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Running title: D-glucose regulates IRS-2 biosynthesis

**The Metabolisable Hexoses D-Glucose and D-Mannose Enhance the Expression of IRS-2 but not of IRS-1 in Pancreatic β-Cells**

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**Key words:** IRS-2, diabetes, β-cells, pancreatic islets

**Abbreviations:** IRS-1 and -2, insulin receptor substrate-1 and -2; CREB, cAMP response element binding protein; TBS-T, Tris-buffered saline/Tween 20; PBS, Phosphate Buffered Saline; HEPES, [4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid];
Abstract

D-glucose regulates maintenance and function of pancreatic β-cells. Several studies have shown that IRS-2, but not IRS-1, is necessary to maintain and sufficient to expand functional β-cell mass. We therefore analyzed the expression of IRS-2 and IRS-1 in β-cells after culture in the presence of various concentrations of D-glucose and other metabolisable or non-metabolisable hexoses. D-glucose increased *Irs-2* transcription and IRS-2 accumulation in a dose-dependent manner (1.6 to 25 mmol/l), with a 3-fold increased plateau after 10 h. In contrast, the expression of IRS-1 remained unaffected. D-glucose also induced phosphorylation of IRS-2 while non-metabolisable hexoses did neither affect expression nor phosphorylation. D-glucose-mediated elevation and phosphorylation of IRS-2 were independent of autocrine insulin action although insulin itself could transiently and slightly enhance IRS-2 expression.
Introduction

Although impaired peripheral insulin sensitivity is thought to be the primary cause for type 2 diabetes, it has been recognized that pancreatic β-cells can prevent the onset of overt hyperglycemia by secreting compensatory amounts of insulin. β-cell mass is regulated through changes in cell number and size by humoral, neural and intraislet factors (Bernard-Kargar and Ktorza, 2001; Bonner-Weir, 2000). Despite its chronically toxic effect D-glucose and some other hexoses are among the most potent stimulators of β-cell proliferation (Bonner-Weir et al., 1989; King and Chick, 1976; King et al., 1978; Swenne, 1992). On the other hand, studies with homo- and heterozygous knock-out mice have revealed that IRS-2 is required for maintenance of β-cell mass (Kubota et al., 2000; Withers et al., 1999; Withers et al., 1998). Elevated levels of IRS-2 in islets were found to prevent diabetes in animal models (Hennige et al., 2003). Our own recent work shows that overexpression of IRS-2 in isolated pancreatic islets is sufficient to induce proliferation and protect human β-cells from D-glucose induced apoptosis (Mohanty et al., 2005).

D-glucose acts synergistically with mitogens such as GH and IGF-I (Cousin et al., 1999; Hügl et al., 1998) which are both known to recruit IRS proteins for signal transduction. Studies by Jahla and co-workers (Jhala et al., 2003) suggest that D-glucose increases cytosolic concentrations of cAMP and Ca^{2+} and thereby activates transcription of Irs-2 via cAMP response element binding protein (CREB). However, the direct link between D-glucose and the regulation of IRS-2 biosynthesis remains to be elucidated.

In order to refine the current picture we determined the expression of IRS-2 in β-cells after stimulation with D-glucose, ex vivo. We demonstrate that the
metabolizable hexoses D-glucose and D-mannose but not the inert L-glucose, D-galactose and D-fructose increase the expression and phosphorylation of IRS-2 in islets and INS-1 cells. IRS-1 remained unaffected by hexose stimulation. D-glucose upregulated the expression of *Irs-2* mRNA after 30 minutes while the increase in IRS-2 protein content was observed with a delay of 2 hours. IRS-2 protein levels remained elevated for at least 48 hours.
Materials and Methods

Chemicals and antibodies

RPMI-1640 and cell culture reagents were purchased from Life Technologies (Basel, Switzerland). Human insulin was a gift from Novo Nordisk (Bagsvaerd, Denmark). Collagenase P (Clostridium histolyticum) was purchased from Roche (Rotkreuz, Switzerland). $[^{32}P]$-deoxycytidine triphosphate was obtained from Amersham Pharmacia Biotech (UK). Polyclonal antibodies against IRS-2 and $\beta$-actin, and a monoclonal antibody against phosphotyrosine were from Santa Cruz Biotechnology (USA). An antibody against a C-terminal peptide of human IRS-1 was generated in rabbits. Peroxidase-conjugated secondary antibodies were from Bio-Rad (Glattbrugg, Switzerland).

