Insulin Receptor Substrate 1-induced Inhibition of Endoplasmic Reticulum Ca\(^{2+}\) Uptake in \(\beta\)-Cells

AUTOCRINE REGULATION OF INTRACELLULAR Ca\(^{2+}\) HOMEOSTASIS AND INSULIN SECRETION*

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To understand the role of the insulin receptor pathway in \(\beta\)-cell function, we have generated stable \(\beta\)-cells (\(\beta\)IRS1-A) that overexpress by 2-fold the insulin receptor substrate-1 (IRS-1) and compared them to vector-expressing controls. IRS-1 overexpression dramatically increased basal cytosolic Ca\(^{2+}\) levels from 81 to 278 nM, but it did not affect Ca\(^{2+}\) response to glucose. Overexpression of the insulin receptor also caused an increase in cytosolic Ca\(^{2+}\). Increased cytosolic Ca\(^{2+}\) was due to inhibition of Ca\(^{2+}\) uptake by the endoplasmic reticulum, because endoplasmic reticulum Ca\(^{2+}\) uptake and content were reduced in IRS1-A cells. Fractional insulin secretion was significantly increased 2-fold, and there was a decrease in IRS1-A insulin content and insulin biosynthesis. Steady-state insulin mRNA levels and glucose-stimulated ATP were unchanged. High IRS-1 levels also reduced \(\beta\)-cell proliferation. These data demonstrate a direct link between the insulin receptor signaling pathway and the Ca\(^{2+}\)-dependent pathways regulating insulin secretion of \(\beta\)-cells. We postulate that during regulated insulin secretion, released insulin binds the \(\beta\)-cell insulin receptor and activates IRS-1, thus further increasing cytosolic Ca\(^{2+}\) by reducing Ca\(^{2+}\) uptake. We suggest the existence of a novel pathway of autocrine regulation of intracellular Ca\(^{2+}\) homeostasis and insulin secretion in the \(\beta\)-cell of the endocrine pancreas.

The insulin-secreting \(\beta\)-cell of the endocrine pancreas has a central role in regulating glucose homeostasis (1, 2). It is now recognized that \(\beta\)-cell failure is a major contributing factor to type 2 diabetes mellitus, thus emphasizing the importance of elucidating the normal mechanisms of insulin secretion (3, 4). Glucose oxidation by the \(\beta\)-cell is essential for insulin secretion. In particular, glucokinase, the first step in glycolysis, has been convincingly shown to be the \(\beta\)-cell glucose sensor (5). \(\beta\)-Cell metabolism of glucose results in an increase in the ATP/ADP ratio leading to closure of the K\(_{\text{ATP}}\) channel, depolarization of the \(\beta\)-cell, and influx of extracellular Ca\(^{2+}\) through voltage-dependent Ca\(^{2+}\) channels. The subsequent increase in intracellular Ca\(^{2+}\) then activates insulin exocytosis. The possibility of other signaling pathways involved in glucose-induced insulin secretion has also been suggested (6–11).

Since the discovery of the insulin receptor in insulin-secreting \(\beta\)-cells by Rothenberg and colleagues (12, 13), a rapidly growing body of evidence indicates that the insulin receptor signaling pathway is active in pancreatic \(\beta\)-cells (14) and plays an important role in \(\beta\)-cell regulation (4, 12–17). Activation of the \(\beta\)-cell insulin receptor (IR) results in rapid tyrosine phosphorylation of the IR \(\beta\)-subunit and the IR substrate proteins (12). Deletion of IR results in neonatal death in mice (18, 19) and leprechaunism in humans (20). Mice with heterozygous null alleles of IR and insulin receptor substrate 1 (IRS-1) (IR/IRS-1\(^{-/-}\)) exhibit hyperinsulinemia and \(\beta\)-cell hyperplasia and develop overt diabetes (21). Knockouts of the IRS-1 and IRS-2 produce different effects. Inactivation of IRS-1 (IRS-1\(^{-/-}\)) leads to mild insulin resistance, hyperinsulinemia, and \(\beta\)-cell hyperplasia with no overt diabetes syndrome (4, 17, 22). In contrast, inactivation of IRS-2 (IRS-2\(^{-/-}\)) results in \(\beta\)-cell failure and causes type 2 diabetes (17). The differential effects of IRS-1 and IRS-2 knockout indicate that the two major IR substrates mediate differential signals in \(\beta\)-cells, but the mechanisms accounting for such differential regulation and for IRS-1 function are still unknown.

