Benfotiamine prevents increased β -amyloid production in HEK cells induced by high glucose

Xiao-Jing Sun¹, Lei Zhao¹, Na Zhao¹, Xiao-Li Pan¹, Guo-Qiang Fei¹, Li-Rong Jin¹, Chun-Jiu Zhong^{1,2}

¹Department of Neurology, Zhongshan Hospital & Shanghai Medical College, Fudan University, Shanghai 200032, China

²State Key Laboratory of Medical Neurobiology, Institute of Brain Science, Fudan University, Shanghai 200032, China

© Shanghai Institutes for Biological Sciences, CAS and Springer-Verlag Berlin Heidelberg 2012

Abstract: Objective To determine whether high glucose enhances β -amyloid (A β) production in HEK293 Swedish mutant (APPsw) cells with A β precursor protein (APP) overexpression, and whether under this condition benfotiamine reduces the increased A β production. **Methods** HEK293 APPsw cells were cultured with different concentrations of glucose for different times. The A β content in the supernatant was determined by ELISA. To investigate the mechanism by which benfotiamine reduced A β production, glycogen synthase kinase-3 (GSK-3) activity and expression were measured after the cells were cultured with 5.5 g/L glucose for 12 h. **Results** With 1.0, 3.0, 4.5, 5.5, 6.5, 7.5, 8.5, or 10.5 g/L glucose, A β production by HEK293 APPsw cells was highest in the presence of 5.5 g/L glucose for 6 and 12 h. The difference in A β content between 5.5 and 1.0 g/L was most marked after incubation for 12 h. Benfotiamine at 20 and 40 μ g/mL significantly reduced A β production in cells incubated with 5.5 g/L glucose for 12 h. Moreover, 40 μ g/mL benfotiamine significantly enhanced the ratio of phosphorylated GSK-3 to total GSK-3, together with consistent down-regulation of GSK-3 activity. **Conclusion** High glucose increases A β production by HEK293 APPsw cells while benfotiamine prevents this increase. This is correlated with the modulation of GSK-3 activity.

Keywords: Alzheimer's disease; β -amyloid; high glucose; benfotiamine; glycogen synthase kinase-3

1 Introduction

Alzheimer's disease (AD) is characterized clinically by progressive cognitive impairment and pathologically by the presence of extracellular senile plaques and intracellular neurofibrillary tangles in the brain. Senile plaques are largely composed of β -amyloid 1–42 (A β ₄₂), a 4-kD peptide derived from A β precursor protein (APP). A β ₄₂ is toxic to neurons. The deposition of A β and the formation of senile plaques are considered to be the primary causes of AD. However, the exact process

of pathogenesis remains to be clarified. Recent evidence from population-based studies has shown a link between diabetes mellitus (DM) and AD^[1–4]. DM significantly increases the risk for cognitive dysfunction, especially AD-type dementia^[5–7]. Also, AD patients have a high risk of developing type II DM^[8,9]. Experimental investigations suggest that abnormal cerebral glucose metabolism is a key etiological factor^[1,24].

Glycogen synthase kinase-3 (GSK-3), a serine/threonine protein kinase, is involved in regulating glucose metabolism and plays an important role in many physiological and pathological processes. Interestingly, recent studies have shown that GSK-3 contributes to AD pathogenesis^[10], and inhibiting GSK-3 activity reduces cerebral A β produc-

Corresponding author: Chun-Jiu Zhong

Tel: +86-21-54237920

E-mail: zhongcj@163.com

Article ID: 1673-7067(2012)05-0561-06

Received date: 2012-01-13; Accepted date: 2012-04-13

tion and accumulation in APP-overexpressing mice^[11]. In a previous study, we demonstrated the beneficial effect of benfotiamine against A β production in an APP/presenilin-1 (APP/PS1) double transgenic mouse model^[12], and this was correlated with the inhibition of GSK-3 activity. Here, we used HEK293 cells with APP Swedish mutant (APPsw) overexpression to determine whether high glucose affects A β production and whether benfotiamine plays a role in its regulation. Furthermore, the possible mechanism by which high glucose and benfotiamine affect A β production was investigated.

