

High Glucose Promotes A β Production by Inhibiting APP Degradation

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Abstract

Abnormal deposition of neuritic plaques is the unique neuropathological hallmark of Alzheimer's disease (AD). Amyloid β protein (A β), the major component of plaques, is generated from sequential cleavage of amyloid β precursor protein (APP) by β -secretase and γ -secretase complex. Patients with diabetes mellitus (DM), characterized by chronic hyperglycemia, have increased risk of AD development. However, the role of high blood glucose in APP processing and A β generation remains elusive. In this study, we investigated the effect of high glucose on APP metabolism and A β generation in cultured human cells. We found that high glucose treatment significantly increased APP protein level in both neuronal-like and non-neuronal cells, and promoted A β generation. Furthermore, we found that high glucose-induced increase of APP level was not due to enhancement of APP gene transcription but resulted from inhibition of APP protein degradation. Taken together, our data indicated that hyperglycemia could promote AD pathogenesis by inhibiting APP degradation and enhancing A β production. More importantly, the elevation of APP level and A β generation by high glucose was caused by reduction of APP turnover rate. Thus, our study provides a molecular mechanism of increased risk of developing AD in patients with DM and suggests that glycemic control might be potentially beneficial for reducing the incidence of AD in diabetic patients and delaying the AD progression.

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Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder characterized by a progressive decline in memory and other cognitive functions. It is estimated that over 35 million people worldwide suffered from the disease and AD-related healthcare cost was \$604 billion in 2010 alone [1,2]. While early-onset AD can be caused by gene mutation in *APP*, *presenilin1* or *presenilin2*, it only accounts for less than 5% of all AD cases [3]. The majority of AD cases are sporadic usually with late-onset, caused by unelucidated mechanisms. Both early-onset AD and late-onset AD are characterized by the common major neuropathological features including neuritic plaques, neurofibrillary tangles, and neuronal loss. Amyloid β protein (A β), the major component of plaques, derives from sequential cleavage of amyloid β precursor protein (APP) by β -secretase and γ -secretase complex.

APP is a type I integral membrane protein, encoded by *APP* gene on chromosome 21 [4–7]. There are three major isoforms of APP derived from alternative splicing, of 695 (APP₆₉₅), 751 (APP₇₅₁) and 770 (APP₇₇₀) amino acids, respectively. APP₆₉₅ is predominant in the neurons of the brain, while APP₇₅₁ and APP₇₇₀ are more ubiquitously expressed in most tissues such as kidney, lung, and muscle [8–10]. APP expression is regulated at

multiple levels including transcription, translation and post-translation [11]. *APP* gene transcription is governed by a complex promoter [12,13] and subjected to the regulation of many transcription factors such as homeobox protein Hox-1.3 and NF- κ B [14,15]. APP expression can be stimulated by interleukin-1, retinoic acid, phorbol esters, growth factors [16–19] and various stresses including heat shock, treatment with ethanol and sodium arsenite [20,21]. In addition, APP is extensively post-translationally modified including N-glycosylation, O-glycosylation, tyrosine sulfation and phosphorylation [22,23] and is detected in various subcellular locations endoplasmic reticulum, Golgi apparatus, and plasma membrane [24–27]. APP is metabolized in lysosomes [28,29], and recent study indicates it also being degraded through ubiquitin-proteasome pathway [30]. The half life of APP is 20–30 minutes [23]. It has been reported that APP degradation and processing is significantly affected by its glycosylation modification [31].

APP can be cleaved by β -site APP cleaving enzyme 1 (BACE1) at two sites of A β domain, Asp¹ and Glu¹¹, generating C-terminal fragments of 99 or 89 amino acids (C99 or C89) respectively [32,33]. C99 is subsequently cleaved by γ -secretase complex in the transmembrane domain, liberating A β and APP intracellular domain. Thus, inhibition of BACE1 and/or γ -secretase activity might be an effective way for AD treatment by reducing A β

production [34–36]. Yet under normal conditions, majority of APP protein are cleaved by α -secretase within the A β domain, generating a secreted N-terminal fragment (sAPP α) and a membrane-bound C-terminal fragment of 83 amino acids (C83) [37,38], which excludes A β generation.