Cellular Ca\(^{2+}\) is a critical element in \(\beta\)-cell regulation. Raising intracellular Ca\(^{2+}\) (\([\text{Ca}^{2+}]_{i}\)) is an obligate step for glucose-induced insulin secretion (1, 23). Abnormal \([\text{Ca}^{2+}]_{i}\), is a common defect in both insulin-dependent type 1 diabetes and insulin-independent type 2 diabetes (24). Altered Ca\(^{2+}\) metabolism has also been reported to affect \(\beta\)-cell function including insulin biosynthesis (25, 26). The endoplasmic reticulum (ER) plays an important role in the regulation of intracellular Ca\(^{2+}\) concentrations (27–29). Endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) is the major calcium pump that sequesters cytosol Ca\(^{2+}\) into ER lumen (28, 30). Thapsigargin, a nonphorboid tumor promoter, specifically inhibits ER Ca\(^{2+}\)-ATPase activity (31). Addition of thapsigargin to pancreatic \(\beta\)-cells leads to elevated cytosol Ca\(^{2+}\) concentration and enhanced short term glucose-stimulated insulin secretion (32). Recent data showed that IRs may directly interact with ER Ca\(^{2+}\)-ATPase (SERCA1 and SERCA2) in a tyrosine phosphorylation-dependent manner in muscle and heart (33). This finding suggests that insulin may via insulin receptor signaling pathway regulate ER Ca\(^{2+}\)-ATPase activity, therefore influencing cellular

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‡ The abbreviations used are: IR, insulin receptor; IRS, IR substrate; ER, endoplasmic reticulum; MTT, 3-(4,5-dimethylthiazol-2-y)-2,5-di-phenyltetrazolium bromide; ANOVA, analysis of variance; HPLC, high performance liquid chromatography; BSA, bovine serum albumin; KRB, Krebs-Ringer buffer; SERCA, sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) transport ATPase.
Ca2+ homeostasis. It is currently unknown whether insulin exerts any regulatory role in β-cell Ca2+ homeostasis.

To dissect the role of IRS-1 in β-cell function, we have overexpressed IRS-1 in an insulin-secreting β-cell line. We show that IRS-1 regulates β-cell Ca2+ homeostasis, insulin biosynthesis, and β-cell proliferation and that elevated expression of IRS-1 induces abnormal Ca2+ homeostasis and β-cell dysfunction.

MATERIALS AND METHODS

Cell Lines and Culture Media—The clonal mouse β-cell line βTC6-F7 and culture conditions were previously described (15, 34). In brief, cells were maintained in high glucose Dulbecco’s modified Eagle’s medium (25 mM glucose; Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 units/ml penicillin, 50 μg/ml streptomycin and incubated at 37 °C in a 10% CO2/90% air humidified atmosphere.

Expression Plasmid—The βTC6-F7 cells were transduced with cationic lipopolyethyleneimine (Life Technologies, Inc.) as described before (15). The transfected cells were selected with 500 μM methyl sulfoxide.

Preparation of Cell lysates—Preparation of cell lysates, immuno precipitation, and Western blotting were performed essentially as described previously (12, 35). Tyrosine-phosphorylated proteins were detected with rabbit polyclonal anti-phosphotyrosine antibody K-18 (kindly provided by Dr. P. Rothenberg, University of Pennsylvania). A secondary antibody, rabbit anti mouse IgG (Sigma, catalog no. sc-2001), was used for c-Myc antibody immunoprecipitation and immunoblotting.

Insulin Assay and β-Cell Metabolism (MTT) Assay—Insulin content and secretion assays were performed essentially as described before (12, 35). The metabolic rate of the β-cells was indirectly measured by the production of formazan, which is produced from 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) as the body. Data were collected from at least 10 individual cells in each measurement at an interval of 4.5 s.

The endoplasmic reticulum Ca2+-ATPase inhibitor thapsigargin was purchased from Biomol (Plymouth Meeting, PA) and dissolved in dimethyl sulfoxide.