2 Materials and methods

2.1 Cell culture HEK293 APPsw cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, New York, NY) with 10% heat-inactivated fetal bovine serum (Gibco, New York, NY) at 37°C under 5% CO₂ and with 1.0, 3.0, 4.5, 5.5, 6.5, 7.5, 8.5, or 10.5 g/L glucose for 6, 12 or 24 h to define the optimal concentration and culture time for cell survival and A β production.

2.2 Drug application Benfotiamine was purchased from Shanghai Rixin Biomedical Co. (Shanghai, China) and dissolved in 50% ethanol. Then 37% HCl was added until a homogenous solution was obtained. HEK293 APPsw cells were grown in 6-well culture plates (35-mm diameter) to 70%–80% confluence in 5.5 g/L glucose. They were further randomly divided into four groups, among which three received 10, 20, or 40 μ g/mL benfotiamine in serum-free medium for 12 h, while one was treated with an equal volume of solvent containing 50% ethanol and 37% HCl for 12 h.

2.3 ELISA assay for A β ₄₂ content The supernatants were collected and assayed for residual A β ₄₂ using an ELISA kit (HS A β 42 Colorimetric ELISA, Invitrogen, New York, NY). Protein concentrations were determined with the BCA assay (Pierce, Waltham, MA). Sandwich ELISA was performed following the manufacturer's instructions. Briefly, after 2- to 10-fold dilution of the medium, 50- μ L samples were added into primary antibody-coated wells and incubated at room temperature for 4 h. After washing, the secondary antibody was added (100 μ L/well) and

incubated for 30 min. After washing, stabilized chromogen was added (100 μ L/well) and incubated for 30 min at room temperature in the dark. The reaction was terminated and the optical density at 450 nm was measured using a 96-well microplate spectrophotometer (Thermo, Waltham, MA).

2.4 Cell viability assay Viability was evaluated by CCK-8 assay using a cell viability kit (Dojindo, Japan) according to the manufacturer's instructions. HEK293 APPsw cells were plated onto 96-well plates at 2×10^5 cells/well. Cells were cultured with 1.0, 3.0, 4.5, or 5.5 g/L glucose for 12 h. Then, the cells were switched to medium containing CCK-8 at a final ratio of 10%. After 2-h incubation at 37°C, the absorbance at 450 nm was measured using a 96-well microplate spectrophotometer.

2.5 Western blot HEK293 APPsw cells were divided into three groups: low glucose control (1.0 g/L glucose), high glucose control (5.5 g/L glucose), and benfotiamine (40 μ g/mL benfotiamine with 5.5 g/L glucose). Cells were harvested in RIPA lysis buffer (Beyotime, China) after 12-h incubation. The cell suspensions were centrifuged at 10 000 rpm for 15 min at 4°C, then the supernatants were collected and their protein concentrations were determined using the BCA assay. Protein samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, UK). The membranes were blocked with 3% bovine serum albumin and incubated with specific antibodies for GSK-3 β (1:1 000), phospho-GSK-3 β (Ser9) (1:1 000), GSK-3 α (1:1 000), phospho-GSK-3 α (Ser21) (1:1 000) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1 000) (all from Cell Signaling, Boston, MA). The membranes were washed with Tris-buffered saline containing 0.05% Tween-20, and then incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (1:2 000; Millipore, Billerica, MA) followed by enhanced chemoluminescence detection (Amersham Pharmacia Biotech). The bands on the radiographic films were scanned and quantified with an image analyzer (Imagequant 5.2, GE Healthcare, UK). The phospho-GSK-3/GSK-3 ratio was calculated. GAPDH was used as

an internal control.