Cell culture

INS-1 cells (passages 83-95) were cultured in RPMI-1640 containing 11 mmol/l D-glucose, 10% (v/v) heat inactivated fetal calf serum, 2 mmol/l L-glutamine, 50 $\mu$g/ml gentamycin, 1 mmol/l sodium pyruvate, 10 mmol/l HEPES (pH 7.3), and 50 $\mu$mol/l $\beta$-mercaptoethanol, at 37°C, under 5% CO$_2$ in a humidified atmosphere (Asfari et al., 1992). 1 x 10$^6$ cells per 20-cm$^2$-surface dish were pre-cultured in the presence of 11 mmol/l D-glucose for 3 days to about 80% confluence prior to the experiments. Glucose deprivation was in supplemented RPMI-1640 containing 1.6 mmol/l D-glucose for 24 h or as specified. A concentration of 1.6 mmol/l of D-glucose was chosen because $\beta$-cells are especially sensitive to culture conditions without D-glucose. Long starvation times up to 24 h at 1.6 mmol/l D-glucose were preferred to allow expression of target proteins to reach basal levels.

Isolation and culture of rat islets
Islets of Langerhans were isolated from 5 days old Zur:SIV rats by collagenase digestion of the pancreas followed by separation on Percoll gradients (Yamamoto et al., 1981). Three batches of approximately 100 islets were handpicked and precultured for 5 days in RPMI-1640 containing 11 mmol/l D-glucose and 10% newborn calf serum prior to glucose deprivation at 3.3 mmol/l D-glucose for 6 h.

**Immunoblot analysis**

INS-1 cells were lysed in ice-cold lysis buffer consisting of 50 mmol/l HEPES (pH 7.5), 140 mmol/l NaCl, 0.5% (v/v) Triton X-100, 1 mmol/l phenylmethylsulfonyl fluoride, 3 μg/ml leupeptin, 3 μg/ml aprotinin, 10 mmol/l sodium fluoride, 1 mmol/l disodium pyrophosphate, and 1 mmol/l sodium orthovanadate. Islets were collected in the same lysis buffer but without Triton X-100 and disrupted by multiple strokes of sonication. Thereafter, Triton X-100 was added to 2% (v/v) for complete protein extraction. Protein concentrations were determined with the bicinchoninic acid assay (Pierce, Rockford, IL). 5 μg INS-1 protein extract, and 20 μg islets extract, respectively, were subjected to SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and transferred onto nitrocellulose membranes (Protran BA 85, Schleicher and Schuell). Signals were visualized by enhanced chemiluminiscence (ECL, Amersham Life Science, UK) and quantified densitometrically.

**Northern blot analysis**

INS-1 cells were seeded onto 150 cm² dishes and cultured for 3 days. Total cellular RNA was isolated (Chomczynski and Sacchi, 1987), electrophoresed through agarose-formaldehyde gels and transferred to nylon membranes (Hybond-NX-RPK 5020 - Amersham Life Science, UK). ³²P-labelled DNA probes complementary to murine IRS-2 (Sun et al., 1995) and human β-actin (Alonso et al., 1986) were
hybridized at 42°C (Amasino, 1986). Specific activity of the probes was around 2 x 10^9 cpm/μg. Blots were analyzed in a phosphoimager (FUJIX BAS2000).

**Insulin determination**

Accumulated insulin in the cell supernatant was determined by a competitive ELISA using rat insulin as standard with a detection limit of 0.1 ng/ml (Kekow et al., 1988; Webster et al., 1990).

**Results**

**D-glucose enhances accumulation of IRS-2, but not of IRS-1 in pancreatic β-cells**

To assess if D-glucose concentration affects the accumulation of IRS-1 and IRS-2 in β-cells the insulinoma-derived β-cell line INS-1 was glucose deprived (for details see materials and methods) prior to exposure to 16 mmol/l D-glucose for up to 24 h. Incubation at 16 mmol/l D-glucose did not alter IRS-1 content whereas IRS-2 was increased up to 3-fold relative to actin (Fig. 1A). Moreover, the increase in IRS-2 was accompanied by post-translational modification(s) as reflected by a delay in protein mobility on SDS-PAGE (lanes 3, 5 and 7). Fig. 1C shows that this shift was lost after incubation of lysates with alkaline phosphatase or omitting phosphatase inhibitors during cell lysis. In contrast, the migration of IRS-1 protein was unaffected by D-glucose.

