The effect of high glucose on APP metabolism and Aβ production

by

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B.Sc., Jilin University, 2009

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Neuroscience)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

April 2013

Abstract

Alzheimer's disease (AD) is the most common neurodegenerative disorder characterized by a progressive decline in memory and cognitive functions. It is the leading cause of dementia. Abnormal accumulation and deposition of amyloid-β protein (Aβ) to form plaques is a pathological hallmark of AD. Aβ, the major component of plaques, derives from sequential cleavage of amyloid-β precursor protein (APP) by β-secretase and γ-secretase. Dysregulation of APP processing and Aβ generation is believed to play an essential role in the pathogenesis of AD.

Diabetes is a complex metabolic disorder characterized by chronic hyperglycemia. Epidemiological studies revealed an elevated risk of developing AD in people with diabetes. However, the underlying mechanisms remain unknown. To identify the role of diabetes in AD pathogenesis, the effect of high glucose on APP metabolism and Aβ generation was investigated using cultured human neuroblastoma cells. In this study, we clearly showed that high glucose treatment significantly increased APP protein level and Aβ generation. Moreover, the increase of APP level was not resulted from the enhancement *APP* gene transcription but due to the inhibition of APP protein degradation. This work indicated that hyperglycemia could promote AD development by increasing APP expression and facilitating APP processing and Aβ production, suggesting glycemic control might be beneficial for AD treatment.

Preface

Aβ40 ELISA was performed together with Ms. Shuting Zhang using samples I had prepared.

Dr. Yili Wu helped confirm the role of high glucose on APP transcription by RT-PCR as shown in Figure 3.2.

The *APP* promoter plasmid, pAPP-Luc, which contains 2.94kb of human *APP* promoter region upstream of the luciferase reporter gene was constructed and functionally tested previously in Dr. Weihong Song's lab (Li et al., 2006).

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Aβ Amyloid-β protein

AD Alzheimer's disease

ADAM A disintegrin and metalloprotease

ADDL Aβ-derived diffusible ligand

AGEs Advanced glycation end products

AICD APP intracellular domain

ANOVA Analysis of variance

Aph-1 Anterior pharynx defective-1

APL-1 APP-related protein

APLP1 Amyloid precursor-like protein 1

APLP2 Amyloid precursor-like protein 2

APP Amyloid precursor protein

APPL Amyloid precursor protein-like

BACE1 β-site APP cleaving enzyme 1

BBB Blood-brain barrier

CHX Cycloheximide

DM Diabetes mellitus

DMEM Dulbecco's modified eagle medium

ELISA enzyme-linked immunosorbent assay

FBS Fetal bovine serum

FTDP-17 Frontotemporal dementia and parkinsonism linked to chromosome 17

GDM Gestational diabetes mellitus

GLUT Glucose transporter

HEK293 Human embryonic kidney 293 cell

HbA1c Hemoglobin A1c

hIAPP Human islet amyloid polypeptide

IFG Impaired fasting glucose

kDa Kilodalton

KPI Kunitz serine protease inhibitor

LTP Long term potentiation

mM Millimolar

NFT Neurofibrillary tangles

NICD Notch intracellular domain

NINCDS-ADRDA National Institute of Neurological and Communicative Diseases and Stroke and the Alzheimer's Disease and Related Disorders Association

OGTT Oral glucose tolerance test

PBS Phosphate-buffered saline

PCR Polymerase chain reaction

Pen-2 Presenilin enhancer-2

PS1 Presenilin 1

PS2 Presenilin 2

PVDF-FL Immobilon-FL® polyvindylidine fluoride

RAGEs Receptor for advanced glycation end products

SH-SY5Y Human neuroblastoma

sAPP$_\alpha$ Secreted APP$_\alpha$ fragment

sAPP$_\beta$ Secreted APP$_\beta$ fragment

TGF-α Transforming growth factor-α

TNF-α Tumor necrosis factor-α

20E2 HEK293 cells stably expressing Swedish mutant APP$_{695}$

Acknowledgements

I would like to thank my supervisor Dr. Weihong Song for giving me this valuable opportunity to purse graduate study in his laboratory. I'm truly grateful for his guidance, supervision and great support throughout my graduate study. I benefit a lot from his suggestions both academically and on life as a graduate student. I would also like to thank my supervisory committee members, Dr. William Jia, Dr. Shernaz Bamji and Dr. Yutian Wang for all their support, suggestions and comments.

I sincerely appreciate the wisdom and help that all the current and past lab members have given to me. I want to especially thank Dr. Yili Wu and Shuting Zhang who kindly helped me carry out some experiments, and Dr. Philip Ly and Dr. Fang Cai who gave me valuable technical training. I also want to thank Dr. Zhe Wang for his helpful suggestions, as well as Xi Liu and Fiona Zhang for their kindly assistance. I'm also grateful for the friendship and joy my colleagues have given to me: Xiaojie Zhang, Mingming Zhang, Juelu Wang, Rebecca Ko, Qian Xu, Jing Zhang, Xiaoling Duan and Bruno Heuculano. Many thanks to Dr. Yili Wu for taking time previewing and commenting this thesis.

Finally, I would like to give my sincerest thanks to my parents and friends. It was their encouragement, spur and love that supported me to get this far.

Chapter 1 General introduction

1.1 An overview of Alzheimer's disease

Alzheimer's disease (AD) is an irreversible neurodegenerative disorder characterized by a progressive decline in memory and cognitive functions. As the leading cause of dementia, AD accounts for two thirds of all cases of senior dementia. At early stage, the clinical symptoms of AD could manifest as personality change and altered behavior including paranoia, delusions, and loss of social appropriateness. It also features deterioration of memory, orientation, reasoning and judgment. At later stage, motor skills and language function are also impaired and the disorder culminates in the death of the individual. After initial diagnosis, AD patients live about 8 years on average with some cases that could last for as long as 20 years.

AD is named after the Germany physician Alois Alzheimer who first described the clinical and pathological features of the disease at the Meeting of the Psychiatrists of South West Germany in 1906 (Alzheimer, 1907). The prevalence of Alzheimer's disease has skyrocketed in the last decades with 36 million people worldwide estimated to be afflicted by the disease and it costs the world $604 billion in 2010 alone (Wimo and Prince, 2010). Aging is the principal risk factor for AD. In an European population, the prevalence of AD increases from 0.3% for individuals at ages 60 to 69 to 10.8% for those aged 80 to 89 (Rocca et al., 1991). Higher prevalence is reported in a US community population with 3% for those aged 65 to74, 18.7% for those aged 75

to 84, and 47.2% for those aged over 85 (Evans et al., 1989). With the prolonged life expectancy, the prevalence of AD is estimated to exceed 100 million by 2050 (Wimo and Prince, 2010). In spite of great efforts in trying to understand the disease, unfortunately, there is still no effective therapy or prevention strategy for AD. The currently available drugs for AD could only, at best, provide symptomatic benefit rather than cure for the disease. Therefore, a better understanding of the pathogenesis of the disorder is urgently needed to develop effective treatments.

1.1.1 Pathological features of Alzheimer's disease

AD is characterized by three main neuropathological changes in the brain: formation of neurofibrillary tangles (NFTs) within neurons, extracellular deposition of amyloid plaques and significant neuronal loss. The structure of the NFTs is first revealed under the electron microscopy by Kidd in 1963 as paired helical filament around the nucleus, compressing the cytoplasm against the cell membrane (Kidd, 1963). The major component of NFTs is hyperphosphorylated microtubule-associated protein tau (Goedert et al., 1988; Grundke-Iqbal et al., 1986; Iqbal et al., 1989; Kosik et al., 1986; Wood et al., 1986). However, the NFTs formation is not a unique pathology of AD as it's also present in patients with frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) which is caused by missense mutations in tau (Hutton et al., 1998).

Neuritic plaques, the unique neuropathological feature of AD, are present in large numbers in the AD brain parenchyma (Glenner, 1983). First described in details by Terry in 1964 using the

electron microscopy, the mature neuritic plaques are composed of a star-shaped extracellular amyloid plaque core and surrounding degenerating axons and dendrites (Terry et al., 1964). The amyloid plaque core is comprised of 5-10nm β-pleated sheet filaments (Miyakawa et al., 1986) that stain with congo red (Mann et al., 1985). The major constituent, amyloid-β protein (Aβ), derives from sequential cleavage of amyloid precursor protein (APP) by β-secretase and γ-secretase complex.

AD is also characterized by substantial neuronal loss in the CA1 region of the hippocampus and entorhinal cortex along with medial temporal lobe volume loss (Gomez-Isla et al., 1996; Price et al., 2001; Rodrigue and Raz, 2004; West et al., 1994). By analyzing 34 AD brains and 17 age-matched nondemented controls, Gomez-Isla *et al.* found that the average total number of superior temporal sulcus neurons was reduced by 53% in AD group compared to control group (Gomez-Isla et al., 1997). Notably, among all the pathological features of AD, neocortical cerebral atrophy has good correlation with clinical symptoms of dementia (Savva et al., 2009). AD was once deemed to be featured with selective degeneration of cholinergic neurons (Whitehouse et al., 1982) which fueled the development of therapies based on cholinesterase inhibition. However, the drugs are only marginally effective in ameliorating the symptoms in some patients. The selectivity of neuronal damage is probably overstated as impairment of catecholamine (Adolfsson et al., 1979) and glutamatergic innervations are also demonstrated in AD brain (Hardy et al., 1987).

1.1.2 Two types of Alzheimer's disease

AD can be divided into two subtypes according to age of onset: early-onset AD with a disease onset in early 40s or late 50s and late-onset AD that develops after 65 years old. The clinical severity of dementia of early-onset and late-onset AD are similar (Lehtovirta et al., 1996). Early-onset AD accounts for less than 5% of all AD cases (Reitz et al., 2011) and can be caused by autosomal dominant gene mutations in *APP* on chromosome 21(Goate et al., 1991), *presenilins 1* on chromosome 14 (Sherrington et al., 1995) or *presenilins 2* on chromosome 1 (Levy-Lahad et al., 1995; Rogaev et al., 1995). These mutations typically result in premature development of cerebral Aβ deposits (Borchelt et al., 1996; Cai et al., 1993; Citron et al., 1992; Citron et al., 1997; Duff et al., 1996; Scheuner et al., 1996; Suzuki et al., 1994) In addition, *APP* gene duplication (Rovelet-Lecrux et al., 2006) and trisomy of Chromosome 21 (Mann, 1988a, b) can also cause early-onset AD. The late-onset AD, on the other hand, does not show an obvious genetic inheritance and probably arises from an interplay between environmental and genetic factors. Many risk factors for sporadic AD have been revealed such as aging, stroke, and diabetes (Ballard et al., 2011; Reitz et al., 2011). APOEε4 was identified as the strongest genetic risk factor for late-onset AD (Bertram and Tanzi, 2008; Corder et al., 1993) with other susceptible genes being identified by genome-wide association studies (Harold et al., 2009; Hollingworth et al., 2011; Lambert et al., 2009).

