

# Intensive insulin treatment induces insulin resistance in diabetic rats by impairing glucose metabolism-related mechanisms in muscle and liver

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## Abstract

Insulin replacement is the only effective therapy to manage hyperglycemia in type 1 diabetes mellitus (T1DM). Nevertheless, intensive insulin therapy has inadvertently led to insulin resistance. This study investigates mechanisms involved in the insulin resistance induced by hyperinsulinization. Wistar rats were rendered diabetic by alloxan injection, and 2 weeks later received saline or different doses of neutral protamine Hagedorn insulin (1·5, 3, 6, and 9 U/day) over 7 days. Insulinopenic-untreated rats and 6U- and 9U-treated rats developed insulin resistance, whereas 3U-treated rats revealed

the highest grade of insulin sensitivity, but did not achieve good glycemic control as 6U- and 9U-treated rats did. This insulin sensitivity profile was in agreement with glucose transporter 4 expression and translocation in skeletal muscle, and insulin signaling, phosphoenolpyruvate carboxykinase/glucose-6-phosphatase expression and glycogen storage in the liver. Under the expectation that insulin resistance develops in hyperinsulinized diabetic patients, we believe insulin sensitizer approaches should be considered in treating T1DM.

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## Introduction

Maintenance of glucose homeostasis depends on insulin-stimulated glucose uptake by muscle and adipose cells, brought about by an increase in levels of plasma membrane (PM) glucose transporter 4 (GLUT4; Huang & Czech 2007). In addition, the liver plays a decisive role in blood glucose homeostasis by maintaining the balance between glucose input (glucose uptake and glycogen synthesis) and output (glycogenolysis and gluconeogenesis; Vidal-Puig & O'Rahilly 2001). Insulin severely inhibits hepatic glucose output, suppressing gluconeogenesis and glycogenolysis, by inhibiting expression and activity of the key enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase; Barthel & Schmoll 2003).

Insulin resistance is involved in the pathogenesis of type 2 diabetes mellitus (T2DM; Björnholm & Zierath 2005). In type 1 diabetes mellitus (T1DM), apart from its well-known pathogenesis, insulin resistance has also been described in both undertreated insulinopenic and well-treated hyperinsulinemic subjects. Insulin resistance in insulinopenic diabetic rats has been associated with impaired glucose disposal in liver and skeletal muscle, similar to T2DM (Camps *et al.* 1992,

Kainulainen *et al.* 1994). However, little is known about insulin resistance in hyperinsulinized diabetic subjects.

Reduced GLUT4 protein expression plays an important role in insulin resistance of skeletal muscle, which has been described in experimental models of insulinopenic diabetes (Camps *et al.* 1992, Kainulainen *et al.* 1994). Conversely, although short-term hyperinsulinemia in diabetic rats increases GLUT4 expression in adipose tissue (Berger *et al.* 1989, Sivitz *et al.* 1989), long periods of hyperinsulinemia in T1DM might induce the contrary, as observed in obese and/or T2DM subjects (Machado *et al.* 1994, Ledwig *et al.* 2002).

In the liver, insulin inhibits gluconeogenesis in an insulin-receptor-mediated phosphoinositide 3-kinase and AKT (PI3K/AKT) dependent manner (Withers *et al.* 1998, Kubota *et al.* 2000, Michael *et al.* 2000). After insulin treatment (Andjelković *et al.* 1997), AKT is known to be translocated into the nucleus, where it can phosphorylate FOXO1. Phosphorylation of FOXO1 leads to nuclear exclusion (Van Der Heide *et al.* 2004) and to inhibition of FOXO1-dependent gene transcription (Biggs *et al.* 1999, Brunet *et al.* 1999), such as the genes of PEPCK and G6Pase proteins (Nakae *et al.* 2001, Altomonte *et al.* 2003).

Independent of the degree of insulin deficiency in diabetes, a tight control of blood glucose levels has been proposed to prevent complications and to improve life expectancy (Purnell *et al.* 1998, DCCT 2001). For that, intensive insulin treatment has been required, leading to peripheral hyperinsulinemia. We believe that hyperinsulinemia might induce insulin resistance; thus, requiring additional care in diabetic subjects. In the light of such concerns, we sought to investigate, in diabetic insulinopenic rats, the effect of different doses of insulin upon insulin sensitivity and the potential molecular mechanisms involved.

## Materials and Methods

### Materials

Neutral protamine Hagedorn (NPH) and regular insulin were purchased from Eli Lilly. Rabbit polyclonal antibodies against p-Tyr, PI3K (p85 $\alpha$ ) and GLUT4 were obtained from Millipore (Billerica, MA, USA). Rabbit polyclonal antibodies against pAKT 1/2/3 Ser473, pAKT 1/2/3 Thr308, AKT 1/2/3 (H-136), IRS-2 (H205), IR $\beta$  (c19), and FOXO1 (H128) were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA), and rabbit polyclonal antibodies against pGSK3- $\alpha/\beta$  (Ser21/9) and GSK-3 $\beta$  (27C10), by Cell Signaling Technology (Beverly, MA, USA). Secondary anti-rabbit IgG, conjugated with HRP, and protein A Sepharose 6MB were obtained from GE Healthcare UK Ltd (Little Chalfont, Buckinghamshire, England). Sodium thiopental was purchased from Cristalia (Itapira, São Paulo, Brazil); alloxan monohydrate, from Sigma-Aldrich; TRIZOL reagent, from Invitrogen (Invitrogen Life Technologies); and TaqMan gene expression (Assay-on-Demand) Rn00689876\_m1 (G6Pase, catalytic), Rn01529014\_m1 (PEPCK1, cytosolic), and Rn01775763\_g1 (glyceraldehyde-3-phosphate dehydrogenase (GAPDH)), from Applied Biosystems (Foster City, CA, USA). All chemicals used in western blotting were purchased from Invitrogen (Invitrogen Life Technologies) and general reagents used in RT-PCR, from Promega.

