

Pancreatic and duodenal homeobox-1 nuclear localization is regulated by glucose in dispersed rat islets but not in insulin-secreting cell lines

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Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; DAPI, 4', 6-diamidino-2-phenylindole; DMEM, dulbecco's modified eagle medium; EDTA, ethylenediaminetetraacetic acid; GFP, green fluorescent protein; HDAC, histone deacetylase; KRBH, krebs ringer bicarbonate hepes; MODY, maturity-onset diabetes of the young; PDX-1, pancreatic and duodenal homeobox-1; SEM, standard error of the mean; SUMO, small ubiquitin-like modifier; T2D, type 2 diabetes; ZDF, zucker diabetic fatty

The transcription factor Pancreatic and Duodenal Homeobox-1 (PDX-1) plays a major role in the development and function of pancreatic β -cells and its mutation results in diabetes. In adult β -cells, glucose stimulates transcription of the insulin gene in part by regulating PDX-1 expression, stability and activity. Glucose is also thought to modulate PDX-1 nuclear translocation but in vitro studies examining nucleo-cytoplasmic shuttling of endogenous or ectopically expressed PDX-1 in insulin-secreting cell lines have led to conflicting results. Here we show that endogenous PDX-1 undergoes translocation from the cytoplasm to the nucleus in response to glucose in dispersed rat islets but not in insulin-secreting MIN6, HIT-T15, or INS832/13 cells. Interestingly, however, we found that a PDX-1-GFP fusion protein can shuttle from the cytoplasm to the nucleus in response to glucose stimulation in HIT-T15 cells. Our results suggest that the regulation of endogenous PDX-1 sub-cellular localization by glucose is observed in primary islets and that care should be taken when interpreting data from insulin-secreting cell lines.

Introduction

The worldwide prevalence of type 2 diabetes (T2D) has reached epidemic proportions as a result of abundant nutrient supply and sedentary lifestyle. T2D is characterized by fasting hyperglycaemia resulting from insufficient insulin secretion from pancreatic β -cells and peripheral insulin resistance. The transcription factor Pancreatic and Duodenal Homeobox-1 (PDX-1) is a homeodomain protein that activates insulin gene transcription as well as other pancreas-specific genes and plays a critical role in both pancreatic development and adult β -cell function (reviewed in¹). Complete PDX-1 deficiency results in pancreatic agenesis in mice² and humans,³ whereas β -cell specific disruption or heterozygous mutations result in T2D.⁴ In humans, mutations in *pdx-1* are associated with the development of maturity-onset diabetes of the young type 4 (MODY 4), a monogenic form of T2D characterized by impaired β -cell function.^{5,6} Other mutations in human

pdx-1 were shown to be associated with the development of adult-onset forms of T2D.⁷⁻⁹

Glucose induces post-translational modifications of PDX-1. These include O-GlcNAcylation,^{10,11} SUMOylation¹² and phosphorylation¹³⁻²⁵ that regulate PDX-1 protein stability,^{18,19,26} its transactivation potential,²⁷ its interaction with co-factors such as the histone acetyl transferase p300,²⁸ and its binding to the A boxes on the insulin gene promoter^{10,11,29} to trigger insulin gene transcription. The PDX-1 homeodomain contains a basic amino acid sequence, RRMKWKK, that also serves as a nuclear localization signal for PDX-1 nuclear import.³⁰ The interest in the regulation of PDX-1 nucleo-cytoplasmic shuttling stems from the observation that β -cell dysfunction in islets from T2D patients correlates with the exclusion of PDX-1 from the nucleus.^{31,32} Similar results were obtained in rodent models of metabolic stress, such as in *db/db* mice,³² high fat diet in mice,^{31,33} ZDF rats³⁴ nutrient infusion in rats,³⁵ and in vitro oxidative and glucolipotoxic stress.³⁵⁻³⁸ Glucose^{16,35} and other factors promoting

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insulin secretion and β -cell survival, including GLP-1,^{31,39,40} TGF- β ,⁴¹ nitric oxide⁴² and insulin^{15,43} increase nuclear PDX-1. The glucose-dependent shuttling of PDX-1 from the cytoplasm to the nucleus was established in vivo in glucose-infused rats³⁵ and in vitro in cultured human islets.¹⁶ In contrast, studies using a number of insulin-secreting cell lines showed that PDX-1 is localized to the nuclear periphery,^{21,25,44} or restricted to the nucleus^{26,30} regardless of the glucose concentration. Since insulin-secreting cells are commonly used as in vitro models for studying the regulation of β -cell gene expression, this study aimed to determine whether PDX-1 nucleo-cytoplasmic shuttling is differently regulated by glucose in primary rat islets vs. the commonly used insulin-secreting MIN6, HIT-T15 and INS832/13 cell lines.

