The Effects of Glucose and Amylin on β-Cell Death
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Abstract
Alzheimer’s Disease (AD) and T2DM are both characterised by the deposition of a locally aggregated protein, β-amyloid in AD and fibrillar IAPP in T2DM. Cdk5 expressed in neurons is activated via the aberrant cleavage of its activator p35 to p25. This Cdk5/p25 complex initiates the apoptotic pathway, causing neurotoxicity and AD. β-cells express Cdk5 and have recently been shown to express the p35 activator, suggesting that the β-cell death mechanism parallels the neuropathology of AD. This study confirms the gene and protein expression of Cdk5, p35 and p25 in pancreatic β-cell lines NIT-1, BTC-6 and INS-1. The effects of chronic glucose stimulation and amylin oligomerisation on the gene and protein expression of Cdk5, p35 and p25 and the induction of β-cell apoptosis in the three cell lines is also studied. Studying these effects would enable the determination of the role of Cdk5/p35 and Cdk5/p25 in the molecular pathway of glucose and/or amylin-induced β-cell death in in vitro and in vivo diabetic conditions. This would establish the β-cell death mechanism and link the pathologies of the previously unassociated diseases, AD and T2DM, enabling application of AD therapeutic interventions to T2DM.

Introduction
Amyloid deposits in pancreatic β-cells have recently gained attention as an important player in the pathogenesis of β-cell death (Khan et al. 1990). Though the mechanism of amylin-induced β-cell toxicity remains unclear, it parallels the neuropathology in Alzheimer’s disease (Leena et al. 2008) as apoptosis of cells occur from a deposition of a locally aggregated protein, the β-amyloid in Alzheimer’s disease and fibrillar IAPP in T2DM.

Cyclin-dependent kinase 5 (Cdk5). is a key protein involved in both diseases Its activators, p35 and p39 are predominantly expressed in neurons and was thought had activity limited to neuronal activities (Wei et al. 2007). In AD, Cdk5 is hyperactivated via aberrant cleavage of its activator p35 to p25. The Cdk5/p25 complex is more stable than its parent Cdk5/p35 complex and this triggers downstream signalling cascades which resulting in tau phosphorylation and increased β-amyloid production and deposition (Wei et al. 2007). β-amyloid production stimulates further cleavage of p35 to p25 triggering a vicious cycle of increased amyloid production and deposition in neurons, validating the importance of the Cdk5/p25 signalling pathway in the pathogenesis of neurodegenerative diseases (Wei et al. 2007).

Recently, p35 expression was also discovered in non-neuronal cells like the pancreatic β-cells and this expression increases when the β-cells are exposed to chronic levels of glucose. This, together with other glucotoxicity-related symptoms such as hyperactivated Cdk5 and amylin deposition in β-cells leading to ultimate β-cell death. (Wei et al. 2007)

The intermediate oligomers of amylin are the most toxic to pancreatic β-cells (Janson et al 1999). However, the mechanism of the amylin-induced-cell toxicity remains unknown. With the discovery of Cdk5 and p35 in pancreatic cells, it can be postulated that, if β-amyloid activated Cdk5/p25 signalling pathway plays an important role in neuron death, the oligomerisation of amylin may also act in the same way to cause pancreatic islet cell death in T2DM.

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This study will attempt to replicate data showing Cdk5 and p35 gene and protein expression in NIT-1, BTC-6 and INS-1 pancreatic β-cells and determine the toxic effects of glucose on the above mentioned cells and its effects on Cdk5 and p35 gene and protein expression. Lastly, the study aims to focus on the toxicity of amylin oligomerisation in the above mentioned cells

**Materials and Methods**

**Cell culture**

Rat INS-1 were cultured in RPMI 1640 medium containing 11.1 mM glucose supplemented with 10% Fetal Bovine Serum (FBS), 1 mM sodium pyruvate, 10 mM Hepes, 50 μM β-mercaptoethanol, 100 μM penicillin and 100 mg/ml streptomycin. BTC-6 cells were propagated in Dulbecco's modified Eagle's medium containing 15% FBS, 100 μM penicillin and 100 mg/ml streptomycin. NIT-1 cells were grown in F12K Medium supplemented with 10% FBS, 100 μM penicillin and 100 mg/ml streptomycin.

**Glucose treatment**

NIT-1, BTC-6 and INS-1 cells were treated with 10, 15, 20, 25 and 30 mM glucose, 2, 5, 10, 15, 20, 30 mM glucose and 12, 15, 20, 25 and 30 mM glucose respectively. The treated cells were subsequently harvested for protein and RNA after 24 hours.

**Western Blotting**

20 micrograms of total protein from each sample was first heat denatured at 95°C for 10 minutes prior to separation in a 12% polyacrylamide gel and transferred onto a nitrocellulose membrane. The membranes were probed with primary antibodies overnight at 4°C and subsequently incubated with a horseradish peroxidase-conjugated secondary antibody for an hour at room temperature. The protein bands were detected using a chemiluminescent Amersham ECL Plus Western blotting detection system.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

1 μg of the previously isolated total RNA was converted to complementary DNA (cDNA) using the High-capacity cDNA Reverse Transcription Kit as per the manufacturer’s protocol. The PCR reaction was carried out with a mixture of 5 ng cDNA, 1× PCR buffer, 0.1 mM dNTP-mix, forward and reverse primers and 0.2 U Taq DNA polymerase (5 U/μl). β-actin mRNA was used as the internal standard.

