Berberine Improves Diabetic Encephalopathy Through the SIRT1/ER Stress Pathway in *db/db* Mice

Hong-Ying Li, Xin-Chen Wang, Yu-Min Xu, Na-Chuan Luo, Si Luo, Xu-Yi Hao, Shu-Yi Cheng, Jian-Song Fang, Qi Wang, Shi-Jie Zhang, and Yun-Bo Chen

Abstract

The association between diabetes and dementia has been well demonstrated by epidemiologic studies. Berberine (BBR) has been reported to ameliorate diabetes and diabetic encephalopathy (DE). However, the mechanism is still unknown. In this study, we employ a diabetic model, *db/db* mice, to explore whether BBR could protect DE through the SIRT1/endoplasmic reticulum (ER) stress pathway. Behavioral results (Morris water maze, Y-maze spontaneous alternation test, and fear conditioning test) showed that oral administration of BBR (50 mg/kg) improved the learning and memory ability. Furthermore, BBR promoted lipid metabolism and decreased fasting glucose in *db/db* mice. Moreover, western blot analysis revealed that BBR increased the synapse- and nerve-related protein expression (PSD95, SYN, and NGF) and decreased the protein expression of inflammatory factors (TNF- α and NF- κ B) in the hippocampus of *db/db* mice. BBR also increased the protein expression of SIRT1 and downregulated ER stress-associated proteins (PERK, IRE-1 α , eIF-2 α , PDI, and CHOP) in the hippocampus of *db/db* mice. Taken together, the present results suggest that the SIRT1/ER stress pathway might be a crucial mechanism in the neuroprotective effect of BBR against DE.

Keywords: berberine, diabetic encephalopathy, SIRT1, ER stress

Introduction

DIABETIC ENCEPHALOPATHY (DE), a diabetic complication, is closely related with the degeneration and dysfunction of the central nervous system.^{1,2} A growing body of evidences identified that both T1DM and T2DM patients exhibit a variety of neuropathological and neurobehavioral changes, including brain infarcts, cerebral white matter hyperintensities, poorer visuospatial construction, planning, visual memory, and speed.^{3–5} Cerebrovascular changes,¹ oxidative stress,⁶ inflammation, neuronal loss, neurotrophic impairment,⁷ increased advanced glycation end products,⁸ and impairments in cerebral insulin signaling systems⁹ are thought to be the reasons for DE. Notably, diabetes-induced changes observed in the aging brain are associated with accelerated brain aging¹⁰ and are the main risk factors for neurodegenerative disorders, such as Alzheimer's disease.¹⁰

Berberine (BBR, structure shown in figure 1), an isoquinoline alkaloid, is derived from the Berberidacea plant family, which has a long history in Chinese and Indian medicines.¹¹ Numerous studies have demonstrated its multiple pharmacological actions, including anti-inflammatory,¹² antiproliferative,¹³ and antihypertensive actions.¹⁴ Moreover, the effects of BBR on insulin

sensitivity and glucose tolerance have shown advantages in metabolic disorders such as hyperglycemia and hyperlipidemia.^{15–18} Furthermore, some studies have identified that BBR has potent neuroprotective effects against diabetic neuropathy.^{19,20} However, further mechanisms still need to be investigated.

Endoplasmic reticulum (ER) is a eukaryotic organelle involved in protein synthesis, folding and trafficking, calcium homeostasis, and lipid and steroid synthesis. Perturbations of ER homeostasis because of certain stress stimuli such as ischemia, nutrient deprivation, oxidative stress, and ER Ca²⁺ depletion lead to accumulation of unfolded or misfolded proteins within the lumen of ER, a condition known as ER stress.²¹ Errors in protein folding in the lumen of the ER can lead to accumulation of ER stress that has been traditionally viewed as an adaptive mechanism, also known as the unfolded protein response (UPR).²² ER stress can be induced by diabetes. Previous studies have shown that cell death mediated by CHOP-dependent ER stress is a mechanism underlying the pathological changes in hippocampal synapses and cognitive impairment associated with hyperglycemia.²³ Interestingly, Wang et al. found that diabetes-induced neuronal ER stress plays a critical role in DE development.²⁴ In

addition, the antidiabetic drug, BBR, has been found to ameliorate ER stress.^{25,26} We assume that the protective effect of BBR on DE might be through inhibiting ER stress.