1.2 Amyloid hypothesis of Alzheimer's disease

Although the familial occurrences of AD have been reported as early as in 1930s (Essen-Moeller,

1946; Schottky, 1932; Van Bogaert, 1940), it was until 1991 that a specific mutation in *APP* gene was first identified to cause AD (Goate et al., 1991). Later, mutations in *presenilins 1* (Sherrington et al., 1995) and *presenilins 2* (Levy-Lahad et al., 1995; Rogaev et al., 1995) were also discovered to cause autosomal dominant AD. Moreover, *APP* duplication (Rovelet-Lecrux et al., 2006) and trisomy of Chromosome 21 (Mann, 1988a, b) can also cause early-onset AD whereas partial trisomy 21 excluding *APP* region does not (Prasher et al., 1998). The notion that specific gene mutations or overexpression typically promote Aβ production or propensity of Aβ to aggregate (Borchelt et al., 1996; Cai et al., 1993; Citron et al., 1992; Citron et al., 1997; Duff et al., 1996; Scheuner et al., 1996; Suzuki et al., 1994) led to the articulation of amyloid hypothesis that posits Aβ deposition as the central and initiating event in the disease process (Hardy and Allsop, 1991; Hardy and Selkoe, 2002; Hardy and Higgins, 1992; Selkoe, 1991). In support of the amyloid hypothesis, APOEε4, the strongest genetic risk factor for late-onset AD (Corder et al., 1993) has been found to be the most potent factor in promoting Aβ deposition (Holtzman et al., 2000) and least effective in clearing Aβ comparing with other APOE alleles (Castellano et al., 2011).

The original amyloid hypothesis has limitations. For example, the amyloid load does not correlate well with clinical symptoms (Giannakopoulos et al., 2003). Indeed, with advanced techniques to detect Aβ burden *in vivo*, amyloid plaques are frequently found to be present in people without apparent cognitive impairment (Aizenstein et al., 2008; Perrin et al., 2009; Pike et al., 2007). Moreover, while tau pathology can cause frontotemporal dementia, no amyloid

plaque-only dementia has been reported. These discrepancies are addressed by a revised amyloid hypothesis which takes into account the smaller, soluble oligomeric Aβ aggregates in the pathological process of AD (Karran et al., 2011).

1.3 Amyloid-β precursor protein

The purification and subsequent determination of amino acid sequence of Aβ (Glenner and Wong, 1984a, b; Masters et al., 1985) led to the identification of amyloid-β precursor protein (*APP*) gene and its localization on chromosome 21 (Goldgaber et al., 1987; Kang et al., 1987; Robakis et al., 1987; Tanzi et al., 1987). APP is a type I integral membrane protein, encoded by *APP* gene on chromosome 21q21.2-3. The *APP* gene has 19 exons spanning more than 170kb. Exon 7 encodes a domain of 57 amino acids homologous to the Kunitz serine protease inhibitor (KPI) in the ectodomain, whereas exon 8 encodes a MRC OX-2 domain of 19 amino acids. The corresponding Aβ region spans portions of the ectodomain and transmembrane domain of APP and is encoded by exon 16 and 17. There are three major isoforms of APP that are derived by alternative splicing of exon 7 and 8: the 695-amino acid form (APP_{695}) which contains neither KPI nor MRC OX-2 domain, the 751-amino acid form (APP_{751}) which has only KPI domain, and the 770-amino acid form (APP_{770}) that contains both domains (Kang et al., 1987; Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988). The Aβ domain is conserved in all three isoforms. In the neurons of the brain, APP_{695} is predominant while APP_{751} and APP_{770} are more ubiquitously expressed in most tissues such as brain, kidney, lung, and muscle (Neve et al., 1988; Tanzi et al., 1988; Zimmermann et al., 1988).

APP belongs to a multigene family which also includes amyloid precursor-like protein 1 (APLP1), amyloid precursor-like protein 2 (APLP2) in mammals (De Strooper and Annaert, 2000), amyloid precursor protein-like (APPL) in *Drosophila* (Martin-Morris and White, 1990) and APP-related protein (APL-1) in the *Caenorhabditis elegans* (Daigle and Li, 1993). Although all these proteins share similar structure and are processed in a similar manner to APP, only APP generates an amyloidogenic fragment since Aβ domain is absent in other members of the superfamily.

The high conservancy of APP in evolution suggests its functional significance. Although many inferred functions have been ascribed to APP, the exact physiological function of APP remains elusive. A plethora of studies indicate that APP has an important role in brain development. In cultured cells, APP was demonstrated to be involved in cell proliferation (LeBlanc et al., 1992) and growth (Majocha et al., 1994; Saitoh et al., 1989) , cell motility (Sabo et al., 2001), neuronal adhesion (Soba et al., 2005), neurite outgrowth (Allinquant et al., 1995; Kibbey et al., 1993; Milward et al., 1992; Qiu et al., 1995), synaptogenesis (Ashley et al., 2005), and cell survival (Perez et al., 1997). These observations are extended by *in vivo* experiments. For example, *In utero* RNA interference targeting full-length APP leads to abnormalities in migration of neuronal precursor cells in rodent embryonic cortex (Young-Pearse et al., 2007). On the other hand, overexpression of wild-type APP in mice increased the size of cortical neurons (Oh et al., 2009). Although APP knock-out mice are viable and fertile displaying only subtle abnormalities (Zheng et al., 1995), double deficiency in APP and its nearest homolog APLP2 leads to early

postnatal lethality along with defective synapses and neurotransmitter release (Heber et al., 2000; Wang et al., 2005; Yang et al., 2005) and mice with triple knock-out of APP, APLP1 and APLP2 die shortly after birth and exhibit high incidence of cortical dysplasias and cranial abnormalities (Herms et al., 2004).These observations suggest potential functional redundancies among the APP family and also highlight its essential role in regulating synaptic formation and function as well as neuron migration during brain development. In adult animals, APP expression is enhanced after traumatic brain injury, indicating APP might be involved in brain repair (Itoh et al., 2009; Leyssen et al., 2005; Murakami et al., 1998; Van den Heuvel et al., 1999). In fact, APP has been suggested to function as a cell surface receptor that can bind to F-spondin which is a secreted signaling glycoprotein implicated in neuronal development and repair (Ho and Sudhof, 2004).

APP is extensively post-translationally modified including N-glycosylation, O-glycosylation, tyrosine sulfation and phosphorylation (Turner et al., 2003; Weidemann et al., 1989) and is detected in various subcellular locations including endoplasmic reticulum, Golgi apparatus, and plasma membrane (Caporaso et al., 1994; Schubert et al., 1991; Shigematsu et al., 1992; Tomimoto et al., 1995). After being synthesized, APP is transported to the plasma membrane through the constitutive secretory pathway and undergoes fast anterograde axonal transport (Koo et al., 1990). Some cell surface APP is internalized and recycled by endosomes. Proteolysis of APP occurs at multiple sites of trafficking routes such as in the secretory pathway, on the plasma membrane, and in the endocytotic compartments (Suzuki et al., 2006). APP undergoes rapid

turnover in cells with an approximate half life of 20-30 minutes (Weidemann et al., 1989).

1.4 APP processing

The essential role of Aβ in AD pathogenesis highlighted the importance of understanding APP processing. Generally, there are two distinct APP proteolytic pathways (Fig 1.1). Under normal conditions, majority of APP protein are cleaved by α-secretase between Lys-16 and Leu-17 within the Aβ domain, generating a secreted N-terminal fragment ($sAPP_{\alpha}$) and a membrane-bound carboxy-terminal fragment C83 (Esch et al., 1990; Sisodia et al., 1990). The C83 fragment can be further cleaved by γ-secretase, releasing p3 fragment and APP intracellular domain (AICD). Since α-secretase cleavage precludes the Aβ generation, this pathway is called non-amyloidogenic pathway. In the amyloidogenic pathway, APP is first cleaved by β-secretase, generating the ectodomain $sAPP_{\beta}$ and a 99-amino-acid fragment of APP carboxy-terminal called C99. C99 is subsequently cleaved by γ-secretase in the transmembrane domain liberating Aβ and AICD. β-secretase can also cleave APP within the Aβ region and generate a C89 fragment, mediating non-amyloidogenic processing (Lee et al., 2003; Li et al., 2006; Liu et al., 2006; Vassar et al., 1999).

The physiological functions of APP metabolites are still elusive. Although AICD has been reported to be involved in transcription activation of a series of target genes (Cao and Sudhof, 2001; Slomnicki and Lesniak, 2008), its physiological function remains controversial (Hebert et al., 2006). $sAPP_{\alpha}$ has been suggested to be neuroprotective (Bandyopadhyay et al., 2007) and

proposed to mediate the principal functions of the APP holoprotein as the mice that exclusively express $sAPP_{\alpha}$ did not exhibit prominent deficits observed in APP knock-out mice (Ring et al., 2007). Recently, a derivative from $sAPP_{\beta}$ is found to bind to the death receptor DR6 and triggers axon pruning and neuronal death (Nikolaev et al., 2009).

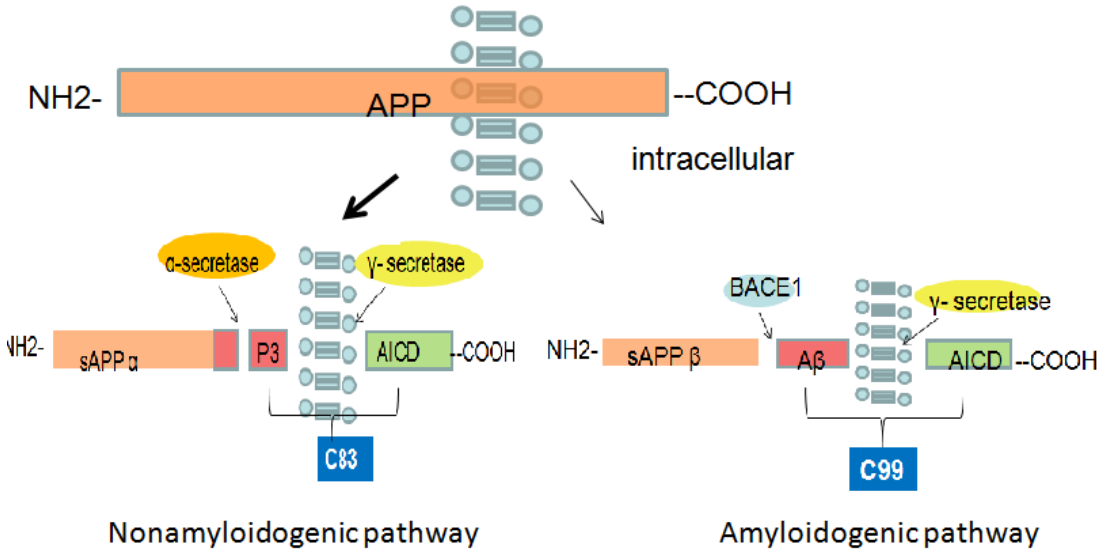

Figure 1.1 APP processing pathways
Under physiological conditions, majority of APP protein are cleaved by α-secretase within the Aβ domain, generating C83 fragment and precludes Aβ generation. In the amyloidogenic pathway, APP is cleaved by BACE1, generating C99 fragment which is further cleaved by γ-secretase to produce Aβ.

