic β cells, insulin acts upon an autocrine loop activating β cell IRs that lead to IRS1 engagement and further enhancement of insulin secretion through a Ca^{+2}-dependent mechanism [11]. Through the same autocrine loop insulin (probably to a lesser extent than IGF-1) activates IRS2, which participates in the control of β cell growth and mitogenesis, most probably during early phases of development or in β cell tumorigenesis [7,16,17], or yet, in the induction of transcription of genes that encode proteins that participate in the physiologic control of β cell function [18].

Ancient studies by Mallaise and his collaborators [19] and recent data [20] indicate that exposure of isolated pancreatic islets to insulin leads to reduced rather than increased insulin secretion. Thus, it seems that when acting upon an undisrupted and non-genetically manipulated pancreatic islet, insulin provides a signal that, through unknown mechanisms, produces a net effect that is inhibitory on its own secretion. In the present study, evidence is provided which indicates that somatostatin and glucagon, secreted under the control of insulin, participate in the paracrine control of insulin secretion, and that paracrine signaling in pancreatic islets provide a more robust signal than the autocrine signal of insulin.

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2. Materials and methods

2.1. Antibodies, chemicals and buffers

Antibodies against IRβ (SC-711), IRS1 (SC-560), IRS2 (SC-1556), somatostatin (SC-7819), and glucagon (SC-7779) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against rat insulin (guinea pig raised) were a kind donation of Dr Leclerq-Meyer (Free University of Brussels, Belgium). Antibodies against phosphotyrosine (py; #05-321) were from UBI (Lake Placid, NY, USA). Somatostatin and glucagon RIA kits were from Phoenix Pharmaceuticals (Belmont, CA, USA) and Linco Research (St. Charles, MI, USA), respectively; insulin was determined by RIA. ^{125}I-protein A Sepharose and ^{125}I-insulin were from Amersham (Buckinghamshire, UK). Protein A Sepharose 6MB 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 PMSF and 0.1 mg/ml aprotinin. Krebs–bicarbonate buffer equilibrated with 95% O_2:5% CO_2, pH 7.4, was used in islet isolation and contained either 2.8 mM or 16.7 mM glucose.

2.2. Phosphorthioate-modified oligonucleotides

Sense and antisense phosphorthioate oligonucleotide specific for IRS1 (sense, 5′ ACC CAC TCC TAT CCC G 3′ and antisense, 5′ CGG GAT AGG AGT GGG T 3′) were produced by Invitrogen (Carlsbad, CA, USA). This sequence was selected among three unrelated pairs of oligonucleotides on the basis of their ability to block IRS1 protein expression as evaluated by immunoblot of total protein extracts of isolated pancreatic islets utilizing specific anti-IRS1 antibodies.

2.3. Experimental animals and islet isolation

All experiments were performed with male Wistar rats (240–260 g) from the University of Campinas Breeding Center. 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. The abdominal cavity was 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 [21]. The University of Campinas Ethical Committee approved all experiments.

2.4. Immunohistochemistry

Pancreata obtained from six rats 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 or double-staining fluorescence methods. Sections were incubated for 30 min with 2% normal rabbit, normal goat or normal mouse sera at room temperature, and then exposed for 12 h in moister chamber at 4%°C to the primary antibodies against IRβ (1/20), IRS1 (1/50), IRS2 (1/50), glucagon (1/50), somatostatin (1/20), or insulin (1/50). For the avidin-peroxidase method, biotinylated secondary antibodies were used in incubations for 2 h at room temperature, which were followed by 1 h incubation with ready-to-use avidin-coupled peroxidase from Vector. The resulting immunocomplexes were detected with 50 mg/100 ml diaminobenzidine – 4 M HCl/0.01 ml/100 ml H_2O_2 dissolved in 5 mM Tris, pH 7.6. For single or double immunofluorescence staining, FITC and rhodamine-conjugated secondary antibodies were employed and analysis and photo-documentation were performed using an Olympus BX60 Microscope and a Zeiss LSM 510 Laser Scanning Confocal Microscope.

2.5. Static secretion studies

Groups of five freshly isolated islets were initially incubated for 6 h at 37°C in RPMI 2.8 mM glucose with no oligonucleotide, or in the presence of either 4 nM sense or 4 nM antisense IRS1 oligonucleotide. RPMI was then replaced and islets were maintained for 1 h under the experimental conditions (RPMI containing 2.8 mM, 11 mM or 16.7 mM glucose, with either no oligonucleotide addition or in the presence of sense or antisense IRS1 oligonucleotide). At the end of incubation time samples of the supernatants of the incubation medium were collected for hormone measurement.

