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Cardiovascular Research 40 (1998) 96–102

Cardiovascular Research

Insulin signalling in heart involves insulin receptor substrates-1 and -2, activation of phosphatidylinositol 3-kinase and the JAK 2-growth related pathway

Licio A. Velloso[a,*], Carla R.O. Carvalho[a], Fernanda A. Rojas[a], Franco Folli[b], Mario J.A. Saad[a]

[a]*Department of Internal Medicine, University of Campinas—UNICAMP, 13081-970 Campinas SP, Brazil*
[b]*Department of Medicine I, H.S. Raffaele, Milan, Italy*

Received 12 August 1997; accepted 17 March 1998

Abstract

Objective: Hyperinsulinemia is a common feature of obesity and hypertension and may be associated with abnormal metabolism and growth of heart muscle and vascular wall. Most of the known actions of insulin were characterised in muscle, adipose tissue and liver. In this study we investigate the initial steps of insulin signalling in rat heart. **Methods:** After insulin infusion in the cava vein of male Wistar rats, the insulin receptor, insulin receptor substrates-1 and -2, phosphatidylinositol 3-kinase activity and Janus kinase (JAK) 2 engagement were studied by immunoprecipitation and immunoblot of heart extracts. **Results:** An insulin load induces rapid autophosphorylation of the insulin receptor which is followed by the phosphorylation of insulin receptor substrates-1 and -2. The phosphorylation of these early intracellular substrates leads to the association of the p85 subunit of phosphatidylinositol 3-kinase and subsequent activation of its catalytic p110 subunit. Besides activation of the lipid metabolising enzyme phosphatidylinositol 3-kinase, the phosphorylation of insulin receptor substrates-1 and -2 engages the intracellular kinase JAK 2 and induces JAK 2–STAT 1 complex formation. **Conclusion:** We demonstrate that the early steps of insulin signalling in heart include the phosphorylation–activation of the insulin receptor, engagement of insulin receptor substrates-1 and -2 with the consequent activation of phosphatidylinositol 3-kinase and the involvement of the recently discovered growth related pathway—JAK 2–STAT 1.

Keywords: Insulin receptor; Insulin receptor substrate-1 and -2; Phosphatidylinositol 3-kinase; JAK 2; Heart hypertrophy; Rat

1. Introduction

Insulin exerts most of its known actions through a heterotetrameric transmembrane protein tyrosine kinase receptor (PTKr), the insulin receptor (IR) [1]. Hormone binding promotes receptor autophosphorylation and triggers a cascade of intracellular events that will lead to several metabolic and growth promoting effects [1]. Most of the early steps of the insulin signalling pathway involve IR substrates-1 and -2 (IRS-1 and IRS-2), two intracellular docking proteins that undergo tyrosine phosphorylation after insulin binding to its receptor [1–3]. The tyrosine phosphorylated IRSs proteins bind to several *src* homology-2 (SH2)-containing molecules, such as phosphatidylinositol 3-kinase (PI3-kinase), Grb 2, SHPTP2, Nck, and Fyn, which will extend the insulin signal inwards in the target cell [4]. Besides IRS-1 and IRS-2 other intracellular proteins as Shc, ecto-ATPase and pp60 are phosphorylated following insulin treatment [5–7].

Most of the studies on insulin signalling were performed on tissues that are traditional targets of insulin action such as liver, muscle and adipose tissue [1,4]. Those studies demonstrated that insulin is a pleotropic messenger, and many of its actions are tissue specific. Therefore, tissues not traditionally responsive to insulin may potentially suffer some regulation by the hormone. Syndromes of insulin resistance and hyperinsulinemia are known to coincide with hypertension, obesity and cardiac hypertrophy, leading to a condition referred to as Syndrome X

*Corresponding author. Tel.: +55 (19) 239 7734; Fax: +55 (19) 239 3114.

Time for primary review 35 days.

0008-6363/98/$ – see front matter
PII: S0008-6363(98)00098-4

[8,9]. Following clinical and epidemiological observations, a pathogenic role for insulin in cardiac disease was proposed [10]. Previous studies attempting to clarify the role of hyperinsulinemia upon hypertension and cardiac disease emphasised abnormalities of lipoprotein metabolism and hyperglycemia as possible effectors [10–12]. A direct effect of insulin on cardiac and vascular muscle growth have also been proposed [13,14], but little is known about its molecular basis. In a recent series of studies we have shown that angiotensin II (AII) induces IRS-1 and IRS-2 phosphorylation in rat heart [15,16]. Moreover, a cross-talk between the insulin and AII signalling systems modulates PI3-kinase activity and involves the intracellular Janus kinase (JAK) 2 [16].