### Animals and treatment

Male Wistar rats (weighing  $\sim$ 260 g) were individually caged in an environment maintained at controlled temperature ( $23 \pm 2^\circ\text{C}$ ), and lighting (12 h light:12 h darkness cycle), and allowed free access to water and standard rodent chow diet (Nuvilab CR-1, Nuvital, Curitiba, Brazil). At week 12 of age, the animals were fasted overnight and rendered diabetic (D) by a single i.v. injection of alloxan (38 mg/kg BW). Non-diabetic control rats (ND) were injected with the same volume of isotonic (0.9% w/v) NaCl solution. The animals with polyuria ( $> 25$  ml/day), glycosuria  $> ++$ , but without ketonuria, were chosen for the study. Two weeks later, diabetic rats were subjected to a 7 day treatment with NPH insulin (I), daily doses of 1.5U (I1.5), 3U (I3), 6U (I6), and

9U (I9), divided into two injections (1/3 of the total dose at 0800 h and the remaining 2/3 at 1700 h), or saline (DS), the same volume twice a day. Blood and tissue collection as well as insulin tolerance tests were performed in anesthetized rats (sodium pentobarbital, 40 mg/kg BW, i.p.), on the day after the end of the treatment. Experimental procedures were approved by the ethics committee for Animal Research of the Institute of Biomedical Sciences of the University of São Paulo (protocol 044/2006).

### Intravenous insulin tolerance test

Tail blood samples were collected in *ad libitum* fed animals before (0 min) and 4, 8, 12, and 16 min after i.v. injection of regular insulin (0.75 U/kg BW). The constant rate for blood glucose disappearance during insulin tolerance test (kITT) was calculated based on the linear regression of the Neperian logarithm of blood glucose concentrations (test strips, Advantage, Roche). The tests were performed from 0900 to 1100 h.

### GLUT4 protein analysis by western blotting

Tissue collection was carried out at 1400 h, in 8 h food-deprived rats. Epididymal fat pad and skeletal muscle gastrocnemius were collected before (right side) and 10 min after (left side) regular insulin injection (4U) in portal vein. For subcellular fractionation, tissue samples were homogenized in sucrose buffer pH 7.4 (10 mmol/l Tris-HCl, 1 mmol/l EDTA and 250 mmol/l sucrose) and subjected to differential centrifugations to obtain PM, microsomal-enriched (M), and total membrane (TM) fractions of adipose tissue (Machado *et al.* 1994) and skeletal muscle (Mori *et al.* 2008). Protein (40  $\mu\text{g}$ ) from each sample was subjected to electrophoresis and immunodetection as described (Zanquetta *et al.* 2006). The blots were quantified by densitometry (ImageQuant TL, Amersham Biosciences UK Limited), and normalized for loading control by densitometry of the lane from the Coomassie stained gel (Ferguson *et al.* 2005). Finally, GLUT4 protein content was expressed as arbitrary unit per g of tissue, taking into account the total protein recovery in the sample. GLUT4 translocation index was calculated as follows: GLUT4 translocation index (%) =  $\text{PM GLUT4} \times 100 / (\text{PM GLUT4} + \text{M GLUT4})$ .

### G6pase and Pepck mRNA analysis by real-time PCR

Liver sampling was carried out at 1400 h, in 8 h food-deprived animals. Total RNA was isolated using Trizol reagent (Invitrogen), following manufacturer's instructions. Total RNA (2  $\mu\text{g}$ ) was used to synthesize cDNA using the IMPRON II Reverse Transcriptase. PCR amplifications were performed by ABI PRISM 7700 heat-cycler detection system (Applied Biosystems), according to the manufacturer's protocol, using specific assay (as described in the section Materials) for each target gene. The level of each gene

**Table 1** Morphometric and metabolic parameters of non-diabetic (ND) and diabetic rats treated with saline (DS) or with neutral protamine Hagedorn insulin at doses of 1.5 (I1.5), 3 (I3), 6 (I6), and 9 (I9) U/day. Basal glycemia and insulinemia were measured in blood collected at the beginning (time 0) of the insulin tolerance test. Data are mean  $\pm$  s.e.m. of 7–13 rats. One-way ANOVA, Student–Newman–Keuls *post hoc* test. Not all comparisons among insulin-treated groups are shown

	ND	DS	I1.5	I3	I6	I9
<b>Parameters</b>						
Body weight gain (g)	7.69 $\pm$ 0.88	1.45 $\pm$ 0.59 <sup>†</sup>	15.4 $\pm$ 6.5 <sup>‡</sup>	32.9 $\pm$ 0.86 <sup>¶</sup>	38.6 $\pm$ 1.20 <sup>¶</sup>	40.9 $\pm$ 4.2 <sup>¶</sup>
Adipose tissue (g)	3.54 $\pm$ 0.18	1.88 $\pm$ 0.20 <sup>†</sup>	2.31 $\pm$ 0.15*	2.85 $\pm$ 0.14 <sup>¶</sup>	3.24 $\pm$ 0.27 <sup>¶</sup>	3.05 $\pm$ 0.26 <sup>¶</sup>
Gastrocnemius (g)	1.77 $\pm$ 0.07	1.42 $\pm$ 0.10*	1.57 $\pm$ 0.07	1.61 $\pm$ 0.05	1.63 $\pm$ 0.05	1.56 $\pm$ 0.06
Glycosuria (mg/24 h)	Undetectable	202 $\pm$ 19.8	133 $\pm$ 24.2 <sup>¶,a</sup>	19.0 $\pm$ 10.0 <sup>¶</sup>	10.9 $\pm$ 4.7 <sup>¶</sup>	6.37 $\pm$ 3.2 <sup>¶</sup>
Basal plasma glucose (mg/dl)	106 $\pm$ 4.6	408 $\pm$ 22.3 <sup>‡</sup>	433 $\pm$ 27.8 <sup>‡,¶,a</sup>	268 $\pm$ 32.8 <sup>‡,¶</sup>	133 $\pm$ 41.3 <sup>¶,a</sup>	118 $\pm$ 41.3 <sup>¶,a</sup>
Basal plasma insulin ( $\mu$ U/ml)	38.8 $\pm$ 1.6	12.6 $\pm$ 0.88*	26.9 $\pm$ 1.5 <sup>b</sup>	94.1 $\pm$ 8.7 <sup>‡,¶</sup>	115 $\pm$ 7.9 <sup>‡,¶</sup>	220 $\pm$ 14.2 <sup>‡,¶,a</sup>

\* $P < 0.05$ , <sup>†</sup> $P < 0.01$ , and <sup>‡</sup> $P < 0.001$  versus ND; <sup>¶</sup> $P < 0.01$ , and <sup>¶</sup> $P < 0.001$  versus DS; <sup>a</sup> $P < 0.01$  and <sup>b</sup> $P < 0.001$  versus I3.

expression was related to a *Gapdh* internal control (Applied Biosystems). All reactions were performed in duplicate and a relative comparison to ND was made with appropriate cDNA. Resulting gene expression data were calculated and analyzed based on the  $2^{-\Delta\Delta C_t}$  method (Pfaffl 2001).

