Inhibition of Cyclin-dependent Kinase 5 Activity Protects Pancreatic Beta Cells from Glucotoxicity*

Mariano Ubeda¹, J. Michael Rukstalis¹, and Joel F. Habener¹

From the ¹Laboratory of Molecular Endocrinology and the ²Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02115 and the ³Howard Hughes Medical Institute, Boston, Massachusetts 02114

Type 2 diabetes (T2D) and Alzheimer disease are degenerative diseases that may share common pathophysiologic mechanisms. Neuronal dysfunction in Alzheimer patients has been linked to overactivity of the cyclin-dependent kinase 5 (CDK5) and its activator p35. Both of these proteins are expressed in the insulin-producing beta cells of the pancreas. Further, glucose enhances p35 gene expression, promoting the formation of active p35/CDK5 complexes that regulate the expression of the insulin gene. In T2D, chronic elevations of glucose, glucotoxicity, impair beta cell function. We therefore postulated that CDK5 and p35 may be responsible for this beta cell impairment and that inhibition of CDK5 might have a beneficial effect. To test this hypothesis, the pancreatic cell line INS-1 was selected as a known in vitro model of glucotoxicity, and roscovitine (10 μM) was used as a CDK5 inhibitor. Chronic exposure of INS-1 cells to high glucose (20–30 mM) reduced both insulin mRNA levels and the activity of an insulin promoter reporter gene. Inhibition of CDK5 prevented this decrease of insulin gene expression. We used DNA binding (gel shift) assays and Western immunoblots to demonstrate that cellular levels of the transcription factor PDX-1, normally decreased by glucotoxicity, were preserved with CDK5 inhibition, as was the binding of PDX-1 to the insulin promoter. Analyses of nuclear and cytoplasmic PDX-1 protein levels revealed that CDK5 inhibition restores nuclear PDX-1, without affecting its cytoplasmic concentration, suggesting that CDK5 regulates the nuclear/cytoplasm partitioning of PDX-1. Using a Myc-tagged PDX-1 construct, we showed that the translocation of PDX-1 from the nucleus to the cytoplasm during glucotoxic conditions was prevented when CDK5 was inhibited. These studies indicate that CDK5 plays a role in the loss of beta cell function under glucotoxic conditions and that CDK5 inhibitors could have therapeutic value for T2D.

Type 2 diabetes mellitus (T2D)² and Alzheimer disease are age-related diseases whose prevalence continues to increase in populations throughout the world. Although an understanding of the pathogenesis of these two diseases is limited, similarities in the pathological alterations in their affected cell types (insulin-producing beta cells in diabetes and neurons in Alzheimer disease) led to the identification of a new signaling pathway in pancreatic beta cells (1). We previously showed that the cyclin-dependent kinase-5 (CDK5) and its activator p35, initially believed to be specific for brain tissue, are also present in pancreatic beta cells. Further, glucose enhances p35 gene expression, promoting the formation of active p35/CDK5 complexes that in turn regulate the expression of the insulin gene (1).

Transient elevations of extracellular glucose promote pancreatic beta cell function and survival (2, 3), whereas chronic elevations of glucose have the opposite effect, impairing beta cell function and survival (4–6). The deleterious effects of chronically elevated glucose are referred to as glucotoxicity. Milder forms of glucotoxicity are referred to as glucose desensitization. Glucotoxicity is a critical component of the pathophysiology of T2D because it impairs both the actions of insulin on peripheral tissues and the secretion of insulin by beta cells. Impairment of insulin secretion contributes to further glucose elevation and resultant glucotoxicity. This self-perpetuating forward feedback mechanism accelerates the progression of diabetes and the appearance of diabetic complications (7).

Because intracellular overactivation of CDK5 is a component of the pathophysiology of Alzheimer disease (8–10) and hyperglycemia results in overactivation of CDK5 in beta cells (1), we investigated the possibility that CDK5 signaling may be involved in T2D. We hypothesized that chronic elevation of glucose may induce overactivation of CDK5 in beta cells and promote functional alterations that result in deficient insulin release. This hypothesis predicts that inhibition of CDK5 signaling during glucotoxic stimulation will have a beneficial effect by preserving the function of pancreatic beta cells. To test this possibility, we inhibited CDK5 activity with roscovitine and examined the effects on INS-1 cells exposed to high glucose, a well-established in vitro model of glucotoxicity. Sustained elevations of glucose concentrations in these cells, for periods as short as 48 h, are known to suppress insulin gene expression (11).