Although recent studies reveal that APP is involved in brain development and repair [39,40], the most well-known function of APP is its pathologic role in AD development. APP gene mutation is the first identified genetic mutation that causes early-onset AD [41]. In addition, elevated APP expression is implicated in the pathogenesis of both early-onset and late-onset AD. First, Down syndrome (DS) patients caused by trisomy of chromosome 21 show higher APP expression in brains compared to control cases and they inevitably develop AD after middle age, although increased expression of other chromosome 21 genes might also contribute to AD pathogenesis, such as *RCAN1* [42–45]. Second, APP overexpression either through gene duplication or mutations in APP promoter can also cause early-onset AD [46,47]. Importantly, recent studies suggest that APP upregulation is also involved in late-onset AD. MiR-106b, which can downregulate APP expression, was found significantly decreased in sporadic AD [48] and marked increase of APP mRNA has been reported in sporadic AD [49]. Taken together, these studies indicate that dysregulation of APP expression and processing plays a central role in AD pathogenesis.

Diabetes mellitus (DM) is a complex metabolic disorder featured by chronic hyperglycemia. Accumulating epidemiological evidence shows a 50–100% increase in risk of developing AD in patients with DM [50,51]. Diabetic patients exhibit cognitive deficits including damaged verbal memory, diminished mental speed and mental flexibility [52,53]. Moreover, recent studies have shown that chronic hyperglycemia, the cardinal feature of diabetes, could accelerate the development of AD pathology in AD model mice. In diabetic mice induced by streptozotocin injection, the formation of both neuritic plaques and neurofibrillary tangles were potentiated [54,55]. On the other hand, it has been reported that 81% of the AD patients show either impaired fasting glucose or frank diabetes [56]. Although a large body of data suggests that AD is highly associated with DM, the underlying mechanisms of this association are still unknown.

In this study, we first examined the effect of high glucose on APP metabolism and A β production *in vitro*. We found that high glucose significantly increased APP level and A β generation. Furthermore, we found that high glucose-induced increase in APP level was not due to enhancement of its gene transcription but resulted from inhibition of its protein degradation. Our data indicated that increased APP level and A β production by high glucose is caused by reduction of APP turnover rate. Thus, our study not only provides a molecular mechanism underlying the increased risk of developing AD in DM patients but also suggests that glycemic control might be potentially beneficial for reducing the incidence of AD in people with DM and delaying the AD progression.

Methods

Cell Culture, High Glucose Treatment and CHX Treatment

Human neuroblastoma cells, SH-SY5Y (ATCC CRL-2266), were maintained in glucose-free Dulbecco's modified eagle medium (DMEM) supplemented with 2.5 mM D-glucose, 10% fetal bovine serum (FBS), 50 units of Penicillin and 50 μ g of Streptomycin (Life Technologies). 20E2 cells, a cell line of human embryonic kidney 293 cells, HEK293 (ATCC CRL-1573), stably expressing Swedish mutant APP₆₉₅ generated in our lab previously

[57], were cultured in glucose-free DMEM supplemented with 5.5 mM D-glucose, 10% FBS, 50 units of Penicillin and 50 μ g of Streptomycin and 50 μ g/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 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. For APP degradation experiment (half-life measurement), 20E2 cells treated with media containing high glucose and cycloheximide (CHX) at 100 μ g/ml (Sigma) were harvested at 0, 15, 30 or 60 minutes for western blot analysis. Similarly, SH-SY5Y cells were treated with high glucose media containing cycloheximide for 30 minutes and then harvested for western blot analysis.

Luciferase Assay

The APP-Luc plasmid, containing 2.94 kb of human APP promoter region upstream of the firefly luciferase reporter gene [32], 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 60 mm plates at the density of 5.0×10^5 cells/ml culture media. On the day of transfection, the cells were grown to approximately 70% confluence and transfected with 4.6 μ g pAPP-Luc plasmid DNA along with 18.4 ng *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).

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 ThermoScriptTM reverse transcriptase (Invitrogen). The newly synthesized cDNA then served as templates and the coding sequence of human APP and β -actin was amplified by Platinum Tag DNA polymerase (Invitrogen) with following primers: APP forward 5'-gctggcctgctgctgaacc; APP reverse 5'-ggcgcggtgtgccagtga; β -actin forward 5'-cgaggatccggacttcgagcaagagatgg; β -actin reverse 5'-cagtctagagaagcatttcggtggcagc. β -actin levels served as an internal control. The PCR products were analyzed in 0.8% agarose gel (Sigma).