#### Insulin signaling proteins analysis by western blotting

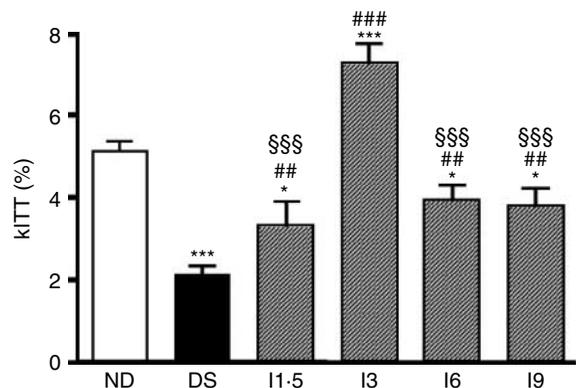
Liver sampling was carried out at 1400 h, in 8 h food-deprived animals. Tissue samples were collected before and 30 s after a 10 U/rat regular insulin injection in the portal vein (Kubota *et al.* 2008). Samples were immediately homogenized in ice-cold solubilization buffer, using a Polytron–Aggregate (Luzern, Switzerland) operated at maximum speed for 10 s. The extracts were incubated at 96 °C for 10 min and then centrifuged at 12 000 *g* at 4 °C for 30 min to remove insoluble material. Equal amounts of total protein (100  $\mu$ g protein) were subjected to electrophoresis, transferred to nitrocellulose membrane, and immunodetected with specific antibodies (pAKT-Ser, pAKT-Thr, pGSK, IRS1, IRS2, and IR $\beta$ ). Chemiluminescence was used to generate autoradiograms. Quantitative analysis of blots was performed by ImageQuant TL (Amersham Biosciences UK Limited).

#### Insulin signaling proteins immunoprecipitation

Liver samples collected as described earlier were homogenized in immunoprecipitation buffer and then centrifuged at 12 000 *g* at 4 °C for 30 min to remove insoluble material. Supernatants were used for immunoprecipitation with anti-IR $\beta$ , anti-IRS1, or anti-IRS2 (Anhê *et al.* 2007). Approximately, 500  $\mu$ g protein from each sample were incubated overnight with 2  $\mu$ g of the specific antibody (see figure legends) and 50  $\mu$ l protein A enriched Sepharose beads. The washed Sepharose beads were treated with Laemmli, subjected to SDS–PAGE and electrotransferred to nitrocellulose membranes. The nitrocellulose membranes were incubated with specific antibodies (phospho-Tyr and PI3K–p85), as described in figure legends and detected as described earlier.

#### Subcellular distribution of FOXO1

Cytosolic and nuclear protein fractions from liver samples were obtained based on Andrews and Faller method (Andrews & Faller 1991). Briefly, samples were pulverized in liquid nitrogen and resuspended in ice-cold PBS with dithiothreitol (DTT; 200  $\mu$ M) and phenylmethylsulphonyl fluoride (PMSF; 200  $\mu$ M). After centrifugation at 1000 *g* (10 min, 4 °C) the supernatant was recovered as the cytosolic fraction. The pellet was incubated (10 min, 4 °C) with hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl, 10 mM KCl, 0.2 mM DTT, 0.2 mM PMSF). NP-40 (10%) was added, and samples were then centrifuged at 15 000 *g* (30 s, 4 °C). High salt extraction buffer (20 mM HEPES pH 7.9, 1.5 mM MgCl, 0.5 mM EDTA, 0.2 mM DTT, 0.2 mM PMSF, 420 mM NaCl) was added to the pellet, providing nucleus lyses after a 20 min incubation at 4 °C. The supernatant yielded by centrifugation at 12 000 *g* (2 min)



**Figure 1** Effect of insulin treatment on insulin sensitivity. Diabetic rats underwent a 7 day treatment with saline (DS) or with NPH insulin (I) at daily doses of 1.5U (I1.5), 3U (I3), 6U (I6), and 9U (I9). Non-diabetic rats (ND) were analyzed as control group. kITT, constant rate for blood glucose disappearance during insulin tolerance test. Data are mean  $\pm$  s.e.m. of 7–13 animals. One-way ANOVA followed by Student–Newman–Keuls. Not all comparisons among insulin-treated groups are shown. \* $P < 0.05$  and \*\*\* $P < 0.001$  versus ND; \*\* $P < 0.01$  and \*\*\* $P < 0.001$  versus DS; \$\$\$ $P < 0.001$  versus I3.

was recovered as nuclear fraction. Cytosolic and nuclear proteins (100 µg) were used for immunodetection of FOXO1, as described in previous sections.

#### Plasma glucose, plasma insulin, and liver glycogen

Plasma and urine samples were assayed for glucose by Glicose Enzimática kit (ANALISA Diagnostica, Belo Horizonte, MG, Brazil). Plasma was also assayed for insulin by Coat-A-Count kit (DPC Diagnostic Products, Los Angeles, CA, USA). Glycogen was extracted from 100 mg of liver samples collected as described earlier. Samples were treated for glycogen breakdown, pelleted with ethanol and saturated Na<sub>2</sub>SO<sub>4</sub>, and resuspended for final measurement of glucose concentration.

#### Statistical analysis

Data are expressed as mean ± S.E.M. The number of animals or experiments is indicated in legends. Comparisons among the groups were made by one-way ANOVA, followed by Student–Newman–Keuls as *post hoc* test. Basal and insulin-stimulated conditions were compared by paired Student's *t*-test.

