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·Original Article·

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

Xiao-Jing Sun[1], Lei Zhao[1], Na Zhao[1], Xiao-Li Pan[1], Guo-Qiang Fei[1], Li-Rong Jin[1], Chun-Jiu Zhong[1,2]

[1]*Department of Neurology, Zhongshan Hospital & Shanghai Medical College, Fudan University, Shanghai 200032, China*
[2]*State Key Laboratory of Medical Neurobiology, Institute of Brain Science, Fudan University, Shanghai 200032, China*

Abstract: Objective To determine whether high glucose enhances β-amyloid (Aβ) production in HEK293 Swedish mutant (APPsw) cells with Aβ precursor protein (APP) overexpression, and whether under this condition benfotiamine reduces the increased Aβ production. **Methods** HEK293 APPsw cells were cultured with different concentrations of glucose for different times. The Aβ content in the supernatant was determined by ELISA. To investigate the mechanism by which benfotiamine reduced Aβ 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, Aβ production by HEK293 APPsw cells was highest in the presence of 5.5 g/L glucose for 6 and 12 h. The difference in Aβ content between 5.5 and 1.0 g/L was most marked after incubation for 12 h. Benfotiamine at 20 and 40 μg/mL significantly reduced Aβ production in cells incubated with 5.5 g/L glucose for 12 h. Moreover, 40 μg/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 Aβ 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 ($A\beta_{42}$), a 4-kD peptide derived from Aβ precursor protein (APP). $A\beta_{42}$ is toxic to neurons. The deposition of Aβ 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 Aβ produc-

Corresponding author: Chun-Jiu Zhong
Tel: +86-21-54237920
E-mail: zhongcj@163.com
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tion and accumulation in APP-overexpressing mice[11]. In a previous study, we demonstrated the beneficial effect of benfotiamine against Aβ 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 Aβ production and whether benfotiamine plays a role in its regulation. Furthermore, the possible mechanism by which high glucose and benfotiamine affect Aβ 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% CO_2 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 Aβ 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 μg/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 $A\beta_{42}$ content The supernatants were collected and assayed for residual $A\beta_{42}$ using an ELISA kit (HS Aβ42 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-μL samples were added into primary antibody-coated wells and incubated at room temperature for 4 h. After washing, the secondary antibody was added (100 μL/well) and incubated for 30 min. After washing, stabilized chromogen was added (100 μL/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 μg/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-3β (1:1 000), phospho-GSK-3β (Ser9) (1:1 000), GSK-3α (1:1 000), phospho-GSK-3α (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-3α and GSK-3β 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 μL), D (10 μL), E (10 μL) and F (10 μL) were mixed and incubated for 3 min at 30°C. The supernatant (5 μL) was immediately added to the reagent mixture and the optical density was measured at 340 nm (SpectraMax 190, Molecular Devices, Sunneyvale, 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 ± 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 Aβ production Different glucose concentrations had complex effects on Aβ production. From 1.0 to 5.5 g/L, Aβ production showed a rising trend, while from 5.5 to 10.5 g/L, it decreased (Fig. 1). Aβ 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 Aβ 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

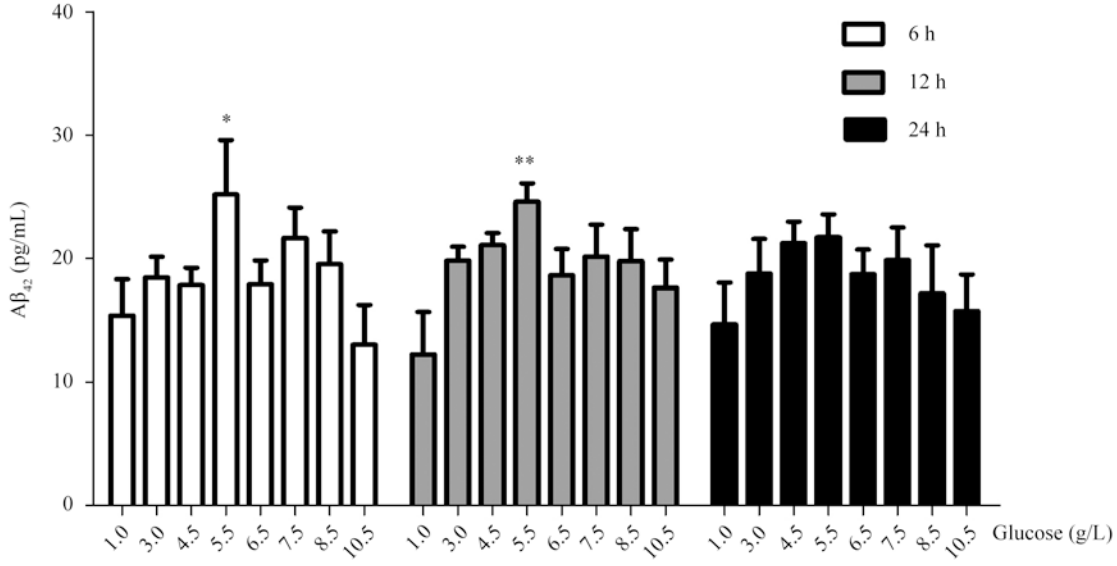

Fig. 1. Effects of glucose on Aβ production by HEK293 APPsw cells. ELISA was performed to determine Aβ production. Statistical results showed that the trend of Aβ production first rose and then dropped with increased concentrations of glucose in the culture medium for 6, 12 and 24 h. Compared with the 1.0 g/L glucose group, Aβ 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 Aβ 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 Aβ production by HEK293 APPsw cells in 5.5 g/L glucose We next studied the effect of benfotiamine at 10, 20, and 40 μg/mL on the Aβ production of cells in 5.5 g/L glucose. The results showed that 20 and 40 μg/mL benfotiamine reduced Aβ production (24.74 ± 1.64 pg/mL at 20 μg/mL and 24.14 ± 1.87 pg/mL at 40 μg/mL group) compared with control (29.96 ± 0.33 pg/mL; P <0.05 for 20 μg/mL, P <0.01 for 40 μg/mL). However, 10 μg/mL 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 Aβ production by assessing the expression and activity of GSK-3 in HEK293 APPsw cells. The results showed that the ratios of phospho-GSK-3α/GSK-3α (0.30 ± 0.08) and phospho-GSK-3β/GSK-3β (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-3α/GSK-3α, P <0.01; 1 ± 0.001 for phospho-GSK-3β/GSK-3β, P <0.05, Fig. 2A, B). Con-