Results and Discussion

Glucose promotes the translocation of PDX-1 from the cytoplasm to the nucleus in dispersed rat islets

We first assessed PDX-1 nucleo-cytoplasmic shuttling by immunocytochemistry in dispersed rat islets exposed for 1, 6 and 24 h to increasing glucose concentrations (0.5 to 16.7 mM). After 1 h of treatment at 0.5 or 2.8 mM glucose, PDX-1 abundance was similar in the cytoplasmic and nuclear compartments (Fig. 1A and D). This distribution did not change after 6 and 24 h of low glucose exposure (Fig. 1B, C and D). Although not significant, there was a tendency for an increase in nuclear PDX-1 in the presence of 5 mM glucose in most cells as shown in Figure 1. After exposure to 16.7 mM

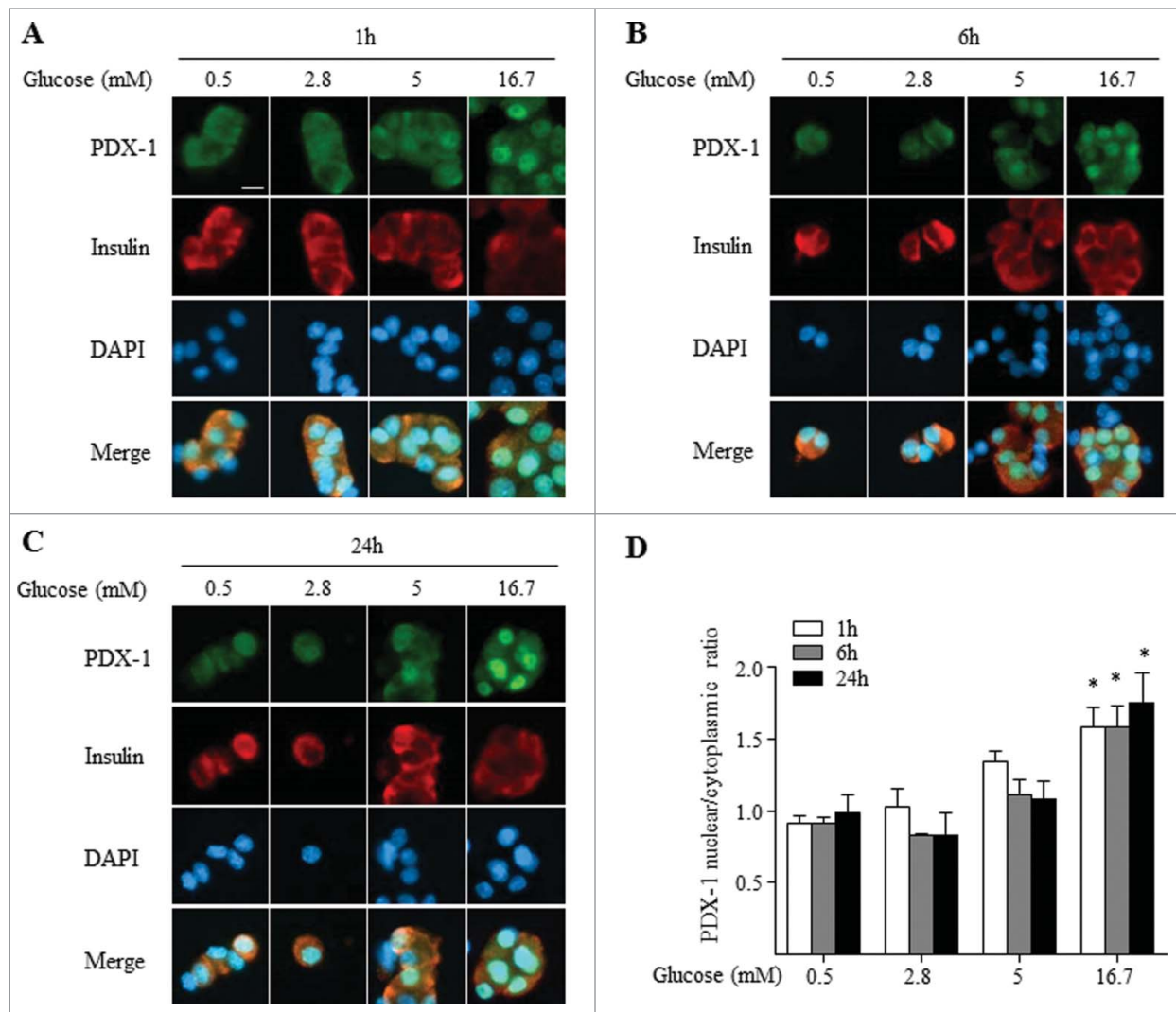


Figure 1. Glucose induces PDX-1 nuclear translocation in dispersed rat islets. PDX-1 (green), insulin (red) and nuclei (DAPI, blue) were visualized by fluorescence microscopy ($\times 20$). Dispersed rat islets were exposed 1 h (A), 6 h (B) and 24 h (C) to increasing glucose concentrations as indicated. (D) Quantification of nuclear/cytoplasmic ratio of 4 replicate experiments. Results are expressed as mean \pm SEM. * $P \leq 0.05$: 16.7 vs 0.5 mM glucose at all time points. Scale bar, 10 μ m.

