Baicalin alleviates diabetes-associated cognitive deficits via modulation of mitogen-activated protein kinase signaling, brain-derived neurotrophic factor and apoptosis

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Abstract. Baicalin is an important active component of the medicinal herb Scutellaria baicalensis Georgi and has shown a variety of pharmacological actions. The present study aimed to evaluate the neuroprotective effects of baicalin against diabetes-associated cognitive deficits (DACD) in rats and to elucidate the potential molecular mechanisms of action. A rat model of diabetes mellitus was prepared by intraperitoneal injection of streptozotocin. After the successful establishment of the diabetic rat model, baicalin (50, 100 and 200 mg/kg) or vehicle was administrated for seven weeks. Learning and memory function were assessed using the Morris water maze test. At the end of the experiment, the activities of acetylcholinesterase (AChE) and choline acetylase (ChAT) were determined using commercial kits. Furthermore, the expression of proteins involved in mitogen-activated protein kinase (MAPK) cascades [extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38], brain-derived neurotrophic factor (BDNF) and apoptosis-associated proteins [caspase-3, B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax)] were determined using western blot analysis. Caspase-3 activity was also analyzed using a commercial kit. The results demonstrated that diabetic rats exhibited decreases in body weight, decreases in the percentage of time spent in the target quadrant and the number of times of crossing the platform in the water maze test, as well as decreases in neuronal survival, ChAT, phosphorylated (p)ERK, BDNF and Bcl-2. Furthermore, diabetic rats showed increases in escape latency and mean path length in the water maze test, increases in the levels of hippocampal AChE, p-JNK, p-p38, caspase-3 and Bax as well as plasma glucose. However, in diabetic rats treated with baicalin, all of the abovementioned observations were obviously reversed. The findings suggested that baicalin exerts neuroprotective effects against DACD via modulation of MAPK cascades, BDNF and apoptosis.

Introduction

It is well established that diabetes mellitus (DM), a common chronic metabolic disorder, has devastating effects on the central nervous system. Cognitive dysfunction is considered to be the most widespread complication of DM and is exacerbated via increasing neuronal apoptosis (1). In 2006, the term ‘diabetes-associated cognitive deficits’ (DACD) was proposed as a novel concept to strengthen the recognition of this disease (2). In addition, a previous study revealed that the incidence of Alzheimer’s disease, which has the characteristics of cognitive deficits, among patients with DM was double of that among patients with a normal glucose metabolism (3), indicating that drugs for alleviating DACD should act via improving glycemic control.

The mitogen-activated protein kinases (MAPKs) are conserved signal-transducing enzymes that have crucial roles in the regulation of cellular function, including proliferation and cellular apoptosis. There are at least three sub-groups of MAPKs: Extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 (4). ERK activation improves cellular survival, while JNK and p38 MAPK promote cell death. In fact, prior studies revealed that the activated form of JNK, phosphorylated (p)-JNK, was markedly augmented at the
protein level in transgenic diabetic animals (db/db mice) (5). Brain-derived neurotrophic factor (BDNF) is an important member of the neurotrophin family of growth factors. It was originally reported to support several facets of central nervous system development, including neuronal survival and the synapse formation (6). In addition, the glucose content has been identified to be modulated by elevated generation of BDNF in the pancreas of diabetic mice (7). Furthermore, it was previously demonstrated that BDNF rapidly enhanced insulin signaling in streptozotocin (STZ)-induced diabetic mice (8), suggesting the hypoglycemic action of BDNF. These findings suggested that MAPK cascades and BDNF provide potential therapeutic targets for the treatment of DM-induced nerve injury.

Baicalin is an important natural product extracted from the plant *Scutellaria baicalensis* Georgi and possesses various pharmacological activities, including as anti-inflammatory (9), anti-oxidative (10) and anti-apoptotic (11) properties. Waisundara et al. (12) found that baicalin significantly diminished hyperglycemia-induced mitochondrial membrane damage in Wistar rats. Li et al. (13) also reported the anti-hyperglycemic effects of baicalin on STZ-nicotinamide induced diabetic rats. However, whether baicalin exerts protective effects against cognitive deficits caused by diabetes has remained elusive. The present study therefore investigated the hypothesis that baicalin protects against DACD and that the neuroprotective effects are associated with the modulation of MAPK signaling and BDNF. The present study focused on the evaluation of the effects of baicalin on DACD and explored the potential molecular mechanisms using an STZ-induced rat model of diabetes mellitus.

Materials and methods

*Experimental animals.* Adult male Wistar rats (8-week-old; Beijing Animal Center, Beijing, China) weighing 200–250 g were used in the present study. They were kept in individual cages under a standard environment (12:12 h light/dark cycle and 50-70% humidity; 37˚C) with free access to water and food. All surgical procedures were performed in strict accordance with the guidelines established by the Animal Care Committee of Central South University (Changsha, China).