Western Blotting

Cells were harvested and lysed in RIPA-DOC lysis buffer (0.05 M Tris-HCl pH 7.2, 0.15 M 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 13,200 rpm 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 polyvinylidene 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°C. Antibody C20 is a polyclonal rabbit antibody made in-house that recognizes the last twenty amino acids of the APP carboxyl-terminus and was used to detect APP protein and C99 fragment. 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 hour, and visualized using an Odyssey Infrared Imaging System (LI-COR Biosciences).

A β ELISA

24 hours after high glucose treatment, conditioned cell culture media of 20E2 cells were collected. 20E2 cells are HEK293 cells that stably express Swedish mutant APP₆₉₅. To prevent degradation of A β , protease inhibitors (AEBSF) were added. The cell media were centrifuged at 2000 rpm for 5 minutes to precipitate cells in the media. The concentration of A β 40 was measured by A β 40 human ELISA kit (KHB3482, Life Technologies) according to the manufacturer's instructions.

Statistics Analysis

Three or more independent experiments were performed. For APP degradation experiment, two-way ANOVA followed by Bonferroni test was used for statistical analysis. The rest of data were analyzed by one-way ANOVA followed by Tukey test. Values were expressed as mean \pm S.E.M. We reported F value and P value, and $P < 0.05$ was considered as statistically significant.

Results

High Glucose Treatment Increases APP Protein Level

To investigate whether hyperglycemia could affect APP level, we treated the human neuroblastoma SH-SY5Y cells with media containing 10 mM or 25 mM glucose to mimic hyperglycemia *in vitro* and compared with cells treated with 2.5 mM glucose media which served as control. The 2.5 mM glucose was used as the equivalent of the physiological extracellular glucose concentration in the human brain [58,59]. We found that 10 or 25 mM glucose treatment significantly increased the level of full-length APP protein to $152.63 \pm 10.78\%$ or $140.59 \pm 6.80\%$ ($F = 10.88$, $p < 0.05$), respectively, comparing with the control at 24-hour time point (Fig. 1A and B). Similarly, the level of full-length APP was significantly increased to $120.52 \pm 4.20\%$ or $146.04 \pm 0.59\%$ ($F = 74.5$, $p < 0.05$), respectively, in cells treated with 10 or 25 mM glucose for 48 hours comparing with the control (Fig. 1C and D).

High Glucose Treatment does not affect APP Gene Transcription

To examine whether the increase in full-length APP protein induced by high glucose treatment was via upregulation of APP gene transcription, we measured APP promoter activity in human neuroblastoma SH-SY5Y cells treated with 10 or 25 mM glucose. pAPP-Luc plasmid was constructed by inserting a 2.94 kb region of human APP promoter into the promoterless vector, pGL3-basic, upstream of the firefly luciferase reporter gene [32]. We found that neither 24-hour nor 48-hour treatment of 10 or 25 mM glucose affected the APP promoter activity (Fig. 2A and B).

Although the luciferase assay is very sensitive to assess APP promoter activity, the 2.94 kb promoter region of APP gene may not reflect the endogenous APP promoter activity. To further investigate if high glucose could affect APP transcription, we examined the level of endogenous APP mRNA in SH-SY5Y cells after 10 or 25 mM glucose treatment. Consistent with the promoter assay data, 24-hour and 48-hour treatment with 10 or 25 mM glucose did not have significant effect on APP mRNA level (Fig. 2C and E). Statistical analysis showed that there was no