glucose, however, PDX-1 was located predominantly in the nucleus already after 1 h and the distribution remained nuclear after 6 and 24 h (Fig. 1; $p \leq 0.05$ at 16.7 vs. 0.5 mM glucose for all 3 time points). These results demonstrate that in dispersed rat islets, PDX-1 undergoes a cytoplasmic to nuclear shift in response to increasing glucose concentrations. These results are similar to previous reports in human islets *ex vivo*¹⁶ and in glucose-infused rats *in vivo*.³⁵

PDX-1 subcellular localization is not regulated by glucose in MIN6 cells

To determine whether insulin-secreting cells respond similarly to glucose as dispersed rat islets, the murine insulinoma MIN6 cell line was exposed for 1, 6 and 24 h to increasing glucose concentrations (0.5 to 16 mM) and endogenous PDX-1 sub-cellular localization was assessed as described above. PDX-1 immunostaining was already predominantly detected in the nucleus at 0.5 and 2 mM glucose and this pattern was maintained at 5, 11 and 16 mM glucose at all time points examined (Fig. 2). Even after 24 h of exposure to a low glucose

concentration (0.5 mM) which started to trigger cell death (as shown by the presence of pyknotic nuclei (Fig. 2C)), PDX-1 was largely nuclear. We asked whether the constitutive nuclear localization of Pdx-1 in MIN6 was due to glucose hyper-responsiveness. To test this we analyzed glucose-stimulated insulin secretion over the same range of glucose concentrations (Suppl. Fig. 1A) and observed that glucose dose-dependently stimulates insulin secretion in MIN6 cells in a similar manner as in isolated islets, as previously published.⁴⁵ Nuclear localization of Pdx-1 in MIN6 cells is consistent with our previous observation²⁶ and in accordance with another reported study using an exogenous PDX-1-GFP fusion protein.³⁰ However, other studies using MIN6 cells reported endogenous PDX-1 in the cytoplasm at low glucose levels and an increase in the nuclear/cytoplasmic ratio in the presence of high glucose.^{15,16,34} In some cases the discrepancy with our data may be due to differences in experimental approaches. For example, Macfarlane et al.¹⁶ measured the localization of specific PDX-1 isoforms by Western blotting. Rafiq et al.¹⁴ found that increasing glucose levels from 3 to 30 mM led to a small increase in the nuclear