1.4.1 α-secretase

Under normal condition, the major APP proteolytic process occurs within the Aβ region, thus precludes the generation of Aβ (Esch et al., 1990; Sisodia et al., 1990). Although the exact identity of α-secretase still needs further investigation, several members of the "A disintegrin and metalloprotease" (ADAM) family such as ADAM9, ADAM10 and ADAM17 (also called TNF-α converting enzyme) have been implicated to act as α-secretases (Asai et al., 2003; Buxbaum et

al., 1998; Koike et al., 1999; Lammich et al., 1999). Some studies suggest that the ADAMs can substitute for each other with regard to α-secretase cleavage (Hartmann et al., 2002; Le Gall et al., 2009).

In addition to APP cleavage, the ADAMs harbor a broad role in cell signaling by releasing the ectodomains of many membrane-anchored proteins. For example, ADAM17 is involved in shedding of transforming growth factor-α (TGF-α) and tumor necrosis factor-α (TNF-α) (Peschon et al., 1998). ADAM10 is crucial in regulating cell-cell adhesion and β-catenin signaling (Maretzky et al., 2005; Reiss et al., 2005).

Enhancing α-secretase cleavage of APP for AD treatment has been proposed (Bandyopadhyay et al., 2007). Overexpression of ADAM10 in transgenic AD mice reduced amyloid plaques and ameliorated the memory deficit (Postina et al., 2004). In another study, intracerebral injection of acitretin which was demonstrated to stimulate ADAM10 expression also reduced Aβ production in APP/PS transgenic mice (Tippmann et al., 2009). However, given the broad signaling pathways that ADAMs regulate, further studies are needed to investigate the safety of this therapeutic strategy.

1.4.2 β-secretase

In 1999, several groups using a variety of techniques identified BACE1 as the β-secretase *in vivo* (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999). BACE1 cleaves

APP at two sites of Aβ domain, Asp+1 and Glu+11, generating C99 and C89 fragment respectively (Li et al., 2006). BACE1 is a type I transmembrane aspartyl protease of 501 amino acids and encoded by *BACE1* gene on Chromosome 11. BACE1 is widely expressed with the highest expression in pancreas and brain (Ehehalt et al., 2002; Yan et al., 1999). The expression level of BACE1 is relatively low (Li et al., 2006; Zhou and Song, 2006) and is under strict regulation (Sun et al., 2012). A number of posttranslational modifications are required for BACE1 maturation such as removal of propeptide (Benjannet et al., 2001; Bennett et al., 2000; Capell et al., 2000; Creemers et al., 2001; Shi et al., 2001), glycosylation (Capell et al., 2000; Charlwood et al., 2001; Haniu et al., 2000; Huse et al., 2003), palmitoylation (Benjannet et al., 2001), and phosphorylation (Capell et al., 2000; Haniu et al., 2000; Walter et al., 2001). BACE1 can be degraded via ubiquitin-proteasome pathway and lysosomal pathway (Koh et al., 2005; Qing et al., 2004).

BACE1 cleavage of APP is essential for Aβ generation as knockout of BACE1 abolished Aβ generation (Cai et al., 2001; Luo et al., 2001; Roberds et al., 2001). Genetic deletion of *BACE1* also rescued memory deficit in transgenic AD mouse model (Ohno et al., 2004). In addition, 50% reduction in BACE1 activity resulted in a remarkable reduction in Aβ plaques and synaptic deficits (McConlogue et al., 2007). All these studies suggested BACE1 as an excellent therapeutic target. Although no major physiological abnormalities were observed in *BACE1* knockout mice in early studies, subtle abnormalities have been recently reported in BACE1-null mice such as alteration in synaptic plasticity (Laird et al., 2005), reduced sodium channel

Na(v)1.1 levels (Kim et al., 2011), hypomyelination (Hu et al., 2006; Willem et al., 2006), axon targeting defects (Rajapaksha et al., 2011), hyperactive behavior (Dominguez et al., 2005), spontaneous seizures (Hu et al., 2010) and schizophrenia-like features (Savonenko et al., 2008), implying that BACE1 has many other biological functions in maintaining health. Moreover, a number of BACE1's substrates other than APP have been identified such as APP homolog proteins APLP1 and APLP2 (Eggert et al., 2004; Li and Sudhof, 2004; Pastorino et al., 2004), low-density lipoprotein receptor-related protein (von Arnim et al., 2005), α2,6-sialyltransferase (Kitazume et al., 2001), P-selectin glycoprotein ligand-1 (Lichtenthaler et al., 2003), interleukin-1 receptor II (Kuhn et al., 2007), voltage-gated sodium channel β2 subunit (Kim et al., 2007; Wong et al., 2005), neuregulin-1 (Hu et al., 2006; Willem et al., 2006) and neuregulin-3 (Hu et al., 2008). Taken together, partial or specific rather than complete inhibition of BACE1 activity may probably serve as a more viable therapeutic strategy.

1.4.3 γ-secretase complex

The final step in the generation and release of Aβ requires **γ** –secretase which is a multi-subunit intramembrane protease complex that cleaves c-terminal fragments of APP. The γ-secretase complex consists of four proteins: presenilin 1 (PS1) or presenilin 2 (PS2), nicastrin, anterior pharynx defective-1 (Aph-1) and presenilin enhancer-2 (Pen-2) (De Strooper, 2003; Edbauer et al., 2003; Francis et al., 2002; Goutte et al., 2002; Kimberly et al., 2003; Steiner et al., 2002; Takasugi et al., 2003; Yu et al., 2000) which are present at equal stoichiometry (Osenkowski et al., 2009; Sato et al., 2007). PS1 and PS2 are homologous proteins that share overall 63%

identity in amino acid sequence and very similar structure. Likewise, human Aph-1 also has two homologues, Aph-1a which has two alternative spliced forms and Aph-1b. Therefore, in total six distinct γ-secretase complexes exist (Shirotani et al., 2004). A number of experiments in knockout mice suggest different complexes have heterogenous biochemical properties and exert divergent biological functions (Dejaegere et al., 2008; Herreman et al., 1999; Ma et al., 2005; Serneels et al., 2005; Serneels et al., 2009). Additional proteins that interact with γ-secretase complex are also identified and are indicated to be involved in the maturation and regulation of complex (Wakabayashi et al., 2009).

PS 1 and PS2 are the first **γ** -secretase subunits identified (De Strooper et al., 1998; Herreman et al., 2000; Zhang et al., 2000) and are proposed to provide the active sites that are needed for γ-secretase processing of APP (Wolfe et al., 1999). Several studies indicate PS2 is less efficient than PS1 in terms of APP cleavage and Aβ production (Bentahir et al., 2006; Lai et al., 2003; Mastrangelo et al., 2005). Thus, it might not be surprising that less than 15 mutations in PS2 have been associated with AD while over 180 FAD-linked PS1 mutations are reported.

In addition to APP, many other substrates have been identified for presenilins (Wakabayashi and De Strooper, 2008). For example, Notch is cleaved by presenilins to release the Notch intracellular domain (NICD) which transduces the Notch signaling (De Strooper et al., 1999; Song et al., 1999; Struhl and Greenwald, 1999). Accordingly, PS1/PS2 double-null mice die at embryonic day 9.5 with multiple developmental defects (Donoviel et al., 1999). Similarly,

administration of γ-secretase inhibitors caused gastrointestinal toxicity in experimental animals due to disruption of Notch signaling (Searfoss et al., 2003; van Es et al., 2005; Wong et al., 2004). Therefore, several approaches have been proposed to specifically inhibit the γ-secretase activity towards Aβ42 production without significantly perturbing Notch signaling such as specific inactivation of the Aph1B γ-secretase (Serneels et al., 2009), modulating the cellular trafficking of γ-secretase (Thathiah et al., 2009), and use of non-steroidal anti-inflammatory drugs (Takahashi et al., 2003; Weggen et al., 2001; Weggen et al., 2003).

1.5 Amyloid-β protein

Amyloid-β protein (Aβ), the major protein constituent of the amyloid fibrils, was first isolated and purified from plaques in AD patient and aged individuals with Down syndrome by Glenner and Wong (Glenner and Wong, 1984a, b). It primarily composed of variants of the 4-kDa peptide that differs in solubility, stability and biological properties. Aβ exhibits a substantial heterogeneity due to proteolysis and additional modifications (De Strooper, 2010). γ-secretase complex can mediate γ-cleavage at different positions of APP, producing a variety of Aβ. $A\beta_{40}$ which ends at residue Val-40 is most abundant and is continuously produced in both healthy and AD affected brains. Historically, many researches were focused on another Aβ form, $A\beta_{42}$, since it aggregates into fibril deposition in amyloid plaques more readily than $A\beta_{40}$ due to its higher hydrophobicity. Moreover, genetic mutations causing early onset AD increased the relative production of this peptide.

As accumulating evidence demonstrates the importance of soluble Aβ assemblies in AD pathogenesis, the contribution of this particular $A\beta_{42}$ species might have been overstated. Aβ can aggregate and form different oligomeric assemblies. It can also, as mentioned above, arrange into cross–β-sheet units and form amyloid fibrils in the deposited plaques. While original amyloid hypothesis focused on deposited Aβ as the major culprit of neuronal damage, increasing evidence suggests smaller, soluble oligomeric species of Aβ could indeed mediate the neurotoxicity. Lambert *et al.* reported that small diffusible Aβ oligomers which they referred to as Aβ-derived diffusible ligands (ADDLs) could inhibit hippocampal long term potentiation (LTP) and cause neuronal death (Lambert et al., 1998). Similarly, impairment of LTP and memory deficits induced by smaller Aβ species such as dimers, trimers and a 56-kDa soluble assembly are also demonstrated in vivo by other groups (Lesne et al., 2006; Shankar et al., 2008; Townsend et al., 2006; Walsh et al., 2002). Moreover, soluble Aβ appears to be a better correlate with dementia and synaptic loss than deposited Aβ in plaques (Lue et al., 1999; McLean et al., 1999). Yet, which Aβ species are most pathogenic and how they mediate neurotoxicity remains to be clarified (Benilova et al., 2012). Since various Aβ species in different aggregation states coexist *in vivo*, it has been proposed that a mixture of Aβ derivatives could each interact with various cellular proteins causing neuronal damage.