2.6. Dynamic secretion studies

Groups of 100 freshly isolated islets were pre-incubated during 6 h at 37°C in RPMI 2.8 mM glucose with no oligonucleotide or in the presence of either 4 nM sense or 4 nM antisense IRS1 oligonucleotide. 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 (maintaining the presence or not of respective oligonucleotides in perfusion solution). Finally, a perfusion buffer containing 11 mM or 16.7 mM glucose was introduced and the presence or absence of respective oligonucleotides was maintained. Samples of perfusate for quantification of insulin were collected at every second minute, starting at the tenth minute following the beginning of perfusion.

2.7. Immunoblot, immunoprecipitation and 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 nM sense or antisense IRS1 oligonucleotide or with no addition of oligonucleotide. Following incubation, islets were lysed in 0.3 ml of boiling buffer A and insoluble material was removed by centrifugation during 20 min at $12\,000 \times g$. Protein extracts from skeletal muscle, adipose tissue and liver were obtained as previously described [22]. Protein quantification in the supernatants was determined by the Bradford method [23]. 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 IRS1-associated PI3-kinase activity assays, groups of 800 pancreatic islets were exposed to experimental conditions and technical procedures were performed as previously described [24]. Visualization of specific protein bands was performed by incubating membranes with ^{125}I-protein A followed by exposure to RX-films. For PIP evaluation in PI3-kinase assays TLC plates were exposed to RX-films.

2.8. Semi-quantitative analysis of somatostatin mRNA

Groups of islets were cultured for 12 h at 37°C in RPMI 1640 medium containing 5.6 mM glucose and 5% FCS. Insulin 10^{-6} M (insulin concentration within the pancreatic islet microenvironment of living animals is believed to oscillate between 10^{-9} and 10^{-7} M in fasting and fed conditions, respectively) [11,25,26], sense or antisense IRS1 phosphorthioate-modified oligonucleotide were added as required. To determine the expression level of glucagon, insulin and somatostatin mRNA, the relative amount of respective products obtained by reverse transcription PCR (RT-PCR) was compared to β-actin mRNA. Total cellular RNA was extracted from groups of 500 islets using Trizol reagent (Invitrogen). Reverse transcription was performed on 1 μg total RNA using Moloney murine leukemia virus-reverse transcriptase (Superscript II) and random hexamers according to manufacturer 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 glucagon (forward, 5′ CGC CAG ATC ATT CCC AGC TTC C 3′; reverse, 5′ CGC CCA AGT TCC TCA GCT ATG G 3′) amplify a 345-bp cDNA fragment, 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, for rat insulin (forward, 5′ ATT GTT CCA ACA TGG CCC TGT 3′; reverse, 5′ TTG CAG TAG TTC TCC AGC T 3′) amplify a 340-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 were 26 for β-actin, glucagon and insulin, and 32 for somatostatin. 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 down 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.

2.9. Statistical analysis

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

3. Results and discussion

Since the first observation of IR, IRS1 and IRS2 expression in pancreatic islets no further progression was achieved on the characterization of the cellular distribution of such elements in endocrine pancreas [1–3]. In the presence of compelling evidence about the inhibitory role of insulin upon its own secretion [19,20] we decided to perform a thorough evaluation of tissue distribution of the participants of the insulin-signaling pathway in sections of pancreas obtained from 8 to 12-week-old Wistar rats, and to investigate the integration of the molecular events triggered by insulin and the functional response to that hormone in undisrupted, genetically non-manipulated pancreatic islets. Using antibodies specific for IR, IRS1, IRS2, insulin, glucagon and somatostatin, and utilizing an array of immunohistochemical methods, a full characterization of the tissue distribution of each of the elements that participate in the early steps of insulin signaling in pancreatic islets was obtained. IR is expressed in all cell types of the islet. However, there is a clear difference in the patterns of expression of the protein when comparing cells from the core of the islets with cells at the periphery (Figs. 1a,d, 2g). A series of double-staining immunohistochemical studies utilizing antibodies against IR in conjunction with either insulin, glucagon or somatostatin antibodies, allowed for mapping the expression of IR predominantly to somatostatin producing cells,