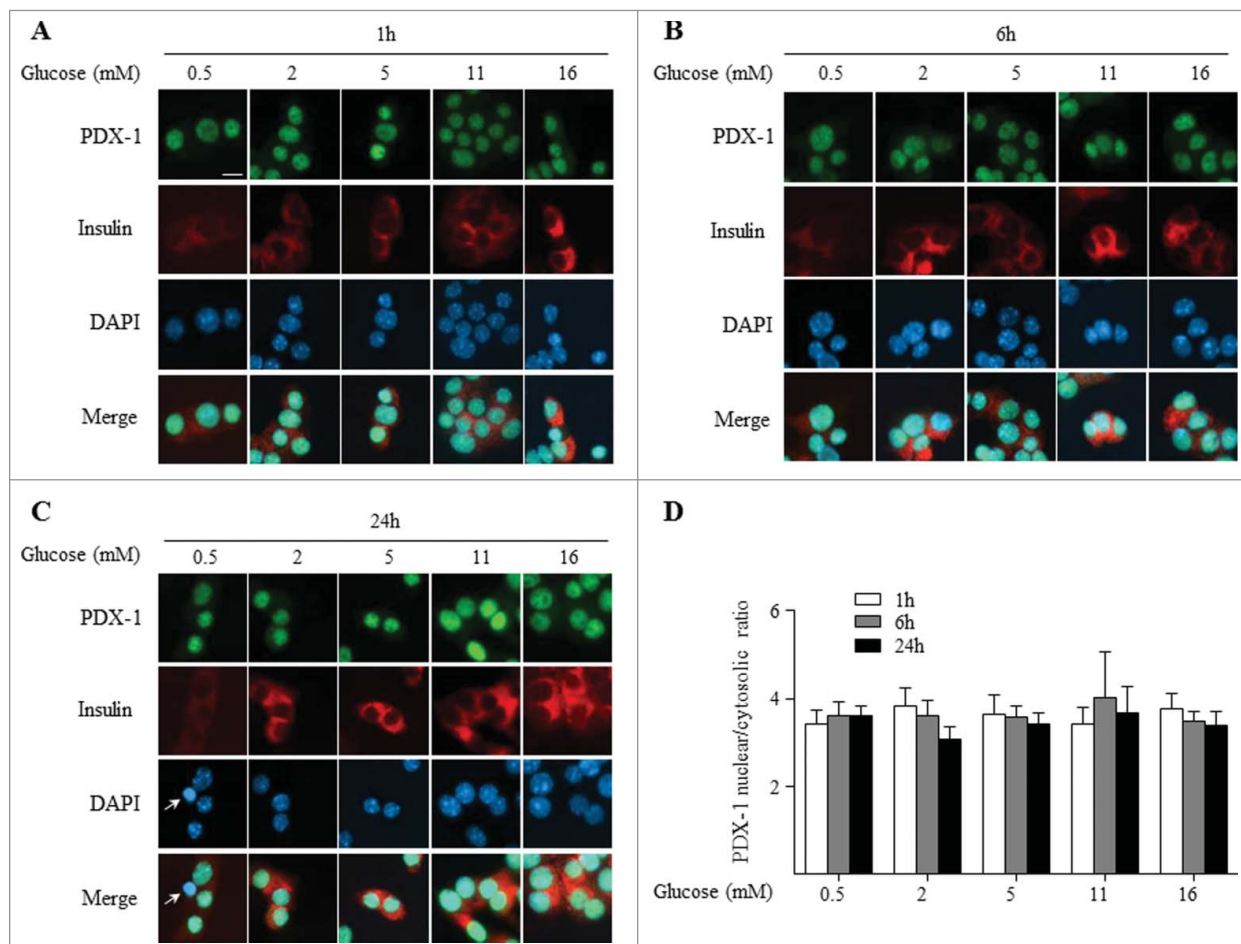


Figure 2. PDX-1 is predominantly localized in the nucleus in MIN6 cells independent of the glucose concentration. PDX-1 (green), insulin (red) and nuclei (DAPI, blue) were visualized by fluorescence microscopy ($\times 20$). MIN6 cells were exposed 1 h (A), 6 h (B) and 24 h (C) to increasing glucose concentrations as indicated. Arrows indicate pyknotic nuclei. (D) Quantification of nuclear/cytoplasmic ratio of 3 replicate experiments. Results are expressed as mean \pm SEM. Scale bar, 10 μ m.

fraction of endogenous PDX-1. Although this differs from our results, we did not test glucose concentrations above the physiological range.

Glucose regulates exogenous but not endogenous PDX-1 nuclear translocation in HIT-T15 cells

To investigate the nucleo-cytoplasmic shuttling of PDX-1 in another rodent insulin-secreting cell line, hamster HIT-T15 cells were exposed for 1, 6 and 24 h to increasing glucose concentrations (0.1 to 11 mM). The glucose concentration range was chosen based on results from insulin secretion assays which revealed a dose-dependent but left-shifted (0.1–16 mM) increase in insulin secretion (Suppl. Fig. 1B), as previously observed.⁴⁶ Similar to our observation in MIN6 cells (Fig. 2), PDX-1 was predominantly localized in the nucleus of HIT-T15 cells following exposure to low glucose (0.1 mM) at all time points, and increasing the glucose concentration had no detectable effect on PDX-1 localization (Fig. 3A–D). As

numerous studies have described the shuttling of ectopically expressed PDX-1 fusion proteins from the cytoplasm or nuclear periphery to the nucleoplasm in response to increasing glucose concentrations,^{14,21,34,44} we tested whether this could be detected in HIT-T15 cells under the same conditions used to study endogenous PDX-1. Similar to our previous results,³⁸ and unlike the endogenous protein (Fig. 3A–D), exogenous PDX-1 was predominantly expressed at the nuclear periphery and in the cytoplasm of HIT-T15 at low glucose concentrations and translocated to the nucleus in response to high glucose (Fig. 3E–F). This indicates that the mechanisms controlling glucose-dependent PDX-1 nuclear translocation are functional in HIT-T15 cells but are not operative with the endogenous protein. We speculate that nuclear import/export or retention signals, such as HDAC1 and HDAC2²⁵ and Importin β 1,³⁴ may become saturated by overexpression of exogenous PDX-1 resulting in accumulation of the protein in the cytoplasm/nuclear periphery.