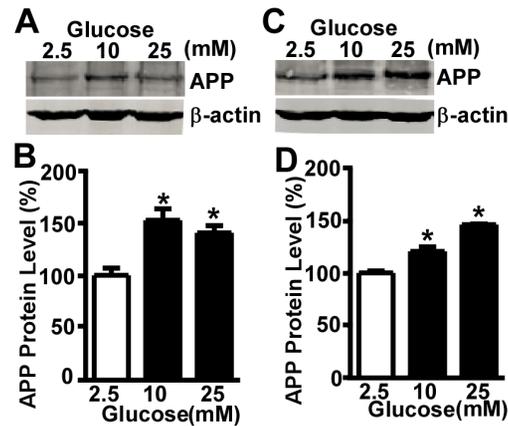


Figure 1. High glucose treatment increases full-length APP protein level. SH-SY5Y cells were treated with different concentration of glucose for 24 hours (A), and 48 hours (C) with 2.5 mM glucose treatment serving as control. The cell lysates were analyzed by Western blot. Full-length APP was detected by C20 antibody. β -actin, serving as internal control, was detected by AC-15 antibody. 24-hour and 48-hour of high glucose treatment significantly increased full-length APP protein level. Quantification of full-length APP after 24-hour treatment of high glucose in SH-SY5Y cells (B). The values are expressed as mean \pm S.E.M, $n = 4$, $*p < 0.05$ by ANOVA. Quantification of full-length APP after 48-hour treatment of high glucose in SH-SY5Y cells (D). The values are expressed as mean \pm S.E.M, $n = 3$, $*p < 0.05$ by ANOVA. doi:10.1371/journal.pone.0069824.g001

significant difference between glucose treatments and control (Fig. 2D and F).

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 high glucose-induced alteration of APP level occurs at post-transcription level. To confirm it, we examined if high glucose treatment has effect on exogenous APP level in 20E2 cells, whose transcription was driven by the CMV promoter and therefore is not subject to transcriptional regulation in human cells. 20E2 cell is a HEK cell line that stably expresses Swedish mutant APP₆₉₅ under the CMV promoter. Since 20E2 derives from a peripheral cell line, we used 5.5 mM glucose, the physiological normal blood glucose at periphery, as control. Like in SH-SY5Y cells, we observed a significant increase in APP protein level in 20E2 cells after 24-hour high glucose treatment (Fig. 3A). The APP protein in cells treated with 10 mM glucose increased to $169.45 \pm 4.94\%$ of that of the control group and to $213.36 \pm 7.33\%$ in 25 mM group ($F = 90.74$, $p < 0.0001$) (Fig. 3B). The protein level in the cells depends on the counterbalance between its production and degradation, and our data (Fig. 3A) indicated that increased APP translation or reduced APP degradation might be involved in high glucose-induced increase of APP expression. APP is degraded through proteasome and lysosome pathways [28–30], and both pathways have been reported to be altered by hyperglycemia [60,61]. Therefore, we next examined if high glucose-induced APP elevation could result from dysregulation of its degradation. We used cycloheximide (CHX) to stop protein synthesis and measured the amount of remaining protein at various time points after 10 or 25 mM glucose treatment. In 20E2 cells, elevated glucose concentration significantly enhanced APP level in a dosage dependent manner ($F = 79.2$, $p < 0.001$). 15 minutes after CHX treatment, $65.57 \pm 1.67\%$ of the initial total APP protein remained in cells

