Insulin signaling proteins in pancreatic islets of insulin-resistant rats induced by glucocorticoid

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ABSTRACT

Chronic administration of glucocorticoids induces insulin resistance that is compensated by an increase in β-cell function and mass. Since insulin signaling is involved in the control of β-cell function and mass, we investigated the content of insulin pathway proteins in pancreatic islets. Rats were made insulin resistant by daily administration of dexamethasone (1mg/kg, i.p.) for 5 consecutive days (DEX), whilst control rats received saline (CTL). Circulating insulin and insulin released from isolated islets were measured by radioimmunoassay whereas the content of proteins was analyzed by Western blotting. DEX rats were hyperinsulinemic and exhibited augmented insulin secretion in response to glucose (P < 0.01). The IRα-subunit, IRS-1, Shc, AKT, p-p70S6K, ERK1/2, p-ERK1/2, and glucocorticoid receptor protein levels were similar between DEX and CTL islets. However, the IRβ-subunit, p-IRβ-subunit, IRS-2, PI3-K, p-AKT and p70S6K protein contents were increased in DEX islets (P < 0.05). We conclude that IRS-2 may have a major role, among the immediate substrates of the insulin receptor, to link activated receptors to downstream signaling components related to islet function and growth in this insulin-resistant rat model.

Key terms: dexamethasone, glucocorticoid, insulin resistance, insulin signaling, pancreatic islets.

INTRODUCTION

Numerous compounds with glucocorticoid activity have been synthesized. Among them, dexamethasone has a 50-fold greater affinity for the glucocorticoid receptor, relative to cortisol. In clinical practice, dexamethasone administration is indicated for the suppression of the in

<response>

Insulin signaling proteins participate in the control of β-cell function and growth (Kulkarni et al., 1999a; Kulkarni et al., 1999b; Otani et al., 2004; Cantley et al., 2007). The insulin pathway includes the insulin receptor that may be constituted of insulin receptor type A (IR-A) and/or insulin receptor type B (IR-B) and the insulin-like growth factor-1 receptor (IGF-1R). The receptors are tetrameric structures composed of ‘half receptors’, each of which in turn comprises an α-subunit, which is predominately an extracellular binding domain, and a β-subunit which is predominately an intracellular domain that has tyrosine kinase activity regulated by ligand binding (Pollak, 2008, Leibiger et al., 2010). The immediate insulin receptor substrates, also known as the adapter proteins, include the insulin receptor substrate (IRS) proteins IRS-1to IRS-6, growth factor receptor binding protein 2 (Grb-2), and some lower-molecular-weight substrates such as Shc, p60, and Gab1 (reviewed in Wirkamäki et al., 1999; Leibiger et al., 2008). The adapter proteins link the activated insulin receptors to downstream effector proteins such as phosphatidylinositol 3-kinase (PI3-K) isoforms, isoforms of protein kinase B (PKB, also called AKT), the mammalian target of rapamycin (mTOR), the S6 ribosomal protein kinase (p70S6K) as well as the phospholipase Cγ (PLCγ) (all these effectors form the metabolic branch of insulin signaling). The receptor substrates may also be linked to the proteins of the mitogen-activated protein kinase (MAPK) pathway, such as extracellular-regulated-signal kinase-1/2 (ERK1/2), which is activated by the proto-oncogenes Ras and Raf (mitogenic branch of insulin signaling) (reviewed in Wirkamäki et al., 1999; Leibiger et al., 2008).

The aim of this study was to investigate the protein content of some important components of the insulin signaling proteins in islets of insulin-resistant rats treated with dexamethasone. By measuring circulating insulin levels and protein content in isolated islets, we sought to elucidate the role of insulin signaling in the development of insulin resistance induced by dexamethasone.

In this study, we investigated the content of insulin signaling proteins in pancreatic islets of rats made insulin resistant by dexamethasone administration. We measured circulating insulin and insulin released from isolated islets and analyzed the content of proteins using Western blotting.