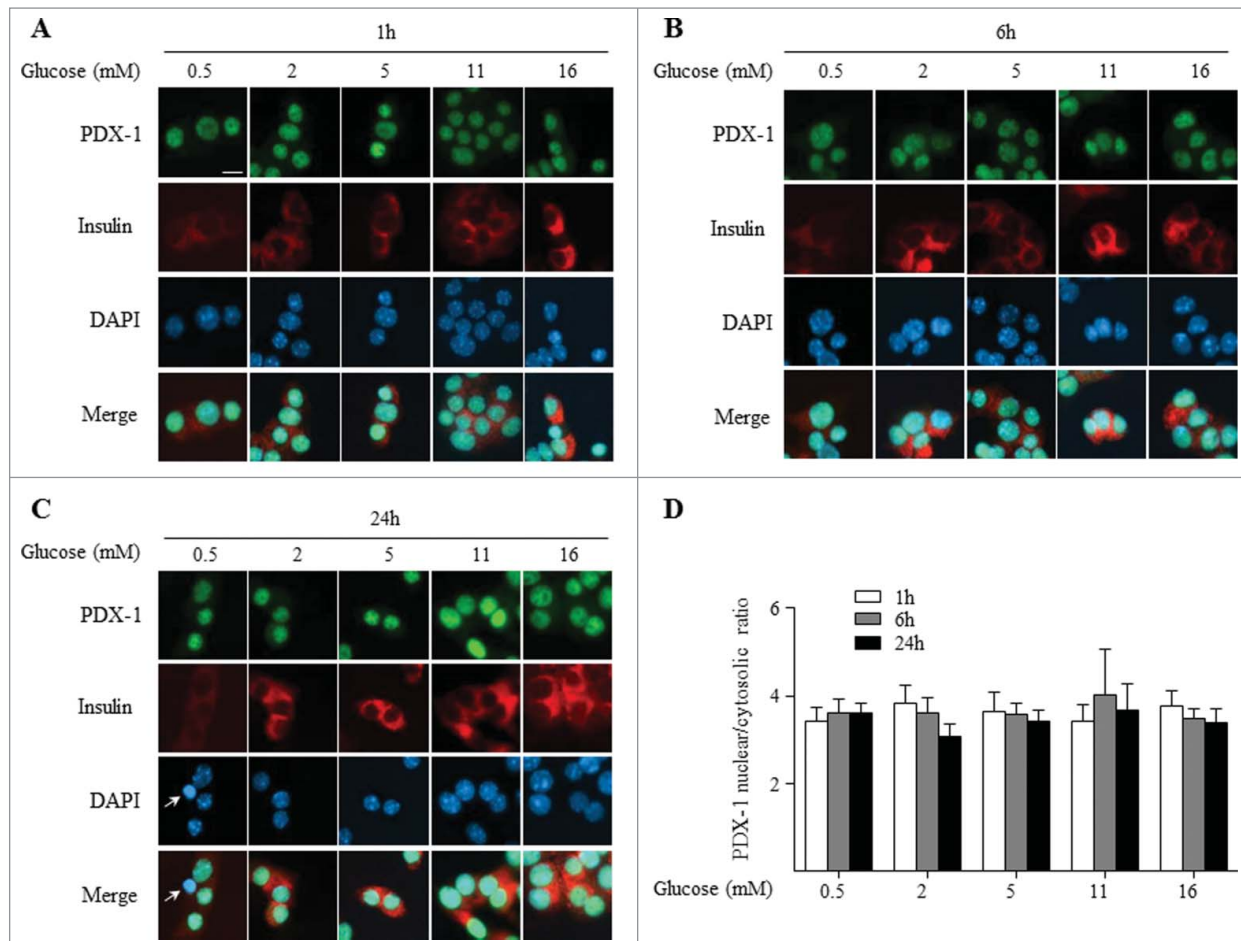


Figure 3. In HIT-T15 cells, endogenous PDX-1 is localized to the nucleus independent of the glucose concentration, whereas ectopically expressed PDX-1 shuttles from the cytoplasm to the nucleus in a glucose-dependent manner. PDX-1 (green), insulin (red) and nuclei (DAPI, blue) were visualized by fluorescence microscopy ($\times 20$). HIT-T15 cells were exposed 1 h (A), 6 h (B) and 24 h (C) to increasing glucose concentrations as indicated. (D) Quantification of nuclear/cytoplasmic ratio of 3 replicate experiments. Results are expressed as mean \pm SEM. (E) HIT-T15 cells were transfected with pcDNA3.1-PDX-1-GFP plasmid and exposed for 24 h to 0.1 or 5 mM glucose. PDX-1 (GFP, green) and nuclei (DAPI, blue) were visualized by confocal microscopy. (F) Quantification of nuclear/cytoplasmic ratio of 3 replicate experiments. Results are expressed as mean \pm SEM. ****** $P \leq 0.01$. Scale bar, 10 μ m.

PDX-1 subcellular localization is not regulated by glucose in INS832/13 cells

As both MIN6⁴⁷ and HIT-T15⁴⁸ cell lines were established by expression of the SV40 T antigen in islets, we wanted to exclude the possibility that T antigen transformation interferes with glucose-dependent nucleo-cytoplasmic shuttling of PDX-1. We analyzed PDX-1 localization in response to glucose in a clonal derivative of the X-ray-induced rat insulinoma INS-1 cells,⁴⁹ INS832/13, that was selected for its glucose-stimulated insulin secretory responsiveness.⁵⁰ INS832/13 cells were exposed for 1, 6 and 24 h to increasing glucose concentrations (0.5 to 16 mM). The glucose concentration range was chosen based on results from insulin secretion assays which revealed a dose-dependent (0.1–16 mM) increase in insulin secretion (Suppl. Fig. 1C), as previously described.⁵⁰ Similar to our observation in MIN6 (Fig. 2) and HIT-T15 (Fig. 3) cells, PDX-1 was predominantly localized in the nucleus of INS832/13 cells following exposure to low glucose (0.5 mM) at all time points, and increasing the glucose concentration had no significant effect on PDX-1 localization (Fig. 4). Although a report by Sayo et al.⁴¹ found that glucose

increased nuclear PDX-1 in the parental INS-1 cells, our data are not inconsistent as glucose also increased total cellular PDX-1 and the shuttling of PDX-1 was not addressed in that study.