To test whether D-glucose can also affect IRS-2 expression in primary β-cells, isolated rat islets were used. As shown in Fig. 1B, after exposure for 6 h to 20 mmol/l D-glucose, islets and INS-1 β-cells responded similarly and both showed significantly increased IRS-2 content. Despite extended efforts and as described by others (Schuppin et al., 1998), IRS-1 levels in rat islets remained below our detection limit (not shown). Based on these observations we consider the insulinoma-derived INS-1
cell line as representative for pancreatic β-cells and all further experiments were performed in this model cell line.

**Glucose enhances Irs-2 transcription and protein accumulation in a time and dose dependent manner**

Northern blot analysis revealed that steady-state levels of Irs-2 mRNA increased slightly in the presence of 16 mmol/l D-glucose within a period of 4 h compared to actin. (Fig. 2A). Irs-2 mRNA increased prior to IRS-2 protein (compare Fig. 1A and 2B with Fig. 2A).

IRS-2 protein levels reached an elevated plateau after 6 to 10 h exposure to 16 mmol/l D-glucose and remained elevated for the entire investigated period of 48 h (Fig. 2B). In contrast, the extent of IRS-2 post-translational modification was transient with a peak at 10 h, after which it slowly declined. Culturing cells in the presence of D-glucose ranging from 5.5 mM to 25 mM resulted in increasingly higher levels of IRS-2 (Fig. 2C).

**Hexoses need to be metabolized to increase IRS-2 protein accumulation**

The biologically inert L-glucose did neither affect cellular IRS-2 content nor its posttranslational modification at 14.4 mmol/l when present at basal levels of 1.6 mmol/l D-glucose for up to 18 h (Fig. 3A). This finding rules out that increased osmolarity affected IRS-2 expression as observed for other genes (Okazaki et al., 1997). Furthermore, we tested other metabolisable and non-metabolisable hexoses at concentrations of 14.4 mmol/l supplemented with 1.6 mmol/l D-glucose to a total hexose concentration of 16 mmol/l. Like D-glucose, also D-mannose increased IRS-2 levels (Fig. 3B, lanes 2, 4, 8, and 10) whereas the metabolically inert D-galactose and
D-fructose (Ashcroft, 1980) did neither increase IRS-2 content nor affect its posttranslational modification (Fig. 3B, lanes 3, 5, 6, 9, 12, and 13). Finally, the non-glycolytic glucose analogues 2-deoxy-D-glucose and 3-0-methyl-glucose (Ashcroft et al., 1973) did not enhance IRS-2 biosynthesis at total hexose concentrations of 16 mmol/l in a period of 24 h (Fig. 3C, lanes 2 and 3).

**Insulin enhances IRS-2 expression but does not act as a long-term mediator for D-glucose**

Since D-glucose induces insulin secretion from β-cells autocrine insulin action could mediate the effect of D-glucose on Irs-2 expression. First we tested if insulin itself increases IRS-2 protein accumulation in INS-1 cells. Fig. 4A shows that 100 nmol/l insulin transiently enhances cellular IRS-2 content, but to a much lesser extent than 16 mmol/l D-glucose. However, the insulin concentration used in these experiments was supraphysiological (see Fig. 4C for comparison). Therefore we challenged INS-1 cells with 16 mmol/l D-glucose and with 100 nmol/l insulin simultaneously or individually and found (Fig. 4B) that D-glucose increased expression and phosphorylation of IRS-2 in the presence of 100 nmol/l insulin. In addition, we preincubated INS-1 cells in the presence of 250 μmol/l diazoxide prior to a D-glucose challenge. Diazoxide blocked glucose-induced insulin secretion (Fig. 4C), however, this did not attenuate the glucose-induced increase of IRS-2 accumulation and phosphorylation (Fig. 4C).
Discussion