In the present work we investigate the early steps of insulin signalling in rat heart, including IR autophosphorylation, IRS-1, IRS-2, JAK 2 and STAT 1 engagement, PI3-kinase association to the IRS proteins and its activation following insulin treatment.

2. Methods

2.1. Antibodies and chemicals

Antisera against IRS-1, IRS-2 and the IR were previously described [3,17]. Antibodies against the p85 subunit of PI3-kinase, against JAK 2 and against STAT 1 were from Santa Cruz (Santa Cruz, CA, USA). Antibodies against phosphotyrosine were from UBI (Lake Placid, NY, USA). ^{125}I-protein A was from Amersham (Buckinghamshire, UK). Protein A Sepharose 6MB was from Pharmacia (Uppsala, Sweden). Rat liver phosphatidylinositol was from Avanti Polar Lipids (Milan, Italy). Chemicals were from Sigma (St. Louis, MO, USA).

2.2. Buffers

Buffer A consisted of 100 mM Tris, 1% sodium dodecyl sulphate (SDS), 50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (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 phenylmethylsulphonic fluoride 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 (28 day old/90–100 g), bred at the animal facilities of the University of Campinas, were used in the experiments. The rats were allowed free access to standard rodent chow and water ad libitum. Food was withdrawn 12 h before the experiments. The rats were anaesthetised by intraperitoneal injection of sodium amobarbital (100 mg/kg body weight), and the experiments were performed after the loss of corneal and pedal reflexes. All experiments involving animals were approved by the University of Campinas ethical committee.

2.4. Tissue extraction, immunoblot and immunoprecipitation

In vivo exposition of the heart to isovolumetric (500 μl) solutions containing either insulin (10^{-7} M for most of the experiments, or at the concentration stated in the legends of respective figures) or saline were performed by abdominal cava vein injection. The injections were performed in a single dose, as a bolus. A fragment of the tip of the heart was excised after 90 s (or as described in time course experiments) and immediately homogenised in freshly prepared, boiling buffer A when intended for immunoblots, or freshly prepared, ice-cold buffer B when intended for immunoprecipitations. Insoluble material was removed by 45 min centrifugation at 50 000 *g* at 4°C. Protein concentration in the supernatants was determined by the Bradford method [18].

For immunoprecipitations, samples containing 3 mg of total protein were incubated with 15 μl of antisera antiIRS-1, -IRS-2, -JAK 2 or -IR at 4°C overnight, followed by the addition of Protein A Sepharose 6MB for 1 h. The pellets were repeatedly washed in buffer C (five times), resuspended in 50 μl of Laemmli sample buffer [19], and boiled for 5 min prior to loading onto the gel. For 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 a 6% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) system in a miniature slab gel apparatus (BioRad, Richmond, CA, USA). Electrotransfer, blotting and signal detection were as previously described [20,21].

2.5. PI3-kinase assay

Aliquots of supernatants of the homogenates intended for immunoprecipitations, and containing equal amounts of total protein, were incubated overnight at 4°C using antibodies against IRS-1 or IRS-2. The immunocomplexes were precipitated with Protein A Sepharose 6MB. In vitro PI3-kinase assays were performed as described [22]. Shortly, The ^{32}P-labelled 3-P-phosphatidylinositol was quantified by the Imagequant software of the PhosphorImager (Molecular Dynamics).

2.6. Data analysis

Results are presented as direct comparisons of bands present in fluorographs from insulin treated or saline treated rats. When needed, quantification was performed by the Imagequant software of the PhosphorImager (Molecu-

lar Dynamics). Student's *t*-test for paired data was employed.

3. Results

The infusion of insulin in the cava vein of rats induced the phosphorylation of proteins present in at least three bands (185, 135 and 95 kilodaltons (kDa) in total extracts of heart, separated by SDS-PAGE, and blotted with antiphosphotyrosine antibodies (Fig. 1). The induction of tyrosine phosphorylation was dose dependent, and highest phosphorylation was achieved at insulin concentration of 10^{-7} M ($n=3$, $p<0.05$ vs. saline treated). Further increase in insulin concentration led to a slight decrease in protein tyrosine phosphorylation (Fig. 1a). Using the optimum concentration of 10^{-7} M insulin, a time-course experiment was performed, and after 90 s of infusion in the cava vein highest phosphorylation was detected (Fig. 1b).