PDX-1 localization is affected by oxidative stress in dispersed rat islets, MIN6 and INS832/13 cells but not in HIT-T15 cells

PDX-1 is excluded from the nucleus of β cells in islets isolated from rats infused with glucose + Intralipid,³⁵ from *db/db* mice or mice fed a high fat – high sucrose diet³¹ and in ZDF rats⁵¹ and in vitro in islets exposed to glucose and palmitate.³⁷ Reactive oxygen species are thought to be the main trigger as oxidative stress leads to PDX-1 nuclear exclusion in HIT-T15 cells³⁷ and anti-oxidant treatment of ZDF rats restores PDX-1 nuclear localization.⁴⁶ To investigate whether PDX-1 nucleo-cytoplasmic shuttling remains sensitive to oxidative stress, we exposed dispersed rat islets (Fig. 5A) or MIN6, INS832/13 and HIT-T15 cells (Fig. 5B) to 50 μ M H₂O₂ in the presence of 16.7 mM glucose for 1 h and examined PDX-1 localization. As a positive control for PDX-1 nuclear exclusion, palmitate prevented glucose-induced PDX-1 nuclear import in isolated rat islets (compare Fig. 5A to Fig. 1C)

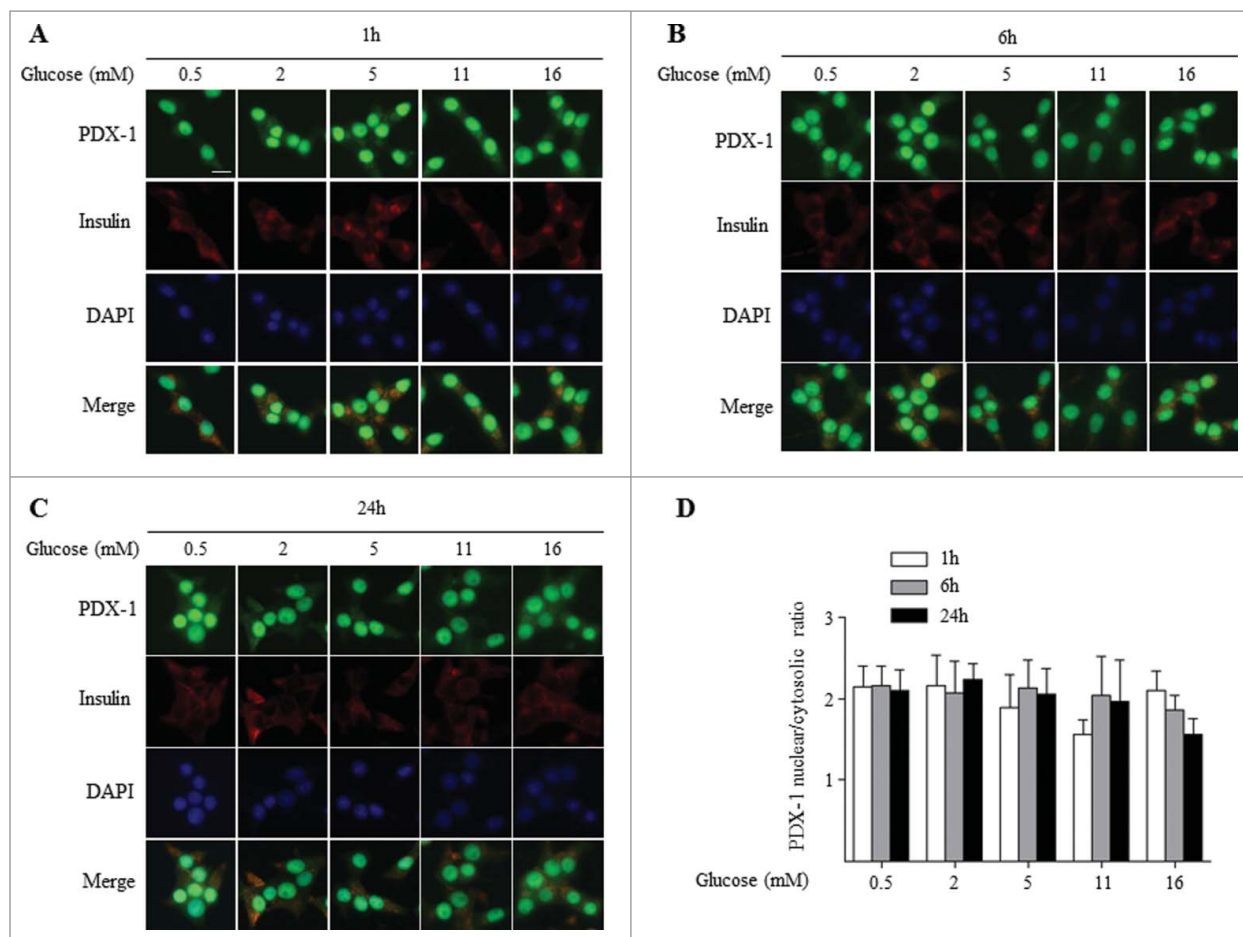


Figure 4. PDX-1 is predominantly localized in the nucleus in INS832/13 cells independent of the glucose concentration. PDX-1 (green), insulin (red) and nuclei (DAPI, blue) were visualized by fluorescence microscopy ($\times 20$). INS832/13 cells were exposed 1 h (A), 6 h (B) and 24 h (C) to increasing glucose concentrations as indicated. (D) Quantification of nuclear/cytoplasmic ratio of 3 replicate experiments. Results are expressed as mean \pm SEM. Scale bar, 10 μ m.

