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Glucose modulates Pax6 expression through the JNK/p38 MAP kinase pathway in pancreatic beta-cells

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ABSTRACT

Aim: The paired and homeodomain-containing transcription factor, paired box 6 (Pax6), has shown to play pivotal roles in beta-cell function, including cell survival, insulin biosynthesis and secretion. The present study investigates the signaling events that regulate the modulation of Pax6 expression by glucose and the role of this modulation in cell survival in rat insulinoma-1E (INS-1E) cells.

Main methods: INS-1E cells were incubated on 1 mM (low) or 25 mM (high) glucose overnight. To elucidate the signaling pathways that regulate Pax6 expression, we utilized specific inhibitors. The siRNA transfection of Pax6 into INS-1E cells was performed by electroporation. The mRNA and protein levels were determined by real-time PCR and Western blotting, respectively.

Key findings: We found that the mRNA and protein levels of Pax6 were reduced by approximately 4-fold in high, compared to low, glucose-treated cells. Staurosporine, the c-Jun N-terminal kinase (JNK) inhibitor SP600125 and the p38 mitogen-activated protein kinase (p38 MAPK) inhibitor SB203580 significantly increased Pax6 levels in high glucose-treated INS-1E cells compared to their respective controls. However, neither calcium ionophore nor the extracellular signal-regulated kinase (ERK) inhibitor U0126 resulted in any alteration in Pax6 protein expression. Further, a siRNA-mediated knockdown of Pax6 significantly decreased the expression of tumor-suppressor phosphatase with tensin homology (PTEN) while increasing cell viability in low glucose-treated INS-1E cells.

Significance: This study addresses the signaling events that regulate the glucose-dependent expression of Pax6 and the role of these events in cell survival in pancreatic beta cells.

Introduction

Glucose homeostasis is principally maintained by the alpha and beta cells of pancreatic islets through the precisely regulated release of glucagon and insulin, respectively. Glucose coordinately recruits a highly sophisticated network of transcription factors and co-activators to the insulin promoter and controls insulin production and secretion. In addition to insulin, glucose regulates the expression of various genes that encode the proteins involved in cellular maintenance, repair, transcription and RNA splicing. The glucose regulation of beta-cell function is an important mechanism by which cells can adapt their metabolism and function to variations in the concentration of this vital nutrient (Poitout et al., 2006; Meugnier et al., 2007; Martinez et al., 2006).

Paired box 6 (Pax6) is a transcription factor with two DNA-binding domains (a paired box and a homeobox) and a proline–serine threonine (PST)-rich transactivation domain at the C terminus (Mishra et al., 2002). Pax6 plays a vital role in the development of the eye, central nervous system and the pancreas (Kozmik, 2008; Sansom et al., 2009; Hart et al., 2013) and is crucial for beta cell maturation through its transcriptional control of key genes that code for the proteins involved in insulin biosynthesis and secretion and in glucose and incretin actions on beta-cells (Gosmain et al., 2012). It has been shown that heterozygous mutations in the Pax6 gene can induce glucose intolerance (Yasuda et al., 2002). The loss of Pax6 in the adult islet cells has been demonstrated to affect the expression of multiple target genes involved in the maintenance of pancreatic endocrine function and glucose handling, resulting in the rapid appearance of diabetic symptoms (Hart et al., 2013). Most recently, it has been shown that Pax6 is involved in CCCTC-binding factor-mediated regulation of beta cell survival (Tsui et al., 2014). Therefore, the present investigation of the mechanisms by which glucose regulates Pax6 in beta-cells should help us understand beta-cell function and its associated disorders.

In the present study, we show that glucose modulates Pax6 expression through the c-Jun N-terminal kinase (JNK)/p38 mitogen activated protein kinase (p38 MAPK) pathways in INS-1E cells. Further, we demonstrate that tumor-suppressor phosphatase with tensin homology (PTEN) is one of the possible targets of Pax6 in INS-1E cells.

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Materials and methods

Chemicals

Staurosporine, nifedipine, SP600125, U0126, ionomycin and SB203580 were purchased from Sigma Chemicals Company, Saint Louis, MO, USA. Cell culture media were obtained from HiMedia Laboratories Pvt. Ltd., Mumbai, India. All of the other chemicals used were of analytical grade and were obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai, India and HiMedia Laboratories Pvt. Ltd., Mumbai, India.

Cell culture

Rat insulinoma-1E (INS-1E) cells were cultured in a humidified atmosphere containing 5% CO_2 in complete medium, composed of RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 2 mM glutamine, 10 mM Hepes, 100 units/ml penicillin, and 100 μg/ml streptomycin. For the glucose regulation experiments, cells were grown in complete medium and then washed with 3× phosphate-buffered saline before being transferred to low (1 mM) or high (25 mM) glucose-containing media for the indicated times. For inhibitor-based assays, cells were treated with specific inhibitors 1 h prior to glucose treatment.