The traditional pathway of insulin signalling in liver, muscle and adipose tissue involves the phosphorylation and activation of the transmembrane IR, the phosphorylation of two intracellular docking proteins, IRS-1 and IRS-2, and the engagement of several SH2 containing proteins. One of these proteins is the lipid metabolising enzyme PI3-kinase, which is known to be involved in vesicular trafficking through the cytoplasm, membrane ruffling, and GLUT 4 translocation towards the cell membrane [4]. Using specific antibodies we detected, by immunoprecipitation, IR phosphorylation in heart, following insulin treatment in vivo. The tyrosine phosphorylated β-subunit of the IR migrated at 95 kDa in SDS-PAGE and corresponded to the 95 kDa band observed in immunoblots of total extracts of heart (Fig. 2a). Highest phosphorylation occurred 90 s after insulin injection in the cava vein ($n=5$, $p<0.05$ vs. saline treated).

The tyrosine phosphorylation of IRS-1 and IRS-2 was

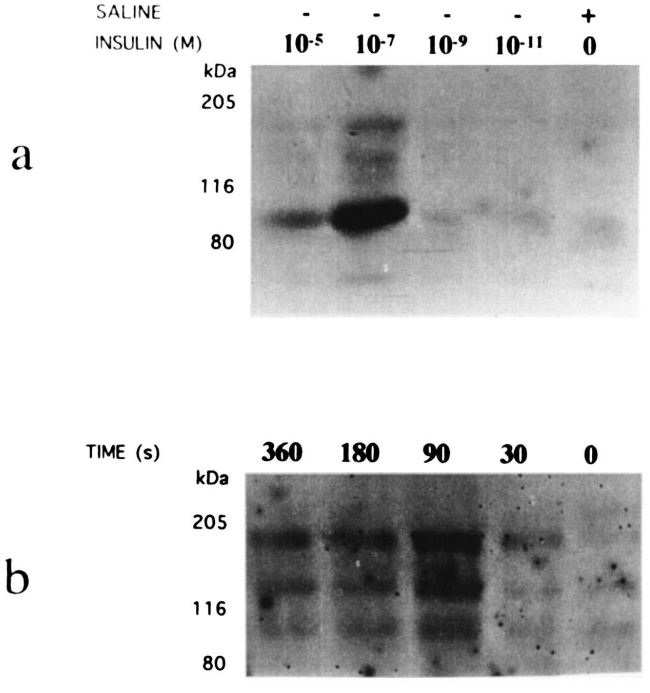

Fig. 1. Autoradiograms of antiphosphotyrosine immunoblots of rat heart total extracts resolved in 6.5% SDS-PAGE. (a) Dose–response of tyrosine phosphorylated proteins of the heart exposed to increasing concentrations of insulin injected through the cava vein. A tip of the heart was excised 90 s after insulin or saline injection (as depicted in the upper margin of the figure) ($n=3$). (b) Time course of insulin-stimulated phosphorylation of proteins in rat heart. Five hundred μl of 10^{-7} M insulin was injected though the cava vein and a tip of the heart was excised after the elapsed time (as depicted in the upper margin of the figure) ($n=3$). Molecular mass standards are depicted in the left-hand margin.

