Glucose- and insulin-induced phosphorylation of the insulin receptor and its primary substrates IRS-1 and IRS-2 in rat pancreatic islets


1. Introduction

Protein tyrosine phosphorylation is an important mechanism of intracellular signalling. Insulin action in target tissues is initiated by binding of the ligand to its receptor (IR), promoting the autophosphorylation of its β subunit and subsequently triggering a cascade of intracellular signalling [1]. Two early substrates of the activated IR are the 165,000-185,000 M₀ IRS-1 and IRS-2 [1,2]. Once phosphorylated, IRS-1/2 bind to and activate a series of SH2 motif-containing substrates, including proteins of the src family, phosphotyrosine phosphatases, such as SHPTP2, and the lipid-modifying enzyme PI 3-kinase [1,3]. Studies attempting to clarify early events in the insulin-signalling pathway were performed mostly in liver, muscle and adipose tissue and, although most of the metabolic actions of insulin are exerted in those tissues, it is widely accepted that insulin also possesses autocrine and paracrine actions in the cells of the pancreatic islets. However, the mechanisms involved in insulin-signalling in the islets of Langerhans are not known.

Recent evidences suggest that the inhibition of tyrosine kinase activity in cultured pancreatic islets leads to a considerable increase in insulin secretion [4]. To address the issue of the involvement of tyrosine phosphorylation-mediated events in the control of islet function and in insulin-signalling in the islets of Langerhans, primary cultures of rat pancreatic islets were examined for the presence of tyrosine-phosphorylated proteins under basal and stimulated conditions.

2. Materials and methods

2.1. Chemicals and antibodies

The reagents and apparatus for SDS-PAGE and immunoblotting were from Bio-Rad (Richmond, CA). Tris, PMSF, EDTA, aprotinin and DTI were from Sigma (St Louis, MO). Sodium amobarbital and insulin were from Eli Lilly (Indiana, IN). Protein A was from Amersham (Buckinghamshire, UK) and Protein A Sepharose 6 MB was from Pharmacia (Uppsala, Sweden). Monoclonal antibodies anti-phosphotyrosine and anti-p85 (PI 3-kinase) were from Upstate (Lake Placid, NY). Antisera anti-IRS-1, IRS-2 and IR were raised in rabbits as previously described [2,5].

2.2. Buffers

Buffer A contained 100 mM Tris, 1% SDS, 50 mM Hepes (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA and 10 mM sodium vanadate. Buffer B was similar to buffer A except that 1% Triton X-100 replaced 1% SDS and 2 mM PMSF and 0.1 mg/ml aprotinin were added. Buffer C contained 100 mM Tris, 10 mM sodium vanadate, 10 mM EDTA and 1% Triton X-100.

2.3. Animals

Male Wistar rats were bred at the animal facilities of the State University of Campinas. The animals used in the experiments were 150-180 g. Food and water ad libitum. Food was withdrawn 12-14 h before islet isolation.

2.4. Islet isolation and culture

Islets were isolated by hand-picking after collagenase digestion of the pancreas following a technique previously described [6]. In most of the experiments, the islets were preincubated overnight at 37°C in a humidified air incubator with 5% CO₂. The culture medium consisted RPMI containing 10% FCS, 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 1.8 mg/ml glucose. Other culture conditions had glucose at physiologic (5.6 mM) or high (11 mM or 22 mM) concentrations. When insulin was required, the hormone was added to a final concentration of 10⁻⁵ M.

2.5. Tissue extracts

Following the exposition to the experimental conditions, groups of 300 islets were either immediately transferred to an Eppendorf tube containing 200 µl of buffer A and boiled for 5 min (when intended for
immunoblots of total extracts) or resuspended in 1 ml of buffer B and immediately transferred to ice (when intended for immunoprecipitations) and homogenized using a polytron PTA 20S homogenize (Brinkmann Instruments, Westbury, NY). The extracts were then centrifuged at 20,000 × g to remove insoluble material. Determination of protein content in the supernatant was performed by the method of Lowry.

Specimens of liver, muscle and adipose tissue used in immunoblotting in parallel to islets extracts were treated as described above for immunoblots except that the fragment of tissue was homogenized in 1.5 ml of buffer A.

2.6. Immunoprecipitations

For immunoprecipitations, samples containing 4 mg of total protein were incubated with 15 μl of antisera anti-IRS-1, IRS-2 or IR at 4°C overnight, followed by the addition of Protein A Sepharose 6 MB for 1 h. The pellets were repeatedly washed in buffer C (5 × ) and resuspended in 50 μl of Laemmli sample buffer and boiled for 5 min prior to loading onto the gel [7].