Real time PCR

Briefly, the total RNA from INS-1E cells was isolated according to instructions from the RNA isolation kit (One step RNA TRIzol Reagent; Biobasic Inc., Markham Ontario, Canada). First-strand cDNA synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., USA) by following the manufacturer's protocol. The real-time amplification of the cDNA was achieved using the Brilliant SYBR Green QPCR Master Mix according to the manufacturer's protocol (Ampliqon A/S; Skovlunde, Denmark). The specific set of primers used for Pax6 and β-actin genes were F: 5′-CCAACGACAATATACCCAGTGTGTC-3′ and R: 5′-TGTTGCTGGCAGCCGTCTTGCGTG-3′ and F: 5'-TTCAACACCCCAGC CATGT-3' and R: 5'-TGGTACGACCAGAGGCATACAG-3', respectively. Fold differences in Pax6 expression were calculated using the formula $2^{\Delta\Delta Ct}$.

Western blotting

Cell extracts from INS-1E cells were prepared in lysis buffer (10 mM Tris, pH 8.0, 140 mM NaCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.5% Nonidet P-40, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride, protease and phosphatases inhibitors). Proteins were separated by SDS-PAGE and subsequently electroblotted onto nitrocellulose membranes. Membranes were blocked for 1 h at room temperature in 1× TTBS (20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Triton X-100) supplemented with 5% Carnation nonfat dry milk. After blocking, membranes were incubated overnight at 4 °C, either with antibodies specific for Pax6, β-actin, cyclin D1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or PTEN (Cell Signaling Technology, USA). Membranes were washed four times for 10 min in TTBS and subsequently incubated for 1 h at room temperature with a secondary horseradish peroxidase-conjugated antibody. Proteins were visualized using the 3,3'-Diaminobenzidine (DAB) chromogen system (Bio-Rad Laboratories, Inc., USA). The densitometric analyses were carried out with lab image platform ver 2.1 software by Kapelan Bio-Imaging GmbH.

siRNAs transfection

Transfection with small interfering RNAs (siRNAs) was accomplished using the Eppendorf Multiporator system under optimized conditions. Silencer negative control (sc-37007) and Pax6 (sc-270113) siRNAs were obtained as annealed oligos from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. The siRNAs were transfected at a final concentration of 200 nM.

Assay of cell viability

Cell viability was determined using a colorimetric, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay. Briefly, Pax6 knockdown INS-1E cells were cultured in serum-free low (1 mM) or high (25 mM) glucose-containing media overnight. Next, the MTT solution was added (0.5 mg/ml) to the cells and incubated for 4 h. The blue formazan products formed in the INS-1E cells were dissolved in DMSO and spectrophotometrically measured at a wavelength of 550 nm.

Data analysis

The values are expressed as means ± standard deviation (SD). Differences between the groups were assessed by one way analysis of variance using the GraphPad Prism 6.0 software package for Windows. Post hoc testing was performed for inter-group comparisons using Tukey's multiple comparison test. Values are statistically significant at $^{**}P < 0.001$ and $^{*}P < 0.05$.

Results

High glucose suppresses Pax6 expression

In the present study, we investigated whether glucose has any role in the regulation of Pax6 expression in INS-1E cells. To test this idea, INS-1E cells were incubated on 1 mM (low) or 25 mM (high) glucose overnight. The mRNA and protein levels were determined by Real Time PCR and Western blotting, respectively. Interestingly, we found that the mRNA and protein levels of Pax6 were reduced by 4-fold approximately in high glucose when compared to low glucose treated cells (Fig. 1A & B). Further, we have also analyzed the expression levels of Pax6 in normal glucose (5.5 mM) and osmotic control (25 mM mannitol) media. The protein levels of Pax6 were significantly reduced in high glucose when compared to normal and osmotic controls (Fig. 1C). To test whether high glucose removal restores Pax6 expression, high glucose-treated cells were switched to low glucose overnight. Interestingly, the removal of high glucose from the culture media restored Pax6 protein expression in INS-1E cells (Fig. 1D). These results reveal that high glucose suppresses Pax6 expression in INS-1E cells.

High glucose suppression of Pax6 is mediated by phosphorylation

To test whether Pax6 levels are regulated by cellular kinases, we treated low or high glucose-grown INS-1E beta-cells with the general protein kinase inhibitor staurosporine. Staurosporine treatment in high glucose-added INS-1E cells significantly increased Pax6 protein levels when compared to cells treated with high glucose alone. However, staurosporine treatment in low glucose did not show any change in Pax6 protein expression when compared to INS-1E cells treated with low glucose alone (Fig. 2). These results indicate that phosphorylation event(s) suppress(es) Pax6 expression in high glucose-treated INS-1E beta-cells.

Calcium signaling is not involved in Pax6 regulation

It has been shown that glucose increases intracellular calcium levels and mediates Ca^{2+}-dependent phosphorylation events in beta-cells (Wollheim and Pozzan, 1984; Alejandro et al., 2010). To identify whether Ca^{2+}-dependent phosphorylation events are involved in the glucose regulation of Pax6 expression in INS-1E cells, we treated low or high