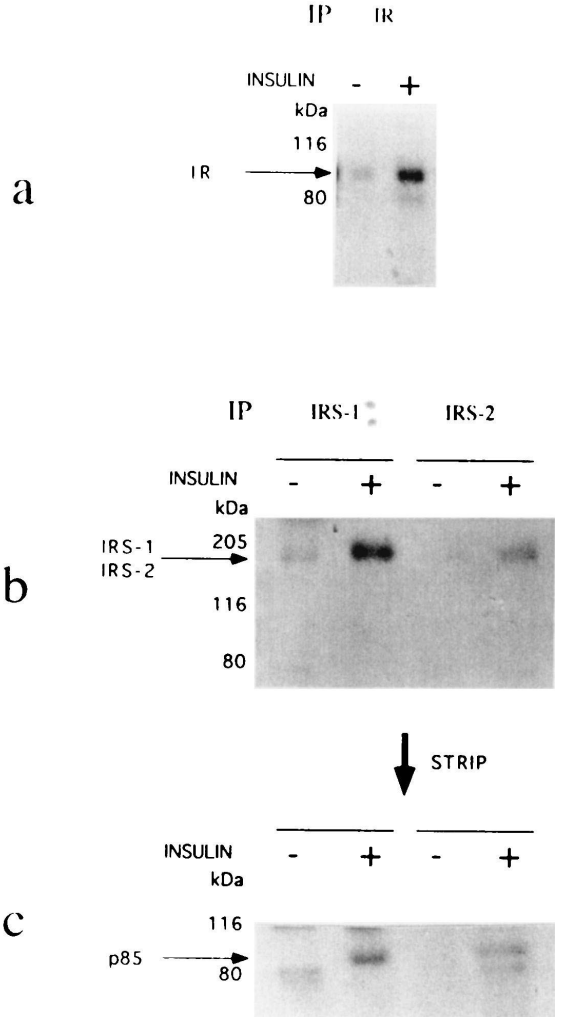

Fig. 2. Autoradiograms of immunoblots of rat heart immunoprecipitates resolved in 6.5% SDS-PAGE. Five hundred μl of 10^{-7} M insulin or saline (as depicted in the upper margins) were injected though the cava vein and after 90 s the hearts were excised and homogenized. Immunoprecipitations were performed using antiIR (a), antiIRS-1 or antiIRS-2 (b and c) antibodies. Blots were performed using antiphosphotyrosine antibodies (a and b). Membranes from IRS-1 and IRS-2 immunoprecipitates blotted with antiphosphotyrosine antibodies were stripped and reblotted with antip85 antibody (c) ($n=5$). Molecular mass standards are depicted in the left-hand margins.

detected in immunoprecipitation experiments. Thus, 90 s after the injection of 500 μl of 10^{-7} M insulin, tyrosine phosphorylated IRS-1 and IRS-2 could be captured in immunoprecipitation experiments using specific antibodies, and detection was performed by separation in SDS-PAGE and blotting with antiphosphotyrosine antibodies (Fig. 2b). The association of the p85 subunit of PI3-kinase with the phosphorylated IRS-1 and IRS-2 was demonstrated by stripping nitrocellulose membranes of IRS-1 and IRS-2 immunoprecipitates and reblotting with antip85 antibodies (Fig. 2c). The insulin-induced association of the p85 subunit of PI3-kinase with IRS-1 and IRS-2 led to a dose dependent activation of the lipid metabolising enzyme. Thus, after a 500 μl load of 10^{-7} M insulin in the cava vein a complex containing maximal activity of PI3-kinase towards the substrate phosphatidylinositol could be precipitated together with IRS-1 (Fig. 3a) and IRS-2 (Fig. 3b) ($n=3$, $p<0.05$ vs. saline treated).

The activation of cellular growth promoting pathways by insulin have been described in tissues such as liver, muscle and adipose tissue [1]. Although in those tissues the activation of metabolic related pathways is predominant, the engagement of Shc, Grb, the downstream MAP kinase cascade and JAK 2 have been fully observed [1,4]. To investigate whether in heart JAK 2 is engaged after insulin treatment, we performed immunoprecipitation experiments using a specific JAK 2 antiserum. The injection of 500 μl of 10^{-7} M insulin induced the phosphorylation of JAK 2 (Fig. 4a) ($n=4$, $p<0.05$ vs. saline treated). Striping and reblotting of nitrocellulose membranes revealed the insulin-induced association of JAK 2 with IRS-1 (Fig. 4c) and IRS-2 (not shown). The amount of JAK 2 captured by the immunocomplex was similar in heart immunoprecipitates from insulin-treated and non-insulin-treated rats (Fig. 4b). The mitogenic activity of JAK 2 is carried towards the nucleus by its association with activators of transcription known as STATs. In THE heart of rats we observed that the treatment with 500 ml of 10^{-7} M insulin induced the association of JAK 2 with STAT 1 (Fig. 4d) ($n=4$, $p<0.05$ vs. saline treated).