2.7. Protein analysis by immunoblotting

Samples of 150 μg of total protein were suspended in 50 μl of Laemmli sample buffer and boiled for 5 min before loading onto 6% SDS-PAGE in a miniature slab gel apparatus. Electrotransfer, blotting and the procedures for analysis were as previously described [8 10].

3. Results and discussion

3.1. Constitutive phosphorylation of proteins in pancreatic islets cultured in low glucose concentration and regulation of tyrosine phosphorylation by glucose and insulin

Over the past decade, there has been a dramatic increase in the understanding of early postreceptor events in insulin action [1,3]. After insulin binds to the α subunit of its receptor, there is an activation of the kinase in the β subunit, thus, enhancing the tyrosine kinase activity of the receptor towards intracellular protein substrates [1,3]. In most cells, this event leads to the subsequent tyrosyl phosphorylation of two cytoplasm proteins with an apparent molecular mass of 165–185 kDa, namely IRS-1 and IRS-2. Phosphorylated IRS-1/2 associate and activate the lipid metabolizing enzyme PI 3-kinase [1–3]. Thus, IR, IRS-1, IRS-2 and PI 3-kinase represent some of the earliest steps in insulin action. These initial steps have been well-characterized in classic insulin target tissues, i.e. liver, muscle and fat [11–13]. However, as no information was available regarding a possible involvement of this pathway in auto- and paracrine-signalling in pancreatic islets, we initially used immunoblotting to search for tyrosine-phosphorylated proteins in isolated rat pancreatic islets.

Groups of 300 islets each were cultured for 12 h in RPMI containing 1.8 mM glucose and pulsed for 90 s with RPMI containing 11 mM glucose as depicted in the upper margin. Three bands detected upon blotting with antiphosphotyrosine antibody are indicated by the arrows in the right-hand margin. Molecular mass standards are depicted in the left-hand margin. The results are representative of three experiments run in duplicate.

Fig. 1. Fluorography of an immunoblot of total extracts from rat pancreatic islets cultured for 12 h in RPMI containing 1.8 or 5.6 mM glucose as depicted in the upper margin. At least three bands were detected upon blotting with antiphosphotyrosine antibody as indicated by the arrows in the right-hand margin. Molecular mass standards are depicted in the left-hand margin. The results are representative of four experiments run in duplicate.

Fig. 2. Fluorography of an immunoblot of total extracts from rat pancreatic islets cultured for 12 h in RPMI containing 1.8 mM glucose and pulsed for 90 s with RPMI containing 11 mM glucose as depicted in the upper margin. Three bands detected upon blotting with antiphosphotyrosine antibody are indicated by the arrows in the right-hand margin. Molecular mass standards are depicted in the left-hand margin. The results are representative of three experiments run in duplicate.
Fig. 3. Fluorography of immunoblots of IRS-1, IRS-2 or IR immunoprecipitates from rat pancreatic islets cultured for 12 h in RPMI containing 1.8 mM glucose and pulsed for 90 s with RPMI containing 11 mM glucose or 10^{-7} M insulin as depicted in the upper margins of A and C. In A, the upper part of the nitrocellulose membrane of IRS-1 and IRS-2 immunoprecipitates was blotted with antiphosphotyrosine antibody while, in B, the lower part of the same membrane was blotted with anti-p85 PI 3-kinase antibody. In C, the nitrocellulose membrane of IR immunoprecipitates was blotted with antiphosphotyrosine antibody and in D the same nitrocellulose membrane was rebotted, after stripping, with anti-IR antibody. Molecular mass standards are depicted in the left-hand margin. The results are representative of three experiments run in duplicate.

Fig. 4. Fluorography of immunoblots of IRS-1 immunoprecipitates from rat pancreatic islets cultured for 12 h in RPMI containing 1.8 mM glucose and pulsed for 30 s with RPMI containing 11 mM glucose or 10^{-7} M insulin as depicted in the upper margin. Three bands detected upon blotting with antiphosphotyrosine antibody are indicated by the arrows in the right-hand margin. Molecular mass standards are depicted in the left-hand margin. The results are representative of two experiments run in duplicate.