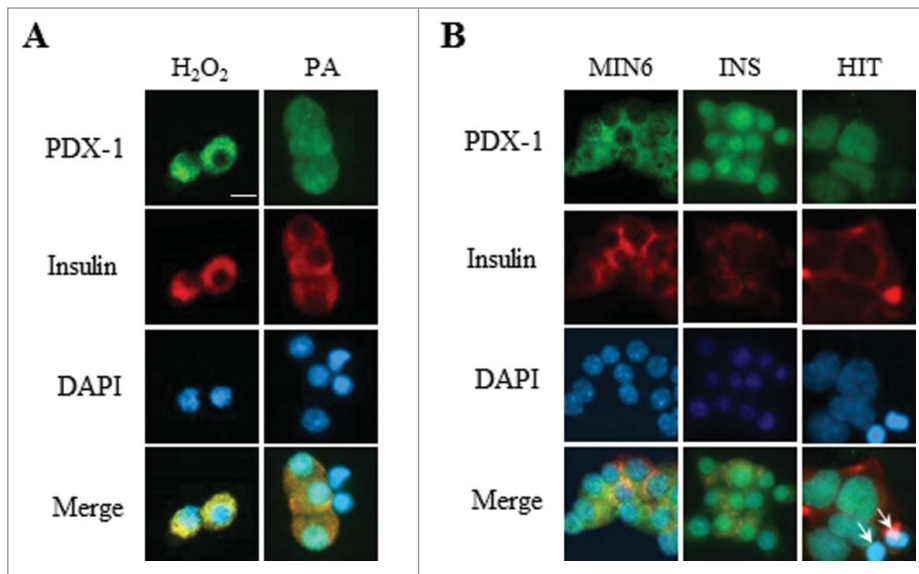


Figure 5. PDX-1 localization is affected by oxidative stress in dispersed rat islets and MIN6 and INS832/13 cells but not in HIT-T15 cells. PDX-1 (green), insulin (red) and nuclei (DAPI, blue) were visualized by fluorescence microscopy ($\times 20$). **(A)** Dispersed rat islets were exposed to 16.7 mM glucose in the presence of 50 μM H_2O_2 for 1 h or 0.5 mM palmitate (PA) for 24 h. **(B)** MIN6, INS832/13 (INS) and HIT-T15 (HIT) cells were exposed to 16.7 mM glucose in the presence of 50 μM H_2O_2 for 1 h. Arrows indicate pyknotic nuclei. Scale bar, 10 μm .

as shown previously.³⁷ H_2O_2 treatment led to PDX-1 nuclear exclusion in dispersed rat islets (compare Fig. 5A to Fig. 1A) and MIN6 cells (compare Fig. 5B to Fig. 2A). H_2O_2 treatment of INS832/13 led to an approximately 3-fold decrease in the nuclear/cytoplasmic ratio of PDX-1 (0.66 ± 0.08 vs 2.1 ± 0.24 , $p < 0.01$; compare Fig. 5B to Fig. 4A). Surprisingly, H_2O_2 exposure did not affect PDX-1 localization in HIT-T15 cells (compare Fig. 5B to Fig. 3A), in contrast to a previous study.³⁷ This discrepancy may be due to the difference in the passage number of HIT-T15 which may affect their susceptibility to oxidative stress. While ours were used at 78–86 passages, cells used by Kawamori et al.³⁷ were of higher passages.

In summary, these data show that PDX-1 nuclear localization is regulated by glucose in dispersed rat islets, consistent with previous *ex vivo*¹⁶ and *in vivo* studies.³⁵ In contrast, using the same experimental conditions we found endogenous PDX-1 sub-cellular localization to be independent of glucose in the insulin-secreting MIN6, HIT-T15 and INS832/13 cells. These data highlight a defect in the mechanism regulating the localization of PDX-1 and its glucose responsiveness in a number of commonly used β -cell lines which we speculate may have arisen during the transformation process. In support of this, PDX-1 is highly expressed in human pancreatic tumors^{52–54} and controls their oncogenic properties.^{55–57} It would be interesting to examine the localization of PDX-1 and its glucose responsiveness in insulin-secreting cell lines under restricted growth conditions. In conclusion, these results raise a note of caution regarding the use of transformed insulin-secreting cell lines to examine the regulation of PDX-1 nucleo-cytoplasmic shuttling.

Materials and Methods

Generation of PDX-1-GFP expression plasmid

A pcDNA3.1-PDX-1-GFP plasmid was generated as previously described.^{38,44} Briefly, after stop codon removal, the mouse PDX-1-cMyc sequence was excised from the pcDNA3.1-PDX-1-cMyc plasmid (provided by Dr. Guy Rutter, Imperial College London, London, United Kingdom) using Hind III and Kpn I and re-inserted into the mammalian expression vector pcDNA3.1-GFP provided by Dr. Marc Prentki (Montreal Diabetes Research Center, CRCHUM, Montreal, QC, Canada).