The suppression of insulin gene expression by glucotoxicity in beta cells involves several transcription factors and different pathophysiological mechanisms. These mechanisms include the translocation of PDX-1 (also known as STF-1, IPF-1, and IDX-1) from the nucleus to the cytoplasm (12), the proteasomal degradation of MafA (RIPE3b1) (13), and the induction of transcription factors such as CCAAT enhancer-binding protein β.

2 The abbreviations used are: T2D, type 2 diabetes; CDK5, cyclin-dependent kinase-5; PBS, phosphate-buffered saline; siRNA, small interfering RNA; ROS, reactive oxygen species.

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1 An investigator with the Howard Hughes Medical Institute. To whom correspondence should be addressed: Laboratory of Molecular Endocrinology, MA General Hospital, THEIR 320, 55 Fruit St., Boston MA 02114. Tel.: 617-726-5190; Fax: 617-726-6954; E-mail: jhabener@partners.org.

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(14) that act as negative regulators of insulin gene transcription (15). We now show that overstimulation of CDK5 in an experimental model of glucotoxicity is involved in the cytoplasmic translocation of PDX-1 during glucotoxicity. In fact, inhibition of CDK5 with roscovitine prevents the loss of insulin gene expression, an effect mediated, at least in part, by the preservation of the nuclear localization of PDX-1 and its corresponding DNA binding and transcriptional activities. These data unveil a new molecular target for therapies aiming to prevent, to cure, or to reduce the pathological alterations of diabetes mellitus.

EXPERIMENTAL PROCEDURES

Chemicals and Antibodies—Tissue culture media, serum, and other cell culture reagents were purchased from Invitrogen. DNA-modifying enzymes were acquired from New England Biolabs (Beverly, MA). Roscovitine was purchased from Calbiochem. A rabbit polyclonal PDX-1 antibody (Hm 253) was generated by Covance Research Products (Philadelphia, PA) against the carboxy-terminal portion of PDX-1, and a Myc tag antibody (9E10) was obtained from BD Biosciences.

Cell Lines and Cell Culture Conditions—Rat INS-1 cells were provided by C. Wollheim from the University of Geneva, Switzerland. We used cell passages 99–110. INS-1 cells were propagated in RPMI 1640 medium containing 10% fetal bovine serum, 10 mM HEPES, 11.1 mM glucose, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 100 μM penicillin G, and 100 μg/ml streptomycin. Glucose concentration was adjusted to the experimental conditions by adding 1 mM glucose to glucose-free RPMI 1640.

RNA Preparation and Northern Blots—Total RNA from INS-1 cells was extracted using a Qiagen RNeasy kit (Valencia, CA). Aliquots of 10 μg of total RNA were lyophilized and denatured at 65 °C in 7.2% formaldehyde and 50% formamide for 5 min. RNAs were then separated on a 0.66 M formaldehyde agarose gel and transferred to a nylon membrane and hybridized with specific rat insulin and actin probes as described previously (16).

Protein Extraction and Western Blot Assays—Nuclear and cytoplasmic extracts were prepared as described previously (17) in the presence of the protease inhibitors pepstatin A (1 mg/ml), leupeptin (10 mg/ml), aprotonin (10 mg/ml), and p-aminobenzamidine (0.1 mM). Protein concentrations were determined using a Bio-Rad reagent kit. For Western blots, 50 μg of nuclear extracts and 200 μg of cytoplasmic extracts of total protein were run on a 10% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane as described previously (1). Membranes were incubated with a primary antibody followed by a horseradish peroxidase-conjugated secondary antibody, and immunoreactive proteins were visualized with the luminescence-enhanced chemiluminescence system (Amersham Biosciences).