Results:

- Dexamethasone treatment resulted in hyperinsulinemia and augmented insulin secretion.
- The IRα-subunit and IRS-1, Shc, AKT, p-p70S6K, ERK1/2, and p-ERK1/2 protein levels were similar between dexamethasone-treated (DEX) and control (CTL) islets.
- The IRβ-subunit, p-IRβ-subunit, IRS-2, PI3-K, p-AKT, and p70S6K protein contents were increased in DEX islets compared to CTL islets.

Conclusions:

- IRS-2 may play a major role in linking activated receptors to downstream signaling components.
- The increased content of insulin signaling proteins in DEX islets suggests a compensatory mechanism to counteract insulin resistance.

Key Terms: dexamethasone, glucocorticoid, insulin resistance, insulin signaling, pancreatic islets.
pathway in pancreatic islets from insulin-resistant rats induced by dexamethasone. We found that IRS-2, but not IRS-1 and Shc protein contents are higher in islets from dexamethasone-treated rats than in control islets. This increase was accompanied by an augmentation in the PI3-K and p-AKT, but not in p-p70\textsuperscript{S6K} and p-ERK1/2 protein levels.

METHODS

Materials

Dexamethasone phosphate (Decadron\textsuperscript{\textregistered}) was from Aché (Campinas, SP, Brazil). The reagents used in the insulin secretion protocol, radiimmunoassay (RIA) and immunoblotting were from Mallinckrodt Baker, Inc. (Paris, Kentucky, France), Merck (Darmstadt, Germany), Sigma (St. Louis, MO, USA) and Bio-Rad (Hercules, CA, USA). The \textsuperscript{125}I-labeled insulin (human recombinant) for RIA assay was purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Anti insulin, anti IR\textalpha-subunit, anti IR\beta-subunit, anti phosphorylated IR\beta-subunit (Tyr 1162/1163) (p-IR\beta) anti IRS-1, anti Shc, anti AKT, anti phosphorylated AKT (Thr 308) (p-AKT), anti p70\textsuperscript{S6K}, anti phosphorylated ERK1/2 (Tyr 204) (p-ERK1/2), anti glucocorticoid receptor \alpha/\beta (GR\alpha/\beta), and anti \alpha-tubulin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti IRS-2, and anti phosphorylated p70\textsuperscript{S6K} (p-p70\textsuperscript{S6K}) was from Cell Signaling Technology (Beverly, MA, USA). Anti PI3-K, and anti ERK1/2 were from Upstate (Lake Placid, NY, USA).

Animals

Experiments were performed on two groups of 10 males Wistar rats (3 months old). The rats were obtained from the University of Campinas Animal Breeding Center and were kept at 24°C on a 12 h light/dark cycle (light period 06:00 – 18:00). The rats had access to food and water ad lib. The experiments with animals were approved by the institutional Campinas State University Committee for Ethics in Animal Experimentation and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23 revised 1996).

Dexamethasone treatment

A group of rats received daily i.p. injection of 1mg/kg b.w. dexamethasone (DEX rats) or saline - NaCl 0.9% - (CTL rats), between 7:30 – 8:30 h, for 5 consecutive days (Rafacho et al., 2008a).

Blood glucose and serum insulin measurement

Blood was collected from the tail tip of fed rats and blood glucose levels were measured with a glucometer ("one touch" - Johnson & Johnson). Immediately afterwards, animals were sacrificed (exposure to CO\textsubscript{2} followed by decapitation) the trunk blood was collected. The serum, obtained by centrifugation, was used to measure the insulin content by RIA, utilizing Guinea-pig anti-rat insulin antibody and rat insulin as standard.