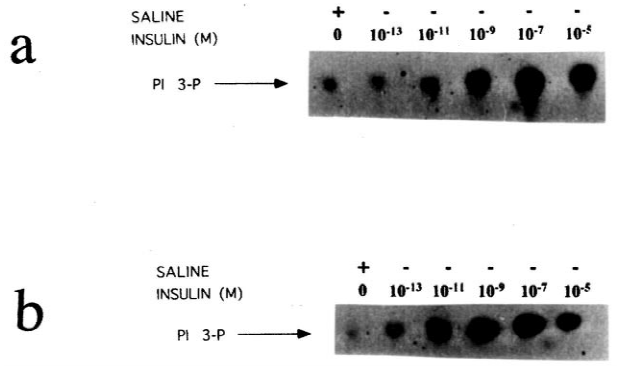

Fig. 3. Autoradiograms of silica TLC plates of IRS-1 (a) and IRS-2 (b) associated PI3-kinase activity. Five hundred μl of saline or insulin (as depicted in the upper margins) were injected through the cava veins of rats and after 90 s the hearts were excised and homogenized. Immunoprecipitations were performed using antiIRS-1 (a) and antiIRS-2 (b) antibodies, and PI3-kinase activity assay was performed as described in Section 2 ($n=3$). The arrow indicates the migration site of PI3-P (phosphatidylinositol 3-phosphate).

4. Discussion

In the present study we show that in rat heart, rapidly after insulin stimulation, the β-subunit of the IR undergoes autophosphorylation. As a consequence both IRS-1 and IRS-2 are as well phosphorylated. Following phosphorylation both IRS-1 and IRS-2 bind to and activate, in a dose dependent manner, the lipid metabolising enzyme PI3-kinase. Moreover, the intracellular kinase JAK 2 is co-precipitated with IRS-1 and IRS-2 and phosphorylated in response to insulin stimulation. The insulin-induced JAK 2 phosphorylation leads to JAK 2–STAT 1 complex formation and directs the insulin signal towards the nucleus.

The insulin-stimulated increase in IRS-1 and IRS-2 associated PI3-kinase activity correlated with insulin stimulation of tyrosine phosphorylation of IRS-1 and IRS-2, and the IR β-subunit. When the data of IR β-subunit, IRS-1 and IRS-2 phosphorylation and PI3-kinase associated activity from a dose–response experiment in heart are expressed as a percentage of maximal stimulation, a close correlation between these parameters is apparent. The ED_{50} values for these parameters were 0.4 μg for IRS-1 and IRS-2 phosphorylation, 0.5 μg for PI3-kinase activity and 0.8 μg for the IR β-subunit phosphorylation. The acute half-maximal stimulation of PI3-kinase in heart occurred at a plasma insulin concentration which is achieved in normal rats during a meal tolerance test or an oral glucose tolerance test (data not shown). Thus, our results show that there is a significant increase in PI3-kinase activity at concentrations of insulin which are needed to regulate glucose disposal by the heart in intact rats. Distinct experimental approaches coming from different tissues or cell culture have demonstrated a correlation between PI3-kinase activity and glucose transport, and also of PI3-kinase activity and glycogen metabolism [1]. Taking together these results, and the data herein presented, we believe that the IRS-1/IRS-2–PI3-kinase associated pathway may be linked to the activation of glucose transport and glycogen synthesis in the heart.

During the last 10 years the cardiovascular effects of insulin were emphasised and insulin resistance/hyperinsulinemia have been implicated in hypertension and atherosclerosis. A higher than expected frequency of myocardial hypertrophy occurs in some genetic insulin states and also in acquired forms of insulin resistance [8,9,23,24]. Although these associations are difficult to explain, they may result, at least in part, from the hyperinsulinemia that occurs in the majority of patients. The pathways used by hyperinsulinemia to induce myocardial hypertrophy are not known but some recent data on