3.2. Phosphorylation of IRS-1, IRS-2 and the β subunit of the IR and association of IRS-12 with the p85 subunit of PI 3-kinase are increased after a pulse of high glucose or exogenous insulin

As the apparent molecular mass of two of the bands were 165-185 kDa and 95 kDa, it was suspected that they could correspond to IRS-1, IRS-2 and the β subunit of the IR, respectively. This hypothesis was tested by performing immunoprecipitations with anti-IRS-1, -IRS-2 or -IR antisera. Groups of 300 islets cultured for 12 h in media containing 1.8 mM glucose were pulsed for 90 s with media containing 11 mM glucose or insulin at a final concentration of 10^{-7} M. Nitrocellulose membranes containing the immunoprecipitates were initially blotted with antiphosphotyrosine antibody. IRS-1, IRS-2 and IR were phosphorylated at the basal condition and underwent an increase in the tyrosine phosphorylation level after both 11 mM glucose or 10^{-7} M insulin pulse. The increases in the phosphorylation levels were 2- and 3-fold for IRS-1 after 11 mM glucose and 10^{-7} M insulin, respectively, 2- and 2.5-fold for IRS-2 after 11 mM glucose and 10^{-7} M insulin, respectively, and 2- and 2.5-fold for IR after 11 mM glucose and 10^{-7} M insulin, respectively (Fig. 3a,c) (data representative of n = 3). The lower part of the membranes from the IRS-1 and IRS-2 immunoprecipitates were blotted with the anti-p85 antibody and the associations of IRS-1 and IRS-2 with the regulatory subunit of PI 3-kinase were detected. The increases in IRS-1-p85 association after 11 mM glucose and 10^{-7} M insulin pulse were 1.5- and 2.5-fold, respectively. The increases in IRS-2-p85 association after 11 mM glucose and 10^{-7} M insulin were 1.5- and 2.3-fold, respectively (Fig. 3b) (data representative of n = 3). The nitrocellulose membranes of the IR immunoprecipitates were rebotted using anti-IR antiserum and the amount of IR was similar in all three conditions (Fig. 3d) (n = 3). These results provide evidence that the tyrosine-phosphorylated band at 165-185 kDa corresponds to the primary substrates of the IR, IRS-1 and IRS-2 and that the degree of tyrosine phosphorylation of the substrates is responsive to short exposition to insulin or high glucose. After longer expositions (60 min or 12 h) to the same concentrations of insulin or glucose, the level of tyrosine phosphorylation of all bands return to nearly basal (data not shown). Shorter exposition to 11 mM glucose does not induce an increase in the phosphorylation of the bands while expositions as short as 30 s to insulin at 10^{-7} M still produce an increase in the tyrosine phosphorylation of all three bands (Fig. 4). Thus, two possible explanations are proposed: (1) Insulin

3.3. Phosphorylation of IRS-1 and IRS-2 and the p85 subunit of PI 3-kinase are increased after a pulse of high glucose or exogenous insulin

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autocrine loop, insulin exerts an inhibitory action upon the secretory activity of the β cell. Although the intracellular mechanism responsible for this regulation is currently unknown, the PI 3-kinase pathway might be involved. Thus, it is possible that glucose-induced insulin secretion by the pancreatic β cell could, via an autocrine activation of the IR-signalling pathway, modulate negatively the β cell’s own function. Experiments with wortmannin, an inhibitor of PI 3-kinase [18], could be useful as a tool for answering this question.

3.3. Relative amounts of IRS-1 and IR in pancreatic islets in comparison to tissues that are traditional targets for insulin action

200 µg of total protein from freshly prepared homogenates of liver, fat and muscle were separated by SDS-PAGE in parallel to the same amount of proteins from rat pancreatic islets cultured for 12 h in RPMI containing 1.8 mM glucose. Nitrocellulose membranes from the electrophoretic transfers were blotted with antisera anti-IRS-1 and -IR. Quantitation by densitometric scanning of the specific bands revealed that the IRS-1 was present in liver, muscle, fat and islets in the following proportions based on arbitrary densitometric units: 100, 85, 78 and 18. For the IR, the relation was 100, 92, 85 and 16, respectively, for liver, muscle, fat and pancreatic islets (n = 2) (Fig. 5).

In summary, isolated rat pancreatic islets possess proteins that are phosphorylated at tyrosine residues. The level of phosphorylation is regulated by glucose and insulin although the effects of glucose occurs possibly through its insulin secretory activity. Three of these proteins were identified as components of the insulin-signalling pathway and may be involved in autocrine and paracrine regulation in the islets of Langerhans.

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