The *db/db* mouse is a useful model of type 2 diabetes (T2D) that results in excessive food consumption, precocious and progressive increase in body weight, hyperglycemia, and hyperinsulinemia.²⁷ The *db/db* mice exhibit pathological characteristics that mimic dementia.²⁸ In this study, *db/db* mice were employed to study the effect of BBR on DE. The mechanism that BBR improved DE might be related to activating SIRT1 and inhibiting ER stress.

Materials and Methods

Animal protocols

The *db/db* mice (7 weeks, female) and age-matched wild-type C57BL/6J-*db/m* mice (7 weeks, female) were purchased from Nanjing Biomedical Research Institute of Nanjing University, Nanjing, China. The mice were randomly divided into four groups (eight mice per group): control group (*db/m*, 0.9% saline), normal intervention group (*db/m* + BBR), diabetic group (*db/db*), and BBR-treated group (*db/db* + BBR). All mice were kept in a specific pathogen-free animal room under controlled temperature ($24^{\circ}\text{C} \pm 1^{\circ}\text{C}$) and humidity (55%–70%). The animals were allowed to acclimatize to the environment where a 12-hour light–12-hour dark cycle was maintained with fresh water *ad libitum* and standard pellet chow for 2 months. BBR (50 mg/kg, 98% purity; Sigma-Aldrich, St. Louis, MO) administration started at 16 weeks of age and lasted for 10 weeks. The doses of BBR were chosen according to previous studies.^{29,30} Because of this dose, the administration mode is more close to the clinical practice than veno-injection or intraperitoneal administration.^{17,31,32} Furthermore, chronic treatment with BBR holds a promising effect for neuronal injury in diabetes, which is well in accordance with earlier studies.³³ Body weights were determined weekly. All experiments were conducted in accordance with the *Guiding Principles for the Care and Use of Laboratory Animals* as adopted and promulgated by the United States National Institutes of Health.

Morris water maze test

After 10 weeks of drug treatment, spatial memory was detected by the Morris water maze test.²⁸ The water maze consists of a white circular pool with a diameter of 100 cm and filled with opaque water ($22^{\circ}\text{C} \pm 1^{\circ}\text{C}$). The pool was divided into four equally spaced quadrants. The acquisition consisted of 4 days (four trials per day) with the platform submerged. During this phase, the platform was located in quadrant IV. The time limit was 60 s/trial with an interval of 30 minutes. If the animal did not find the platform, it was placed on it for 10 seconds. The retention phase started a day after the acquisition phase was completed, the platform was removed from the pool and the mice were permitted to swim freely for 60 seconds to search for the platform. The time spent in the target quadrant was taken to indicate the level of memory retention that had taken place after learning. During each trial, the distance taken to find the hidden platform (path length in cm) and percent time spent in each quadrant of the pool during probe trials of the mouse were recorded using a video-tracking system (EthoVision 2.0; Noldus Information Technology, Leesburg, VA).

Y-maze spontaneous alternation test

Each mouse was placed at the end of one arm facing the center and allowed to explore the maze freely for 5 minutes without training, reward or punishment, while the experimenter remained out of sight. Entries into each arm were scored and alternation behavior was defined as a complete cycle of consecutive entrances into each of the three arms without repetition. The percentage of spontaneous alternation was defined as the number of actual alternations divided by the possible alternations [(# alternations)/(total arm entries – 2) $\times$ 100].

Conditioned fear test

Fear conditioning test was performed as described previously.³⁴ Briefly, a mouse was placed in a plexiglas training chamber whose floor had a stainless grid for shock delivery. On the training day, mice were placed in the test chamber and allowed to explore for 2 minutes. The conditioned stimulus (CS) was presented for 30 seconds (a white noise 80 dB sound) and followed immediately by a mild foot shock (2 seconds, 0.7 mA) that served as the unconditioned stimulus (US). After 2 minutes, the mice received a second CS-US pairing. The freeze frame monitor system was used to control the timing of CS and US presentations. Freezing behavior was measured through the computer program. Each training chamber was cleaned with 95% ethyl alcohol before placement of a mouse. Twenty-four hours after training, the mouse was placed again in the training chamber for 3 minutes without foot shock. Each animal's freezing behavior was scored during the 3-minute observation period. Freezing scores are expressed as the percentage of total observations during the 3-minute test.