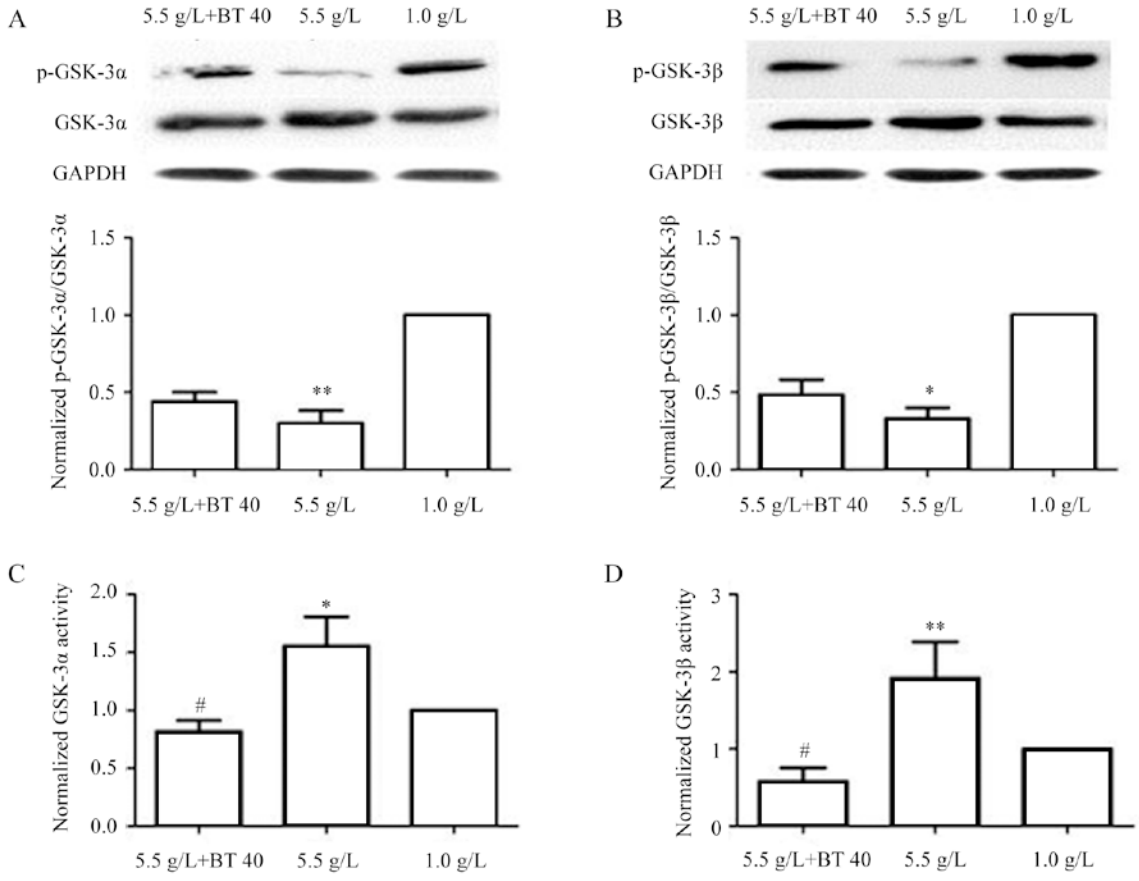

Fig. 2. High glucose up-regulates while 40 μg/mL benfotiamine (BT 40) down-regulates GSK-3 activity in HEK293 APPsw cells. A and B: The ratios of phospho (p)-GSK-3α (Ser21)/GSK-3α (A) and p-GSK-3β (Ser9)/GSK-3β (B) in the high glucose group (5.5 g/L) were significantly low than those in the lower glucose group (1.0 g/L). There was a trend for both ratios to increase in high glucose after benfotiamine treatment (40 μg/mL, P >0.05). C and D: Activity of GSK-3α (C) and GSK-3β (D) in high glucose was significantly increased compared with that in low glucose. After benfotiamine treatment (40 μg/mL), the enzymatic activity of GSK-3α (C) and GSK-3β (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-3α (1.56 ± 0.25) and GSK-3β (1.92 ± 0.47) in the high glucose control was higher than that in the low glucose control (1 ± 0.001 for GSK-3α, P <0.05; 1 ± 0.001 for GSK-3β, P <0.01). Furthermore, 40 μg/mL benfotiamine decreased the enzymatic activity of GSK-3α (0.82 ± 0.97) and GSK-3β (0.58 ± 0.18) compared with the control group in high glucose control (P <0.05, Fig. 2C, D).

4 Discussion

Limiting Aβ 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 Aβ 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 Aβ 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 Aβ 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 Aβ 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 Aβ 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-3α and GSK-3β 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 Aβ production in HEK293 APPsw cells. Our data indicated that the inhibitory effects of benfotiamine on Aβ 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 Aβ production, the modulation of GSK-3 activity by benfotiamine could be beneficial in AD therapy.

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