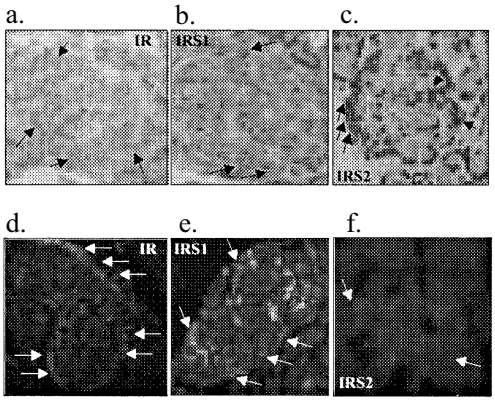

Fig. 1. Immunohistochemical characterization of tissue distribution of proteins that participate in insulin signaling in pancreatic islets of rats. a: IR single staining detected by immunoperoxidase; b: IRS1 single staining detected by immunoperoxidase; c: IRS2 single staining detected by immunoperoxidase; d: IR single staining detected by FITC-labeled immunofluorescence; e: IRS1 single staining detected by FITC-labeled immunofluorescence; f: IRS2 single staining detected by FITC-labeled immunofluorescence. The expression of IR (a and d) occurs widely in pancreatic islets with apparent higher expression in cells of the periphery (arrows in a and d). The same pattern of IR expression is observed in single-primary antibody IRS1 and IRS2 staining (b and e, c and f, respectively), with arrows depicting the preferential peripheral expression.

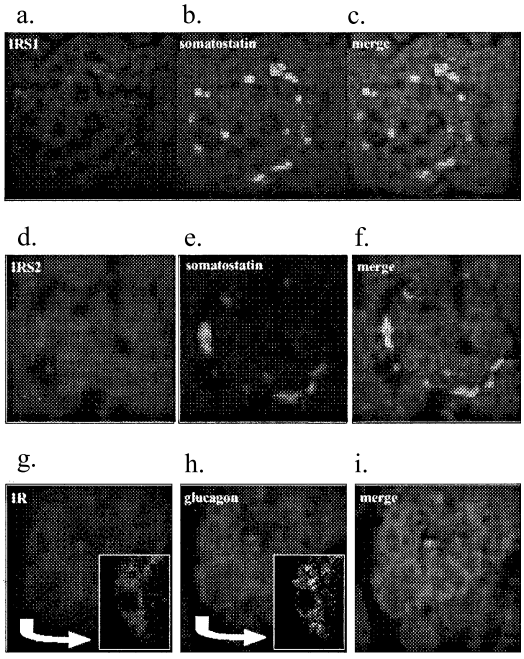

Fig. 2. Double immunofluorescence staining for IRS1 and somatostatin (a–c); IRS2 and somatostatin (d–f); and IR and glucagon (g–i). Note that both IRS1 and IRS2 are widely distributed in islet cells; however, there is a clear preferential co-localization with somatostatin (c and f). IR is, as well, widely distributed in pancreatic islets, and some cells clearly stain for both IR and glucagon (g–i). In the inset in h the granules of glucagon are apparent in a cell expressing IR.

however some co-localization with glucagon was detected (Fig. 2g–i). The characteristics of IRS1 expression in rat pancreatic islets are the same as for IR. Thus, IRS1 is widely expressed in pancreatic islets (Fig. 1b,e), and employing double stain technique evaluated by conventional and confocal microscopy most of the expression was revealed to occur in δ cells (Fig. 2a–c). Finally and similarly, IRS2 is present in all cells of the islets with highest expression occurring at the periphery (Fig. 1c,f) and depicting an impressive co-localization with somatostatin (Fig. 2d–f). Therefore, based on the characteristics of tissue distribution of IR and its substrates, it is suggested that both autocrine and paracrine signaling of insulin shall play a role in whole islet function. Since all studies on which proteins of the insulin-signaling pathway were knocked out were undertaken in mice, we performed immunohistochemical evaluation of IR, IRS1 and IRS2 in pancreas of CBA/J mice and detected a similar pattern of specific protein distribution as in rat islets (not shown).