Endoplasmic Reticulum Ca2+-Uptake—Endoplasmic reticulum Ca2+ uptake was measured as described (40, 41). Briefly, the cells were trypsinized and washed twice with KRB without Ca2+ or glucose (0.1% BSA). Then, they were permeabilized with 20 μg/ml digitonin (Sigma) in KRB without Ca2+ or glucose (0.1% BSA) at 37 °C for 10 min. The cells were then incubated for 30 min in TES buffer (100 mM TES, pH 7.2, 100 mM KCl, 2.5 mM MgCl2, 0.2 mM EGTA, 5 μg/ml of Ca (ICN, Lisle, IL), 5 μCi/ml of H2O (ICN) for diffusible space correction). The cell-associated fraction of the total cellular content of 3H-labeled proteins. The 3H-labeled insulin immuno reactive material was immuno precipitated with 3 μl of undiluted guinea pig anti-bovine insulin serum (ICN) and protein A-Sepharose CL-4B beads (Amersham Pharmacia Biotech). The radioactivity was determined by liquid scintillation counting. Normal guinea pig serum (Linco, St. Charles, MO) was used as background control. Recovery of insulin immuno reactive material was more than 60% under the assay condition as determined with 125I-labeled insulin.

RNA Protection Assay—RNA protection assays were performed according to Ambion’s protocol (Ambion, Austin, TX). Briefly, the cells were grown in culture medium for 2 days and then trypsinized and washed twice with Dulbecco’s phosphate-buffered saline. To 4 × 106 cells, 0.4 ml of Ambion’s lysis/denaturation solution (direct protect kit,
FIG. 1. Western analysis of the IRS-1 protein and tyrosine phosphorylation of IRS-1. A, Western analysis. Cells were lysed as described under "Materials and Methods." For c-Myc epitope detection (lanes 1 and 2), 250 μg of total protein were immunoprecipitated and blotted with c-Myc antibody 9E10. For IRS-1 detection (lanes 3–6), 16 μg of total protein were loaded in duplicate and blotted with IRS-1 antibody. Positions of IRS1-Myc and endogenous IRS-1 are indicated to the right. Eleven clones were analyzed. Data shown are from a representative clone IRS1-A. B, quantitation of IRS-1 protein. IRS-1 protein levels by immunoblots were quantitated with a PhosphorImager and expressed as a percentage of the IRS-1 level in parental βTC6-F7 cells. Data are shown as mean ± S.E., n = 6, from three independent experiments. *, p = 0.02. C, tyrosine phosphorylation. Cells were treated with or without insulin (15). Twenty μg of total protein were loaded onto SDS gels for phosphotyrosine and IRS-1 Western analysis. Anti-phosphotyrosine (α-PY) detection is shown in the upper panel. The IRS-1 protein was detected in duplicate blots with α-IRS1 antibody and is shown in the lower panel. Chinese hamster ovary-T is a cell line overexpressing the insulin receptor and is used as positive control (lanes 7 and 8). D, quantitation of IRS-1 tyrosine phosphorylation. Radioactivity of the protein bands was quantitated with a PhosphorImager. The IRS-1 signal was normalized to the p120 band (IRS1/p120) to correct for small sample loading variation. Data were collected from two experiments with n = 6 and are shown as mean ± S.E. **, p = 0.001. Cell lines were as follows: βIRS1-A, stable transfected expressing the tagged IRS1-Myc protein; βTC6-F7, parental cell; NEO, βTC6-F7 cells transfected with vector only.

Results

Overexpression of IRS-1 in β-Cells—We overexpressed IRS-1 in a clonal β-cell line βTC6-F7. The exogenous IRS-1 had a c-Myc tag at its C terminus and migrated slightly slower than the endogenous IRS-1 in SDS-gels (Fig. 1A, lanes 1, 3, and 4). β-IRS1-A, one of the 11 stable transfecants tested, expressed IRS-1 protein two times (199 ± 56%) more than the controls (p = 0.02) (Fig. 1B). Fifty percent of the increase was contributed by the exogenous IRS1-Myc; the other 50% was from the endogenous IRS-1. Addition of 100 nM insulin led to rapid tyrosine phosphorylation of IRS-1 in the 160–180 kDa range that co-migrated with the IRS-1 protein (Fig. 1C, upper gel, lanes 2, 4, and 6) (also see Ref. 12). A 120-kDa protein (p120) was also heavily tyrosine-phosphorylated in the β-cells. The abundance of p120 and its extent of tyrosine phosphorylation are relatively stable in the three β-cell lines (Fig. 1C and data not shown), and it was therefore used to normalize the level of IRS-1 phosphorylation. Normalized level of IRS-1 tyrosine phosphorylation in the βIRS1-A cells was 2-fold higher than that in the control cells (Fig. 1, C, upper gel, lane 4, and D). This is proportional to the elevated IRS-1 protein level in the βIRS1-A cells. These data demonstrated that the excess IRS-1 was tyrosine-phosphorylated to the same extent as the endogenous substrate upon insulin stimulation.