Isolation of islet and static secretion protocols

Islets were isolated by collagenase digestion of the pancreas. Insulin content and secretion were measured as described in detail previously (Giozzet et al., 2008; Rafacho et al., 2008a). Briefly, after islet isolation, groups of five islets were first incubated for 1 h at 37 °C in 1 mL Krebs-bicarbonate buffer solution containing 5.6mmol/L glucose, supplemented with 0.5% of bovine serum albumin and equilibrated with a mixture of 95% O\textsubscript{2}; 5% CO\textsubscript{2}, pH 7.4. The medium was then replaced by 1 mL fresh buffer solution containing 5.6 or 11.1 mmol/L glucose and incubated for a further 1 h period. At the end of the incubation, the supernatant was collected and appropriately stored at –20°C for subsequent measurement of insulin content by RIA, as described above.

Protein extraction and immunoblotting

Protein extraction and immunoblotting were carried out as previously reported (Rafacho et al., 2008b, Rafacho et al., 2010b). Pools of isolated islets were homogenized in ice-cold cell lysis buffer (Cell Signaling, MA, USA). Protein concentration from total cell lysate was determined by the Bradford method, according to the manufacturer (Bio-Rad, CA, USA). Protein obtained from islets (100 μg) was used for each experiment. Immunoblotting experiments were performed at least 6 times using different samples (each sample consisting of islets obtained from one rat). After 2h blocking in 5% non-fat milk solution at room temperature, immunodetection was performed following an incubation with rabbit polyclonal IR\alpha-subunit (1:1000 dilution), rabbit polyclonal IR\beta-subunit (1:1000 dilution), goat polyclonal IRS-1 (1:750 dilution), rabbit polyclonal IRS-2 (1:500 dilution), rabbit polyclonal Shc (1:1000 dilution), mouse monoclonal PI3-K (1:1000 dilution), rabbit polyclonal AKT (1:1000 dilution), goat polyclonal p-AKT (1:1000 dilution), rabbit polyclonal p-IR\beta-subunit (1:1000 dilution), goat polyclonal p-IR\alpha-subunit (1:500 dilution), rabbit polyclonal IRS-1 (1:750 dilution), rabbit polyclonal IRS-2 (1:500 dilution), rabbit polyclonal Shc (1:1000 dilution), mouse monoclonal PI3-K (1:1000 dilution), rabbit polyclonal AKT (1:1000 dilution), rabbit polyclonal p-AKT (1:500 dilution), mouse monoclonal p70\textsuperscript{S6K} (1:1000 dilution), mouse monoclonal p-p70\textsuperscript{S6K} (p-p70\textsuperscript{S6K}), p-ERK1/2 (1:500 dilution), mouse monoclonal GR\alpha/\beta, p-ERK1/2 (1:1000 dilution), rabbit polyclonal AKT (1:1000 dilution), rabbit polyclonal p-AKT (1:500 dilution), mouse monoclonal p-ERK1/2 (1:500 dilution) and mouse monoclonal α-tubulin (1:1000 dilution) antibody. Membranes were then exposed to specific secondary peroxidase-conjugated antibody (anti IgG (H+L)–HRP, Calbiochem, Darmstadt, Germany) at room temperature, and visualized by chemiluminescence (SuperSignal, Pierce Biotechnology Inc., Rockford, IL, USA). The bands were quantified using the Scion Image software (ScionCorp., Frederick, MD, USA).

Statistical analysis

Results are expressed as the means ± S.E.M. of the indicated number (n) of experiments. Statistical analyses were performed using Student’s t-test and when necessary Welch’s corrected t-test was applied. P < 0.05 was considered statistically significant.