Supported by evidence that IRS1 participates in the control of insulin secretion through PI3-kinase-modulated Ca^{+2} influx [13,14], and that IRS2 may be more importantly related to cell growth and mitogenesis [15], we decided for evaluating the role of IRS1 in autocrine and paracrine insulin signaling by blocking IRS1 protein synthesis utilizing phosphorthioate-modified antisense oligonucleotides specific for IRS1. Islet specific expression of IR and IRS1 is approximately 0.3-fold that observed in tissues that traditionally respond to insulin stimulus, such as liver, adipose tissue and skeletal muscle (Fig. 3a,b). Nonetheless, the exposure of isolated islets to either 10^{-6} M insulin or 16.7 mM glucose produces effects that are similar to those observed in liver, muscle and fat when

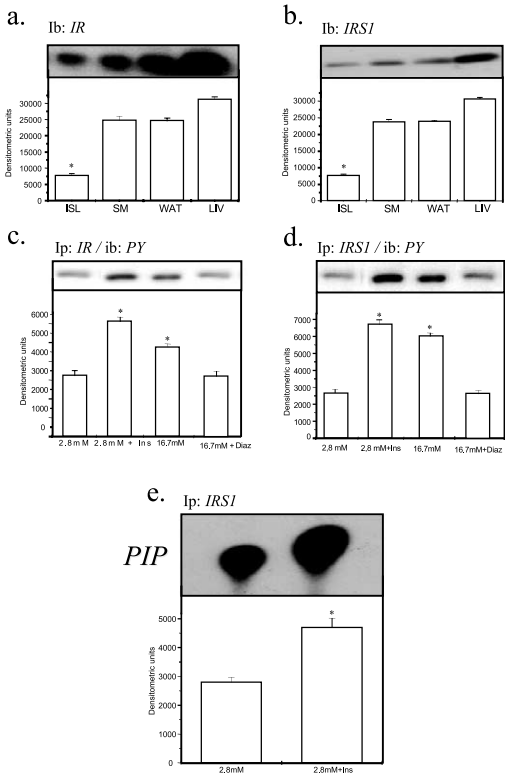

Fig. 3. The expression of IR (a) and IRS1 (b) is quantitatively compared in islets (ISL), skeletal muscle (SM), white adipose tissue (WAT) and liver (LIV) of rats. Two hundred μg of total protein extracts from each tissue were separated by SDS–PAGE, transferred to nitrocellulose membranes and blotted (ib) with anti-IR (a) or anti-IRS1 (b) antibodies. Islets express between 0.2 and 0.3-fold of IR ($n=4$, $^*P<0.05$ as compared to SM, WAT and LIV), and 0.2 and 0.3-fold of IRS1 ($n=4$, $^*P<0.05$ as compared to SM, WAT and LIV) of the respective amounts expressed by tissues that are traditional targets for insulin action. Tyrosine phosphorylation of IR and IRS1 is evaluated by immunoprecipitation (ip) of pancreatic islet protein extracts with anti-IR (c) and anti-IRS1 (d) antibodies, and immunoblotting (ib) with anti-phosphotyrosine (py) antibodies. Insulin and high glucose (16.7 mM) promoted significant induction of tyrosine phosphorylation of IR (c) ($n=4$, $^*P<0.05$, as compared to islets maintained in 2.8 mM glucose) and IRS1 (d) ($n=4$, $^*P<0.05$, as compared to islets maintained in 2.8 mM glucose), while diazoxide (Diaz) was capable of impairing high glucose-induced IR (c) or IRS1 (d) tyrosine phosphorylation. Insulin induces the activation of IRS1-associated PI3-kinase (e). Immunoprecipitates collected from protein extracts obtained from control (2.8 mM glucose) and insulin-treated isolated islets were assayed for PI3-kinase activity and analyzed following TLC resolution. The exposure of isolated islets to 10^{-6} M insulin induces an increase of 1.7-fold in IRS1-associated PI3-kinase activity ($n=3$, $^*P<0.05$). PIP, phosphorylated phosphoinositol.