1.6 Association between Alzheimer's disease and diabetes mellitus

1.6.1 An overview of diabetes mellitus

Diabetes mellitus (DM) is a complex metabolic disorder characterized by chronic hyperglycemia and associated with macrovascular and microvascular complications. Depending on the degree of the disorder, the clinical presentations range widely from being asymptomatic to polyuria and polydipsia to ketoacidosis or coma. With prolonged duration of hyperglycemia, microvascular complications (retinopathy, nephropathy and neuropathy) may be developed, leading to visual disturbance, renal failure and gangrene. Diabetes also increases the risks for stroke and myocardial infarction by accelerating and exacerbating arteriosclerosis which constitutes the major cause of the mortality of diabetic patients.

According to the World Health Organization's report released in September 2012, 347 million people worldwide currently suffer from diabetes mellitus with 90% of the cases being type 2 diabetes (WHO, 2012). In 2030, the global prevalence of diabetes is projected to be 439 million individuals, constituting 7.7% of the world population (Farag and Gaballa, 2011).

The current diagnostic criteria for diabetes mellitus are based on the fasting and postprandial plasma glucose level. Individuals are diagnosed to have diabetes if they meet one of the following criteria: (i) fasting plasma glucose level of ≥126 mg/dL (7.0 mmol/L); (ii) 2-h value of ≥200 mg/dL (11.1 mmol/L) in 75 g oral glucose tolerance test (OGTT); or (iii) casual plasma

glucose level of≥200 mg/dL (11.1 mmol/L). Fasting plasma glucose level of <110 mg/dL (6.1 mmol/L) and 2-h value of <140 mg/dL (7.8 mmol/L) in OGTT is defined as normal type. Values between the diabetic and normal are defined as borderline type (American Diabetes Association, 2012). In addition, an elevated level of Hemoglobin A1c (HbA1c) that≥6.5% is now also considered as an indication of diabetes mellitus. While direct blood glucose measurement provides current plasma glucose level, HbA1c results reflect the level of plasma glucose over a course of 2-3 months.

Based on its etiology, diabetes mellitus is classified into four groups: type 1 diabetes, type 2 diabetes, Gestational diabetes and diabetes due to other specific mechanisms and diseases (American Diabetes Association, 2012). Type 1 diabetes is characterized by an absolute deficiency in insulin derived from autoimmune destruction of β-cells in the pancreas. Type 2 diabetes, which accounts for about 90% of all diabetes cases around the world is characterized by combinations of decreased insulin secretion and insulin resistance. Gestational diabetes mellitus (GDM) is defined by the American Diabetes Association as “any degree of glucose intolerance with onset or first recognition during pregnancy” (Metzger et al., 2007). Unlike other types of diabetes, GDM utilizes different diagnostic criteria as the normal physiological blood glucose level is altered during pregnancy. Other specific types of diabetes are caused through mechanisms other than the ones above, such as nonimmune-mediated injury to the pancreatic β -cells or the pancreas as a whole and dysregulation of hormones with opposing effect against insulin.

1.6.2 Epidemiological and experimental evidence of the association between Alzheimer's disease and diabetes

It was demonstrated as early as in 1922 by Miles and Root that there is a cognitive impairment in people with diabetes (Miles Wr, 1922). Later a large number of studies confirmed that diabetic patients suffer from damaged verbal memory, diminished mental speed and mental flexibility (Brands et al., 2005; Cukierman et al., 2005; Stewart and Liolitsa, 1999; Strachan et al., 1997).

Accumulating epidemiological evidence shows an elevated risk of Alzheimer's disease in people with diabetes. In a systematic review of longitudinal population-based studies that compare the incidence of dementia between diabetic and nondiabetic groups, Biessels *et al.* reported an increase in risk of AD of 50-100% in diabetic individuals with no obvious relation to ethnic origins as shown in table 1.1 (Biessels et al., 2006). Similarly, in a more recent review that particularly focused on the risk of incident Alzheimer's disease in diabetic patients, Kopf *et al.* also reported an elevated risk of AD in people with diabetes (Kopf and Frolich, 2009). He also pointed out that studies with larger sample size, ascertainment of early diabetes and strict diagnosis for dementia subtype are prone to report a positive association between the two diseases.

Table 1.1 Increased risk of Alzheimer's disease in patients with diabetes mellitus

Country	Follow-up (years)	Patients(patients with diabetes/total number of patients)	Relative Risk* (95%CI)	Reference
Netherlands	2.1	692/6370	1.9 (1.2–3.1)	(Ott et al., 1999)
UK	2.4	25/376 §	OR1.4 (1.1–17.0)	Brayne et al., 1998)
Japan	7	70/828	2.2 (1.0–4.9)	(Yoshitake et al., 1995)
USA(Hawaii)	2.9	900/2574 §	1.7 (1.0–2.8)	(Peila et al., 2002)
Canada	5	503/5574 §	1.2 (0.8–1.8)	(MacKnight et al., 2002)
Sweden	4.7	114/1301	HR1.3 (0.8–1.9)	(Xu et al., 2004)
USA	6.9	1455/75000 §	SMR1.6 (1.3–2.0)	(Leibson et al., 1997)
USA	5.5	231/1138 §	HR2.4 (1.8–3.2)	(Luchsinger et al., 2005)
USA	5.5	27/824 §	HR1.7 (1.1–2.5)	(Arvanitakis et al., 2004)

Diabetes increased risk of developing AD by 50-100%. Results were adjusted for age and sex mostly for education, and vascular risk factors. Diagnoses for AD were made according to NINCDS-ADRDA (McKhann et al., 1984). §Number at follow-up, all the rest are numbers at baseline *Relative risks unless otherwise stated. Abbreviations: OR, odds ratio; HR, hazard ratio; SMR, standard morbidity ratio. Table adapted from Biessels *et al.*2006.

Furthermore, studies in experimental animal models also demonstrated a strong association between diabetes and Alzheimer's disease. Crossing APP23 mice, a well-established AD mouse model, with two lines of diabetic mice (ob/ob and NSY mice) exacerbated the Alzheimer-like cognitive dysfunction (Takeda et al., 2010). In another transgenic AD mouse model predisposed

to develop tau pathology, the onset of experimental diabetes by streptozotocin administration potentiated tau deposition and NFT formation (Ke et al., 2009). Similarly, induction of diabetes by streptozotocin injection exaggerated AD neuropathology in APP transgenic mice, including tau phosphorylation and Aβ deposits (Jolivalt et al., 2010).

1.6.3 Common pathologies in Alzheimer's disease and diabetes

AD and diabetes share a number of pathologies such as impaired glucose metabolism, amyloidogenesis, brain atrophy, enhanced formation of advanced glycation end products (AGEs), oxidative stress, mitochondria dysfunction, inflammation and hypercholesterolemia (Sims-Robinson et al., 2010). Each, alone or in synergy with other factors, contributes to the initiation and/or progression of the two disorders. Each aspect has been under intensive studies with a large body of publications. For the purpose of introducing background information that is most relevant of this study, I will mainly introduce abnormalities in glucose metabolism including hyperglycemia in AD and briefly touch on other aspects.

1.6.3.1 Impaired glucose metabolism

Diabetes is featured by systematic impairment of glucose metabolism and subsequent chronic hyperglycemia. AD, on the other hand, also exhibits metabolic abnormalities that resemble diabetes (Hoyer, 2004). Deficits in cerebral glucose utilization associated with insulin resistance in AD patients are well observed and studied over decades. Overall, global cerebral metabolic rate for glucose is 20–25% lower in AD compared with healthy controls (Cunnane et al., 2011). The most severely affected cerebral regions include the posterior cingulate, posterior temporal

and anterior occipital lobes in early stages, and later on, spread to the prefrontal cortex (Foster et al., 1984; Friedland et al., 1985; Mosconi et al., 2008).

Accumulating evidence demonstrated that patients with AD suffered from hyperglycemia. In a community-based controlled study, 81% of the AD patients exhibited either impaired fasting glucose (IFG) (fasting glucose concentration 110-125 mg/dL) or frank diabetes (fasting glucose concentration≥126 mg/dL) (Janson et al., 2004). Similar increase in plasma glucose concentration in AD patients compared with control group is also observed in other studies (Carantoni et al., 2000; Meneilly and Hill, 1993; Razay G, 2007).

1.6.3.2 Other shared pathology of AD and diabetes

Generation of amyloidogenic peptides and subsequent aggregation into insoluble plaques is a shared pathological characteristic in diabetes and AD (Chiti and Dobson, 2006). Analogous to amyloid plaques in AD brains, islet amyloid deposits composed of human islet amyloid polypeptide (hIAPP) are present in the pancreas of majority of diabetic patients (Clark et al., 1988; Cooper et al., 1987; Westermark et al., 1987). hIAPP fibrils share a similar molecular structure with Aβ fibrils (Luca et al., 2007) and are suggested to exert toxicity by similar mechanisms (Haataja et al., 2008). Diabetic patients also exhibit subcortical atrophy in brain that correlates with impaired cognition (Akisaki et al., 2006; Manschot et al., 2006). In addition, increased level of AGEs (Srikanth et al., 2011), mitochondria dysfunction associated with oxidative stress (Moreira et al., 2007), inflammation (Donath and Shoelson, 2011; Wyss-Coray,

2006), and hypercholesterolemia (Harris and Milton, 2010; Mooradian, 2009) are also important pathologies that are common to both AD and diabetes.

1.7 Role of glucose in cognition

The brain is in high demand of energy in order to maintain its functions and glucose is the main cerebral energy substrate. While the brain only makes up for 2% of the total body weight, it consumes at least 25% of the circulating glucose under resting conditions (Magistretti, 1999). While shortage of glucose supply can cause severe brain damage as seen in stroke, chronic hyperglycemia is also detrimental to brain function. Therefore, balanced glucose metabolism is required for optimal brain function.

1.7.1 Glucose uptake in human brain

To reach neurons in the brain, glucose must across the blood-brain barrier (BBB) and cell membrane. This process is mediated by facilitative glucose transporters (GLUTs). 14 members of the GLUT family have been identified in human with different substrate specificity, binding affinity and tissue distribution (Scheepers et al., 2004). The predominant glucose transporters in brain are GLUT 1 and GLUT3 while other transporters are also expressed in brain at much lower levels (Vannucci et al., 1997).