2.6 Enzyme activity assay HEK293 APPsw cells were divided into three groups as above. Cells were harvested after 12 h treatment, and the activity of GSK-3 α and GSK-3 β was determined by a kit (Genmed, Boston, MA) according to the manufacturer's instructions. Briefly, the cells were rinsed twice with reagent A and then homogenized with the cell lysis buffer reagent B. After 30-min incubation on ice, the homogenized mixture was centrifuged twice at 10 000 g for 10 min at 4°C, then the supernatants were collected and protein concentrations were determined using the BCA assay. Reagents C (65 μ L), D (10 μ L), E (10 μ L) and F (10 μ L) were mixed and incubated for 3 min at 30°C. The supernatant (5 μ L) was immediately added to the reagent mixture and the optical density was measured at 340 nm (SpectraMax 190, Molecular Devices, Sunnyvale, CA) every 30 s for 3 min. The enzyme activity was determined by calculating the difference between the absorbance values at 0 and 180 s. The assay was repeated twice for each sample.

2.7 Statistical analysis All data are shown as mean $\pm$

SEM. Student's *t*-test was used to evaluate statistical significance. All statistical analyses were performed using Origin 7.0 (OriginLab, Northampton, MA).

3 Results

3.1 Effects of glucose concentration on A β production

Different glucose concentrations had complex effects on A β production. From 1.0 to 5.5 g/L, A β production showed a rising trend, while from 5.5 to 10.5 g/L, it decreased (Fig. 1). A β production by HEK293 APPsw cells incubated with 5.5 g/L glucose (25.18 ± 4.4 pg/mL at 6 h; 24.58 ± 1.51 pg/mL at 12 h) was higher than that with 1.0 g/L (15.4 ± 2.92 pg/mL at 6 h, 12.25 ± 3.40 pg/mL at 12 h; $P < 0.05$ for 6 h, $P < 0.01$ for 12 h; Fig. 1). Although the A β production of cells with 5.5 g/L glucose for 24 h showed a rising trend, there was no significant difference between the 5.5 g/L (21.71 ± 1.87 pg/mL) and 1.0 g/L glucose groups (14.71 ± 3.31 pg/mL, $P > 0.05$; Fig. 1).

3.2 Effects of glucose on the viability of HEK293 APPsw cells

The OD values under the glucose concentrations used showed no significant differences (0.74 ± 0.01

Fig. 1. Effects of glucose on A β production by HEK293 APPsw cells. ELISA was performed to determine A β production. Statistical results showed that the trend of A β production first rose and then dropped with increased concentrations of glucose in the culture medium for 6, 12 and 24 h. Compared with the 1.0 g/L glucose group, A β production by cells incubated with 5.5 g/L glucose for 6 and 12 h was higher ($P < 0.05$ for 6 h, $P < 0.01$ for 12 h). There were no significant differences in A β production between cells with 1.0 g/L and 5.5 g/L glucose treatment for 24 h.

for 1.0 g/L, 0.76 ± 0.01 for 3.0 g/L, 0.78 ± 0.02 for 4.5 g/L, and 0.78 ± 0.02 for 5.5 g/L; $P > 0.05$). This showed that glucose had no significant effect on cell proliferation and growth within this limited range of concentration.

3.3 Benfotiamine prevented increased A β production by HEK293 APPsw cells in 5.5 g/L glucose We next studied the effect of benfotiamine at 10, 20, and 40 μ M on the A β production of cells in 5.5 g/L glucose. The results showed that 20 and 40 μ M benfotiamine reduced A β production (24.74 ± 1.64 pg/mL at 20 μ M and 24.14 ± 1.87 pg/mL at 40 μ M group) compared with control (29.96 ± 0.33 pg/mL; $P < 0.05$ for 20 μ M, $P < 0.01$ for

40 μ M). However, 10 μ M benfotiamine had no effect (28.47 ± 1.67 pg/mL).

3.4 Benfotiamine decreased GSK-3 activity in 5.5 g/L glucose We further investigated the mechanism by which benfotiamine modulates A β production by assessing the expression and activity of GSK-3 in HEK293 APPsw cells. The results showed that the ratios of phospho-GSK-3 α /GSK-3 α (0.30 ± 0.08) and phospho-GSK-3 β /GSK-3 β (0.33 ± 0.07) in 5.5 g/L glucose controls were decreased compared with those in 1.0 g/L glucose controls (1 ± 0.001 for phospho-GSK-3 α /GSK-3 α , $P < 0.01$; 1 ± 0.001 for phospho-GSK-3 β /GSK-3 β , $P < 0.05$, Fig. 2A, B). Con-