living animals are challenged by insulin. Thus, increases of 2.0 and 2.5-fold in IR ($P<0.05$) and IRS1 ($P<0.05$) tyrosine phosphorylation, respectively, are induced after 90 s islet exposure to 10^{-6} M insulin (Fig. 3c,d). Exposure of islets to high glucose concentration (16.7 mM) for 3 min also induced significant increase in IR and IRS1 tyrosine phosphorylation (Fig. 3c,d). The effect of glucose upon the activation of elements participating in insulin-signaling pathway seems to be mediated by secreted insulin, since it is blunted if isolated islets are incubated in the presence of 100 μM diazoxide (Fig. 3c,d). Following insulin-stimulated phosphorylation of IR and IRS1 a series of intracellular branches of the insulin-signaling cascade may be induced. Among them the modulation of the activity of the lipid metabolizing enzyme PI3-kinase seems to play a particularly important role in the control of insulin secretion [27–29]. Once activated PI3-kinase catalyzes the incorporation of phosphate at the 3′ position of membrane-bound phosphoinositols generating PI3,4P2 and PI3,4,5P3, which serve as docking sites for downstream signaling molecules, and for connecting the insulin signal with stimuli delivered by other hormones, growth factors and cytokines [30]. Exposure of isolated islets to 10^{-6} M insulin for 5 min induces a significant increase (1.7-fold, $P<0.05$) in IRS1-associated PI3-kinase activity (Fig. 3e), reproducing in that way the characteristics of the molecular response to insulin observed in traditional target tissues.

In order to evaluate the property of phosphorthioate-modified oligonucleotides antisense to block IRS1 protein synthesis, three different sequences were tested. Groups of 300 islets were incubated in RPMI containing 2.8 mM glucose in the presence of 4 nM sense or antisense oligonucleotides or in the absence of oligonucleotides. Incubation times were either 3 h or 6 h. Based on the results obtained, the sequence 5′ CGG GAT AGG AGT GGG T 3′ for antisense IRS1 and its respective sense were selected for the studies. Six hours incubation in the presence of the selected antisense oligonucleotide was sufficient to promote 45% reduction in IRS1 expression in isolated islets (Fig. 4a) and was utilized as standard pre-incubation time in all experiments.

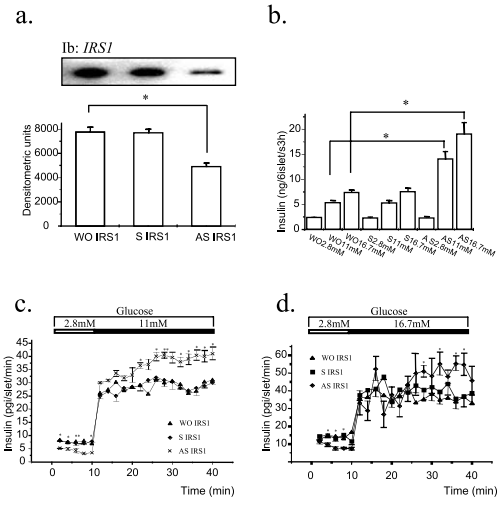

Fig. 4. Treatment of isolated islets with phosphorthioate antisense IRS1 oligonucleotide promotes a reduction of 40% in protein expression of IRS1 (a) ($n=4$, $^*P<0.05$, as compared with islets non-exposed to antisense oligonucleotide) as detected by immunoblot (ib) performed on nitrocellulose transfers of SDS–PAGE separations of 0.2 mg total protein extracts from islets treated with 4 nM sense (S IRS1) or antisense (AS IRS1) oligonucleotide, or exposed to no oligonucleotide (WO IRS1). In static insulin secretion studies (b), a significant increase in glucose-stimulated insulin secretion is detected in islets treated with IRS1 antisense oligonucleotide (AS) [$n=5$, $^*P<0.05$, as compared with islets not exposed to oligonucleotides (WO)]. The values 2.8 mM, 11 mM and 16.7 mM refer to the concentration of glucose in incubation media and S refers to IRS1 sense oligonucleotide treated islets. During dynamic secretion studies (c and d), significant rise in the rate of glucose-induced insulin secretion is detected in islets exposed to IRS1 antisense oligonucleotides and submitted to either 11 mM (c) or 16.7 mM (d) glucose concentration ($n=5$, $^*P<0.05$, when comparing IRS1 oligonucleotide antisense treated islets with islets exposed to no oligonucleotide).