RESULTS

Insulin-resistant rats

As observed previously (Rafacho et al., 2008a, Rafacho et al., 2009) DEX rats exhibited marked fasting and fed hyperinsulinemia. This augmentation was 8.6-fold in DEX
compared to CTL rats (fed serum insulin values were 4.3 ± 0.2 and 36.9 ± 2.1 ng/dL for CTL and DEX rats, respectively; n = 10, P < 0.01). Blood glucose levels were similar between the two groups of rats (99.7 ± 1.5 and 108.8 ± 4.2 mg/dL for CTL and DEX rats, respectively; n = 10). As expected (Rafacho et al., 2008a; Rafacho et al., 2010b), islets isolated from DEX rats also secreted more insulin than CTL rats in response to 5.6 or 11.1 mmol/L glucose concentrations (Fig 1; n = 10 wells, P < 0.001). The insulin values were 0.56 ± 0.04 and 2.21 ± 0.18 ng/islet.mL⁻¹.h⁻¹ for 5.6 mmol/L glucose and 8.42 ± 0.36 and 29.37 ± 1.24 ng/islet.mL⁻¹.h⁻¹ for 16.7 mmol/L glucose for CTL and DEX islets, respectively.

**Insulin receptor and immediate adapter proteins**

Substrates from the insulin receptor, also known as adapter proteins, such as IRS and Shc proteins are involved in the control of β-cell function and growth. The protein content of IRα-subunit, IRβ-subunit, IRS-1, IRS-2, Shc and p-IRβ-subunit proteins were investigated in pancreatic islets lysates by Western blotting. The levels of IRα-subunit were similar between DEX and CTL islets (Fig. 2A). No alteration in the expression of IRS-1 and the two subunits of the low-molecular-weight substrate Shc was noticed in DEX, compared to CTL islets (Figs. 2C,E,F, respectively; n = 6). However, an augmentation of 209% for IRβ-subunit, 60% for the IRS-2 and 148% for p-IRβ-subunit protein contents were observed in DEX, compared to CTL islets (Figs. 2B,D,G, respectively; n = 6, P < 0.05). The ratio between p-IRβ-subunit protein was not changed in DEX, compared to CTL islets (data not shown).

**Downstream signaling components**

We next measured the levels of some downstream effectors of insulin signaling such as PI3-K, AKT, p70S6K and ERK1/2 proteins. The protein content of AKT and ERK1/2 was not altered between DEX and CTL rat islets (Figs. 3B and F, respectively; n = 6). However, an augmentation in PI3-K (30%) and in p70S6K (34%) protein levels in DEX, compared to CTL islets was observed (Fig. 3A and D, respectively; n = 6, P < 0.05). We also measured the content of phosphorylated AKT, p70S6K and ERK1/2 proteins. The former increased 31% in DEX, compared to CTL islets (P < 0.05), but no significant alterations were observed with p-p70S6K and p-ERK1/2 proteins (Figs. 3C,E,G, respectively). The ratio values between the phosphorylated and the total content for the above proteins revealed a significant increase of 58% for AKT (P < 0.05) in DEX, compared to CTL islets (0.91 ± 0.05 and 1.44 ± 0.18 for CTL and DEX respectively).

**Glucocorticoid receptor**

The glucocorticoid receptor modulates the gene transcription activity. We next investigate whether this protein could be altered in islets from DEX rats. Figure 4A shows that the islet content of GRα/β protein was similar between DEX and CTL groups (n = 6).

**DISCUSSION**

Insulin maintains blood glucose concentration within narrow limits by regulating the uptake of glucose in peripheral tissues (muscle and fat) as well as regulating hepatic glucose output. For this purpose, pancreatic β-cells secrete adequate amounts of insulin to face to the respective blood glucose levels, a process often referred to as the stimulus-secretion coupling (Weir et al., 2001). Under the pathological condition of insulin resistance, induced or not by administration of glucocorticoids, both the uptake of glucose by muscle and fat tissues and the hepatic glucose output are impaired, which results in increased demand for insulin to maintain the glycemia at physiological range (Weir et al., 2001; Nicod et al., 2003; Burén et al., 2008). The increase in insulin secretion and in β-cell mass are among the adaptive compensations in the endocrine pancreas that counteract the peripheral insulin resistance and guarantee the high levels of circulating insulin (Weir et al., 2001; Rafacho et al., 2009). In the present study we made rats insulin resistant by 5 days of dexamethasone administration. These insulin-resistant rats (DEX) exhibited hyperinsulinemia and increased glucose-stimulated insulin secretion, which agreed with the endocrine pancreas compensations that occur under insulin resistance to maintain glycemia at normal or near-physiological ranges (Weir et al., 2001; Rafacho et al., 2008a).