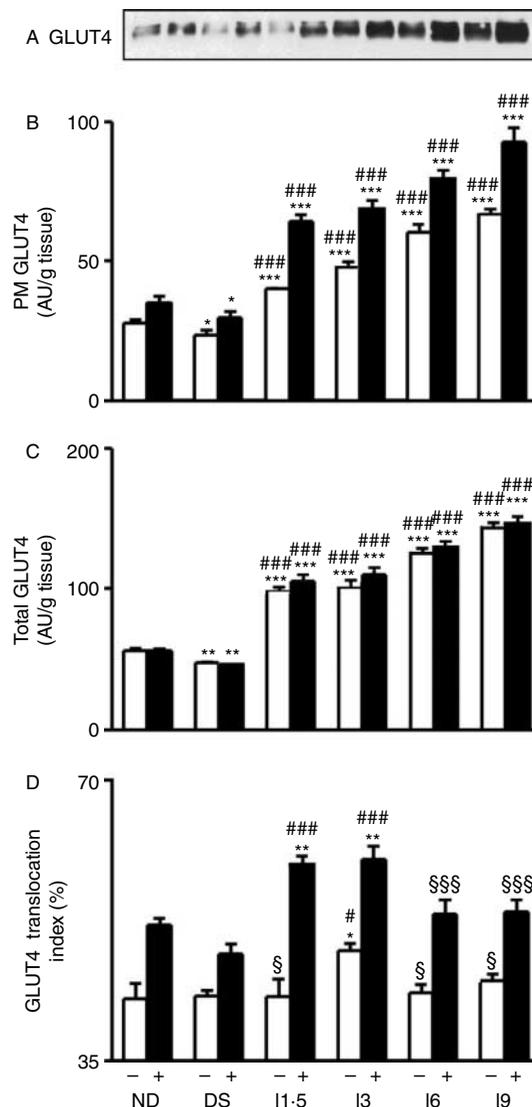
## Results

#### Morphometric and metabolic parameters of diabetic rats treated or not with insulin

Table 1 shows that diabetes induced lower body weight gain (~19% of ND value,  $P < 0.01$ ), and decreased adipose (53% of ND value,  $P < 0.01$ ) and muscle (80% of ND value,  $P < 0.05$ ) tissue weights. As expected, high plasma glucose (approximately fourfold,  $P < 0.001$ ) and low plasma insulin (1/3,  $P < 0.05$ ) levels, as well as pronounced glycosuria were observed in saline-treated diabetic animals. Increased body weight ( $P < 0.001$ ) and fat mass ( $P < 0.01$ ), were found in I3-, I6-, and I9-treated diabetic rats, compared with saline-treated rats. Insulin treatment reduced glycosuria and plasma glucose in diabetic rats in a dose-dependent manner. Higher doses of I6 and I9 were able to decrease plasma glucose levels to values similar to those found in ND rats. However, this glycaemic control was achieved with plasma insulin levels three- and six-fold higher than ND, respectively, for I6 and I9 ( $P < 0.001$ ).

#### Effect of different doses of insulin replacement on insulin sensitivity of diabetic rats

As indicated by the insulin tolerance test (Fig. 1), diabetic rats showed a significant decrease in insulin sensitivity (41% of ND,  $P < 0.001$ ). The 1.5, 6, and 9U doses increased ( $P < 0.05$  to  $P < 0.01$  versus DS) the kITT, but did not restore it to ND value ( $P < 0.05$  versus ND). Treatment with 3U of insulin maximally increased the kITT value (3.5-fold versus DS,  $P < 0.001$ ), reaching value approximately twofold higher than



**Figure 2** Effect of insulin treatment on basal and insulin-stimulated GLUT4 protein content in white adipose tissue. Diabetic rats underwent a 7 day treatment with saline (DS) or with NPH insulin (I) at daily doses of 1.5U (I1.5), 3U (I3), 6U (I6), and 9U (I9). Non-diabetic rats (ND) were analyzed as control group. Periepididymal fat pad was collected before (–) and 10 min after (+) insulin injection (4U/rat). GLUT4 protein was assessed in plasma membrane (PM) and microsomal (M) fractions. (A) Typical autoradiogram of GLUT4 protein in PM samples according to the sequence presented at the bottom. (B) PM GLUT4 protein content expressed as arbitrary units (AU) per g of tissue. (C) Total GLUT4 content (PM+M) expressed by g of tissue. (D) Translocation index, showing the PM GLUT4 content as a percentage of the total GLUT4. Data are mean ± S.E.M. of six animals. One-way ANOVA followed by Student–Newman–Keuls was used to compare groups under the same condition (basal or insulin-stimulated). Not all comparisons among insulin-treated groups are shown. Paired Student's *t*-test showed a significant difference between basal (–) and insulin-stimulated (+) conditions for all groups in panels B and D (not shown). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  versus ND; # $P < 0.05$  and ### $P < 0.001$  versus DS; § $P < 0.05$  and §§§ $P < 0.001$  versus I3.

that observed in I1.5-, I6-, and I9-treated rats ( $P < 0.001$ ), and even higher than that observed in ND rats (50%,  $P < 0.001$ ).

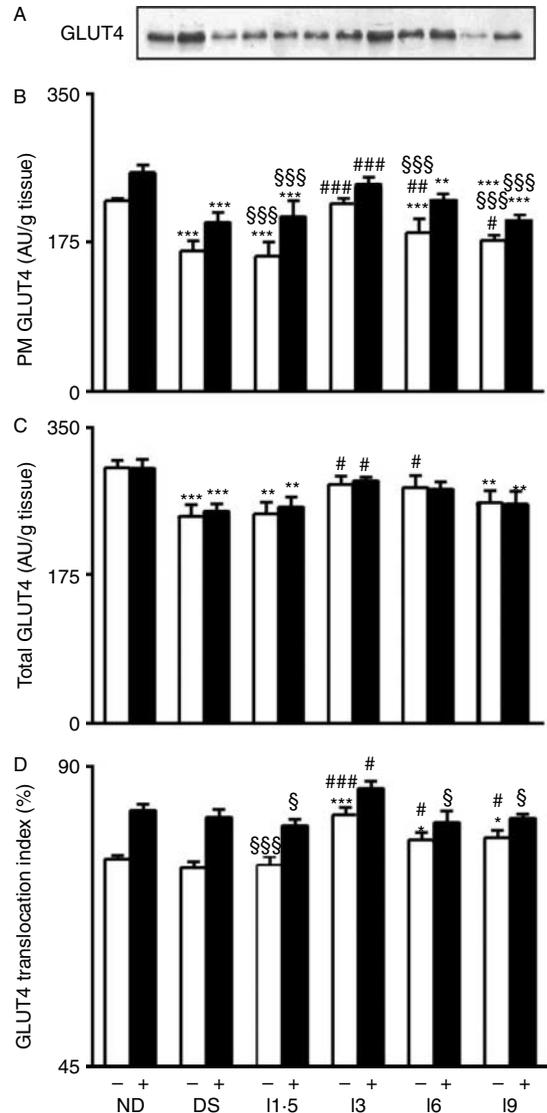
### I3 treatment increases adipose and muscle GLUT4 content and translocation

Figure 2 shows GLUT4 protein analysis in white adipose tissue. Figure 2B shows that diabetes decreased the GLUT4 content in PM ( $P < 0.05$ ), and insulin treatment induced a dose-dependent increase in GLUT4 in both basal and insulin-stimulated conditions ( $P < 0.001$  versus ND and DS). A similar pattern is observed in total GLUT4 protein, measured in samples containing total cellular proteins (Fig. 2C). By relating PM GLUT4 content to total GLUT4 content (both expressed in AU/g of tissue), the percentage of total GLUT4 present in the PM at the moment of tissue withdrawal, referred to as translocation index (Fig. 2D), was obtained. Although the translocation index was not altered by diabetes, I3-treated group was the only one to show both basal and insulin-stimulated translocation index higher than DS and ND ( $P < 0.05$  to  $P < 0.001$ ). Importantly, I6 and I9 treatments decreased this parameter ( $P < 0.001$ ) compared with I3. Finally, acute effects of insulin were observed in all groups (panels B and C), comparisons not shown in the figure.