In static insulin secretion studies, the treatment of isolated islets with antisense oligonucleotide promoted no significant change in the rate of insulin secretion of islets incubated in 2.8 mM glucose (Fig. 4b). However, when islets were exposed to high glucose concentrations (11 mM or 16.7 mM), significant increases in glucose-induced insulin secretion were observed (2.0 and 2.8-fold, respectively, $P < 0.05$; Fig. 4b). No changes in insulin secretion were promoted by exposure of islets to IRS1 sense oligonucleotide (Fig. 4b). Similarly, during dynamic insulin secretion studies the blockade of IRS1 expression was accompanied by a significant increase in glucose-induced insulin secretion both when islets were exposed to a medium containing 11 mM or 16.7 mM glucose (Fig. 4c,d, respectively). Of particular interest, it was observed that treatment with antisense IRS1 oligonucleotide coincided with a reduction of insulin secretion rate in islets exposed to 2.8 mM glucose, and that the increased rate of insulin secretion under high glucose condition was more pronounced after 15 min of 11 mM or 16.7 mM glucose exposure (Fig. 4c,d), which might suggest that intermediary steps in signaling shall participate in the observed phenomenon. Determination of insulin-induced insulin mRNA expression in pancreatic islets showed that exposure of isolated islets to exogenous insulin at a concentration of 10^{-6} M for 6 h produces a significant decrease (0.65-fold, $n = 4$; $P < 0.05$, as compared to islets maintained in 2.8 mM glucose without exogenous insulin) in insulin mRNA species, while blockade of IRS1 expression by antisense oligonucleotide treatment for 6 h preceding and during exogenous insulin treatment could partially inhibit the capacity of exogenous insulin to negatively modulate insulin mRNA expression (0.9-fold, $n = 4$; n.s. vs. islets maintained in 2.8 mM glucose without exogenous insulin).

Since higher expression levels of IR and IRS1 were detected in cells located at the periphery of the islets (mostly in δ cells), we decided to investigate the role of IRS1 blockade upon the pattern of secretion of glucagon and somatostatin in isolated islets exposed to low (2.8 mM) or high (16.7 mM) glucose concentrations. Although insulin is known to enhance the inhibitory effect of glucose upon glucagon secretion [31], the mechanisms responsible for the isolated effect of insulin upon α cells is controversial. It is well-known that insulin increases the content of ATP in α cells [32], and that patients and animal models with insulin-deficient diabetes mellitus present a blunted suppression of glucagon secretion, such as they are relatively or absolutely hyperglucagonemics [33,34]. Thus insulin may act directly upon α cells participating in the fine control of glucagon secretion. Previous attempts to characterize the presence of IR or functional binding sites for insulin in α cells were not successful [35], and in the present series of experiments we were also unable to provide undisputed evidence for that fact. However, the pattern of staining for IR, IRS1 and IRS2 observed in more than 60 different sections evaluated during these studies (Fig. 2g–i), plus the characteristics of glucagon secretion in IRS1 depleted islet (Fig. 5a) and the modulation of glucagon mRNA expression in the presence of exogenous insulin and under the blockade of IRS1 (Fig. 5c) strongly suggest that IR is present and functional in glucagon-secreting cells.

Similarly to the control of glucagon secretion, the effects of glucose and insulin upon somatostatin release are not completely understood. Most studies to date provide evidence for glucose-induced inhibition of somatostatin release [31], while