Insulin signaling components may modulate the β-cell function and mass (Kulkarni et al., 1999a; Kulkarni et al., 1999b; Otani et al., 2004; Cantley et al., 2007). Herein, we showed that IRβ-subunit, p-IRβ-subunit, and IRS-2 protein contents are augmented in pancreatic islets from DEX rats (Fig. 2). It has been demonstrated that the secreted insulin may be essential for insulin exocytosis or even have a positive effect on its own release (Aspinwall et al., 1999). Islets from mice with a systemic knockout of IRS-1 (Kulkarni et al., 1999b), or with a β-cell knockout of insulin receptor (IR) (Kulkarni et al., 1999a; Otani et al., 2004), IGF-1R (Kulkarni et al., 2002), or with

![Figure 1](https://example.com/image.png) **Figure 1. Increased glucose-stimulated insulin secretion in DEX rats.** Cumulative static insulin secretion from isolated islets in response to basal or stimulating glucose concentrations. Insulin release was higher in islets from DEX rats at both 5.6 and 16.7 mmol/L glucose. Data are means ± S.E.M. *significantly different vs CTL. n = 10 wells, P < 0.05 for unpaired Student t-test.
Figure 2. Increase of IRβ-subunit, p-IRβ-subunit and IRS-2 protein levels in DEX islets. Protein levels of IRα-subunit (A), IRβ-subunit (B), IRS-1 (C), IRS-2 (D), two subunits of Shc (E,F), p-IRβ-subunit (G), and representative control blot for α-tubulin (H). Note the significant increase in IRβ-subunit, IRS-2 and p-IRβ-subunit protein contents in islet lysates from DEX rats. The protein levels of IRα-subunit, IRS-1 and Shc proteins were similar between DEX and CTL islets. The figures are representative immunoblots performed at least six times on separate islet extracts. Data are means ± S.E.M. *significantly different vs CTL. \( P < 0.05 \) for unpaired Student \( t \)-test.
Figure 3. Increase of PI3-K, p-AKT and p70S6K protein levels in DEX islets. Protein levels of PI3-K (A), AKT (B), p-AKT (C), p70S6K (D), p-p70S6K (E), ERK1/2 (F), p-ERK1/2 (G), and representative control blot for α-tubulin (H). Note the significant increase in PI3-K, p-AKT and p70S6K protein contents in islet lysates from DEX rats. The protein levels of AKT, ERK1/2 and p-ERK1/2 proteins were similar between DEX and CTL islets. The figures are representative immunoblots performed at least six times on separate islet extracts. Data are means ± S.E.M. *significantly different vs CTL. $P < 0.05$ for unpaired Student $t$-test.
an islet cell knockout of IRS-2 (Cantley et al., 2007), exhibit a marked defect in insulin secretion in response to glucose. However, overexpression of IRS-2 in isolated rat islets leads to increased basal and glucose-stimulated insulin secretion (Mohany et al., 2005). These data emphasize the importance of IR and IRS proteins for the adequate control of insulin secretion in pancreatic β cells. Although we cannot rule out the participation of IRS-1, the increase in IRβ-subunit, p-IRβ-subunit and IRS-2 protein levels in DEX islets may exert a positive role on the augmented insulin secretion observed in our insulin-resistant rats induced by dexamethasone.