Down-regulation of Insulin Content and Secretion by Overexpression of IRS-1—Nine of the transfected clones that showed detectable c-Myc signal and increased IRS-1 levels, including βIRS1-A, were tested for their insulin content. They all exhibited lowered insulin content. Data from the βIRS1-A clone are shown in Fig. 2. The βIRS1-A cells had significantly lowered insulin content: 29.0 ± 2.9 ng/10⁵ cells versus 114 ± 2.2 ng/10⁵ cells (Neo control), p = 0.0001 (Fig. 2A). Net insulin secretion was also reduced 61% at 0 mM glucose (G0) and 58% at 15 mM glucose (G15) (Fig. 2B). Insulin secretion of both βIRS1-A and control cell lines was glucose- and extracellular calcium-dependent. Removal of extracellular Ca²⁺ and addition of 1 mM EGTA completely abolished glucose-stimulated insulin secretion. Addition of either the phosphatidylinositol 3-kinase inhibitor wortmannin (100 nM) or the p70 ribosomal S6 protein kinase inhibitor rapamycin (100 nM) did not change insulin content and secretion of βIRS1-A cells (data not shown).

Although the net amount of insulin secreted by the IRS-1-overproducing cell lines was reduced as mentioned above, glucose-stimulated fractional insulin secretion (the ratio of secreted insulin/total insulin content, expressed as a percentage) was significantly increased in βIRS1-A cells (Fig. 2C). This increased fractional insulin secretion was glucose-dependent. At 0 mM glucose, fractional insulin secretion of the βIRS1-A cells (4.3 ± 0.9%) was not significantly different from that of the Neo control (3.0 ± 0.6%) (p > 0.5). At glucose concentrations above 1 mM (stimulatory glucose concentrations for this β-cell line), fractional insulin secretions were increased more than 2-fold compared with the Neo control (Fig. 2C) (p < 0.04). These data indicate that overexpression of IRS-1 may enhance the capacity
of the β-cell to secrete insulin under stimulatory glucose concentrations. Neither wortmannin (100 nm) nor rapamycin (100 nm) affected glucose-stimulated fractional insulin secretion (data not shown).

Glucose Responsiveness of the β-Cells—To determine whether the reduced insulin content and secretion were due to reduced glucose sensitivity, we examined glucose responsiveness of the IRS-1-overproducing cells. [3H]Leucine incorporation into total protein at 0 mM glucose (G0) was 3.4 ± 0.4 × 10^6 dpm/mg of protein (βIRS1-A) and 3.0 ± 0.3 × 10^6 dpm/mg of protein (control), and at 16 mM glucose (G16), it was 5.7 ± 0.5 × 10^6 dpm/mg protein (βIRS1-A) and 5.6 ± 0.5 × 10^6 dpm/mg of protein (control). However, insulin biosynthesis was significantly reduced as judged by insulin-specific fractional biosynthesis (insulin/total trichloroacetic acid-precipitable proteins) (Fig. 4B) at both basal and glucose-stimulated states. Insulin biosynthesis was reduced 66% in the βIRS1-A cells compared with the control. This down-regulation is specific to the translational regulation. Insulin mRNA levels in βIRS1-A and control cells were similar as measured with the RNA protection assay (42, 43): 100 ± 19% (control) versus 132 ± 60% (βIRS1-A) (*p < 0.01). These data demonstrated that overexpression of IRS-1 in β-cells decreased the rate of insulin biosynthesis at the translational level but did not change the steady-state insulin mRNA level.