Glucose is transported from circulating blood, across BBB, into cerebral interstitial spaces by GLUT1, following concentration gradient. First cloned from human HepG2 hepatoma cells (Mueckler et al., 1985), GLUT1is ubiquitously expressed throughout the human tissues. Besides

its high expression in brain, GLUT1 is also expressed in erythrocytes (Kalaria et al., 1988) and other blood-tissue barriers such as retina and placenta (Harik et al., 1990). In brain, GLUT1is detected as two isoforms of different molecular weight: the 55kDa form exclusively present in endothelial cells of blood-brain barrier and the 45kDa form expressed in all other neural cells (Maher et al., 1993b). The two isoforms are encoded by the same gene and their difference in molecular weight is due to varying extent of glycosylation (Birnbaum et al., 1986). The transportation of glucose across BBB is mediated by GLUT1 55kDa form. The expression of GLUT1 can be regulated by glucose as high glucose concentration decreased GLUT1 mRNA and protein level in cultured cells (Klip et al., 1994).

Once across the BBB and reaching cerebral interstitial spaces, glucose can be uptaken by neurons via its high affinity transporter, GLUT3. GLUT3 is cloned from human fetal skeletal muscle (Kayano et al., 1988) and is the predominant neuronal glucose transporter (Bondy et al., 1992; Nagamatsu et al., 1992). GLUT3 is also present at lower level in heart, placenta, and platelets (Craik et al., 1995; Shepherd et al., 1992) but not in BBB (Maher et al., 1993b). GLUT3 expression appears to be regulated by neuronal activity. Chronic depolarization which is known to stimulate oxidative metabolism increased GLUT3 expression (Maher and Simpson, 1994). Also, enhanced hypothalamic signal induced by water deprivation and streptozotocin-induced diabetes promotes GLUT3 expression (Maher et al., 1993a; Vannucci et al., 1994).

1.7.2 Physiological level of extracellular glucose in human brain

Presumably due to blood brain barrier and high metabolic rate, the concentration of extracellular brain glucose is substantially lower than that in circulating blood. Using quantitative microdialysis in anesthetized human, Langemann *et al*. reported the extracellular glucose is approximately 1.7mM in human brain (Langemann et al., 2001). Another group reported a similar result---a baseline value of 1.7±0.9mM glucose in awake human (Reinstrup et al., 2000). In addition, by zero-flow technique, the glucose level in brain dialysate of conscious human under basal fasting conditions was calculated to be 1.57±0.76mM (Abi-Saab et al., 2002).

Furthermore, studies in both experimental animals and human subjects have found that the level of extracellular glucose in brain closely parallels the changes in plasma glucose. Using glucose microelectrodes, Silver and Erecinska observed that extracellular brain glucose increased during hyperglycemia and decreased during hypoglycemia in rats: while the concentration of extracellular glucose in brain is 2.4±0.1mM in normoglycemic rat, it rose to 4.5±0.4mM during hyperglycemia induced by intraperitoneal injection of glucose and dropped to 0.16±0.03mM during hypoglycemia induced by insulin administration (Silver and Erecinska, 1994). Similar phenomenon was also observed in human by intracerebral microdialysis: in euglycemia when plasma glucose is around 5.5mM, the corresponding value in brain is around 1mM; when plasma glucose increased to 11.5mM during hyperglycemia clamp, extracellular glucose in brain increased to approximately 1.6mM; during hypoglycemia with 3mM glucose in plasma the glucose in brain declined to about 0.3mM (Abi-Saab et al., 2002). The lower value of brain

glucose reported here is probably due to the specific microdialysis methods employed as a higher reading was obtained using the zero-flow method in the same study.

1.7.3 Effect of hyperglycemia on cognition

While sufficient supply of glucose is required for normal brain function, studies in both animals and human proved that excessive blood glucose is toxic and detrimental on cognition. In streptozotocin-induced diabetic rats which are severely hyperglycemic, both spatial learning and hippocampal long-term potentiation are impaired compared with control (Biessels et al., 1996). Also，in old rats high blood glucose level was found to correlate with poor spatial learning (Blokland and Raaijmakers, 1993).

Many studies have demonstrated a strong association between chronic hyperglycaemia and cognitive impairment. For example, Perlmuter *et al.* found diabetic patients with higher HbA1c levels showed a poorer performance in a serial learning task (Perlmuter et al., 1984). Similarly, Jagusch *et al*. found an inverse correlation between HbA1c levels in diabetic patients and performance in a memory test (Jagusch et al., 1992). In the Zutphen population study, increased errors on Mini-Mental State Examination are reported in diabetic men with higher fasting blood glucose levels (Kalmijn et al., 1995). Moreover, hyperglycemia also has a negative impact on cognitive function in nondiabetic individuals. Rolandsson *et al.* found episodic memory is inversely associated with fasting blood glucose level in nondiabetic women (Rolandsson et al., 2008). Perhaps the most compelling evidence came from a longitudinal study of elderly women

in which high fasting blood glucose level was found to significantly increase the risk of developing cognitive impairment after multivariable adjustment (Yaffe et al., 2009).

While some study showed memory facilitation after acute increase in blood glucose to 225 mg/dl by hyperglycemic clamp in individuals with very mild AD (Craft et al., 1993), it should be noted that acute and chronic hyperglycemia may induce different effects on cognition.

1.8 Hypothesis and specific aims

1.8.1 Hypothesis

Diabetes is a great risk factor for development of Alzheimer's disease. Yet the underlying mechanism is not known. Hyperglycemia is a pathological feature that forms the basis of diabetes and amyloidogenesis is central to AD pathogenesis. I hypothesize that high level of glucose could up-regulate APP level and promote Aβ production. This study is designed to investigate if hyperglycemia could enhance amyloidogenesis.

1.8.2 Specific aims

Specific aim 1: To determine the effect of high glucose on APP protein level and Aβ production

Specific aim 2: To examine the effect of high glucose on APP transcription

Specific aim 3: To define the effect of high glucose on APP degradation

Overall, this study may provide a new insight into the link between diabetes mellitus and Alzheimer's disease and therefore offers a new perspective for therapeutic interventions of AD.

Chapter 2 High glucose treatment increases APP protein level and Aβ production

Given the central role of APP processing and Aβ generation in AD pathogenesis, we first examined if hyperglycemia, the prominent characteristic of diabetes that also present in AD, could affect APP and Aβ generation. We found that high glucose treatment significantly increased full-length APP protein level in human neuroblastoma SH-SY5Y cells and high glucose also increased Aβ40 production in 20E2 cells, a human embryonic kidney 293 (HEK293) cell line stably expressing Swedish mutant APP_{695} (Qing et al., 2004).

2.1 Materials and methods

2.1.1 Cell culture and high glucose treatment

Human neuroblastoma SH-SY5Y cells were maintained in glucose-free Dulbecco's modified eagle medium (DMEM) supplemented with 2.5mM D-glucose, 10% fetal bovine serum (FBS), 50 units of Penicillin and 50 μg of Streptomycin (Life Technologies). 20E2 cells, a cell line of HEK293 cells stably expressing Swedish mutant APP_{695} (Qing et al., 2004), were cultured in glucose-free DMEM supplemented with 5.5mM D-glucose, 10% FBS, 50 units of Penicillin and 50 μg of Streptomycin and 50 μg/ml geneticin. All cells were maintained at 37°C in an incubator containing 5% CO2. High glucose media were prepared by adding additional glucose to the media above to 10 and 25 mM glucose. The osmotic pressure was adjusted with D-mannitol (Fisher). The cells were treated with high glucose media for 24 or 48 hours.

2.1.2 Western blot

Cells were harvested and lysed in RIPA-DOC lysis buffer (0.05M Tris-HCl pH 7.2, 0.15M NaCl, 0.1% SDS, 1% sodium deoxycholate, and 1% triton x-100) supplemented with complete protease inhibitor (Roche Diagnostics). Lysates were sonicated and centrifuged at 13,200rpm for 10 minutes to pellet the cellular debris.The supernatant was then diluted in 4X SDS-sample buffer and boiled. After resolved in 8% tris-glycine SDS-PAGE, the proteins were transferred to polyvindylidine fluoride (PVDF-FL) membranes (Immobilon-FL, Millipore, MA, USA). For immunoblot analysis, membranes were blocked with 5% non-fat milk dissolved in phosphate-buffered saline (PBS) for 1 h and incubated in primary antibodies overnight at 4℃. Anti-APP antibody C20 is a polyclonal rabbit antibody made in-house that recognizes the last twenty amino acids of the APP carboxyl-terminus. Monoclonal antibody AC-15 (Sigma) was used to detect β-actin. After incubation, the membranes were washed in PBS with 0.1% Tween-20 and incubated with secondary antibodies, IRDye™ 680 goat anti-rabbit antibody or IRDye™ 800CW-labelled goat anti-mouse antibody (LI-COR Biosciences) at room temperature for 1 h, and visualized using an Odyssey Infrared Imaging System (LI-COR Biosciences).

2.1.3 Human Aβ40 ELISA

24 hours after high glucose treatment, conditioned cell culture media were collected. To prevent degradation of Aβ, protease inhibitors (AEBSF) were added. The cell media were centrifuged at 2000rpm for 5 minutes to precipitate cells in the media. The concentration of Aβ40 was

measured by Aβ40 human ELISA kit (KHB3482, Life Technologies) according to the manufacturer's instructions.

2.2 Results

2.2.1 High glucose treatment increases APP protein level

To investigate whether hyperglycemia could affect APP level, we treated the human neuroblastoma SH-SY5Y cells with culture media containing 10mM or 25mM glucose to mimic hyperglycemia *in vitro* and compared with cells treated with 2.5mM glucose media which serves as control. The 2.5mM glucose was used to approximate the physiological extracellular glucose concentration in the brain (Abi-Saab et al., 2002; Reinstrup et al., 2000). We found that 10 and 25mM glucose treatment significantly increased the level of full-length APP protein to 152.63±10.78%, 140.59±6.80% ($p<0.05$) comparing with the control (Fig.2.1 A,B) at 24-hour time point. Similarly, the expression of full-length APP was significantly increased to 120.52±4.20% or 146.04±0.59% ($p<0.05$), respectively, in cells treated with 10 or 25mM glucose for 48 hours comparing with the control (Fig.2.1C,D).