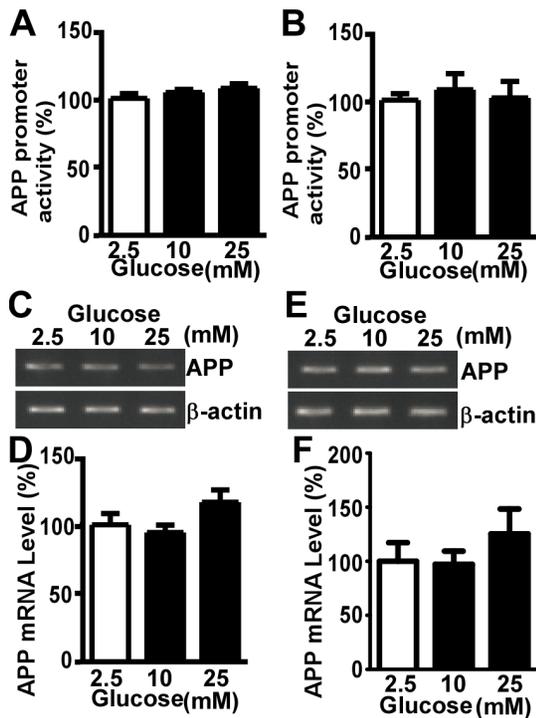


Figure 2. High glucose treatment does not affect APP transcription. The 2.94 kb human APP promoter was transfected into SH-SY5Y cells and treated with high glucose for 24 hours (A) and 48 hours (B). 2.5 mM glucose serves as control. Luciferase assay was performed. High glucose treatment did not affect APP promoter activity. All the promoter data shown are results of 4 independent experiments, with each condition performed in triplicates. The values are expressed as mean \pm S.E.M. $n=4$, by ANOVA. SH-SY5Y cells were treated with different concentration of glucose for 24 hours (C) and 48 hours (E). 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 (D) and after 48-hour treatment of high glucose (F). The values are expressed as mean \pm S.E.M. $n=7$, by ANOVA. Quantification of full-length APP after 24-hour treatment of high glucose (D) and after 48-hour treatment of high glucose (F). The values are expressed as mean \pm S.E.M. $n=5$, by ANOVA. doi:10.1371/journal.pone.0069824.g002

treated with 5.5 mM glucose while $85.72 \pm 4.87\%$ of initial APP persisted in 10 mM glucose treated cells ($p < 0.001$) (Fig. 3C, D and E). At 30 minutes, cells cultured in 5.5 mM glucose media contained $39.94 \pm 0.21\%$ of the initial total APP while cells treated with 10 mM glucose contained $59.57 \pm 3.28\%$ of initial total APP ($p < 0.001$) (Fig. 3C, D and E). At 60 minutes time point, the levels of APP were reduced to $20.91 \pm 0.08\%$ and $48.38 \pm 0.88\%$ of controls in cells treated with 5.5 mM and 10 mM glucose, respectively ($p < 0.001$) (Fig. 3C, D and E). Similarly, in SH-SY5Y cells, 30 minutes after CHX treatment, $52.25 \pm 5.07\%$ of the initial total APP protein remains in cells treated with 2.5 mM glucose while $70.48 \pm 2.95\%$ APP persists in 10 mM glucose treated cells, and $82.43 \pm 6.19\%$ APP persists in 25 mM glucose treated cells ($F = 6.58$, $p < 0.05$) (Fig. 3F and G). Taken together, our data suggested that high glucose slowed down the APP protein degradation in both neuronal-like cells and non-neuronal cells.

High Glucose Treatment Enhances C99 and A β Production

APP is cleaved by BACE1 to generate C99 fragments and C99 is subsequently processed by γ -secretase to produce A β . Since the level of full-length APP was increased after high glucose treatment, we further examined whether high glucose treatment also has an impact on APP processing and A β production. We measured the level of C99 fragment and A β_{40} in the 20E2 cells. After 24-hour treatment, the level of C99 fragment significantly increased to $112.61 \pm 2.57\%$ in cells treated with 10 mM glucose and to $138.33 \pm 2.18\%$ in cells treated with 25 mM glucose ($F = 52.46$, $p < 0.001$) (Fig. 4A and B). Moreover, the A β_{40} level increased to $133.21 \pm 3.69\%$ at 10 mM glucose compared with control and to $142.49 \pm 4.21\%$ when treated with culture media containing 25 mM glucose ($F = 31.66$, $p < 0.001$) (Fig. 4C). Thus, high glucose treatment markedly enhanced the level of C99 and A β .

Discussion

Diabetes has been shown to nearly double the risk of AD development [50,51]. Yet the molecular mechanism underlying this association is not known. As a cardinal pathology of diabetes, chronic hyperglycemia is found to be inversely correlated with cognitive function [62,63]. Meanwhile, AD is associated with hyperglycemia [56,64,65], indicating that hyperglycemia may play a role in AD pathogenesis.

Previous report has shown that people with diabetes have higher APP level in platelets than controls [66]. However, the role of high glucose in regulating expression of APP in neuronal cells remains elusive. Therefore, we first examined the effect of high glucose on APP expression in human neuroblastoma cells. We found that the level of APP protein is significantly increased by high glucose treatment for 24 or 48 hours (Fig. 1). 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 treatment with osmolarity-unadjusted media induced a similar increase in APP protein as that by osmolarity-adjusted media (data not shown). Our data indicated that increased APP protein level by high glucose treatment is independent of high glucose-induced alteration of the osmotic pressure.