Increased β-Cell Cytosolic Ca^{2+} Level—An increase in intracellular Ca^{2+} is recognized as a key obligatory step in insulin secretion and has been implicated mechanistically in insulin release (1, 23). Calcium content in ER has been shown to affect insulin biosynthesis. Reduced ER Ca^{2+} content results in decreased rate of translation initiation (25). It is currently unknown whether insulin receptor signaling affects β-cell Ca^{2+} homeostasis. To investigate that, we measured cytosolic Ca^{2+} levels in βIRS1-A and control cells using fura-2 Ca^{2+} indicator as described under “Materials and Methods.” As shown in Fig. 5A, cytosolic free [Ca^{2+}] in the βIRS1-A cells was increased more than 3-fold both in basal (G0) and 15 mM glucose-stimulated (G15) conditions: 278 ± 39 nm (βIRS1-A) versus 81 ± 18 nm (control) at G0 (*p < 0.001) and 739 ± 121 nm (βIRS1-A) versus 209 ± 42 nm (control) at G15 (p < 0.001). The peak value upon glucose stimulation, however, was not different between the two cell lines: 1886 ± 614 nm (βIRS1-A) versus 1634 ± 512 nm (control).

To determine whether this increased cytosolic [Ca^{2+}] is specific to insulin receptor signaling, we examined cytosolic Ca^{2+} levels in the insulin receptor-overproducing cells (Fig. 5B). We have previously established β-cell lines overproducing the wild type IR (the IR-S2 cells) or the kinase-deficient IR (the AK-S2 cells) (15). Compared with control cells, expression level of IR had previously been established (15). Compared with control cells, expression level of IR (the IR-S2 cells) or the kinase-deficient IR (the AK-S2 cells) (15). Compared with control cells, expression level of IR was 4-fold and 2-fold higher in the IR-S2 and AK-S2 cells, respectively (15). Cytosolic [Ca^{2+}] in control cell line βTC6-F7 and NEO was at 90 ± 9 and 88 ± 8 nm, respectively. The cells overexpressing the kinase-deficient insulin receptor (AK-S2) had a [Ca^{2+}] of 92 ± 11 nm similar to the controls. The cells...
overproducing the wild type IR (IR-S2) had a significantly elevated Ca\(^{2+}\) level: 135 ± 16 nM (p < 0.01 compared with controls, a 50% increase). These data demonstrated that elevated cytosol [Ca\(^{2+}\)] is insulin receptor kinase-dependent. Overexpression of IR and IRS-1 both increased cytosol [Ca\(^{2+}\)] in \(\beta\)-cells.

ER Ca\(^{2+}\)-ATPase plays an important role in regulating cytosol [Ca\(^{2+}\)]. It sequesters cytosolic Ca\(^{2+}\) into endoplasmic reticulum in an ATP-dependent manner (44–46) therefore lowering [Ca\(^{2+}\)]. Thapsigargin is a specific inhibitor of the ER Ca\(^{2+}\)-ATPase (31, 47). Addition of thapsigargin to the \(\beta\)-cells prevented ER Ca\(^{2+}\) uptake, therefore leading to elevated cytosol free [Ca\(^{2+}\)] (32). As shown in Fig. 4C, addition of 200 nM thapsigargin to the control \(\beta\)-cell \(\beta\)TC6-F7 led to an increase in [Ca\(^{2+}\)]; from the basal level of 107 ± 7 nM to 184 ± 11 nM (p < 0.05) (Fig. 5, C and D). In the \(\beta\)IRS1-A cells, however, thapsigargin had no effect on cytosol free [Ca\(^{2+}\)]; 223 ± 35 nM (basal) versus 194 ± 36 nM (200 nM thapsigargin). Because addition of thapsigargin raised [Ca\(^{2+}\)], to the same level as that caused by overexpression of IRS-1, these data indicated that altered ER Ca\(^{2+}\) uptake may be the major cause of elevated [Ca\(^{2+}\)], in IRS-1-overproducing cells.

Inhibition of Endoplasmic Reticulum Ca\(^{2+}\) Uptake—To determine whether endoplasmic reticulum Ca\(^{2+}\) uptake is affected by IRS-1, we directly measured ER Ca\(^{2+}\) uptake with permeabilized \(\beta\)-cells. Calcium uptake by endoplasmic reticulum can be directly measured with radioactive \(^{45}\)Ca\(^{2+}\). We assayed ER Ca\(^{2+}\) uptake at two Ca\(^{2+}\) concentrations: 100 nM (basal condition) and 500 nM (equivalent to glucose-stimulated \(\beta\)-cells). As shown in Fig. 6, Ca\(^{2+}\) uptake in \(\beta\)IRS1-A was significantly reduced at both Ca\(^{2+}\) concentrations compared with control. At 100 nM [Ca\(^{2+}\)], ER Ca\(^{2+}\) uptake for the control \(\beta\)IRS1-A cell was 4.28 ± 0.89 and 2.77 ± 0.22 nmol/mg of protein, respectively (a reduction of 35%) (p < 0.05). At 500 nM [Ca\(^{2+}\)], the uptake was 11.42 ± 1.65 (control) and 7.15 ± 0.90 (\(\beta\)IRS1-A) nmol/mg of protein (p = 0.01), a reduction of 37%. Similar ER Ca\(^{2+}\) uptake results were also obtained from two additional IRS-1-overproducing clones (data not shown). These data clearly demonstrated that overexpression of IRS-1 reduced endoplasmic reticulum Ca\(^{2+}\) uptake and therefore lowered ER Ca\(^{2+}\) content.