Oral glucose tolerance test and insulin tolerance test

The mice were fasted overnight and then 1 g/kg glucose solution was orally administered for the oral glucose tolerance test (OGTT). Glucose levels in tail blood were determined at 0, 15, 30, 60, 90, and 120 minutes after oral administration. Blood glucose levels were determined using the ACCU-CHEK Advantage glucose analyzer (Roche Diagnostics, Basel, Switzerland). Insulin tolerance test (ITT) was performed on a different day. Humulin R (0.75 U/kg) (Eli Lilly and Co., IN) in saline was administered through

FIG. 1. The chemical structure of BBR. BBR, berberine.

FIG. 2. BBR ameliorates diabetes-induced cognitive dysfunction shown by the Morris water maze test in *db/db* mice. **(A)** Escape latency of five consecutive days of the test. **(B)** The swimming paths of respective groups on the first and fifth day. **(C)** Crossing times of the target platform in the probe trial. **(D)** Time spent in the target quadrant in the probe trial. **(E)** The swimming speed in the probe trial. Data represent mean $\pm$ SEM ($n = 8$ per group). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus *db/m*; $^*p < 0.05$ versus *db/db*. SEM, standard error of the mean.

injection into the peritoneal cavity. Blood samples were obtained by the tail clip method at various time points (0, 30, 60, 90, and 120 minutes).

Fasting-refeeding protocol

Mice were fasted for 24 hours and then re-fed through gavage with 300 mL (0.33 kcal) of an Ensure (Abbott Laboratories) solution containing 1.1 kcal/mL, with 20% cal-

ories from fat, 13% from carbohydrates, and 18% from proteins.³⁵ Blood samples were collected after fasting and 1 and 2 hours after refeeding to measure insulin.

Serum biomarker analysis

After a 10-week administration of BBR, all mice were sacrificed by cervical dislocation. Serum was collected from the whole blood by centrifugation (12,000 rpm, 4°C, for 15

minutes). Serum insulin levels were determined by enzyme-linked immunosorbent assay (ELISA; CUSABIO, Wuhan, China). The levels of serum triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were measured by commercial kits (Nanjing Jiancheng Institute of Biotechnology, Nanjing, China).

Western blot analysis

Hippocampus tissue samples from each group were homogenized with lysis buffer plus 1 mM PMSF and protease inhibitor cocktail. Protein concentrations were determined using a BCA protein assay kit. Equal amounts of total protein extract (20 μ g per well) from each sample were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. The membranes were immersed in TBST with 3% BSA for 1.5 hours. Then, the membranes were incubated overnight at 4°C with one of the following specific primary antibodies (1:1000): anti-PERK, anti-p-PERK, anti-eIF-2 α , p-eIF-2 α , anti-IRE1 α , anti-p-IRE1 α , anti-CHOP, anti-PDI, anti-SIRT1, anti-NGF, anti-PSD95, anti-SYN, anti-TNF- α , anti-NF- κ B, and anti- β -actin. The membranes were followed by incubation with horseradish peroxidase conjugated anti-rabbit antibody or anti-mouse antibody at room temperature in 2 hours. A superenhanced chemiluminescence reagent (ECL; Applygen Technologies Inc., Beijing, China) was added to the membrane for visualizing the target bands. Intensity of the bands was analyzed with ImageJ software, and the results were presented as a ratio between the intensity of target proteins. All these antibodies were purchased from Cell Signaling Technology, Inc. (Boston, MA).