Functional β-cell mass can increase to compensate for insulin resistance and thereby prevents the onset of overt hyperglycemia (Bonner-Weir, 2000). However, with time β-cells often fail and type 2 diabetes develops (Leahy and Weir, 1991; Ling et al., 1996). Hyperglycemia exerts dual and opposing effects on β-cells. Chronically elevated glucose levels result in pathological protein glycosylation, enhanced Fas-receptor expression and superoxide-mediated activation of uncoupling protein 2 (Liu et al., 2000; Maedler et al., 2001; Ristow et al., 2003). On the other hand, glucose stimulates β-cell growth and has been found to be anti-apoptotic (Bonner-Weir, 2000; Bonner-Weir et al., 1989; Hoorens et al., 1996). These opposing effects of D-glucose could depend on IRS-2 levels in β-cells. Various reports have shown that Irs-2 is required for the maintenance of β-cell mass in vivo. We have shown previously that upregulation of IRS-2 in isolated islets stimulates β-cell proliferation and inhibits D-glucose-induced apoptosis (Mohanty et al., 2005). Furthermore, D-glucose and IGF-I synergistically increase proliferation of β-cells. (Kubota et al., 2000; Withers et al., 1999; Withers et al., 1998). The present study provides further evidence that IRS-2 is the link between D-glucose and its effects on β-cells.

We found that D-glucose upregulates transcription of Irs-2 and increases IRS-2 protein levels for at least 48 hours while Irs-1 remained unaffected. This finding correlates well with reports showing D-glucose-induced β-cell growth to last for 96 h in hyperglycemic rats (Bonner-Weir et al., 1989). Our observations also support previously published data (Jhala et al., 2003) that showed D-glucose-dependent activation of the transcription factor CREB which can bind to a cAMP response element (CRE) in the IRS-2 promoter. However, our results directly link D-glucose
and IRS-2 levels in β-cells. We could confirm that D-glucose induces phosphorylation of CREB at Ser133 within 3-5 minutes and so did insulin (not shown), but only D-glucose induced sustained upregulation of IRS-2. Thus, activation of CREB at Ser133 might not be sufficient for a prolonged and strong increase of IRS-2 levels.

A new and original finding of the present study is that only metabolisable hexoses can increase cellular IRS-2 content. Metabolic inert hexoses and analogues of D-glucose were ineffective (Fig. 3). Thus, neither hexose translocation through glucose transporters (3-0-methyl-glucose) nor the initial phosphorylation by hexokinase (2-deoxy-D-glucose) were sufficient to enhance IRS-2 accumulation indicating that the trigger for IRS-2 accumulation requires glycolysis.

Incubation of INS-1 cells at elevated concentrations of metabolisable hexoses induced a transient phosphorylation of IRS-2 but not IRS-1. This raises the question which kinase(s) is(are) involved. Since D-glucose was able to induce phosphorylation of IRS-2 even if endogenous insulin secretion was prevented with diazoxide, the tyrosine kinases of the insulin and IGF-I receptor, respectively, are unlikely to be the mediators of this effect. We therefore suggest that metabolisable hexoses induce cytosolic kinases that are known to phosphorylate IRS proteins, such as protein kinase C (PKC), JNK or mammalian target of rapamycin (mTOR) (for review see (Pirola et al., 2004)). Indeed, a recent study has identified IRS-2 as a target of mTOR-dependent Ser/Thr phosphorylation in INS-1 cells (Briaud et al., 2004). These authors show that chronically elevated D-glucose concentrations can induce proteosomal degradation of IRS-2 which points to a possible mechanism underlying glucotoxicity. However, the mechanisms underlying this switch from upregulation to degradation of IRS-2 in β-cells by elevated D-glucose concentrations remain to be determined. It is conceivable that ER-stress-dependent activation of c-Jun N-terminal kinase (JNK) plays a role. A
recent study shows that obesity-induced ER-stress can repress IRS proteins in liver and fat (Ozcan et al., 2004). In addition, ER-stress-induced apoptosis and β-cell failure is a long discussed concept that could underlie hyperglycemia-induced β-cell exhaustion (Oyadomari et al., 2002). We are currently investigating the intracellular mechanisms, which lead to upregulation and phosphorylation of IRS-2 by D-glucose.
Acknowledgments

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References


proliferation and protects human beta-cells from hyperglycemia-induced apoptosis. 


beta-cell lines is involved in mediating serum-stimulated beta-cell growth. *Diabetes*, 47, 1074-1085.