Mice knockout for IR, specifically in β cells, show a decrease in β-cell mass in an age-dependent manner (Kulkarni et al., 1999a). In addition, the global knockout of IRS-2 lead to a type 2 diabetes mellitus-like phenotype due to reduced β-cell mass (Withers et al., 1998; Kubota et al., 2000). A similar reduction in β-cell mass was observed in mice with ablation of IRS-2 in β-cells by a pancreas-restricted knockout, using the pancreatic-duodenal homeobox factor-1 (PDX-1)-promoter-driven Cre system (Cantley et al., 2007). Nevertheless, β-cell proliferation significantly increases in rat islets overexpressing IRS-2 whilst IRS-1 seems to be less effective (Mohany et al., 2005). These results demonstrate the participation of IR and IRS proteins in the regulation of β-cell growth. The increased levels of IRβ-subunit, p-IRβ-subunit and IRS-2 proteins in islets from DEX rats may favor the augmentation in the β-cell mass and proliferation that is found in this insulin-resistant model (Rafacho et al., 2009, Rafacho et al., 2010b, Rafacho et al., 2011).

We also observed higher levels of the PI3-K, p-AKT, and p70S6K, but not of the AKT, p-p70S6K, ERK1/2, and p-ERK1/2 in islets from DEX rats (Fig. 3). These proteins are among the several downstream effectors of insulin signaling and also modulate the β-cell function and growth (Vasavada et al., 2006). Overexpression of AKT in mice leads to a marked increase in β-cell mass and proliferation (Bernal-Mizrachi et al., 2001). Although our present results showed similar AKT levels between DEX and CTL rats, we demonstrated that the phosphorylated levels of AKT increases in DEX islets, which agreed with previous observations (Rafacho et al., 2009), and may support the increase of β-cell function and proliferation observed in DEX rats. Similarly, p70S6K has also been demonstrated to exert a positive effect on β-cell function and growth (Pende et al., 2000) supporting our observation, at least in part, of an increased p70S6K protein levels in DEX islets. The ERK1/2 proteins are the effectors of the MAPK signaling pathway, a mitogen branch of insulin signaling, but total and phosphorylated levels of these proteins were found similar in both groups. Thus, ERK1/2 proteins seem not to be the major signal for pancreatic β-cell mass expansion that was observed previously in this model (Rafacho et al., 2008b, Rafacho et al., 2009, Rafacho et al., 2010b, Rafacho et al., 2011). Based on these data we suggest that insulin signaling effectors such as PI3-K and AKT may have the major positive role in the endocrine pancreas adaptations developed by insulin-resistant rats.

Glucocorticoid receptor (GR) is a ligand-activated transcription factor that upon ligand binding dissociates from the heat shock proteins, translocates into the nucleus and bind as homodimer to GR responsive elements in promoter regions of glucocorticoid responsive genes, modulating gene transcription (Schäcke et al., 2002). In the present study, we did not detect differences in GR protein levels between DEX and CTL islets (Fig. 4). Although this does not exclude GR as a key regulator of gene transcription in islets from DEX rats, we are tempted to suggest that insulin signaling may exert a role in this process. Circulating insulin is significantly elevated in DEX rats after 24 h of dexamethasone treatment and remains high for the follow 4 days in our DEX model (Rafacho et al., 2011). Insulin stimulates amino acid uptake in cells, inhibits protein degradation and promotes protein synthesis (Saltiel and Kahn, 2001). Thus, it is feasible that insulin modulates the intracellular pro-protein synthesis events and modulates the increase of insulin secretion and β-cell mass through activation of IRS-2 /PI3-K/AKT pathway.

In summary, dexamethasone induces insulin resistance that leads to an increase in circulating insulin levels and enhancement of glucose-induced insulin secretion. IRS-2, but not IRS-1, and Shc protein contents are higher in islets from dexamethasone-treated rats than in control islets. This increase was accompanied by an augmentation in the PI3-K and p-AKT, but not in p-p70S6K and p-ERK1/2 protein levels. We conclude that IRS-2 may have a major role among the immediate substrates of the insulin receptor to link activated receptors to downstream insulin signaling components related to islet function and growth in this insulin-resistant rat model.

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