Immunoprecipitation and Kinase Assay—Determination of CDK5 activity in cellular extracts from INS-1 cells grown in normal (5 mM) and high (30 mM) concentration of glucose was achieved as reported previously (1). Briefly, immunoprecipitated kinase complexes were incubated with histone H1 substrate in the presence of [γ-32P]ATP. Samples were run on 10% SDS-PAGE gels and subjected to analysis by autoradiography.

Transient Transfections and Luciferase Assays—Adherent INS-1 cells grown to 80–90% confluence in 24-well plates were transfected using Lipofectamine 2000 (Invitrogen). Cells were rinsed twice in serum-free culture medium before the addition of the transfection mix that contained 250 ng of reporter (−410 Ins-Luc). Cells were then incubated at 37 °C for 4 h before replacing the normal medium with medium containing different concentrations of glucose as indicated in the figure legends and results. Firefly luciferase activity was determined using a luciferase assay kit from Promega (Madison, WI). All experiments were carried out in triplicate (n = 3) on at least three different occasions.

Electrophoretic Mobility Shift Assays—DNA-protein binding assays were carried out by analyzing 10 μg of nuclear extracts and 10 fmol of the radiolabeled double-stranded DNA probe on non-denaturing polyacrylamide gels. The sequence of the E2/A3 element used was: 5’-GATCTTAG-ACCTAGGTAAATTATATTAAACAGGGGCGAGA-TGCCGATGAGAG-3’. The gels were run at 180 V in 0.5× Tris borate-EDTA buffer, and the radiolabeled proteins were detected by exposure of the dried gels to x-ray film.

Inhibition of CDK5 Expression with siRNAs—Specific depletion of CDK5 was achieved by using small (21 bp) interfering double-stranded ribonucleotides (siRNA) specifically designed to target the coding sequence of the rat CDK5 mRNA. The targeted sequence was 5’-GUUCAGCCUCUGGAGAUUUT-3’. A corresponding 21-bp scramble siRNA was used as control. These siRNAs were synthesized, high pressure liquid chromatography-purified, and annealed (Dharmacon Research Inc., Lafayette, CO). INS-1 cells were transfected with the corresponding ribonucleotides using Lipofectamine 2000 as described above. The specific depletion of CDK5 protein was evaluated by its detection using Western blot assays.

Immunocytochemistry—Detection of nuclear and cytoplasmic PDX-1 in individual cultured cells was achieved by the following procedure. Cells grown on microscope slides were fixed in 4% paraformaldehyde (in PBS) for 10 min at room temperature and rinsed in PBS 3 × 5 min. Nonspecific binding was blocked by 3% normal donkey serum for 45 min at room temperature. Slides were then incubated with primary antibody at a 1:5000 dilution for PDX-1 antibody and 1:500 for Myc antibody overnight at 4 °C. Slides were then washed in PBS (3 × 5 min) to eliminate excess antibody. Incubations with secondary antibodies (Cy3 and Cy2) for 45 min at room temperature were followed by final washes in PBS (3 × 5 min), and slides were then mounted using fluorescence mounting medium (Kirkgaard & Perry Laboratories, Gaithersburg, MD). Cells were visualized using an epifluorescence microscope (Carl Zeiss, Inc., New York, NY) equipped with a TEC-470 CCD camera (Optronics International, Chelmsford, MA).

Statistical Analysis—Data are presented as the mean ± S.E. An analysis of variance for repeated measures was used to estimate the significance of differences in the measured values within and between groups followed by Fisher’s f test. Values of p < 0.05 were considered statistically significant.
RESULTS