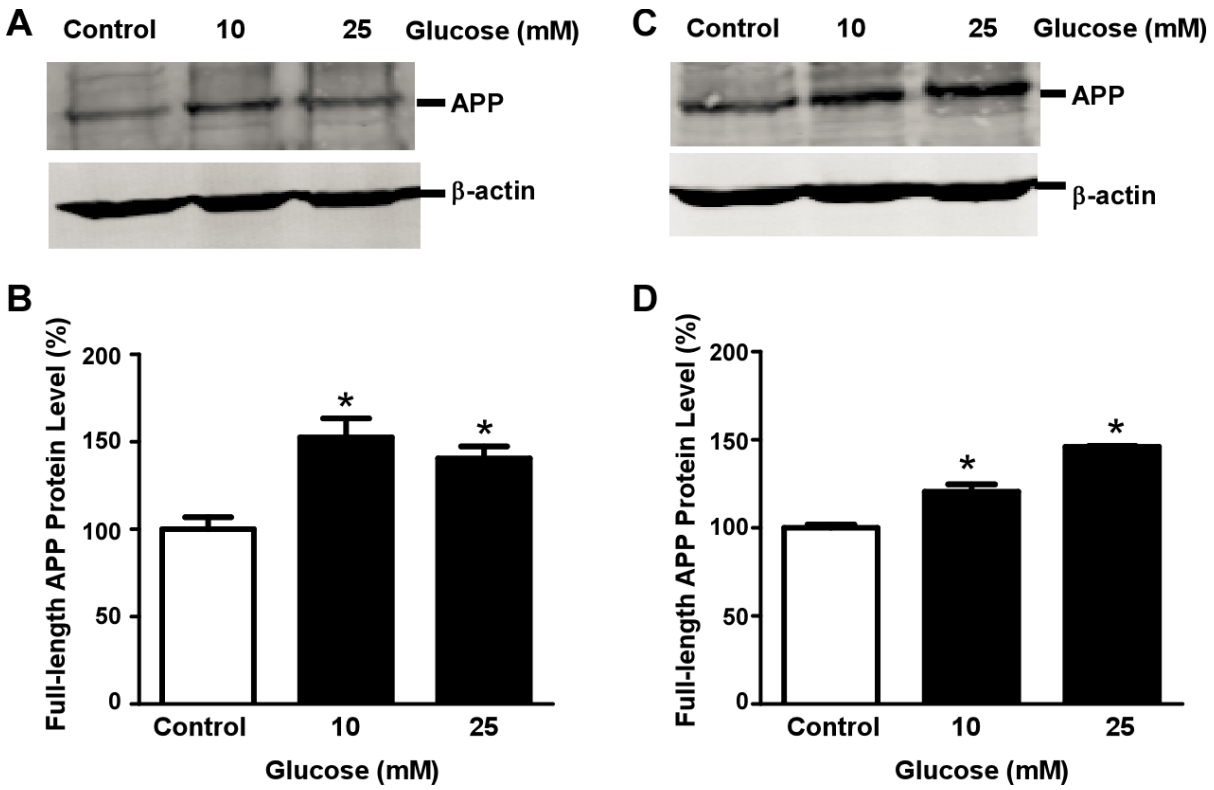

Figure 2.1 High glucose treatment increases full-length APP protein level

SH-SY5Y cells were cultured and treated with different concentration of glucose for 24 hours**(A),** and 48 hours**(C).** 2.5mM glucose treatment serves as control. The cell lysates were analyzed by Western blot. Full-length APP was detected by C20 antibody. β-actin, serving as internal control, was detected by AC-15 antibody. 24- and 48h-high glucose treatment significantly increased full-length APP protein level. Quantification of full-length APP after 24-hour treatment of high glucose by Image J**(B)** The APP protein level was normalized by β-actin. The values are expressed as mean±S.E.M, n=4,*$p<0.05$ by ANOVA. Quantification of full-length APP after 48-hour treatment of high glucose by Image J **(D)** The APP protein level was normalized by β-actin .The values are expressed as mean±S.E.M, n=3,*$p<0.05$ by ANOVA.

2.2.2 High glucose treatment enhances Aβ production

Since the level of full-length APP was increased after high glucose treatment, we wanted to know whether high glucose treatment also has impact on Aβ production. Therefore, we measured the level of $A\beta_{40}$ in the conditioned media of 20E2 cells, a HEK cell line that stably expresses Swedish mutant APP_{695}. Since 20E2 derives from a peripheral cell line, we used 5.5mM glucose, which is the physiological normal blood glucose at periphery, as control. After 24-hour treatment

of 10mM glucose the $A\beta_{40}$ level increased to 133.21±3.69% ($p<0.0001$) (Fig.2.2). When treated with culture media containing 25mM glucose $A\beta_{40}$ production increased to 142.49±4.21% ($p<0.0001$) (Fig.2.2). Taken together, high glucose treatment markedly enhanced $A\beta_{40}$ production.

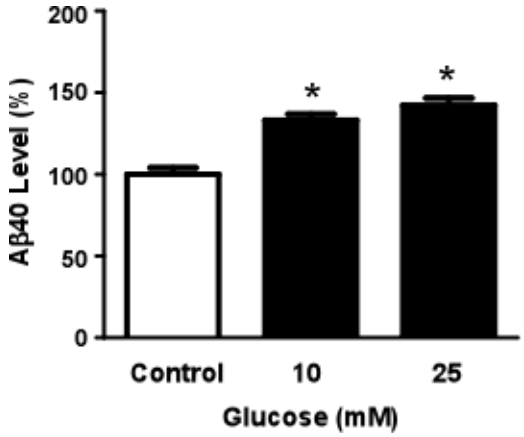

Figure 2.2 High glucose treatment increases $A\beta_{40}$ production
The 20E2 cells were cultured and treated with different concentrations of glucose for 24 hours. Media containing 5.5mM glucose serves as control. $A\beta_{40}$ level in conditioned media of 20E2 cells was measured by ELISA. The values are expressed as mean±S.E.M, n=4, *$p<0.0001$, by ANOVA.

2.3 Conclusion and discussion

We found high glucose treatment increased the level of full-length APP protein in cultured human neuroblastoma cells and facilitated the production of Aβ40 in 20E2 cells. Our results indicate that hyperglycemia in diabetic patients might enhance the expression and amyloidogenic processing of APP and promote development of Alzheimer's disease.

In human brain, the basal concentration of glucose in interstitial spaces which neurons are directly exposed to is around 2mM (Abi-Saab et al., 2002; Langemann et al., 2001; Reinstrup et

al., 2000). The normal plasma glucose concentration in peripheral is about 5.5mM. While many commercial culture media contains glucose around 25mM, it significantly departs from physiological glucose levels, raising the risk of inducing nonphysiological responses. In supporting of this notion, Kleman *et al.* demonstrated that the metabolic environment of cultured cells has a significant impact on neuronal viability and response to metabolic stress (Kleman et al., 2008). Therefore, application of glucose within concentration that is physiologically relevant can more accurately detect the *in vivo* response.

It is known that the expression of APP can be stimulated by various cellular stress conditions (Abe et al., 1991; Dewji et al., 1995). To avoid the potential confounding effect of osmotic pressure, the osmolarity of all the treatment media was adjusted with D-mannitol. In fact, we found that the osmotic pressure does not affect APP expression as treatment with osmolarity-unadjusted media induced increase in APP protein to a similar extent as that by osmolarity-adjusted media (unshown data).

The increase in Aβ could result from enhanced APP level, yet, it is possible that high glucose also upregulates β-secretase and γ-secretase expression and/or activity. Hyperglycemia can lead to elevated oxidative stress (Vincent et al., 2004) and both BACE1 and PS1 expression are found to be stimulated by oxidative stress accompanied by an increase in Aβ production (Oda et al., 2010; Quiroz-Baez et al., 2009; Tamagno et al., 2008; Tong et al., 2005). Therefore, it may be helpful to investigate the effect of high glucose treatment on BACE1 and PS1 in future studies.

Chapter 3 High glucose treatment does not affect *APP* transcription

Since our data demonstrated that high glucose significantly increased the level of APP protein and Aβ production, we further investigated the underlying mechanism of this phenomenon. First, we examined glucose's effect on *APP* promoter activity and gene transcription. Our result showed that high glucose treatment did not significantly affect the promoter activity or the mRNA level of *APP* gene.

3.1 Materials and methods

3.1.1 Cell culture and high glucose treatment

SH-SY5Y cells were maintained as described in Section 2.1.1 and treated with media containing 10mM and 25mM glucose for 24 or 48 hours. The osmotic pressure was adjusted with D-mannitol (Fisher).

3.1.2 Luciferase assay

The pAPP-Luc plasmid, containing 2.94kb of human *APP* promoter region upstream of the firefly luciferase reporter gene (Li et al., 2006), was used for luciferase assay to determine the activity of APP promoter. The transfection was performed using Lipofectamine 2000 (Invitrogen). One day prior to transfection, SH-SY5Y cells were seeded onto 60mm plates at the density of 5.0×10^5cells/ml culture media. On the day of transfection, the cells were grown to approximately 70% confluence and transfected with 4.6ug plasmid DNA along with 18.4ng *Renilla* luciferase vector pCMV-Rluc (Promega) to control transfection efficiency. Four hours after transfection, the cells were seeded to 24-well plates and grown overnight. 16 to 18 hours

later, the culture media were changed to conditional media with different glucose concentration. The cells were treated for 24 or 48 hours and then harvested. The luciferase assay was performed according to the protocol for Dual-Luciferase Reporter Assay system (Promega) using a luminometer (Fluoroskan Ascent, ThermoLab Systems).

3.1.3 Semi-quantitative reverse transcription PCR

RNA was extracted from cells using TRI-Reagent (Sigma). An equal amount of RNA samples were used as templates to synthesize the first strand cDNA with ThermoScript™ reverse transcriptase (Invitrogen). The newly synthesized cDNA then served as templates and the coding sequence of human *APP* was amplified by Platinum *Tag* DNA polymerase (Invitrogen) in a 20-μl reaction with following primers:

APP forward 5'- GCTGGCCTGCTGGCTGAACC;

APP reverse 5'-GGCGACGGTGTGCCAGTGAA;

β -actin levels served as an internal control. The PCR products were analyzed in 0.8% agarose gel (Sigma).

3.2 Results

3.2.1 High glucose treatment does not affect *APP* gene promoter activity

To examine whether the increase in full-length APP protein induced by high glucose treatment was via enhancement of *APP* gene transcription, we first determined *APP* promoter activity in human neuroblastoma SH-SY5Y cells treated with high glucose. We used pAPP-Luc plasmid which was constructed by inserting a 2.94kb region of human *APP* promoter into the

promoterless vector pGL3-basic upstream of the firefly luciferase reporter gene (Li et al., 2006). The SH-SY5Y cells were transfected with the plasmid and treated with different glucose. 24 hours or 48 hours after the treatment, the cells were harvested and the promoter activity was determined by luciferase assay. We found that neither 24-hour nor 48-hour treatment of high glucose changed the *APP* promoter activity (Fig 3.1).