**Amylin treatment**

Cells on coverslips were treated with 100 nM human amylin, which was “pre-aged” at room temperature for 0, 15, 30, 45, 60 or 120 min. The treated coverslips were then fixed after 6, 24 or 48 hours. Each treatment was performed in duplicates. TUNEL assays were later performed on the cells according to the manufacturer’s instructions.

**Results**

1. **Endogenous Expression of Cdk5 and Its Activator p35 in Pancreatic β-cells**

   To determine the presence of endogenous transcription of Cdk5 and p35 mRNA in INS-1, BTC-6 and NIT-1 pancreatic cell islets, RT-PCR was performed on RNA obtained from the cell lines using Cdk5 and p35 specific primers. The 3 insulin-secreting pancreatic β-cell lines have endogenous Cdk5 and p35 gene expression.

2. **The Effects of Varied Glucose Stimulation on the Expression of Cdk5 and p35 in NIT-1 Pancreatic β-cells.**

   RT-PCR and Western blots results show that increasing glucose concentrations had no effect on expression of Cdk5, p35 and p25 at both the gene and protein level.

3. **The Effects of Varied Glucose Stimulation on the Expression of Cdk5 and p35 in BTC-6 Pancreatic β-cells.**

   RT-PCR and Western blots show that increasing glucose concentrations had no effect on expression of Cdk5, p35 and p25 for BTC-6 cells in both the transcriptional and translational level.

4. **The Effects of Varied Glucose Stimulation on the Expression of Cdk5 and p35 in INS-1 Pancreatic β-cells.**
An increase in glucose concentration in INS-1 cells affects p35 expression at the translational level and not at the transcriptional level. Cdk5 and p25 gene and protein expression are not affected by increased glucose concentrations.

5. The Effects of Varied Glucose Stimulation on cell death in INS-1 Pancreatic \( \beta \)-cells.

The previous result showed an increase in the p35 protein expression in the INS-1 pancreatic cells in response to increasing glucose concentration treatments. The Cdk5/p35 complex is part of the apoptotic pathway in neuron death in Alzheimer’s disease and is also postulated to be part of the apoptotic pathway in \( \beta \)-cell death in T2DM. To determine the effects of varied glucose on \( \beta \)-cell death in INS-1 cells, Western blots were performed. An increase in cleaved caspase 3 protein expression suggests an increased activation of the apoptotic pathway which is indicative of increased cell death.

Fig 3B: Densitometric quantification of NIT-1 varied glucose treated cells using \( \alpha \)-tubulin to normalize the protein levels from three biological replicates (n=3)

Fig 5B: Densitometric quantification of BTC-6 varied glucose treated cells using \( \alpha \)-tubulin to normalize the protein levels from three biological replicates (n=3)

Fig 7B: Densitometric quantification of INS-1 varied glucose treated cells using \( \alpha \)-tubulin to normalize the protein levels from three biological replicates (n=3)

Fig 8B: Densitometric quantification of cleaved caspase 3 using \( \alpha \)-tubulin to normalize the protein levels from two biological replicates (n=2). Error bars correspond to the standard error of mean of each treatment condition.
A Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was carried out to ascertain the number of dying cells for 0, 15, 30, 45, 60 and 120 minutes. Cells were then exposed to the hIAPP for 6, 24 and 48 hours. In all three incubation periods, maximum cell death rate occurred at t=60mins. The cell death rate becomes more pronounced with longer incubation periods of amylin treatment.

![Figure 9. The effects of hIAPP oligomerisation on cell death in NIT-1 β-cells (6hr)](image)

![Figure 10. The effects of hIAPP oligomerisation on cell death in NIT-1 β-cells (24hr)](image)

![Figure 11. The effects of hIAPP oligomerisation on cell death in NIT-1 β-cells (48hr)](image)

**Discussion**

It was essential to first determine the endogenous expression of these three proteins in the three cell lines used in this study, to determine the presence of a Cdk5/p35 pathway in all 3 insulinoma cell lines. Determining the mechanism of glucose and amylin-induced cell death would pave the way to assessing the combined effects of glucose and amylin treatment on β-cell death and its possible mediation by Cdk5/p25. The effect of combined glucose and amylin would enable the confirmation of this study’s proposed molecular mechanism of β-cell death under *in vitro* conditions and the determination if the combined effect of chronic glucose and toxic amylin oligomers had a more pronounced effect on p35 cleavage to p25 and subsequent β-cell death.

The proposed mechanism of cell death under *in vitro* conditions needs to be replicated in primary cell cultures and *in vivo* conditions to ensure the confirmation of this mechanism under true diabetic conditions. Once the role of Cdk5 and its activators are established under *in vitro* and *in vivo* conditions of T2DM, inhibitors of certain components of the cell death pathway can be used to study its potential in the inhibition or reversal of the detrimental effects of glucose and amylin-induced cell death. One such inhibitor is the Cdk5 inhibitory peptide (CIP), which has previously been shown to halt overexpression of Cdk5 in Aβ-mediated neuronal cell death (Zheng et al, 2005). In conclusion, elucidating similarities in the role of Cdk5 and its activators in the mechanisms of cell death in both Alzheimer’s disease and T2DM is thus beneficial in the implementation of therapeutic interventions currently modelled under a seemingly different disease.

**References**

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