Fig. 2. High glucose up-regulates while 40 μ M benfotiamine (BT 40) down-regulates GSK-3 activity in HEK293 APPsw cells. **A and B:** The ratios of phospho (p)-GSK-3 α (Ser21)/GSK-3 α (A) and p-GSK-3 β (Ser9)/GSK-3 β (B) in the high glucose group (5.5 g/L) were significantly low than those in the lower glucose group (1.0 g/L). There was a trend for both ratios to increase in high glucose after benfotiamine treatment (40 μ M, $P > 0.05$). **C and D:** Activity of GSK-3 α (C) and GSK-3 β (D) in high glucose was significantly increased compared with that in low glucose. After benfotiamine treatment (40 μ M), the enzymatic activity of GSK-3 α (C) and GSK-3 β (D) was inhibited compared with the high glucose control group. * $P < 0.05$, ** $P < 0.01$ vs low glucose (1.0 g/L); # $P < 0.05$ vs high glucose (5.5 g/L).

sistently, the activity of GSK-3 α (1.56 ± 0.25) and GSK-3 β (1.92 ± 0.47) in the high glucose control was higher than that in the low glucose control (1 ± 0.001 for GSK-3 α , $P < 0.05$; 1 ± 0.001 for GSK-3 β , $P < 0.01$). Furthermore, 40 $\mu\text{g/mL}$ benfotiamine decreased the enzymatic activity of GSK-3 α (0.82 ± 0.97) and GSK-3 β (0.58 ± 0.18) compared with the control group in high glucose control ($P < 0.05$, Fig. 2C, D).

4 Discussion

Limiting A β production could become an important therapeutic target in AD^[13]. Recent studies have shown that AD is associated with perturbed glucose metabolism^[14,15]. Mild to moderate impairments of cognitive functions have been reported in patients with Type I^[16] and Type II DM^[17,18]. DM appears to be a significant risk factor for AD in several epidemiological studies^[4,19]. The previous studies have reported that high glucose can increase A β deposition and tau phosphorylation in the experimental animal brain^[1,10]. Here, we demonstrated that different concentrations of glucose had a non-linear effect on A β production in HEK293 APPsw cells, manifested by a rising trend in the presence of glucose ranging from 1.0 to 5.5 g/L and a decreasing trend under glucose concentrations ranging from 5.5 to 10.5 g/L (Fig. 1). This may be due to reduced viability of HEK293 APPsw cells caused by glucose >5.5 g/L^[25]. Therefore, our study indicates that high glucose within a limited range of concentrations significantly enhances A β production in HEK293 APPsw cells.

Our previous study reported that benfotiamine significantly reduces the formation of amyloid plaques in APP/PS1 mice^[12]. Here, we further demonstrated the inhibitory effect of benfotiamine on A β production by HEK293 APPsw cells under high glucose (5.5 g/L). It has been well documented that AD is associated with abnormal glucose metabolism^[21,26]. Experimental and clinical studies have demonstrated the beneficial effect of benfotiamine in preventing diabetic neuropathy^[27,28]. Thus, our study has major implications for preventing the pathological progress of AD by antagonizing the mechanism of impaired glucose metabolism.

Mechanistically, GSK-3 was first identified as a kinase involved in modulating glycogen metabolism. GSK-3 is a ubiquitous serine/threonine protein kinase involved in multiple physiological and pathological processes^[12]. Specifically, GSK-3 is a key contributor to the insulin signaling cascade and the molecular pathogenesis of diabetes^[29,30]. Interestingly, GSK-3 also contributes to the pathogenesis of AD^[10,20]. It reduces A β production and accumulation in APP-overexpressing mice by inhibiting GSK-3 activity^[11]. Benfotiamine increases the phosphorylation of Akt, an upstream kinase of GSK-3, in counteracting glucose toxicity and ischemic diabetes^[22,23], which suggests that benfotiamine may also regulate GSK-3 activity. Our previous study reported that benfotiamine significantly enhances the phosphorylation levels and reduces the enzymatic activity of both GSK-3 α and GSK-3 β in APP/PS1 mice, suggesting that a GSK-3-dependent pathway is involved in the beneficial effects of benfotiamine^[12]. The current study further demonstrated that benfotiamine decreased the activity of GSK3 *in vitro*.