Inhibition of CDK5 by Roscovitine Prevents the Down-regulation of Insulin mRNA Expression Caused by Chronic High Glucose—To test the hypothesis that CDK5 inhibition may protect insulin-producing beta cells from glucotoxicity, we established cultures of INS-1 cells in different concentrations of glucose and examined insulin mRNA levels using Northern blot analysis. A dose-dependent reduction of insulin mRNA was observed after incubating the cells in glucotoxic conditions for 48 h (20 or 30 mM glucose) (Fig. 1A).3 These data corroborated previous findings in cultured beta cell lines (11). We confirmed that this reduction of insulin mRNA levels was the result of a transcriptional response by measuring the activity of a luciferase reporter construct driven by the insulin promoter sequence (−410 of the rat insulin-1 gene). The activity of this reporter was also attenuated by elevations in the concentration of glucose in a dose-dependent manner (Fig. 1B) confirming previously reported findings (11). We next determined that in these experimental conditions, sustained elevation of glucose concentration produces an increase of CDK5 activity that remains elevated over the 48 h duration of these experiments (Fig. 1C). This phenomenon is mediated by increased expression of the p35 activator and not by changes in CDK5 protein levels (Fig. 1C). To test the effect of CDK5 inhibition on insulin gene expression, we studied the effect of the CDK5 inhibitor roscovitine on the glucotoxicity-induced down-regulation of insulin mRNA. Roscovitine, 10 μM, was added to confluent cultures of INS-1 cells under conditions previously optimized to achieve specific inhibition of CDK5 activity (1). Under these conditions, roscovitine almost completely prevented the down-regulation of insulin mRNA induced by high glucose (Fig. 1D). Densitometry analysis and statistical comparison of this set of data demonstrate that the effect of roscovitine is statistically significant (Fig. 1E).

Inhibition of CDK5 Preserves the Binding of PDX-1 DNA to the Insulin Promoter—Earlier studies identified that a mechanism by which glucotoxicity decreases insulin gene expression in beta cells is the depletion of transcription factors essential for both tissue-specific and glucose-

3 It is important to note that the fasting glucose levels in rats are typically 7.5 mM, 50% higher than they are in humans (5.5 mM) so that 30 mM glucose in rat INS-1 cells is equivalent to 20 mM glucose in human cells.
induced insulin gene expression (4, 5, 11). One such transcription factor is PDX-1, a pancreas- and duodenum-specific homeotic transcription factor required for pancreas formation and insulin gene expression (18). PDX-1 binds to the A-elements of the insulin promoter and enhances insulin gene expression by interacting with other transcription factors that bind to E-boxes within the promoter. Glucotoxicity has been shown to impair the binding of PDX-1 to the insulin promoter (4, 11). Forced overexpression of PDX-1 under glucotoxic conditions prevents, at least in part, the down-regulation of insulin gene expression (19). Therefore, we investigated the role that inhibition of CDK5 has on PDX-1 binding to the E2/A3 motif of the insulin promoter containing the E2 and A3 elements. We observed that prolonged exposure (48 h) of INS-1 cells to glucose results in a dose-dependent decrease of DNA binding to the E2/A3 element (Fig. 2A). This decrease was specific for DNA-protein complexes (Fig. 2A, complexes C and D), whereas other complexes did not change (Fig. 2A, complexes A and B). By using an antibody for PDX-1, we determined that complex D, and to a lesser extent, complex C, contained DNA-PDX-1 binding complexes. The antibody attenuated the corresponding bands and resulted in the appearance of a new supershift band (Fig. 2B, SS). We then investigated whether inhibition of CDK5 with roscovitine affects the binding of PDX-1 to DNA (Fig. 2C) under glucotoxic conditions. We observed that inhibition of CDK5 preserves PDX-1 binding to the E2/A3 element under conditions of glucotoxicity. The intensities of complexes C and D (Fig. 2C) increased when the cells were concomitantly treated with the CDK5 inhibitor roscovitine.