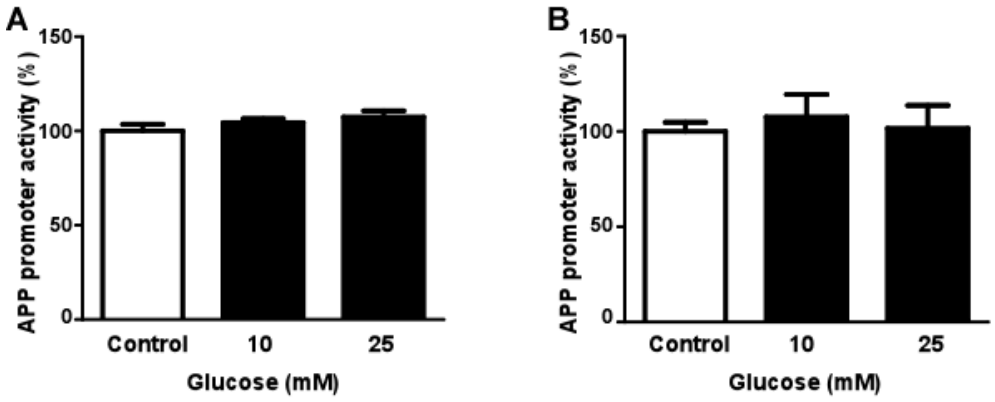

Figure 3.1 High glucose treatment does not affect *APP* gene promoter activity
The 2.94 kb human APP promoter was transfected into SH-SY5Ycells and treated with high glucose for 24 hours **(A)** and 48 hours **(B).** 2.5mM glucose serves as control. Luciferase assasy was performed. High glucose treatment did not affect APP promoter activity. All the data shown are results of 4 independent experiments, with each condition performed in triplicates. The values are expressed as mean±S.E.M. n=4, by ANOVA.

3.2.2 High glucose treatment does not affect *APP* transcription

We also determined the endogenous APP mRNA level in human neuroblastoma SH-SY5Y cells after high glucose treatment. Consistent with the promoter data, 24-hour high glucose treatment did not have a significant effect on APP mRNA level (Fig 3.2A, B). Similarly, at 48 hours, there was not significant change in APP mRNA level (Fig 3.2C, D).

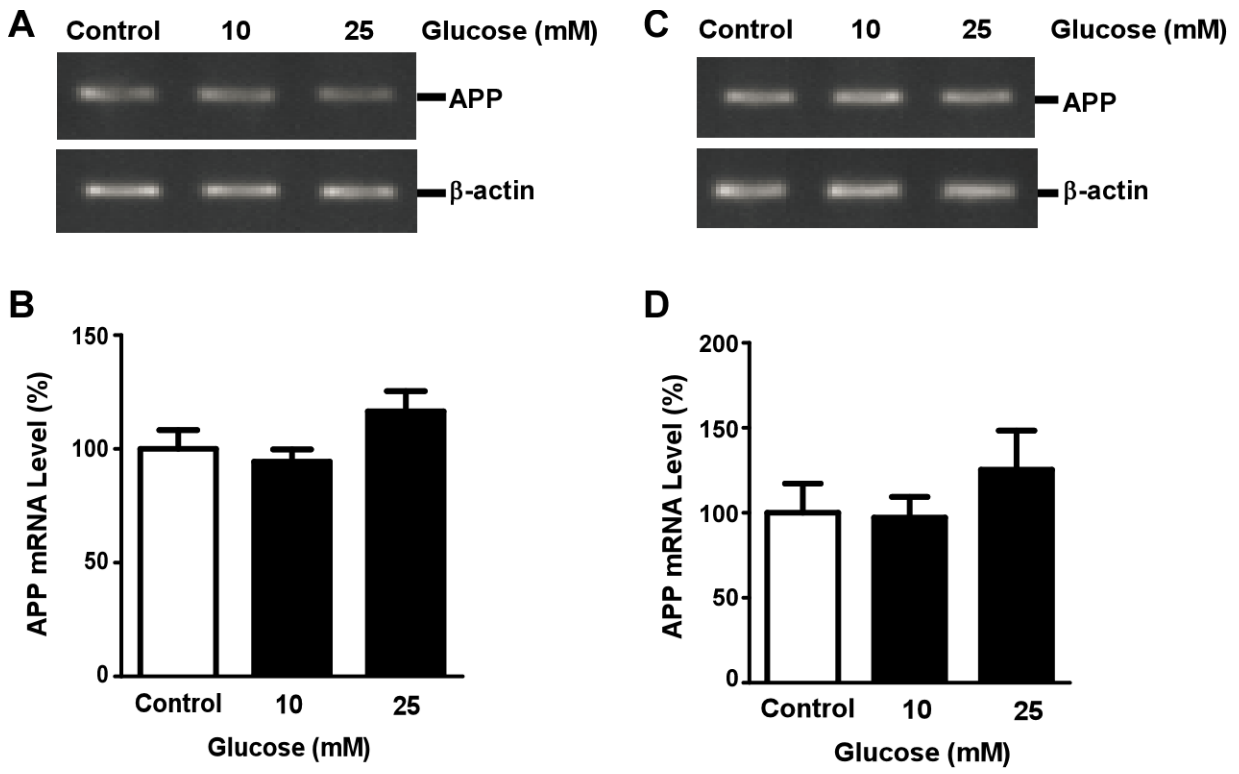

Figure 3.2 High glucose treatment does not affect APP transcription
SH-SY5Y cells were treated with different concentration of glucose for 24 hours**(A)** and 48 hours**(C).** RNA was extracted and APP mRNA level was measured by semi-quantitative PCR with specific primers. β-actin served as an internal control. 24-hour and 48-hour treatment of high glucose did not significantly affect APP mRNA. Quantification of full-length APP after 24-hour treatment of high glucose by Image J**(B)** The APP mRNA level was normalized by β-actin. The values are expressed as mean±S.E.M, n=7, by ANOVA. Quantification of full-length APP after 48-hour treatment of high glucose by Image J, normalized by β-actin**(D)** The values are expressed as mean±S.E.M, n=5, by ANOVA**.**

3.3 Conclusion and discussion

Our result showed that high glucose treatment did not affect *APP* gene transcription in human neuroblastoma cells as it didn't significantly increase the *APP* promoter activity or *APP* mRNA level.

The human *APP* promoter lacks CAAT and TATA box, and displays characteristics of a

housekeeping gene (La Fauci et al., 1989; Salbaum et al., 1988). It contains a high GC region, a heat shock element, a cAMP-responsive element and consensus binding sequences for the transcription factor AP-1, SP-1, homeobox protein Hox-1.3, GATA-1and NF-κB (D. Goldgaber, 1991; Grilli et al., 1995; La Fauci et al., 1989; Salbaum et al., 1988). The proximal *APP* promoter region that is sufficient for high levels of expression in various cell lines extends to about 100bp upstream of the transcription start site (Lahiri and Robakis, 1991; Pollwein et al., 1992; Quitschke and Goldgaber, 1992). APP expression can be stimulated by interleukin-1, retinoic acid, phorbol esters, growth factors (Goldgaber et al., 1989; Konig et al., 1990; Mobley et al., 1988; Yoshikai et al., 1990) and various stresses such as heat shock, treatment with ethanol and sodium arsenite (Abe et al., 1991; Dewji et al., 1995). To our knowledge, no publication has reported the effect of high glucose on APP expression. High glucose is known to lead to excessive production of reactive oxygen species in neurons (Russell et al., 2002). In agreement with our result, previous study reported that reactive oxygen species didn't activate the *APP* promoter in neurons (Yang et al., 1998a).

Chapter 4 High glucose treatment inhibits APP degradation

Previous results have shown an increase in APP protein level yet without significant change in mRNA level. This dissociation between mRNA and protein level suggests that the alteration of APP level occurs at post-transcription level. Since the level of one protein in the cells depends on the counterbalance between its production and degradation, we wanted to see if APP turnover is affected by high glucose treatment. With cycloheximide (CHX), a protein synthesis inhibitor, we examined the APP degradation rate in the presence of high glucose in cultured cells. Compared to cells maintained in control media, cells cultured with high glucose media showed a reduction in APP degradation rate which may explain the increase in APP protein level under high glucose.

4.1 Materials and methods

4.1.1 Cell culture and drug treatment

SH-SY5Y cells were maintained in media containing 2.5mM glucose as described in Section 2.1.1 and seeded into 35mm plates. When grown to approximately 80% confluence, the cells were treated with media containing high glucose and cycloheximide at 100ug/ml (Sigma) and harvested 30minutes later for western blot analysis.

4.1.2 Western blot

Western blot was performed as previously described in Section 2.1.2. Briefly, the cell lysates were denatured in sample buffer and resolved in 8% SDS-PAGE followed by transfer to PVDF membranes. After block with 5% non-fat milk, the membrane was incubated with anti-APP antibody C20 and anti-Actin antibody AC-15 at 4℃ overnight. After washing the membrane was

then incubated with secondary antibodies at room temperature for 1 hour and scanned by Odyssey system.

4.2 Results

4.2.1 High glucose treatment inhibits APP degradation

Previous results demonstrated that APP holoprotein was increased after high glucose treatment. Yet, APP transcription was not affected as glucose treatment didn't have a significant effect on *APP* promoter activity or APP mRNA level. Since protein degradation is an important player of protein expression regulation. We then examined whether high glucose affected APP protein degradation. SH-SY5Y cells were cultured in 2.5mM glucose media and seeded into several 35mm plates at equal amount. When the confluence reached approximately 80%, the culture media were replaced by media containing 100ug/ml CHX and different concentration of glucose. The cells were harvested 30 minutes after the treatment and analyzed by western blot. The result shows that 30 minutes after treatment, 52.25±5.07% of the initial total APP protein remains in cells that were cultured in 2.5mM glucose media while 70.48±2.95% of the initial total APP persists in cells that received 10mM glucose, and 82.43±6.19% of the initial APP persists in cells that received 25mM glucose ($p<0.05$) (Fig. 4.1). Therefore, high glucose slowed down the APP degradation.

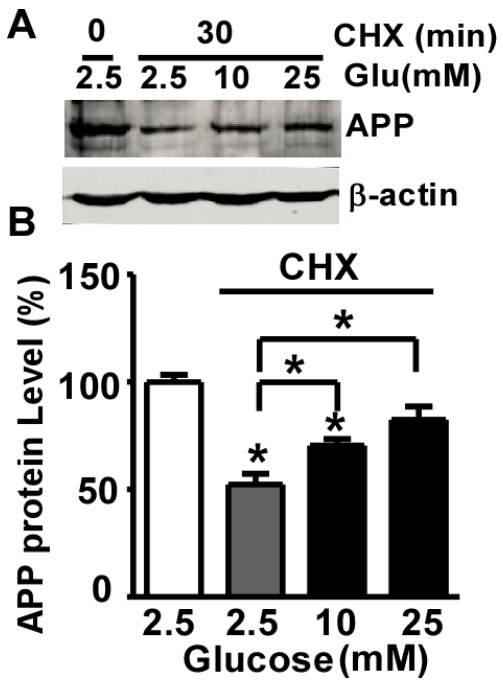

Figure 4.1 High glucose treatment inhibits APP protein degradation
SH-SY5Y cells were treated with 100ug/ml cycloheximide (CHX) and 2.5mM (served as control), 10mM, 25mM glucose. The cells were harvested at 30min post-treatment and the cell lysates were analyzed by Western blot **(A)**. Full-length APP was detected by C20 antibody. β-actin, serving as internal control, was detected by AC-15 antibody. Quantification of western blot by Image J**(B)**. APP protein level was normalized by β-actin and was plotted as a percentage of the APP amount at 0h. The values are expressed as mean±S.E.M. n=3, *p<0.05, by ANOVA.