In conclusion, this study is the first to demonstrate that high glucose alters A β production in HEK293 APPsw cells. Our data indicated that the inhibitory effects of benfotiamine on A β production by HEK293 APPsw cells under high glucose (5.5 g/L) may be through decreasing the activity of GSK-3. Since GSK-3 is a key enzyme in glucose metabolism and involved in A β production, the modulation of GSK-3 activity by benfotiamine could be beneficial in AD therapy.

Acknowledgements: We thank Meiyu Hu for critical technical guidance. This work was supported by grants from the National Natural Science Foundation of China (81071019), the International Cooperation Foundation of Shanghai Municipality, China (10430709600) and the Outstanding Academic Leaders Foundation in Shanghai, China (11XD1401500).

References:

- [1] Jolivald CG, Lee CA, Beiswenger KK, Smith JL, Orlov M, Torrance

- MA, *et al.* Defective insulin signaling pathway and increased glycogen synthase kinase-3 activity in the brain of diabetic mice: parallels with Alzheimer's disease and correction by insulin. *J Neurosci Res* 2008, 86(15): 3265–3274.
- [2] Pasquier F, Boulogne A, Leys D, Fontaine P. Diabetes mellitus and dementia. *Diabetes Metab* 2006, 32(5 Pt 1): 403–414.
 - [3] Arvanitakis Z, Wilson RS, Bienias JL, Evans DA, Bennett DA. Diabetes mellitus and risk of Alzheimer disease and decline in cognitive function. *Arch Neurol* 2004, 61(5): 661–666.
 - [4] Peila R, Rodriguez BL, Launer LJ. Type 2 diabetes, APOE gene, and the risk for dementia and related pathologies: The Honolulu-Asia Aging Study. *Diabetes* 2002, 51(4): 1256–1262.
 - [5] Bosco D, Fava A, Plastino M, Montalcini T, Pujia A. Possible implications of insulin resistance and glucose metabolism in Alzheimer's disease pathogenesis. *J Cell Mol Med* 2011, 15(9): 1807–1821.
 - [6] Biessels GJ, Kappelle LJ. Increased risk of Alzheimer's disease in Type II diabetes: insulin resistance of the brain or insulin-induced amyloid pathology. *Biochem Soc Trans* 2005, 33(Pt 5): 1041–1044.
 - [7] Liu Y, Liu F, Grundke-Iqbal I, Iqbal K, Gong CX. Brain glucose transporters, O-GlcNAcylation and phosphorylation of tau in diabetes and Alzheimer's disease. *J Neurochem* 2009, 111(1): 242–249.
 - [8] Mosconi L, Pupi A, De Leon MJ. Brain glucose hypometabolism and oxidative stress in preclinical Alzheimer's disease. *Ann N Y Acad Sci* 2008, 1147: 180–195.
 - [9] Li L, Holscher C. Common pathological processes in Alzheimer disease and type 2 diabetes: a review. *Brain Res Rev* 2007, 56(2): 384–402.
 - [10] Takashima A. GSK-3 is essential in the pathogenesis of Alzheimer's disease. *J Alzheimers Dis* 2006, 9(3 Suppl): 309–317.
 - [11] Rockenstein E, Torrance M, Adame A, Mante M, Bar-on P, Rose JB, *et al.* Neuroprotective effects of regulators of the glycogen synthase kinase-3 β signaling pathway in a transgenic model of Alzheimer's disease are associated with reduced amyloid precursor protein phosphorylation. *J Neurosci* 2007, 27(8): 1981–1991.
 - [12] Pan X, Gong N, Zhao J, Yu Z, Gu F, Chen J, *et al.* Powerful beneficial effects of benfotiamine on cognitive impairment and beta-amyloid deposition in amyloid precursor protein/presenilin-1 transgenic mice. *Brain* 2010, 133(Pt 5): 1342–1351.