**Cellular Fractionation Indicates That the Inhibition of CDK5 Preserves the Accumulation of PDX-1 in the Nucleus**—To investigate the mechanism responsible for the increase in PDX-1 binding activity in response to the inhibition of CDK5 activity, INS-1 cells were cultured at low (5 mM) and high (30 mM) glucose for a 48-h period in the presence or absence of roscovitine (10 μM). Nuclear and cytoplasmic fractions were prepared, and protein extracts were subjected to Western blot analysis. The results of these studies showed that inhibition of CDK5 restores the level of PDX-1 in the nucleus (Fig. 3A) that is normally reduced by chronic exposure to high glucose. This effect was specific for PDX-1 since the level of another nuclear protein (poly(ADP-ribose)polymerase (PARP)) was unaffected (Fig. 3A). Denitometry quantitative analysis demonstrates that the effect is statistically significant (Fig. 3B). To examine the glucose specificity of our observations, the INS-1 cells were co-cultured in glucose (5 mM) plus mannitol (15 mM), the latter a non-metabolizable glucose analog, to block glucose metabolism. Mannitol had no effect on the levels of nuclear PDX-1, indicating that glucotoxicity is dependent on glucose metabolism (Fig. 3B, lower panel). No significant changes of cytoplasmic PDX-1 were observed in these experiments (Fig. 3C).
Translocation of PDX-1 from the Nucleus to the Cytoplasm Is Blocked by the inhibition of CDK5 Activity—The selective depletion of PDX-1 in the nucleus in response to glucotoxicity and its reversal by roscovitine suggest that inhibition of CDK5 prevents the translocation of PDX-1 from the nucleus to the cytoplasm induced by glucotoxicity. Earlier studies indicated that when beta cells are exposed to chronic high glucose or oxidative stress, levels of nuclear PDX-1 decrease due to a translocation to the cytoplasm (12). Therefore, we tested the possibility that the inhibition of CDK5 prevents translocation of PDX-1 from the nucleus to the cytoplasm. We prepared a Myc-tagged construct of PDX-1 and examined its cellular distribution in response to glucotoxicity and treatment with roscovitine. Chronic stimulation of INS-1 cells with high glucose increased cytoplasmic and decreased nuclear staining of both the Myc-tagged PDX-1 and the endogenous PDX-1 (Fig. 4A). Inhibition of CDK5 with roscovitine restored the accumulation of PDX-1 in the nucleus and decreased cytoplasmic staining. The proportion of cells with nuclear and cytoplasmic staining was scored for ~1000 cells/treatment in three independent experiments (Fig. 4B). Glucotoxicity significantly increased the frequency of cells with detectable cytoplasmic PDX-1, and inhibition of CDK5 with roscovitine prevented such an increase. Further, the inhibition of CDK5 restored the frequency of cells positive for nuclear PDX-1 to that of cells unexposed to high glucose (Fig. 4B). No changes were observed when roscovitine was applied to INS-1 cells grown in 5 mM glucose. To further evaluate the specificity of such an effect, we used a set of double-stranded ribonucleotides designed to interfere with the CDK5 mRNA sequence (siRNAs) to induce its specific degradation. First, we tested the efficacy and specificity of this approach by determining the level of CDK5 and β-actin proteins after siRNA administration (Fig. 5A). Then, we studied the effect of CDK5 siRNA on the intracellular distribution of endogenous and Myc-tagged PDX-1 after transfection of the corresponding expression vector. Quantitative analysis of the data indicates that CDK5 siRNA prevents the translocation of PDX-1 from the nucleus to the cytoplasm induced by glucotoxicity (Fig. 5B). No effect was observed at normal glucose concentrations. These results further corroborate the findings observed with pharmacological inhibition of CDK5 kinase activity and support the notion that sustained activation of CDK5 is a new pathophysiological mechanism that contributes to the impairment of beta cell function due to chronic exposure to high glucose.

FIGURE 3. Inhibition of CDK5 signaling prevents the depletion of nuclear PDX-1 without affecting the cytoplasmic concentration. A, Western blot analysis of nuclear extracts from INS-1 cells treated with roscovitine (10 μM) and exposed to chronic high glucose (30 mM) demonstrates that inhibition of CDK5 prevents the decrease in nuclear PDX-1 induced by glucotoxicity. PARP, poly(ADP-ribose)polymerase. B, densitometric quantification and statistical analysis demonstrate that inhibition of CDK5 significantly changes the nuclear distribution of PDX-1 in INS-1 cells exposed to high glucose. Mannitol (15 mM), a non-metabolizable analog of glucose, had no effect on nuclear levels of PDX-1, indicating that the specificity of glucose toxicity requires glucose metabolism (lower panel) Dens units, density units. C, similar studies performed with the cytoplasmic extracts did not detect any changes in cytoplasmic PDX-1. Three independent experiments were performed with duplicate samples. INS-1 cells were maintained at two different concentrations of glucose (5 and 30 mM) for 48 h. Both sets of experiments were treated with roscovitine or vehicle control for the duration of the experiment.
DISCUSSION