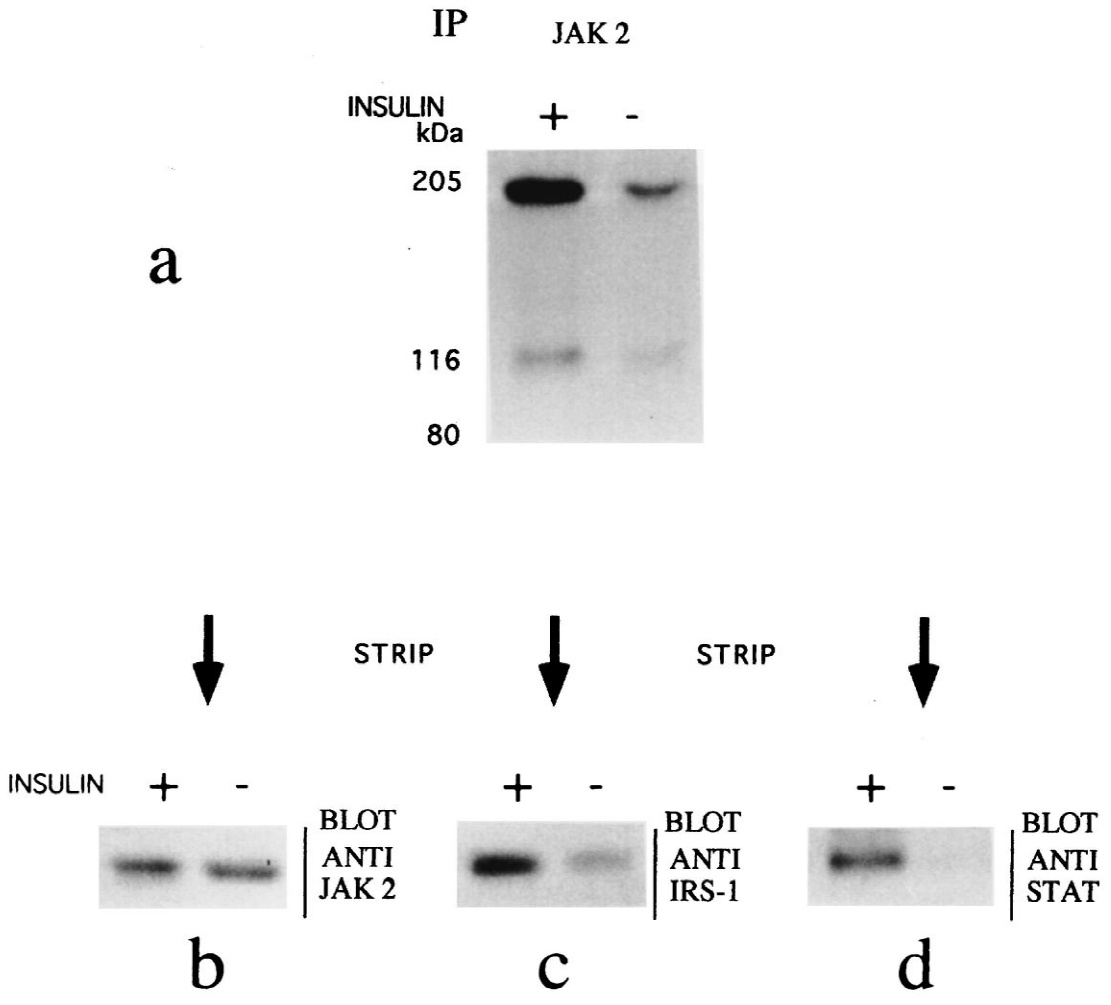

Fig. 4. Autoradiograms of immunoblots of rat heart immunoprecipitates resolved in 6.5% SDS-PAGE. Five hundred μl of 10^{-7} M insulin or saline (as depicted in the upper margins) were injected though the cava vein and after 90 s the hearts were excised and homogenized. Immunoprecipitations were performed using antiJAK 2 antibodies. Tyrosine phosphorylated proteins associated to JAK 2 and stimulated by insulin were detected by blotting nitrocellulose membranes with antiphosphotyrosine antibodies (a). The membranes were stripped and reblotted with antiJAK 2 antibodies (b), antiIRS-1 antibodies (c) and antiSTAT 1 antibodies (d) ($n=4$). Molecular mass standards are depicted in the left-hand margin.

transgenic models of insulin resistance [25–27] and our present results may contribute to formulation of an initial characterisation of these effects. Our results showing that insulin is able to induce JAK 2 tyrosine phosphorylation in heart is of interest, since JAK 2 is involved in growth promoting pathways stimulated by different peptides [16,27,28].