Statistical analyses

Experimental values are given as mean $\pm$ SEM. All statistical analyses were performed with SPSS 19.0 statistical software (IBM, Endicott, NY). Two-way analysis of variance (ANOVA) was applied to analyze differences in data for the biochemical parameters among the different groups, followed by Dunnett's significant *post hoc* test for pair-wise multiple comparisons. The level of statistical significance for all tests was $p < 0.05$.

Results

BBR improves cognitive impairment in db/db mice

In the Morris water maze test, the time to find the hidden platform declined progressively during the five training days (Fig. 2A, B). Compared with the db/m group, the period of time to find the hidden platform remarkably increased in the db/db group. However, the escape latency of the BBR-treated group was significantly shortened than the db/db group (Fig. 2A). The typical swimming traces on the first day and fifth day are shown; the db/db group showed a chaotic and longer swimming path, which improved by BBR treatment (Fig. 2B). The number of platform crossings and the time spent in target quadrant were significantly increased when compared with db/db mice (Fig. 2C, D). The swimming speed of the db/db group significantly decreased compared with the db/m group, but no obvious difference was observed between the db/db group and BBR-treated group (Fig. 2E). In the Y-maze spontaneous alternation test, the db/db group showed less willingness to explore new environments, which was reversed by BBR (Fig. 3A). In the fear conditioning test, the db/db group presented less freezing time than the db/m group ($p < 0.05$, Fig. 3B). This change was ameliorated after treatment with BBR. These results demonstrated that treatment with BBR remarkably reversed the cognitive deficits in db/db mice.

BBR promotes lipid metabolism in db/db mice

In this study, BBR was given to mice once daily from 16 weeks of age and lasted for 10 weeks. The weekly body weights from each group were recorded. As shown in Figure 4A, a large increase of body weight was observed in the db/db group when compared with the db/m group. However, a 10-week administration of BBR lowered the body weight in db/db mice. BBR did not affect the body weight of the db/m group. Based on this observation, we further detected the levels of TG, TC, HDL-C, and LDL-C in the serum. As seen in Figure 4B–E, BBR decreased TG, TC, and LDL-C and increased HDL-C in db/db mice. These results indicated that BBR could attenuate metabolic disorder in db/db mice.

FIG. 3. BBR ameliorates diabetes-induced cognitive dysfunction shown by the Y-maze spontaneous alternation test and fear conditioning test. (A) Y-maze spontaneous alternation test. (B) Fear conditioning test. Data represent mean $\pm$ SEM ($n = 8$ per group). # $p < 0.05$, ## $p < 0.01$ versus db/m; * $p < 0.05$, ** $p < 0.01$ versus db/db.

FIG. 4. BBR promotes lipid metabolism in *db/db* mice. Effect of BBR on serum lipids in *db/db* mice. (A) Weekly body weight. (B) TC. (C) TG. (D) HDL-C. (E) LDL-C. Data represent mean $\pm$ SEM ($n=8$ per group). [#] $p<0.05$, ^{##} $p<0.01$, ^{###} $p<0.001$ versus *db/m*; ^{*} $p<0.05$, ^{**} $p<0.01$ versus *db/db*. HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride.

BBR decreases fasting glucose in *db/db* mice

An OGTT was performed to dynamically assess glucose homeostasis. The BBR-treated *db/m* group exhibited a similar glucose excursion to the *db/m* group. However, *db/db* mice showed glucose intolerance as evident from the significantly higher glucose excursion and area under the curve during the OGTT. After a 10-week administration of BBR, however, *db/db* mice were slightly less glucose intolerant (Fig. 5A). We also performed an ITT. The BBR-treated *db/m* group still showed similar glucose excursion to the *db/m* group. *db/db* mice and BBR-treated *db/db* mice showed no significant difference in the ITT; however, fasting glucose was decreased

by BBR treatment (Fig. 5B). This was also reflected in the fasting insulin levels (Fig. 5C) that were significantly better in the BBR-treated group compared with the *db/db* group. Taken together, these findings suggested that BBR treatment can decrease blood glucose, which is not achieved by increasing insulin sensitivity.