Figure Legends
Fig. 1. D-glucose increases IRS-2, but not IRS-1 protein accumulation and phosphorylation in β-cells. IRS-1 and IRS-2 levels were assessed in lysates from INS-1 cells or pancreatic rat islets by Western blot analysis (A) INS-1 cells were D-glucose deprived at 1.6 mmol/l for 24 h before D-glucose was increased to 16 mmol/l for the indicated times. (B) INS-1 cells, and approximately 100 rat islets, were D-glucose deprived at 3.3 mmol/l for 6 h before D-glucose was increased to 20 mmol/l for additional 6 h. The presented immunoblots are representative of three independent experiments. (C) Cell lysates from glucose-deprived (1.6 mmol/l) or glucose-stimulated (16 mmol/l) INS-1 cells were prepared with (lane 1 and 4) or without phosphatase inhibitors (2, 3, 5 and 6). In addition, two lysates (3 and 6) were exposed to alkaline phosphatase at 37° C for 30 min.

Fig. 2. D-glucose elevates IRS-2 transcript and protein content in a time- and dose-dependent manner. (A) Glucose deprived INS-1 cells were exposed to either 1.6 or 16 mmol/l D-glucose for 0, 15, 30 min, and 1, 2 and 4 h, respectively. Total RNA was isolated and the expression of Irs-2 and actin mRNAs was assessed by Northern blotting. Each lane contains 15 μg of total RNA. Signal intensities of IRS-2 (top) and β-actin (middle) were quantified using a phosphoimager. The lower panel shows ethidium bromide-staining of 18S rRNA. The relative abundance of IRS-2 compared to actin is indicated below the autoradiographs. (B) Glucose deprived INS-1 cells were exposed to 16 mmol/l D-glucose for up to 48 h before IRS-2 abundance was assayed on immunoblots. (D) INS-1 cells were exposed for 24 h to 1.6, 5.5, 11.0, or 25.0 mmol/l D-glucose, respectively, before IRS-2 abundance was assayed on immunoblots.
Fig. 3. Metabolisable hexoses stimulate IRS-2 accumulation in a dose- and time-dependent manner in INS-1 cells. IRS-2 levels were determined in lysates from hexose-induced INS-1 cells by immunoblotting. (A) 14.4 mmol/l D- or L-glucose were added for the indicated times to INS-1 cells after culturing in 1.6 mmol/l D-glucose. (B) INS-1 cells were deprived at 1.6 mmol/l D-glucose for 24 h before exposure for 10 or 24 h to additional 14.4 mmol/l D-glucose (lanes 2, 8), L-glucose (3, 9), D-mannose (4, 10), D-galactose (5, 11), and D-fructose (6, 12), respectively, or maintained at 1.6 mmol/l glucose (1, 7, 13, 14). (C) INS-1 cells were cultured at 1.6 mmol/l D-glucose before they were exposed for 10 h to additional 14.4 mmol/l 2-deoxy-D-glucose (lane 2), 3-0-methyl-glucose (3), and D-glucose (4), respectively, or maintained at 1.6 mmol/l D-glucose (1).

Fig. 4. Insulin enhances the expression of IRS-2 in INS-1 cells but does not act as a mediator for D-glucose. (A, B and C) Cells were cultured in 1.6 mmol/l D-glucose for 16 h. Thereafter, the medium was adjusted to the indicated conditions. Lysates were analysed by Western blotting. (A) Comparison of IRS-2 expression after stimulation with 16 mmol/l D-glucose or with 100 nmol/l insulin in the presence of 1.6 mmol/l D-glucose. (B) Comparison of IRS-2 expression after stimulation with 16 mmol/l D-glucose alone or with 100 nmol/l insulin in the presence of either 1.6 or 16 mmol/l D-glucose. (C) Glucose-deprived INS-1 cells were either maintained at 1.6 mmol/l D-glucose or were exposed to 16 mmol/l D-glucose in the presence or absence of 250 mol/l diazoxide to suppress insulin secretion. The accumulated insulin concentrations in supernatant at the end of the stimulation periods are indicated below the corresponding lanes.
**Fig. 1**

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- **IRS-1**
- **IRS-2**
- **Actin**

**B**

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**C**

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**IRS-2**
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| rel. abundance    | 1.0 | 0.5  | 1.0  | 1.2 | 1.0 | 1.5 |

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B

C

IRS-2

actin

IRS-2

actin

IRS-2

actin
Fig. 4

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