Recently, several reports have demonstrated complex intracellular interactions between the insulin signalling pathway and other hormone signalling systems. Thus, IGF-1 and IL-4 acting through PTKr require IRS-1 for their downstream signalling and potentially modify the insulin signal [29,30]. Moreover, AII and epinephrine acting through G-protein-coupled receptors may modify or have their signals modified by insulin [16,31]. Finally, growth hormone, erythropoietin and IL-9, acting through members of the cytokine-1 receptor family interact with the insulin signalling pathway [27,28,32]. The interaction of members of the IGF-1 receptor family and the insulin signalling pathway occurs directly through their own receptors and early substrates, as the receptors have intrinsic tyrosine kinase activity [29,30]. However, members of the G-protein-coupled receptor family and cytokine-1 receptor family require a kinase to mediate the interaction. Several pieces of evidence demonstrate that members of the JAK family are engaged in the cross-talk between many of these signalling pathways [16,27,28]. Our results, showing that insulin stimulates JAK 2 phosphorylation and STAT 1 association, may contribute to further characterisation of the cross-talk between insulin- and other hormone-signalling pathways in heart.

The findings described herein show a direct effect of insulin in heart with engagement of early responsive elements involved in the insulin signalling pathway. The signal is started by the rapid phosphorylation of the IR which induces the phosphorylation of its two major intracellular substrates, IRS-1 and IRS-2. Following phosphorylation, both IRS-1 and IRS-2 may be co-precipitated

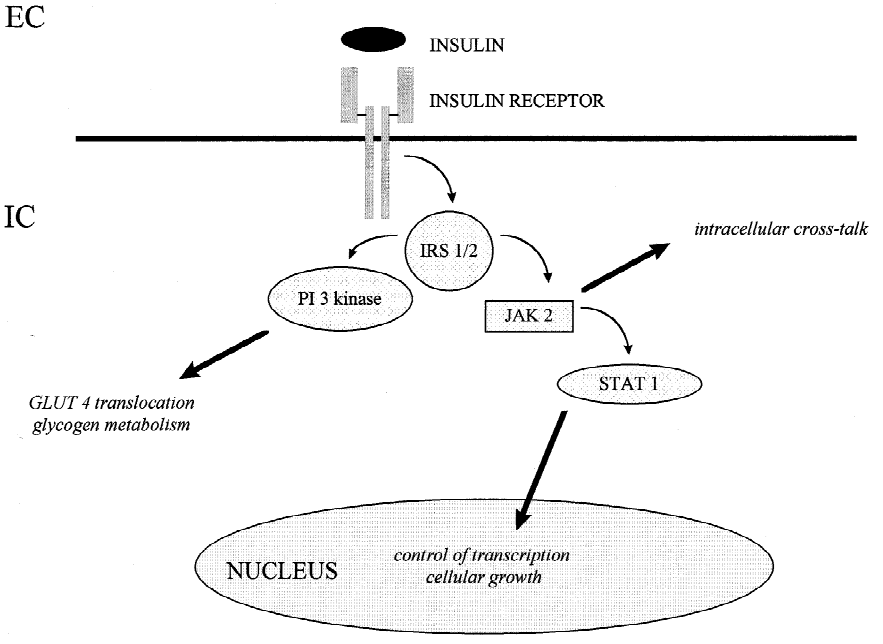

Fig. 5. Summary of findings of the present and previous ([15,16]) studies on insulin signalling in rat heart. The activation of the insulin receptor by insulin binding leads to IRS-1 and IRS-2 phosphorylation. Once phosphorylated IRS-1/2 bind to and activate PI3-kinase which directs the signal towards glycogen metabolism and stimulation of glucose transport. Moreover, phophorylated IRS-1/2 engages JAK 2 leading to STAT 1 activation and nuclear signalling. A role for JAK 2 on intracellular cross-talk is proposed. EC, extracellular; IC, intracellular.

with the p85 subunit of the lipid metabolising enzyme PI3-kinase, which is activated in response to insulin. Finally, we demonstrate that the intracellular kinase JAK 2 is also engaged following an insulin load, associates with STAT 1, and potentially integrates the insulin signal towards growth promotion, and signalling pathways of other messengers. A summary of the findings of the present study and findings from previous studies [15,16] are presented in Fig. 5. We believe that the delivery of the insulin signal to the nucleus by the JAK 2–STAT 1 pathway, plays an important role in hyperinsulinemia associated cardiac hypertrophy. It will be of interest to investigate these signalling systems in the hearts of animal models of hyperinsulinemia, cardiac hypertrophy and hypertension.

Acknowledgements

The authors thank Mr. L. Janeri and Mrs. C da Silva for technical assistance. This work was supported by grants from FAPESP and CNPq.

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