It has been shown that high glucose leads to excessive production of reactive oxygen species in neurons [67] and increased oxidative stress could activate NF- κ B, which is involved in regulation of APP transcription [15]. Thus, we examined whether high glucose affects the activity of APP promoter region containing NF- κ B sites. We found that both 24-hour and 48-hour high glucose treatment did not affect APP promoter activity (Fig. 2). In agreement with our result, previous study reported that reactive oxygen species did not activate the APP promoter in neurons [68]. To further confirm the effect of high glucose on global APP transcription, we measured APP mRNA level in cells treated with high glucose media. Consistently, high glucose treatment had no significant effect on the level of APP mRNA (Fig. 2). Our work first clarified that high glucose has no effect on APP transcription, at least with short term treatment.

Our result indicates that the increase in APP level induced by high glucose treatment occurs at posttranscriptional level, caused by enhanced APP translation or reduced APP degradation. APP protein undergoes rapid turnover as more than 70% of newly synthesized APP is intracellularly degraded [69,70]. It is conceivable that reduction in APP degradation could lead to a significant increase in APP level, which is highly supported by the following evidence. First, it has been reported that proteasome and lysosome pathway are altered by hyperglycemia [60,61] and APP is

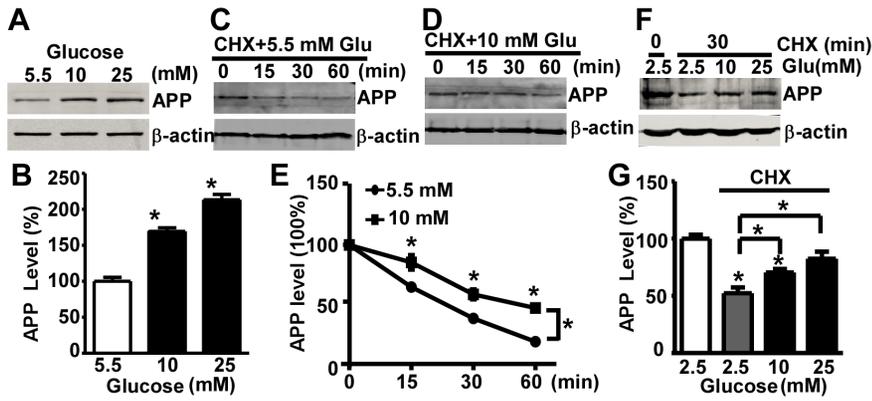


Figure 3. High glucose treatment inhibits APP protein degradation. 20E2 cells were treated with 5.5 mM, 10 mM or 25 mM glucose for 24 hours, and the cell lysates were analyzed by Western blot (A). Full-length APP was detected by C20 antibody. β -actin, serving as internal control, was detected by AC-15 antibody. The level of APP protein was quantified by Image J (B). The values are expressed as mean \pm S.E.M. $n = 3$, $*p < 0.0001$, by ANOVA. For APP degradation experiment, 20E2 cells were treated with culturing media containing 100 μ g/ml CHX along with 5.5 mM or 10 mM glucose. The cell lysates were harvested at 0, 15, 30 or 60 minutes after treatment and analyzed by Western blot (C, D). Quantification of APP protein by Image J (E). APP protein level was plotted as a percentage of the amount at 0 minute. The values are expressed as mean \pm S.E.M. $n = 3$, $*p < 0.001$, by two-way ANOVA. The APP degradation experiment was also conducted using SH-SY5Y cells treated with 100 μ g/ml CHX along with 5.5 mM, 10 mM or 25 mM glucose for 30 minutes. The cell lysates were analyzed by Western blot (F). The level of APP protein was quantified by Image J (G) and was plotted as a percentage of the amount at 0 minute. The values are expressed as mean \pm S.E.M. $n = 3$, $*p < 0.05$, by ANOVA. doi:10.1371/journal.pone.0069824.g003

degraded through both pathways [28–30]. In addition, high glucose might affect APP modification, including glycosylation, phosphorylation and ubiquitination, which could lead to alteration of APP degradation and A β production. Our data confirmed that high glucose treatment prolonged APP half-life from 25 minutes (5.5 mM) to around 60 minutes (10 mM) in HEK293 cells. Same trend was found in human neuroblastoma cells. The mechanism whereby high glucose could inhibit 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 [23]. Studies using mannosidase inhibitors [71,72] and mutation of glycosylation sites [73] demonstrated a significant impact of APP glycosylation on its trafficking and processing. Interestingly, it has been reported that APP degradation pathway could be altered by its glycosylation state which may subsequently induce conformational changes [74]. Moreover, N- and O-glycosylation predispose APP protein to Thr668 phosphorylation