4.3 Conclusion and discussion

Using CHX to stop protein synthesis, we found the degradation rate of full-length APP protein is slower in the presence of high glucose. In our system, the half life of APP is about 30 minutes, which is similar to the previous report by the other group (Weidemann et al., 1989).

The mechanism underlying the inhibitory effect of high glucose on APP degradation remains to be clarified. It is possible that glycosylation modification induced by high glucose treatment is one of the mediators. APP is a glycoprotein that undergoes N-glycosylation and O-glycosylation

during its passes through the endoplasmic reticulum and Golgi apparatus (Weidemann et al., 1989). Many studies have showed that glycosylation has a great impact on the intracellular trafficking and processing of APP (Georgopoulou et al., 2001). For example, abnormal APP glycosylation induced by mannosidase inhibitors resulted in a significant increase in the cellular APP and decrease in secreted APP (McFarlane et al., 1999). Similarly, mutation of the N-glycosylation sites of APP caused a reduction of its secretion (Yazaki et al., 1996). In addition, N-glycosylation has also been found to be required for axonal sorting of APP (Tienari et al., 1996). Recently, it has been reported that APP degradation pathway could be altered by its glycosylation state (Hare, 2006). The author proposed that abolishment of glycosylation may cause conformational changes that expose previously inaccessible proteolytic sites. Beyond N- and O-glycoslation, ε-glycation which is a non-enzymatically mediated reaction between ε-position at lysines and reducing sugars is also proposed to be involved in APP modification and interfere with its degradation (Schmitt, 2006). Taken together, high glucose may affect the susceptibility of APP to degradation through interfering with its intracellular trafficking and/or alteration of its conformation by glycosylation modification.

Chapter 5 General discussion and future directions

5.1 General discussion

Hyperglycemia may promote AD pathogenesis through interactions between multiple factors and pathways. AD is revealed to be accompanied with exacerbated oxidative stress (Gabbita et al., 1998; Lovell and Markesbery, 2001; Nunomura et al., 1999; Sayre et al., 1997), inflammation (Wyss-Coray, 2006), increased AGEs and mitochondrial dysfunction (Moreira et al., 2010). AD is also associated with high blood glucose (Carantoni et al., 2000; Janson et al., 2004; Razay G, 2007). Elevated blood glucose can cause excessive production of reactive oxygen species that lead to attack to proteins, nucleitides, lipids and subsequent damage of their biological activities (Russell et al., 2002; Sies, 1985; Vincent et al., 2004). In support of this notion , mice feeding on a high glucose diet developed hyperglycemia and enhanced oxidative stress (Folmer et al., 2002). Mitochondria are more vulnerable to this oxidative stress since the mitochondrial genome does not contain protective histones and disruption of the electron-transport chain components can exacerbated the situation by generating more free radicals (Yu et al., 2008). Hyperglycemia can cause mitochondrial damage through increased association between mitochondrial fission protein dynamin-regulated protein 1 and pro-apoptotic proteins Bim and Bax, ultimately resulting in cell apoptosis (Leinninger et al., 2006). On the other hand, Excessive glucose can also accelerate the formation of AGEs (Bucala and Cerami, 1992) which are present at 3-fold more in plaques of AD brains than age-matched controls (Vitek et al., 1994) and are also present in hippocampal neurons and astrocytes of AD brains (Sasaki et al., 2001). AGEs can act synergistically with

oxidative stress to cause protein damage (Mullarkey et al., 1990). Also, AGE modification has been shown to accelerate Aβ aggregation (Vitek et al., 1994). In addition, AGEs can induce proinflammatory cytokines release by activating their receptors (RAGEs), leading to inflammation (Berbaum et al., 2008). Although many molecular mechanisms have been studied, the exact role of hyperglycemia in APP regulation, the key molecule in AD pathogenesis, remains elusive. In this study, we investigated the effect of high glucose on APP metabolism and Aβ production.

Our finding that high glucose increased the expression of APP protein is in agreement with a previous report that people with diabetes have higher APP level in platelets than controls (Nomura et al., 1994). We also found that APP transcription was unaffected by high glucose treatment in spite of an increase in APP protein level. By using protein synthesis inhibitor, we found that the rate of APP turnover was decreased after high glucose treatment. APP protein undergoes rapid turnover as more than 70% of newly synthesized APP is intracellularly degraded (Caporaso et al., 1992; Knops et al., 1992). It is conceivable that reduction in APP degradation could lead to a significant increase in APP level. In support of this idea, it is recently reported that disruption of APP degradation by interfering with its ubiquitination resulted in APP accumulation and Aβ overproduction (Kaneko et al., 2010).

The mechanisms whereby alterations in ambient glucose concentrations could affect APP metabolism is unclear. The entry of glucose into neurons requires facilitative glucose

transporters and GLUT3 is the major neuronal glucose transporter. It has been shown that the expression level and/or translocation to plasma membrane of GLUT3 is enhanced during hyperglycemia (Boileau et al., 1995; Merriman-Smith et al., 2003). Thus, it is quite plausible that high extracellular glucose concentrations could lead to an increase in intracellular glucose levels. APP is modified by N-glycosylation and O-glycosylation during maturation (Weidemann et al., 1989). Studies using mannosidase inhibitors (McFarlane et al., 1999; Tienari et al., 1996) and mutation of glycosylation sites (Yazaki et al., 1996) demonstrated a significant impact of APP glycosylation on its trafficking and processing. Interestingly, it has recently been reported that APP degradation pathway could be altered by its glycosylation state which may subsequently induce conformational changes (Hare, 2006). Moreover, N- and O-glycosylation predispose APP protein to Thr668 phosphorylation which, in turn, directs APP to axonal transportation (Ando et al., 1999). The intracellular trafficking of APP is known to be an important determinant for its processing (Andersen et al., 2005; El Ayadi et al., 2012). Therefore, high glucose may affect APP processing and Aβ production through glycosylation modification of APP which leads to alterations in intracellular trafficking and/or the conformation of the protein. In addition to modifying APP *per se*, high glucose could also affect APP metabolism through regulating molecular chaperones that interact with APP such as glucose-regulated protein 78 (Yang et al., 1998b), C-terminus Hsp70 interacting protein (Kumar et al., 2007) and ubiquilin-1(Stieren et al., 2011). These cytosolic and endoplasmic reticulum chaperones are able to both stabilize and promote degradation of APP, and thus have a significant impact on APP processing and Aβ production (Hoshino et al., 2007; Kumar et al., 2007; Stieren et al., 2011) .

AD is associated with build-up of disease-relevant proteins resulting from malfunction of protein degradation system (Morawe et al., 2012). Increased APP is found in AD brains compared with controls (Preece et al., 2004). On the other hand, excessive APP is known to be able to cause AD. APP overexpression either through gene duplication or mutations in *APP* promoter causes early-onset AD (Brouwers et al., 2006; Rovelet-Lecrux et al., 2006). Moreover, the level of APP expression has been found to be inversely correlated with the age of disease onset (Brouwers et al., 2006). Our results show that the impaired degradation of APP is involved in the increase of APP protein after high glucose treatment. Thus, compromised removal of APP protein and enhanced Aβ production induced by high glucose may serve as a molecular link between diabetes and Alzheimer's disease.

The physiological function of APP is still unclear. APP has been suggested to be involved in diverse cellular processes such as cell adhesion, axon pruning and synaptogenesis. Interestingly, one study, with closer relevance to our study, suggests APP could regulate plasma insulin and glucose as APP knockout mice had higher plasma insulin and lower plasma glucose than the wild-types (Needham et al., 2008). Therefore, it is possible that dysregulated APP level resulted from high blood glucose could exacerbate the hyperglycemic condition in AD patients, forming a vicious loop.

The strong association between Alzheimer's disease and diabetes has been demonstrated by accumulating epidemiological and experimental evidences with many underlying mechanisms

being proposed. Our study investigated the effect of hyperglycemia on APP metabolism and Aβ production by using cultured human neuronal-like cells. Our finding showed that high glucose treatment significantly increased the level of full-length APP protein and Aβ production in cultured cells and the accumulation of APP is not due to enhancement of transcription but through inhibition of APP degradation. The finding of this study revealed a new pathway through which diabetes accelerates the development of AD. Also, our study suggests that incorporation of glucose-lowing treatment could be beneficial for AD patients.

5.2 Future directions

In our study, we clearly showed that high glucose treatment promotes APP expression mediated by inhibition of APP degradation, which might lead to Aβ overproduction. Yet, other mechanisms might also be involved in high glucose induced abnormal APP processing and Aβ generation. For example, high glucose treatment may affect APP processing by modulating the expression and/or activities of enzymes involved. In fact, it has been demonstrated by our lab as well as other groups that oxidative stress can potentiate BACE1 expression and Aβ production (Quiroz-Baez et al., 2009; Tamagno et al., 2008; Tong et al., 2005). The same effect of oxidative stress is also observed on γ-secretase (Oda et al., 2010). Since chronic hyperglycemia could result in oxidative stress (Vincent et al., 2004), it is very likely that hyperglycemia can exert an impact on BACE1 and γ-secretase as well. Assessment of the effect and the extent of their contribution could be helpful to determine the impact of hyperglycemia on AD pathogenesis in a broader view. In addition, as mentioned before, since APP trafficking plays an important role in amyloidogenic

processing, examination of the localization and trafficking dynamics of APP under high glucose exposure will provide better understanding of the underlying mechanism.

It will also be informative to examine the pathological consequences of elevated APP and Aβ under hyperglycemia. Previous studies identified a mitochondrial targeting signal at the N-terminus of APP protein and suggested that the accumulation of APP in mitochondria could lead to mitochondrial dysfunction (Anandatheerthavarada et al., 2003; Devi et al., 2006). Accordingly, while mitochondrial APP is very low in control brains, it is present in significantly higher level in AD brains (Devi et al., 2006). Moreover, in both transgenic mice and AD patients, Aβ can directly interact with Aβ-binding alcohol dehydrogenase in the mitochondria resulting in generation of ROS, mitochondrial dysfunction and cell death (Lustbader et al., 2004). Therefore, it might be interesting to examine the interaction between mitochondria and APP and Aβ in our system.

Furthermore, *in vivo* studies by using experimental animals would provide us with deeper insight into the effect of hyperglycemia on AD pathogenesis and underlying mechanisms.

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