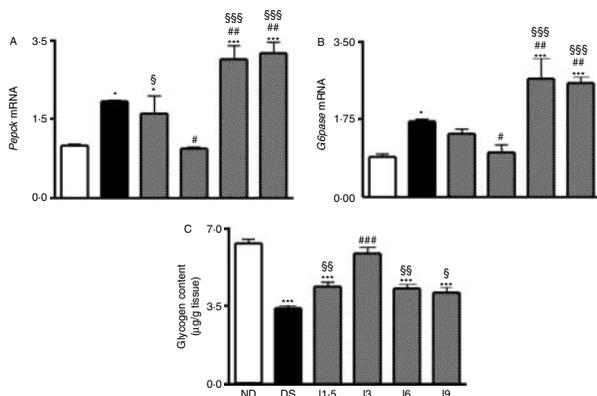
Figure 3 shows GLUT4 protein analysis in gastrocnemius skeletal muscle. Figure 3B shows that diabetes decreased GLUT4 in PM ( $P < 0.001$ ) in both basal and insulin-stimulated conditions. I3-treated diabetic animals were able to increase GLUT4 protein ( $P < 0.001$  versus DS), under both conditions, to values similar to ND; and higher ( $P < 0.001$ ) than those observed in I1.5-, I6-, and I9-treated rats. A similar pattern of results was found in total GLUT4 protein, measured in samples containing TM proteins (Fig. 3C). Only I3- and I6-treated rats were able to restore the total GLUT4 content to ND levels. Figure 3D shows the translocation index. Although diabetes did not alter this parameter, once again I3-treated animals showed the maximal increase, reaching values higher than those observed in I1.5-, I6-, and I9-treated rats ( $P < 0.05$  to  $P < 0.01$ ). In addition, as in adipose tissue, acute insulin effect upon GLUT4 translocation to PM (Fig. 3B and C) was observed in all experimental conditions (statistics not shown in the figure).

### I3 treatment represses *Pepck* and *G6pase* gene expression and enhances glycogen content in liver

Similar regulations of the mRNA expression of *Pepck* (Fig. 4A) and *G6pase* (Fig. 4B) enzymes were observed. Diabetes increased *Pepck* and *G6pase* mRNA content (approximately twofold,  $P < 0.05$ ), and I3 treatment decreased both to levels of ND rats. Importantly, I6 and I9 treatments had the opposite effect, increasing these mRNAs to values higher than those observed in ND and DS ( $P < 0.01$  to  $P < 0.001$ ). As a consequence of the regulation of these enzymes, glycogen storage was conversely modulated



**Figure 3** Effect of insulin treatment on basal and insulin-stimulated GLUT4 protein content in skeletal muscle. Diabetic rats underwent a 7 day treatment with saline (DS) or with NPH insulin (I) at daily doses of 1.5U (I1.5), 3U (I3), 6U (I6), and 9U (I9). Non-diabetic rats (ND) were analyzed as control group. Gastrocnemius was collected before (-) and 10 min after (+) insulin injection (4U/rat). GLUT4 protein was assessed in plasma membrane (PM) and microsomal (M) fractions. (A) Typical autoradiogram of GLUT4 protein in PM samples according to the sequence presented at the bottom. (B) PM GLUT4 protein content expressed as arbitrary units (AU) per g of tissue. (C) Total GLUT4 content (PM+M) expressed by g of tissue. (D) Translocation index, showing the PM GLUT4 content as a percentage of the total GLUT4. Data are mean  $\pm$  S.E.M.  $n = 6$ . One-way ANOVA followed by Student–Newman–Keuls was used to compare groups under the same condition (basal or insulin-stimulated). Not all comparisons among insulin-treated groups are shown. Paired Student's *t*-test showed a significant difference between basal (-) and insulin-stimulated (+) conditions for all groups in panels B and D (not shown). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  versus ND; # $P < 0.05$  and ### $P < 0.001$  versus DS; \$ $P < 0.05$  and \$\$\$ $P < 0.001$  versus I3.



**Figure 4** Effect of insulin treatment on liver phosphoenolpyruvate carboxykinase (*Pepck*) and glucose-6-phosphatase (*G6pase*) mRNA expression, and on glycogen content. Diabetic rats underwent a 7 day treatment with saline (DS) or with NPH insulin (I) at daily doses of 1.5U (I1.5), 3U (I3), 6U (I6), and 9U (I9). Non-diabetic rats (ND) were analyzed as control group. *Pepck* and *G6pase* mRNAs were related to GAPDH as internal control, and expressed as arbitrary units. Data are mean  $\pm$  S.E.M. of six to seven animals. One-way ANOVA, Student–Newman–Keuls as *post hoc* test. Not all comparisons among insulin-treated groups are shown. \* $P < 0.05$  and \*\*\* $P < 0.001$  versus ND; # $P < 0.05$ , ## $P < 0.01$ , and ### $P < 0.001$  versus DS; § $P < 0.05$ , §§ $P < 0.01$ , and §§§ $P < 0.001$  versus I3.

(Fig. 4C). Diabetes decreased glycogen content to  $\sim 50\%$  of ND ( $P < 0.001$ ), I3 treatment increased it to a value similar to that observed in ND, and I6 and I9 decreased it again (respectively,  $P < 0.01$  and  $P < 0.05$  versus I3).