which, in turn, directs APP to axonal transportation [75]. The intracellular trafficking of APP is known to be an important determinant for its processing [76,77]. Therefore, high glucose may affect APP processing and A β production through glycosylation and phosphorylation of APP which lead to alterations in intracellular trafficking and/or the conformation of the protein. In our western blots of full length APP, we did observe an upper APP band which could be glycosylated form. It is possibly caused by the following reasons. First, it has been shown that the signal of modified APP including glycosylated APP is much weaker than unmodified APP. Thus, normally, modified APP may not be easy to be detected. Second, the glycosylated APP has similar migration rate as unmodified APP or differentially modified APP. Elevated level of APP may mask the slight difference of the migration between glycosylated APP and unmodified APP. Although the glycosylated APP has not been detected, it is still possible that this modification contributes to the reduction of APP degradation and the elevation of A β level. In addition, impairment of proteasome or lysosome by high glucose may also contribute to the decrease of APP turnover rate. Moreover, as C99 and A β are degraded through proteasome pathway, high glucose-induced proteasomal impairment may inhibit C99 and A β degradation, which further leads to elevated level of C99 and A β [78–81]. Furthermore, the degradation of A β may also be reduced by high glucose-induced inhibition of insulin-degrading enzyme (IDE), which not only catalyzes the catabolism of insulin but also has been demonstrated to degrade A β both *in vivo* and *in vitro* [82–85]. Further study is needed to clarify the underlying mechanism.

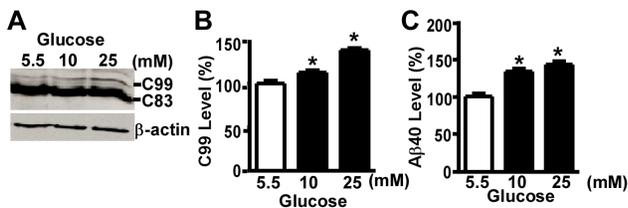


Figure 4. High glucose treatment increases C99 and A β_{40} production. The 20E2 cells were cultured and treated with different concentrations of glucose for 24 hours. Media containing 5.5 mM glucose served as control. The cell lysates were analyzed by western blot (A). C99 was detected by C20 antibody. β -actin, serving as internal control, was detected by AC-15 antibody. Quantification of C99 after 24-hour treatment of high glucose in 20E2 cells (B) The values are expressed as mean \pm S.E.M, $n = 3$, $*p < 0.001$ by ANOVA. The level of A β_{40} in conditioned media of 20E2 cells was measured by ELISA (C). The values are expressed as mean \pm S.E.M, $n = 4$, $*p < 0.001$, by ANOVA. doi:10.1371/journal.pone.0069824.g004

Increased APP expression contributes to A β overgeneration. Consistently, our data showed that high glucose-induced APP upregulation was highly associated with A β overproduction. The increase in A β production could result from enhanced APP level, yet, it is possible that high glucose may also upregulate β -secretase and γ -secretase expression and/or activity contributing to A β overproduction. Hyperglycemia is known to cause elevated oxidative stress [86] and both BACE1 and presenilin 1 expression are known to be stimulated by oxidative stress accompanied by an increase in A β production [87–90]. Therefore, it could be helpful to further investigate the effect of high glucose on BACE1 and

presenilin1. Furthermore, *in vivo* studies by using experimental animals may provide deeper insight into the effect of hyperglycemia on AD pathogenesis and underlying mechanisms.

In conclusion, we found that high glucose increases APP level through inhibition of its degradation and facilitates A β production. Our study provides a potential molecular mechanism underlying the association between diabetes and AD with the implication that glycemic control might be potentially beneficial for reducing the incidence of AD development in patients with DM and delaying the AD progression. Future *in vivo* studies will be essential to determine the effect of hyperglycemia on AD pathogenesis and the

beneficial effect of glycemic control on reducing the incidence of AD development or slowing down the progression of AD.

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Author Contributions

Conceived and designed the experiments: YY YW WS. Performed the experiments: YY YW SZ. Analyzed the data: YY YW WS. Contributed reagents/materials/analysis tools: WS. Wrote the paper: YY YW WS.

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