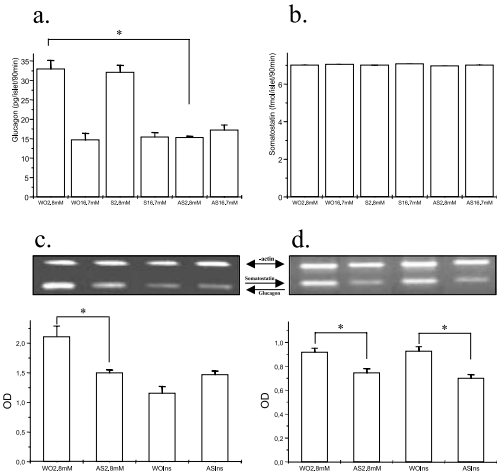

Fig. 5. Static glucagon (a) and somatostatin (b) secretion studies, and glucagon (c) and somatostatin (d) mRNA expression evaluated by RT-PCR. A significant fall in steady-state glucagon (a) secretion is induced by IRS1 antisense oligonucleotide (AS) treatment [$n = 4$, $^*P < 0.05$, as compared with islets exposed to no oligonucleotides (WO)]. No significant variation of somatostatin (b) secretion is detected by the present method ($n = 4$). For a and b, 2.8 mM and 16.7 mM correspond to glucose concentrations in incubation media, antisense (AS), sense (S) and no oligonucleotide treatment (WO). In c, treatment with IRS1 antisense (AS) oligonucleotide induced a significant fall in glucagon mRNA expression, which tended to increase under the stimulus of exogenous insulin ($n = 5$, $^*P < 0.05$, as compared with islets exposed to no oligonucleotides). In d, treatment with IRS1 antisense (AS) oligonucleotide induced a significant fall in somatostatin mRNA expression in islets maintained either in culture media with low glucose (2.8 mM) or in the presence of 10^{-6} M insulin (Ins) ($n = 5$, $^*P < 0.05$ as compared with islets exposed to no oligonucleotides). Sense oligonucleotides exerted no effect upon glucagon and somatostatin mRNA expression (not shown).

only a few studies have attempted to characterize the effects of insulin upon its secretion. In most of those studies no significant modulation inputted by insulin was detected [36,37]. In the present studies the blockade of IRS1 promoted no significant changes in the rate of somatostatin secretion under either low or high glucose concentrations (Fig. 5b). Nevertheless, the partial blockade of IRS1 protein expression promoted a significant fall in somatostatin mRNA expression in pancreatic islets (Fig. 5d). Since the secretion amounts of somatostatin in isolated islets are extremely low, the detectability of traditional RIAs to estimate subtle variations in hormone secretion may be not optimal. The present results suggest that insulin, by acting through IRS1, may stimulate somatostatin mRNA expression and possibly participate in the control of the δ cell's hormone secretion.

As a whole the present studies provide evidence that the blockade of IRS1 expression promotes significant increase in glucose-induced insulin secretion. As most studies to date have evidenced a stimulatory role of insulin upon its own secretion in isolated β cells [13,14,38], we suspect that the phenomenon reported herein might be due to paracrine interactions between α, β and δ cells within the islets. Based on that, a model for explaining the participation of IRS1 on paracrine and autocrine insulin signaling, and the functional feedback exerted by glucagon and possibly by somatostatin, is presented (Fig. 6). Under steady-state low glucose concentration, high glucagon and low somatostatin secretions accompany a low basal rate of insulin secretion. Once exposed to increased glucose concentration the rate of insulin secretion is

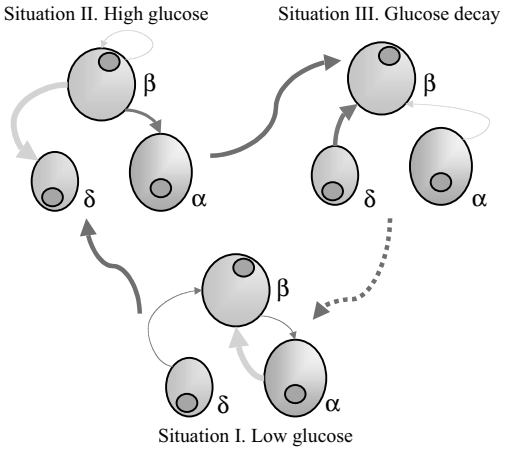

Fig. 6. Proposed model to explain autocrine and paracrine effects of insulin in pancreatic islets based on the present findings. Under steady-state low glucose concentration (situation I), high glucagon levels promote a facilitative tonus upon the pancreatic β cell, while low levels of insulin and somatostatin prevail. Once glucose levels increase (situation II), high amounts of insulin exert a moderate inhibitory action upon α cells and a potent stimulatory effect upon δ cells. Also, a discrete β cell positive-feedback stimulus occurs. Finally, during glucose fall (situation III), an inhibitory tonus provided by somatostatin may participate in the fine tuning of insulin secretion. Light gray arrows, stimulus; dark gray arrows, inhibition; thick arrows, potent effect; thin arrows, discrete effect.

promptly stimulated and acts in concert with glucose to blunt glucagon release and possibly to promote a discrete increase in somatostatin secretion. The reduced tonus of glucagon and increased microenvironment concentration of somatostatin may play an important role in fine control of glucose-induced insulin secretion. When glucose levels start to fall the tonus provided by the combination of low-glucagon–high-somatostatin might accelerate insulin-secretion-rate reduction. Hampering of insulin signaling in pancreatic islets by blocking IRS1 expression leads to a β cell inhibitory effect, which is apparently overcome by a more robust signal of reduced somatostatin accompanied by an inversion in the pattern of glucagon secretion.

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