Inhibition of \(\beta\)-Cell Proliferation—The insulin receptor signaling pathway is also implicated in mitogenic regulation (49, 50). To determine how overexpression of IRS-1 affects \(\beta\)-cells growth, we used the \(^{3}H\)thymidine incorporation assay (51) to assess \(\beta\)-cell proliferation (Fig. 7). \(\beta\)IRS1-A cells exhibited a
32 ± 4% (n = 6) decrease in the rate of cell proliferation as measured by [3H]thymidine incorporation (6,842 ± 6513 dpm/mg DNA) compared with the passage-matched βTC6-F7 control cells (10,114 ± 890 dpm/mg DNA) (p = 0.02) in the presence of 10% serum. In the absence of serum, the growth rate of IRS1-A was reduced 40% compared with the control (1,895 ± 167 dpm/mg DNA, IRS1-A versus 3,183 ± 274 dpm/mg DNA, control; n = 6) (p = 0.007). No increase in β-cell apoptosis was observed when measured with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay (52) (data not shown). These data demonstrated that IRS-1 negatively regulates β-cell proliferation.

DISCUSSION

Earlier studies had suggested that the insulin receptor may be present in β-cells and that it could function to regulate insulin secretion. For many years, however, this concept was viewed as controversial in the absence of definitive evidence identifying the insulin receptor in the β-cells. Our group has recently demonstrated that the various components of the insulin receptor signaling pathway are indeed present in β-cells, including the insulin receptor and IRS-1 (12, 13). Furthermore, we had shown that glucose-induced insulin secretion activates the β-cell surface insulin receptor tyrosine kinase and its intracellular signal transduction pathway and had proposed that...
this represented a novel autocrine mechanism for the regulation of β-cell function (12). However, the physiological role of this pathway in the β-cell has been difficult to elucidate because the β-cell is not a classical insulin target tissue, and there is scant evidence that insulin regulates its own secretion. Very recently, it has been shown that one role of the insulin receptor signaling pathway in β-cells is regulation of β-cell growth because disruption of IRS-2 leads to β-cell deficiency at birth and diabetes, and it has been proposed that IRS-2-dependent signaling pathways are involved in β-cell neogenesis, proliferation, and survival (17). In contrast, mice heterozygous for null alleles of the insulin receptor and IRS-1 become diabetic and develop β-cell hyperplasia (21). Other studies have also shown that insulin receptor signaling in the β-cell can regulate insulin gene transcription, as well as autoregulation of protein synthesis via PHAS-1 phosphorylation (14, 16). Thus, the insulin receptor signaling pathway of the β-cell appears to have multiple physiological effects.

To identify the role of IRS-1 in insulin secreting β-cells, we overexpressed IRS-1 in a clonal β-cell line BTC8-F7. Our data demonstrate that IRS-1 is involved in regulating Ca2⁺ homeostasis, insulin secretion, insulin biosynthesis, and β-cell proliferation and that elevated expression of IRS-1 induces β-cell failure. This is the first study to demonstrate that a 2-fold overexpression of IRS-1 in β-cells increases β-cell cytosol Ca2⁺ levels and reduces ER Ca2⁺ content. These findings are significant because the ER is one of the major compartments for intracellular Ca2⁺ storage. Elegant earlier experiments have shown that the ER is actively involved in regulating intracellular Ca2⁺ in the nanomolar range (27, 28). In β-cells, it is widely established that glucose stimulation results in an increase in intracellular Ca2⁺, a required step for insulin secretion. Typically, basal Ca2⁺ concentrations are in the 80–100 nM range, and following glucose stimulation, they increase 3–5-fold. Once the stimulation is removed, the ER sequesters excess cytosolic Ca2⁺, and the Ca2⁺ levels return to baseline. The ER Ca2⁺-ATPase is the main pump responsible for Ca2⁺ uptake into the ER. Its biochemical characteristics have been extensively described, and it is implicated in the regulation of intracellular Ca2⁺ homeostasis. Recently, two isoforms of the Ca2⁺-ATPase, SERCA2 and SERCA3, have been localized to the islet. Furthermore, SERCA3 expression is reduced in the GK rat, a nonobese model of type 2 diabetes (44–46).