BBR protects synapse and neuroinflammation in the hippocampus of *db/db* mice

As shown in Figure 6, the protein expression of neurotrophic factors, including PSD95, SYN, and NGF, sharply

FIG. 5. BBR decreases fasting glucose in *db/db* mice. (A) OGTT. (B) ITT. (C) Serum insulin level. Data represent mean $\pm$ SEM ($n=8$ per group). # $p < 0.05$, ### $p < 0.001$ versus *db/m*; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus *db/db*. ITT, insulin tolerance test; OGTT, oral glucose tolerance test.

decreased in *db/db* mice, while after the treatment with BBR, these proteins regain the normal level. Furthermore, we also detected inflammatory cytokines in the hippocampus (Fig. 7). BBR significantly decreased the expression of TNF- α and NF- κ B in *db/db* mice.

BBR activates SIRT1 and attenuates ER stress in the hippocampus of *db/db* mice

As shown in Figure 8, the protein expression of SIRT1 decreased in the *db/db* group compared with the *db/m* group,

but increased in the BBR-treated group. Next, we measured the protein levels of the two ER stress transducers, PERK and IRE-1 α . There was an increase in active forms of the effectors of UPR (p-PERK, p-IRE-1 α) in the *db/db* group compared with the *db/m* group, while they decreased after the administration of BBR. To further evaluate the consequences of ER stress, we next measured the protein expression of eIF-1 α , CHOP, and PDI. Both p-eIF-1 α and CHOP in the *db/db* group are upregulated compared with the *db/m* group, while downregulated after the intervention of BBR. The expression of PDI sharply decreased in the *db/db* group, but increased

FIG. 6. BBR protects synapse and nerve in the hippocampus of *db/db* mice. Western blot analysis of protein expression in the hippocampus of *db/db* mice. (A) PSD95. (B) SYN. (C) NGF. Data represent mean $\pm$ SEM ($n=8$ per group). # $p < 0.05$, ## $p < 0.01$ versus *db/m*; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus *db/db*.

FIG. 7. BBR anti-inflammatory effect in the hippocampus of *db/db* mice. Western blot analysis of protein expression in the hippocampus of *db/db* mice. (A) NF-κB. (B) TNF-α. Data represent mean ± SEM ($n = 8$ per group). [#] $p < 0.05$, ^{##} $p < 0.01$ versus *db/m*; ^{*} $p < 0.05$ versus *db/db*.

FIG. 8. BBR activates SIRT1 and attenuates ER stress in the hippocampus of *db/db* mice. Western blot analysis of protein expression in the hippocampus of *db/db* mice. (A) SIRT1. (B) p-PERK/PERK. (C) p-IRE1α/IRE1α. (D) p-eIF2α/eIF2α. (E) CHOP. (F) PDI. Data represent mean ± SEM ($n = 8$ per group). [#] $p < 0.05$, ^{##} $p < 0.01$ versus *db/m*; ^{*} $p < 0.05$, ^{**} $p < 0.01$ versus *db/db*. ER, endoplasmic reticulum.

under the effect of BBR. These results indicated that the effect of BBR on protecting DE might be related to the SIRT1/ER stress pathway.

Discussion

In this study, we demonstrated that administration of BBR improved diabetes-associated cognitive decline in *db/db* mice. A 10-week administration of BBR protected learning and memory, promoted lipid metabolism, and decreased blood glucose in *db/db* mice. For mechanism study, BBR increased the synapse and nerve-related protein expression and decreased the protein expression of inflammatory factors in the hippocampus of *db/db* mice. In addition, BBR activated SIRT1 and attenuated ER stress, which might be key mechanisms of the neuroprotective effect of BBR.

DE is a series of neurochemical, neurophysiological, and structural abnormalities.^{36,37} Prolonged exposure to high plasma glucose leads to neuropathological complications not only in the peripheral nervous system but also in the central nervous systems. Diabetic patients display reduced psychomotor efficiency, cognitive flexibility, and rapid information processing.³⁸ Furthermore, they seem to double the probability of developing Alzheimer's disease and other forms of dementia.³⁹ Recently, BBR has been reported to have a neuroprotective effect in diabetes-induced cognitive impairment.⁴⁹ In our present study, we employed a diabetic model, *db/db* mice, to study the mechanism of the neuroprotective effect of BBR. Behavioral results (Morris water maze test, Y-maze spontaneous alternation test, and fear conditioning test) showed that BBR (50 mg/kg) significantly improved the learning and memory deficits in *db/db* mice, which was in accord with previous studies.⁴¹