### I3 maximally increases insulin signaling in liver

Figure 5A shows that diabetes decreased basal and insulin-stimulated tyrosine phosphorylation of IR $\beta$  ( $P < 0.05$ ). Maximal upregulation of basal and insulin-stimulated tyrosine phosphorylation of IR $\beta$  was achieved with 3U of insulin. Compared with I3 effect, I6 and I9 treatments decreased the IR $\beta$  phosphorylation in basal and insulin-stimulated conditions ( $P < 0.05$  to  $P < 0.001$ ). Figure 5B demonstrates that diabetes did not alter basal IRS2 tyrosine phosphorylation, but this parameter was equally upregulated by all doses of insulin treatment ( $P < 0.001$  versus ND and DS). Insulin-stimulated IRS2 tyrosine phosphorylation was decreased in D rats ( $P < 0.001$ ), and I3, I6, and I9 treatments increased this phosphorylation, with maximal effect observed in I3 dose ( $P < 0.001$  versus DS). Figure 5C shows that, compared with IRS2 phosphorylation, IRS2 in association with PI3K was similarly regulated, showing maximal improvement in I3-treated rats, both under basal and insulin-stimulated conditions ( $P < 0.05$  to  $P < 0.001$  versus I1.5, I6, and I9). Figure 5D shows that, despite unchanged threonine phosphorylation of AKT in DS animals, I3 treatment induced the maximal increase in this parameter, mainly under insulin-stimulated condition ( $P < 0.001$  versus I6 and I9). Similar results were observed for serine phosphorylation of AKT (Fig. 5E), except for the fact that

maximal effects of I3 were more evident under basal condition. Figure 5F shows diabetes-induced reduction in basal and insulin-stimulated GSK3 phosphorylation ( $P < 0.001$  versus ND). Under basal condition, maximal recovery of GSK3 phosphorylation was observed in I3-treated rats ( $P < 0.001$  versus I1.5 and I6;  $P < 0.05$  versus I9). Under insulin-stimulated condition, GSK3 phosphorylation increased with I3 ( $P < 0.001$ ), but also with I6 and I9 ( $P < 0.05$ ) treatments. Finally, in accordance with the representative immunoblots of total IR $\beta$  (5A), total IRS2 (5B), total AKT (5D and 5E), and total GSK3 (5F) protein contents, no differences were observed among the groups.

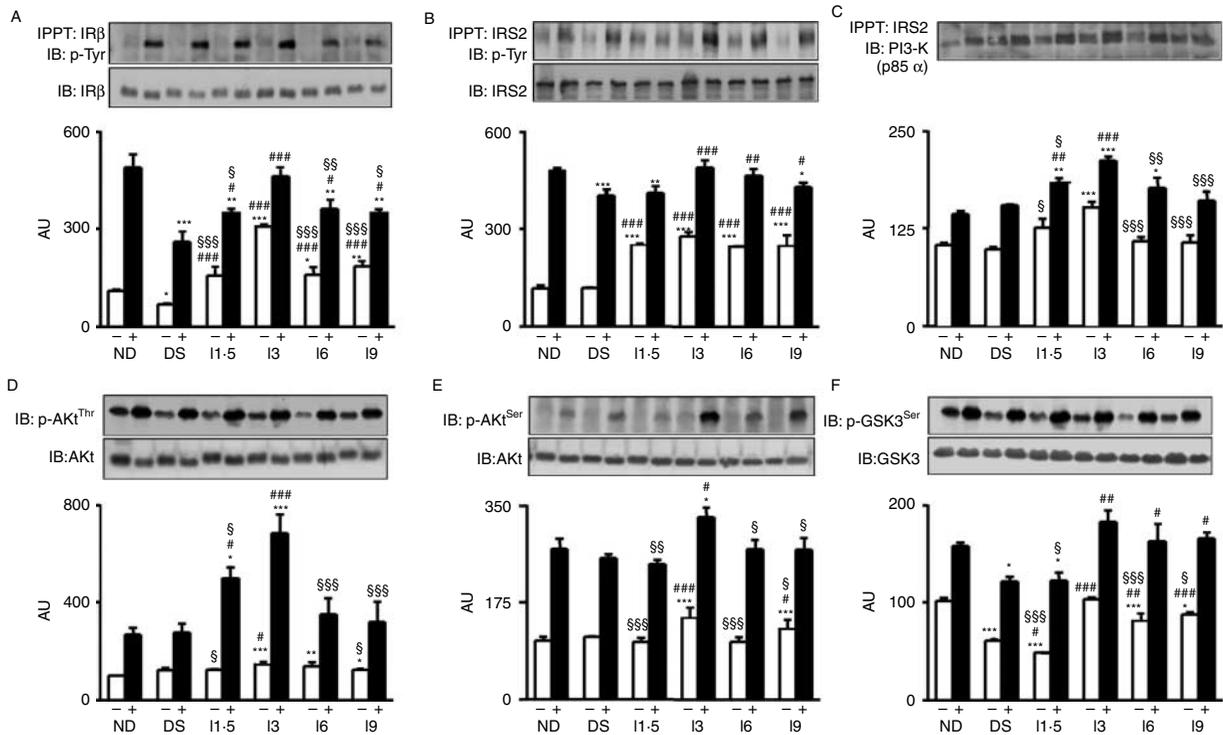
### I3 recovers cytoplasmic accumulation of FOXO1 in the liver

Insulin-mediated reduction in *G6pase* and in *Pepck* is achieved by cytoplasmic arresting of the transcription factor FOXO1 (Mounier & Posner 2006). Increased cytoplasmic accumulation of FOXO1 is facilitated by its phosphorylation via AKT. To correlate our findings of *Pepck* and *G6pase* mRNA contents with insulin signaling, we then assessed FOXO1 relative content in cytoplasm as a parameter of its cytoplasmic arresting. Figure 6 shows the cytoplasmic and nuclear content of FOXO1. The cytoplasmic/nuclear level of FOXO1 is decreased in D rats (46%,  $P < 0.001$ ). Replacement with high doses of insulin (6 and 9 U/day) resulted in partial recovery of cytoplasmic/nuclear levels of Foxo1 ( $\sim 72\%$  of ND values,  $P < 0.05$ ). However, 3U of insulin totally recovered cytoplasmic/nuclear FOXO1 level, to a value similar to that found in ND rats, and significantly higher than that observed in rats treated with 1.5, 6, and 9U of insulin ( $P < 0.05$ ).