Our observations that thapsigargin, an ER Ca2⁺-ATPase-specific inhibitor, increased cytosolic Ca2⁺ levels in the control cells, but not in the IRS-1-overproducing cells (Fig. 5, C and D) suggest that ER Ca2⁺-ATPase in the βIRS1-A cells could have been suppressed by IRS-1 overproduction. This is also strongly supported by the observed decrease in βIRS1-A ER Ca2⁺ uptake (an indirect measurement of Ca2⁺-ATPase) and the fact that Ca2⁺ release from the ER (induced by A23187; data not shown) is not changed in βIRS1-A cells. A possible explanation for IRS-1-induced inhibition of ER Ca2⁺-ATPase is based on an elegant study by Kahn and co-workers (33), who have identified SERCA1 in skeletal muscle and SERCA2 in cardiac muscle as novel IRS-1- and IRS-2-binding proteins. Importantly, this interaction is insulin-dependent (maximal at 100 nM insulin and at 5 min of stimulation), and requires tyrosine phosphorylation of IRS-1. Whether there is a similar interaction between IRS-1 and the β-cell ER Ca2⁺-ATPase still remains to be determined, although our data suggest that such an interaction could result in decreased ER Ca2⁺-ATPase activity, leading to reduced ER Ca2⁺ uptake in β-cells and increased cytosolic Ca2⁺ concentrations.

Our study provides a novel functional link between the IRS-1 signaling pathway and the stimulus-secretion pathway in β-cells, which we believe to be physiologically significant. We postulate that under basal conditions in the β-cell this pathway is not activated. However, once glucose or other secretagogues stimulate insulin secretion, the released insulin will feed back to the β-cell insulin receptor and activates the associated signal transduction pathway. Increased signaling results in IRS-1 tyrosine phosphorylation (12) and subsequent inhibition of ER Ca2⁺ uptake as shown by this study. Decreased Ca2⁺ fluxes into the ER can then increase cytosolic Ca2⁺ and further facilitate the maintenance of increased Ca2⁺ levels due to secretagogue-induced Ca2⁺ influx from the extracellular space. We believe that our studies have identified a novel pathway of autocrine regulation of intracellular Ca2⁺ homeostasis and insulin secretion in the β-cell of the endocrine pancreas.

Insulin secretion and cellular insulin content were significantly reduced in the cells overproducing IRS-1. This is most likely the consequence of reduced insulin biosynthesis. Elevated expression of IRS-1 inhibits insulin biosynthesis at the translational level as measured by the [³H]leucine labeling assay. This inhibition is likely due to the reduced ER [Ca2⁺] found in the IRS-1-overproducing cells. A similar finding that ER Ca2⁺ affects insulin biosynthesis has been reported before (25, 26). The insulin mRNA level in the IRS-1-overexpressing cells was similar to the controls as measured with RNA protection assay. It suggests that IRS-1 may not be involved in mediating activation of insulin gene transcription in βIRS1-A cells. A recent report suggests that IRS-2 may mediate signals for insulin gene transcription (16).

Augmented expression of IRS-1 also leads to inhibition of β-cell growth. IRS1-A cells exhibited a 32 ± 4% decrease in the rate of cell proliferation as measured by [³H]thymidine incorporation compared with the control. These observations are supported by the finding that loss of IRS-1 function leads to β-cell hyperplasia and hyperinsulinemia in the IRS-1 knockout animals (21, 48). β-Cell function is also regulated by the insulin receptor substrate IRS-2. Loss of IRS-2 function results in β-cell failure, including reduced β-cell growth and decreased insulin secretion. Deletion of IRS-2 leads to development of diabetes in transgenic mice (4, 17). It appears that the insulin receptor signaling differentially regulates β-cell function via different substrates. The net output of insulin receptor signaling in β-cells may therefore depend on the relative strength of different substrate signals. Abnormalities in the insulin receptor substrates, e.g. altered IRS expression levels, may directly contribute to β-cell failure in diabetes.

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