T2D is a complex metabolic disorder that is characterized by glucotoxicity and lipotoxicity and a pathognomonic defect in T2D is insulin resistance (IR).⁵ Altered lipid homeostasis can give rise to lipotoxicity, a key feature of the metabolic syndrome and T2D.⁵ In this study, the metabolic impairment was observed in *db/db* mice. BBR reversed these changes by decreasing susceptibility to T2D/IR. This effect of BBR on diabetes is consistent with previous studies.

Central nervous system inflammation is considered a primary driver of brain IR in dementia. T2DM has been considered as an inflammatory disease.⁴² This increased level of inflammation has been attributed to the effects of poorly controlled hyperglycemia that triggers NF- κ B activation and the release of proinflammatory cytokines.⁴³ Recent studies indicate that inflammation and neuronal cell death are implicated in diabetes-associated learning and memory deficits.^{24,44} The involvement of neuronal apoptosis in DE has been demonstrated in diabetic animal models, and evidence of classical apoptosis is associated with decreased neuronal densities and learning and cognitive deficits.⁴⁵ Attenuation of apoptosis of neurons in the hippocampus and cerebral cortex ameliorates the cognition deficits of diabetic rats.⁴⁵ In this study, we found that NF- κ B and TNF- α were significantly increased in the hippocampus of *db/db* mice, and NGF, SYN, and PSD95 were significantly downregulated. BBR alleviated these changes, which might be related to its effect on IR.

SIRT1 plays a key role in governing cellular stress management. It regulates inflammation, stress resistance, and DNA damage repair by deacetylating intracellular signaling

molecules and chromatin histones.^{46,47} Several studies have reported that BBR exerts protective effects through SIRT1 signaling under various stressful conditions.^{48,49} Gomes et al. reveal that SIRT1-mediated stimulation of mitochondrial biogenesis is involved in the prevention of BBR on diet-induced IR.⁵⁰ These observations provide evidence that SIRT1 plays a pivotal role of BBR on DE in *db/db* mice. In addition, some studies indicate that SIRT1 participates in the ER stress response related to inadequate nutrient uptake and its activation protects cardiomyocytes from ER stress-induced cell death.⁵¹ Activation of SIRT1 protects cardiac cells from ER stress through deacetylation of eukaryotic initiation factor 2 ($eIF-2\alpha$).⁵² Accumulating evidences reveal that the anti-inflammatory effect of BBR may be related to its ability of modulating ER stress-related pathways.²⁶ We next explored the effect of BBR on ER stress-associated proteins. In addition, we found that BBR could decrease ER stress in the hippocampus of *db/db* mice. These findings indicated that SIRT1/ER stress signaling might play an important role in the neuroprotective effect of BBR.

Conclusion

In summary, we found that the natural product, BBR, may ameliorate DE by decreasing fasting glucose, activating SIRT1, and inhibiting ER stress. However, further evidences are still needed to confirm this phenomenon. These data could be useful for explaining the underlying mechanism of BBR on DE.

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Author Contributions

S.-J.Z. designed the study. H.-Y.L., X.-C.W., Y.-M.X., N.-C.L., S.L., X.-Y.H., S.-Y.C., J.-S.F., and Q.W. conducted the experiment. S.-J.Z. and H.-Y.L. contributed to initial data analysis and interpretation and drafted the initial manuscript. S.-J.Z. and Y.-B.C. supervised all aspects of the study, critically reviewed and revised the manuscript, and approved the final manuscript as submitted.

Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Shi-Jie Zhang
Institute of Clinical Pharmacology
Guangzhou University of Chinese Medicine
Guangzhou 510405
China

E-mail: zsj19891122@gmail.com

Yun-Bo Chen
Institute of Clinical Pharmacology
Guangzhou University of Chinese Medicine
Guangzhou 510405
China

E-mail: ybchengz@gzucm.edu.cn

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