## Discussion

This study was designed to investigate: 1) whether hyperinsulinization of diabetic rats, required for good glycemic control, induces insulin resistance, and 2) the potential territories and molecular mechanisms involved. The main finding of our study was that, in fact, high doses (6 and 9 U/day) of insulin induced important insulin resistance, whereas the intermediate dose of 3 U/day induced maximal improvement on insulin sensitivity, although glycemic control was not achieved. Furthermore, because of altered GLUT4 expression/translocation in skeletal muscle and PEPCK/G6-Pase expression in the liver, these territories were pointed out as being involved in the modulations of insulin sensitivity.

In this study, we used alloxan for diabetes induction because it is known to lead ketosis-prone diabetes in rat that resembles human T1DM (Federiuk *et al.* 2004). Besides, alloxan is as effective as streptozotocin and the same grade of  $\beta$ -cell loss can be achieved with both drugs; thus, no differences are to be expected between the models (Lenzen 2008). Although insulin resistance is pathognomonic of T2DM, it was also observed in streptozotocin-treated rats



**Figure 5** Effect of insulin treatment on liver insulin signaling. Diabetic rats underwent a 7 day treatment with saline (DS) or with NPH insulin (I) at daily doses of 1.5U (11.5), 3U (13), 6U (16), and 9U (19). Non-diabetic rats (ND) were analyzed as control group. Liver samples were collected before (–) and after (+) insulin injection (10U/rat) in portal vein. Aliquots containing the same amount of protein were immunoprecipitated (IPPT) with anti-Irβ (A) and anti-IRS2 (B and C) antibodies and immunoblotted (IB) with anti-phosphotyrosine antibody (A and B). Membrane with IRS2 immunoprecipitated was stripped and reprobed with anti-PI3K antibody (subunit p85 $\alpha$ ) for association analysis (C). For total protein analysis, membranes were stripped and reprobed with respective antibodies. At the top of each panel, representative autoradiogram of phosphorylated protein (upper) and total protein content (lower). (A) Tyrosine phosphorylation of insulin receptor IR $\beta$ -subunit; (B) tyrosine phosphorylation of IRS2; (C) IRS2 and PI3K association; (D) threonine (Thr) phosphorylation of AKT; (E) serine (Ser) phosphorylation of AKT; (F) serine phosphorylation of GSK3. In panels A, B, D, E, and F graphs correspond to the amount of phosphorylated proteins, and the amount of total proteins are not shown in graphs since they did not vary among the groups. Data are mean  $\pm$  s.e.m. of six animals. One-way ANOVA followed by Student–Newman–Keuls was used to compare groups under the same condition (basal or insulin-stimulated). Not all comparisons among insulin-treated groups are shown. Paired Student's *t*-test revealed significant differences between basal (–) and insulin-stimulated (+) conditions for all groups (not shown), except for the total protein content. \* $P$ <0.05, \*\* $P$ <0.01, and \*\*\* $P$ <0.001 versus ND; # $P$ <0.05, ## $P$ <0.01, and ### $P$ <0.001 versus DS; \$ $P$ <0.05, \$\$ $P$ <0.01, and \$\$\$ $P$ <0.001 versus 13.

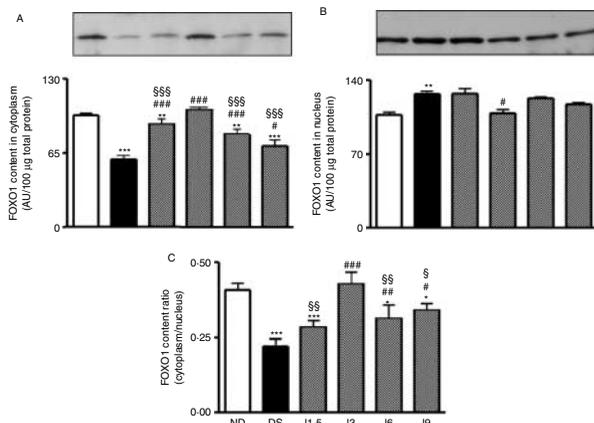
(Blondel & Portha 1989), and we now show the same effect in alloxan-treated rats.

Some metabolic alterations, as observed here, have already been pointed out to contribute to insulin resistance in hypoinsulinemic diabetic rats, such as deficient GLUT4 expression and translocation in skeletal muscle (Liu *et al.* 2010) and increased hepatic glucose output (Blondel & Portha 1989, Burcelin *et al.* 1995). Now, we show in the liver of diabetic rats that: 1) GSK3 activation decreased, contributing to impaired glycogen synthesis and 2) nuclear content of FOXO1 decreased, which concurs with the increased expression of FOXO1-dependent genes, such as *Pepck* and *G6pase*, contributing to enhanced gluconeogenesis and glucose outflow. In this regard, excess glucocorticoids and free fatty acids were demonstrated as able to impair

insulin-induced suppression of endogenous glucose production in diabetic rats (Bitar 2001). Besides, hyperglycemia *per se* may also be involved in the insulin resistance observed in untreated diabetic rats.

In this study, the most important findings are related to insulin resistance as a consequence of therapeutic intervention. Insulin resistance has been described in diabetic insulin-treated patients (DeFronzo 1997), and despite its impact on glycemic control, the inadvertent insulin resistance can be deleterious. In fact, intensive insulin therapy in T1DM has already been correlated with an increased incidence of some complications of diabetes such as microangiopathy (Uruska *et al.* 2010).

A focus of this study was determining molecular mechanisms involved in the insulin therapy-induced changes



**Figure 6** Effect of insulin treatment on liver FOXO1 protein expression and subcellular localization. Diabetic rats underwent a 7 day treatment with saline (DS) or with NPH insulin (I) at daily doses of 1.5U (I1.5), 3U (I3), 6U (I6), and 9U (I9). Non-diabetic rats (ND) were analyzed as control group. (A) Western blotting analysis of FOXO1 protein in cytoplasm: at the top, representative autoradiogram and at the bottom, cytoplasmic FOXO1 content expressed as AU/100 µg of total protein subjected to electrophoresis. (B) Western blotting analysis of FOXO1 protein in nucleus: at the top, representative autoradiogram and at the bottom, cytoplasmic FOXO1 content expressed as AU/100 µg of total protein subjected to electrophoresis. (C) Cytoplasmic/nuclear FOXO1 content ratio. Data are mean  $\pm$  S.E.M. of six animals. One-way ANOVA, Student–Newman–Keuls as *post hoc* test. Not all comparisons among insulin-treated groups are shown. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  versus ND; # $P < 0.05$ , ## $P < 0.01$ , and ### $P < 0.001$  versus DS; § $P < 0.05$ , §§ $P < 0.01$ , and §§§ $P < 0.001$  versus I3.

in insulin sensitivity. Adipose tissue and skeletal muscle were our first focus, since impaired glucose disposal in these tissues is currently involved in insulin resistance. Taking this into consideration, we investigated how the range of insulin dosages used herein would interfere with GLUT4 expression and translocation into PM, which have been currently involved in insulin resistance (Wallberg-Henriksson & Zierath 2001).