Rat islet isolation, dispersion and glucose stimulation

All procedures using animals were approved by the Institutional Committee for the Protection of Animals at the Center Hospitalier de l'Université de Montréal. 250–275 g male Wistar rats (Charles River) were housed on a 12 h light/dark cycle with free access to water and standard laboratory chow. Islets were isolated as previously described.³⁵ After overnight recovery, islets were dispersed by treatment with Trypsin – 0.05 EDTA (Invitrogen) for 5 min and plated on poly-L-lysine-coated (Sigma) coverslips. Dispersed islets were cultured in RPMI-1640 media (Invitrogen) supplemented with 10 % Fetal Bovine Serum (FBS; Invitrogen) and 5.5 mM glucose for 24 h. Media was then changed to 0.5 % fatty acid-free BSA (Equitech-Bio) and 2.8 mM glucose for 2 h after which cells were treated with glucose in the presence or absence of palmitate (Sigma) or H_2O_2 (Invitrogen) as described in RESULTS.

MIN6, INS832/13 and HIT-T15 cell culture, transfection and glucose stimulation

MIN6 cells (passage 25–30; provided by Dr. Jun-ichi Miyazaki, Osaka University Graduate School of Medicine, Suita, Osaka, Japan) were maintained in Hyclone DMEM (Thermo-Fisher Scientific) containing 25 mM glucose and supplemented with 10 % FBS and 0.005 % β -mercaptoethanol. INS832/13 cells (passages 50–56; provided by Dr. Christopher Newgard, Duke University School of Medicine, Durham, NC) were maintained in RPMI-1640 containing 11 mM glucose and supplemented with 10 % FBS. For PDX-1 localization studies MIN6 and INS832/13 culture medium was changed to 5.5 mM glucose overnight then to 2 mM glucose and 0.5 % BSA for an additional 2 h after which cells were exposed to glucose in the presence or absence of H_2O_2 as described in RESULTS. For MIN6 static insulin secretion assays cells were cultured overnight in culture medium containing

5.5 mM glucose and then pre-incubated twice for 30 min in Krebs Ringer bicarbonate HEPES buffer (KRBH) + 0.1 % BSA supplemented with 1 mM glucose prior to stimulation with 1, 5, 9, 12, 16 or 20 mM glucose. For INS832/13 static insulin secretion assays cells were pre-incubated in culture medium containing 1 mM glucose for 2 hours and then in KRBH + 0.1 % BSA supplemented with 1 mM glucose for 1 hour prior to stimulation with 1, 2, 5, 11 and 16 mM glucose. Each condition was run in triplicate. Intracellular insulin content was measured after acid-alcohol extraction. Insulin was quantified using an AlphaLISA immunoassay kit (PerkinElmer). HIT-T15 cells (passages 74–86; provided by Dr. R. Paul Robertson, Pacific Northwest Diabetes Research Institute, Seattle, WA, USA) were maintained in RPMI-1640 containing 11.1 mM glucose and supplemented with 10 % FBS. Medium was changed to 1 mM glucose for 24 h then to 0.1 mM glucose + 0.5 % BSA for an additional 2 h prior to treatment with glucose in the presence or absence of H₂O₂ as described in RESULTS. For static insulin secretion assays cells were cultured overnight in 0.1 mM glucose and then pre-incubated twice for 30 min in KRBH + 0.1 % BSA supplemented with 0.1 mM glucose prior to stimulation with either 0.1, 0.5, 1, 1.5, 2, 5, 11 or 16 mM glucose and treated as described above. Transfection was performed using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions. Following transfection in RPMI-1640 supplemented with 11.1mM glucose and 10 % FBS the medium was changed to 1 mM glucose and after 24 hours to 0.1 mM glucose + 0.5 % BSA for an additional 2 h prior to treatment with either 0.1 or 5 mM glucose.

Immunohistochemistry

PDX-1 immunostaining was performed as previously described.²⁶ Cells were incubated with rabbit anti-PDX-1 (Abcam) and guinea pig anti-insulin (Dako) antibodies followed by Alexa Fluor® 488 (Invitrogen) and Rhodamine (Jackson ImmunoResearch) fluorophore-conjugated secondary antibodies. Coverslips were then mounted using VectaShield mounting

media containing DAPI for nuclei staining (VectorLab). Images were acquired and quantified using a fluorescence microscope (×20) and Zen Imaging Software (Carl Zeiss). Quantification was performed on 3 acquired images of each of the 3–4 independent experiments, and counting 5 cells per image. For exogenous PDX-1 immunostaining, GFP signal was visualized using a Leica confocal microscope (×40).

Statistics

Results are expressed as mean ± SEM and significance was tested using Student's paired *t* test or a 2-way ANOVA with Bonferroni *post hoc* adjustment for multiple comparisons, as appropriate, using GraphPad InStat (GraphPad Software). A value of *p* < 0.05 was considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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