The studies presented herein demonstrate that in beta cells, long term exposure to high glucose results in a corresponding sustained elevation of CDK5 activity. This elevation is due to an increased expression level of its p35 activator without changes in the level of CDK5 protein. We also show that inhibition of CDK5 activity with roscovitine protects pancreatic beta cells from some of the deleterious effects of chronic exposure to supraphysiological concentrations of glucose (glucotoxicity). It is generally believed that many of the alterations associated with diabetes mellitus are induced by the glucotoxic milieu associated with the disease. In T2D, chronic hyperglycemia contributes to both insulin resistance and the functional impairment of beta cells. This circumstance creates a positive forward feedback mechanism responsible for further metabolic deterioration and the eventual appearance of secondary vascular complications. Investigations of beta cell glucotoxicity have utilized several in vitro (4, 11, 20) and in vivo (21, 22) experimental models that collectively demonstrate decreased rates of insulin release, diminished insulin production, and a reduction in insulin gene expression. In these studies, we have corroborated previous findings in INS-1 cells, indicating that the expression of insulin mRNA is remarkably reduced when the cells are cultured in medium containing a high glucose concentration for a period as short as 48 h. Although this model may not represent the full complexity of pathophysiological alterations associated with diabetes mellitus, it has several advantages over other in vitro models of glucotoxicity. These other models require longer exposure times to high glucose, and therefore, multiple in vitro cell passages are also required. Selective toxicity on actively replicating beta cells may allow for selection of certain cell populations better adapted to survive in high glucose. Because in vivo, beta cells have a very low rate of replication, we consider that our data obtained with confluent INS-1 cells may better represent the pure functional alterations characteristic of glucotoxicity.

Glucotoxicity-induced reductions in the levels of transcription factors PDX-1 and MafA (RIPE3b1) in the nuclei of beta cells were identified as potential mediators of impaired insulin production (4, 5, 11). Our findings suggest that the inhibition of CDK5 under conditions of glucotoxicity preserves insulin gene expression by a mechanism that involves the restoration of the DNA binding and nuclear localization of PDX-1. We did not consider in these studies the involvement of MafA because its nuclear depletion during glucotoxicity depends on proteasomal degradation and not on a translocation mechanism such as the one we demonstrate for PDX-1. We found that the restoration of the nuclear levels of PDX-1 by inhibition of CDK5 occurs by preventing the translocation of PDX-1 from the nucleus to the cytoplasm. These findings support the notion that overactivation of CDK5

FIGURE 4. To investigate the effect of CDK5 inhibition on the intracellular localization of PDX-1 at different glucose concentrations, the cellular distributions of both endogenous PDX-1 and a Myc-tagged PDX-1 protein were evaluated by immunofluorescence. A, localization of a Myc-tagged PDX-1 fusion protein was detected by immunocytochemistry in INS-1 cells after transfection. Culture conditions and roscovitine treatments are indicated in the figure. The fate of endogenous PDX-1 was detected in the same cells by using a PDX-1-specific antibody (Ab). B, the cells with clear cytoplasmic or nuclear PDX-1 were counted, and the results were statistically analyzed. For these experiments, ~1,000 cells were scored for each one of the treatments on three independent occasions to allow for statistical analysis. Statistical significance is depicted as * (p < 0.05) when compared with control values and # (p < 0.05) when compared with glucotoxic values.
The mechanism responsible for nuclear depletion of both PDX-1 and MafA during glucotoxicity seems to require the formation of reactive oxygen species (ROS) (12, 13, 24). Because ROS formation and oxidative stress are also characteristic pathogenic alterations in Alzheimer disease, it may be that in beta cells, glucotoxicity-induced oxidative stress is also linked to the pathogenic alterations caused by CDK5 activation. In favor of this possibility are earlier findings, indicating that in human neuroblastoma-derived cells, the induction of reactive oxygen species results in an elevation of p35/CDK5 activity (25). Based on these findings, we suggest that CDK5 activation during chronic exposure to supraphysiological concentrations of glucose may be linked to the generation of ROS. For these reasons, we are currently focusing our investigation on the relationship between CDK5 activation and the generation of ROS species in beta cells.

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