In adipose tissue, we found that increasing doses of insulin progressively upregulate total cellular GLUT4 content, as well as its translocation to the PM, which does not match with the dose effects observed in insulin sensitivity. In contrast, in transgenic mice overexpressing GLUT4 selectively in adipose tissue, whole-body insulin sensitivity improves, but this may be accounted for by the fact that in transgenic mice the GLUT4 protein increase is enormous (10–20 times), and the plasma insulin levels show a compensatory decrease (Shepherd *et al.* 1993, Carvalho *et al.* 2005). On the other hand, the present results also indicate a progressive increase in local insulin sensitivity in adipose tissue, which is in agreement with the progressive fat mass gain observed. Actually, weight gain and even obesity have been related to intensive insulin treatment of type 1 diabetic subjects (DCCT 2001). In contrast to what we observed in adipose tissue, GLUT4 expression and translocation in skeletal muscle of diabetic rats

reached the maximal improvement with the intermediate insulin dose of 3U; and high doses of 6U and 9U decreased the GLUT4 translocation. This profile of hyperinsulinemia-induced modulation of GLUT4 in muscle is in accordance with that observed in whole-body insulin sensitivity, and reveals the muscle participation in hyperinsulinemia-induced insulin resistance, as observed in untreated-diabetic rats.

Increased hepatic and renal glucose outflow are known to play important roles in hyperglycemia. This may occur due to insulin resistance, as in T2DM (Meyer *et al.* 1998, Gerich 2010) or lack of insulin, as in T1DM (Blondel & Portha 1989, Burcelin *et al.* 1995). Insulin inhibits glucose outflow mostly by decreasing gluconeogenesis; but also by decreasing breakdown and increasing synthesis of glycogen (Blondel & Portha 1989, Burcelin *et al.* 1995). Several indirect and direct mechanisms have been described as involved in insulin-regulated inhibition of hepatic glucose production (Girard 2006). However, three mechanisms have been pointed out: 1) inhibition of PEPCK, key enzyme for gluconeogenesis; 2) stimulation of GSK3, key enzyme for glycogen synthesis; and 3) inhibition of G6Pase, responsible for glucose-6-phosphate dephosphorylation, key step to obtain glucose, which is able to cross throughout the GLUT2 transporter (O'Brien *et al.* 2001). In this context, we found that the intermediate dose of 3U maximally reduced *Pepck* and *G6pase* expression and increased serine phosphorylation of GSK3, highlighting the participation of liver in the maximal effect of this insulin dose on whole-body insulin sensitivity improvement. Notably, all these parameters were impaired by the doses of 6U and 9U of insulin, despite the better glycemic control promoted by such high doses. In accordance with all these enzymes regulations, hepatic glycogen content was highest in 3U-treated diabetic rats, and decreased in 6U- and 9U-treated rats, reinforcing the effects of insulin therapy upon hepatic insulin sensitivity and; consequentially, on glucose outflow. According to that, increased fat accumulation secondary to excessive insulin replacement has already been proposed as able to induce liver insulin resistance (Liu *et al.* 2009).

Mechanisms of PEPCK, G6Pase and GSK3 regulation are related to intracellular insulin signaling which was also investigated. After insulin binding to its receptor (IR), tyrosine autophosphorylation of IR is a key step to trigger intracellular insulin signaling (Taniguchi *et al.* 2006). Our data show that insulin-induced tyrosine phosphorylation of IR is reduced in liver of untreated and 6U- and 9U-treated diabetic rats, being maximally upregulated in the liver of 3U-treated diabetic rats. Sequential events downstream of IR phosphorylation, such as IRS2/PI3K association (an important step for insulin-mediated inhibition of gluconeogenesis) (Taniguchi *et al.* 2006, Guo *et al.* 2009), were concordantly regulated. In addition, AKT phosphorylation in both serine and threonine also revealed the same regulation: i.e. decreased in insulinopenic diabetic rats, maximal upregulation in 3U-treated diabetic rats, and decreased again in 6U- and 9U-treated rats.

The present results in the liver suggest that regulation of IRS2 by insulin, irrespective of IRS1, is enough to control

levels of PEPCK and G6Pase expression, as well as GSK3 activation. Although it has been initially suggested that IRS1 is linked to glucose homeostasis, while IRS2 would be related to lipid metabolism (Taniguchi *et al.* 2005), recent studies using *Irs2* knockout mice have shown hepatic insulin resistance and increased gluconeogenesis, showing the importance of IRS2 in hepatic glucose metabolism. In addition, an important difference between IRS1 and IRS2 signaling branches has recently been determined, showing that IRS2, but not IRS1 deficient mice present hepatic insulin resistance in insulinopenic situations such as fasting (Kubota *et al.* 2008). This corroborates the results currently observed in our insulinopenic diabetic rats.

Insulin-induced suppression of PEPCK and G6Pase expression involves the phosphorylation/activation of AKT and the downstream repression of FOXO1 transcriptional activity by decreasing its nuclear content (Yeagley *et al.* 2001, Van Der Heide *et al.* 2004). In accordance with that, we now show that high insulin doses of 6U and 9U decreased AKT activation, and increased nuclear FOXO1 accumulation, which concur with the enhanced PEPCK and G6Pase expression observed. In accordance with our findings, it has been demonstrated that the absence of IRS2 in hepatocytes results in decreased AKT and increased PEPCK and G6Pase expression (Valverde *et al.* 2003).

Altogether, data collected in diabetic rats herein show that insulin therapy with high doses of insulin (necessary to achieve good glycemic control) induced insulin resistance, whereas an intermediate dose optimally improved insulin sensitivity, but did not normalize glycemia. Both up- and downregulations of insulin sensitivity were accompanied by concordant modulations of GLUT4 expression and translocation in skeletal muscle, and insulin signaling, PEPCK/G6Pase expression, and glycogen storage in the liver. These results highlight the participation of muscle and liver in the glucose homeostasis of insulin-treated diabetic rats. Accordingly, the expectation of insulin resistance development in hyperinsulinized diabetic patient must be kept in mind, and insulin sensitizer approaches should be considered in these states.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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