 - [13] Steinhlil ML, Turner RS, Gaut JR. ELISA analysis of beta-secretase cleavage of the Swedish amyloid precursor protein in the secretory and endocytic pathways. *J Neurochem* 2002, 80(6): 1019–1028.
 - [14] Janson J, Laedtke T, Parisi JE, O'Brien P, Petersen RC, Butler PC. Increased risk of type 2 diabetes in Alzheimer disease. *Diabetes* 2004, 53(2): 474–481.
 - [15] Luchsinger JA, Tang MX, Shea S, Mayeux R. Hyperinsulinemia and risk of Alzheimer disease. *Neurology* 2004, 63(7): 1187–1192.
 - [16] Brands AM, Biessels GJ, de Haan EH, Kappelle LJ, Kessels RP. The effects of type 1 diabetes on cognitive performance: a meta-analysis. *Diabetes Care* 2005, 28(3): 726–735.
 - [17] Stewart R, Liolitsa D. Type 2 diabetes mellitus, cognitive impairment and dementia. *Diabet Med* 1999, 16(2): 93–112.
 - [18] Awad N, Gagnon M, Messier C. The relationship between impaired glucose tolerance, type 2 diabetes, and cognitive function. *J Clin Exp Neuropsychol* 2004, 26(8): 1044–1080.
 - [19] Lovestone S. Diabetes and dementia: is the brain another site of end-organ damage. *Neurology* 1999, 53(9): 1907–1909.
 - [20] Avila J, Hernandez F. GSK-3 inhibitors for Alzheimer's disease. *Expert Rev Neurother* 2007, 7(11): 1527–1533.
 - [21] Geert JB, Salka S, Eric B, Carol B, Philip S. Risk of dementia in diabetes mellitus: a systematic review. *Lancet Neurol* 2006, 5: 64–74.
 - [22] Gadau S, Emanueli C, Van LS, Graiani G, Todaro M, Meloni M, *et al.* Benfotiamine accelerates the healing of ischaemic diabetic limbs in mice through protein kinase B/Akt-mediated potentiation of angiogenesis and inhibition of apoptosis. *Diabetologia* 2006, 49(2): 405–420.
 - [23] Marchetti V, Menghini R, Rizza S, Vivanti A, Feccia T, Lauro D, *et al.* Benfotiamine counteracts glucose toxicity effects on endothelial progenitor cell differentiation via Akt/FoxO signaling. *Diabetes* 2006, 55(8): 2231–2237.
 - [24] Ke YD, Delerue F, Gladbach A, Götz J, Ittner LM. Experimental diabetes mellitus exacerbates tau pathology in a transgenic mouse model of Alzheimer's disease. *PLoS One* 2009, 4(11): e7917.
 - [25] Russell JW, Golovoy D, Vincent AM, Mahendru P, Olzmann JA, Mentzer A, *et al.* High glucose-induced oxidative stress and mitochondrial dysfunction in neurons. *FASEB J* 2002, 16(13): 1738–1748.
 - [26] Jolivald CG, Hurford R, Lee CA, Dumaop W, Rockenstein E, Masliah E. Type 1 diabetes exaggerates features of Alzheimer's disease in APP transgenic mice. *Exp Neurol* 2010, 223: 422–431.
 - [27] Balakumar P, Chakkarwar VA, Singh M. Ameliorative effect of combination of benfotiamine and fenofibrate in diabetes-induced vascular endothelial dysfunction and nephropathy in the rat. *Mol Cell Biochem* 2009, 320: 149–162.
 - [28] Head KA. Peripheral neuropathy: pathogenic mechanisms and alternative therapies. *Altern Med Rev* 2006, 11(4): 294–329.
 - [29] Coghlan MP, Culbert AA, Cross DA, Corcoran SL, Yates JW, Pearce NJ, *et al.* Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. *Chem Biol* 2000, (7): 793–803.
 - [30] Ring DB, Johnson KW, Henriksen EJ, Nuss JM, Goff D, Kinnick TR, *et al.* Selective glycogen synthase kinase 3 inhibitors potentiate insulin activation of glucose transport and utilization *in vitro* and *in vivo*. *Diabetes* 2003, (52): 588–595.