*Establishment of diabetic rat model and drug treatment.* The rat model of diabetes was established by intraperitoneal (i.p.) injection of a single dose of 65 mg/kg STZ (Sigma-Aldrich, St. Louis, MO, USA), freshly dissolved in citrate buffer (pH 4.4; 0.1 M). At 48 h post-STZ injection, blood samples were acquired and plasma glucose levels were assessed using an enzymatic glucose oxidase peroxidase diagnostic kit (Span Diagnostic Chemicals, Surat, India). Animals with fasting plasma glucose levels >250 mg/dl (14) were deemed to be diabetic and selected for the subsequent experiment. Animals were randomly divided into five groups each consisting of eight rats: 1) the control group, which was injected with citrate buffer only and received physiological saline treatment (0.1 ml/100 g i.p.); 2) the vehicle group, in which diabetes had been induced by STZ injection and which received physiological saline treatment (0.1 ml/100 g i.p.); and the baicalin groups, comprising diabetic rats which were administered baicalin at doses of 3) 50, 4) 100 and 5) 200 mg/kg baicalin (Sigma-Aldrich, St. Louis, MO, USA; purity, >95%; freshly dissolved in physiological saline; i.p. injection), respectively. Starting from the third day of the experiment until the seventh week, the control and diabetic groups received vehicle or baicalin treatment.

Upon finalization of the drug treatment period, the animals from the different groups were subjected to the Morris water maze test to evaluate their learning and memory function over five consecutive days. Blood samples and hippocampal tissues were then collected for subsequent biochemical analysis.

*Morris water maze test.* At seven weeks post-STZ injection, the cognitive function of the rats was assessed by the Morris water maze test as previously described (15) The Morris water maze consisted of a circular water tank (inner diameter, 90 cm; height, 50 cm), equipped with a digital pick-up camera (V2.20; Nikon Corporation, Tokyo, Japan) 180 cm above the water surface. The tank was filled with water, which was made opaque by adding a white-colored dye (10 g/L; Shanghai Xinruan Technology, Co., Ltd., Shanghai, China). The tank was divided into four equal quadrants, which were labeled as North, South, East and West. These cues were constant throughout the experiment. A round escape platform was placed ~2 cm below the water surface. The navigation test was performed over four consecutive days. In the test, the escape latency (s) and path length (cm) to find the platform were determined. Furthermore, the swimming speed was calculated by dividing the path length by the time to find the platform. On the fifth day, the escape platform was removed from the tank and each animal was subjected to a spatial probe test. The number of times the rat crossed the target quadrant (where the platform was once hidden) and the time spent in the former platform quadrant within 60 sec were measured.

*Assessment of acetylcholinesterase (AChE) and choline acetylase (ChAT) activities.* After baicalin treatment for seven weeks, the activities of AChE and ChAT in the hippocampi of animals from different groups were analyzed using respective commercial kits (kit no. A023 for AChE and kit no. A079-1 for ChAT; Nanjing Jiancheng Biotechnology Institute, Nanjing, China).

*Western blot analysis.* The rats from each group were sacrificed by decapitation at seven weeks post-STZ injection. For western blot analysis, the hippocampi were homogenized at a ratio of 1:5 (w/v) in cold radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride). After centrifugation at 13,200 x g for 20 min at 4˚C, the supernatant was collected and divided into aliquots, which were stored at -80˚C.

Thirty micrograms of protein were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA). After being blocked in 5% defatted milk for 1 h at room temperature, the membranes were probed respectively with the following primary antibodies: Rabbit anti-ERK (1:300; cat. no. sc-292838), mouse anti-phospho (p)-ERK (Tyr204) (1:200; cat. no. sc-377400), goat anti-JNK (1:200; cat. no. sc-46006), mouse anti-p-JNK (Thr183/Tyr185) (1:200; cat. no. sc-81502), rabbit anti-p38 MAPK (1:200; cat. no. sc-353), rabbit anti-p-p38 MAPK (1:200; cat. no. sc-101758), rabbit anti-BDNF (1:200; cat. no. sc-20981), rabbit anti-Bax (1:200; cat. no. sc-526), rabbit anti-Bcl-2 (1:200; cat. no. sc-492), rabbit anti-caspase-3
Baicalin ameliorates learning and memory deficits in STZ-induced diabetic rats. After seven weeks of baicalin or vehicle treatment, mice performed the Morris water maze test in order to demonstrate learning and memory function. As shown in Fig. 1A, no significant difference was observed between any of the groups on the first day. From the second day onwards, a longer escape latency was found in STZ-treated rats (P<0.01) when compared to that in the control group. Baicalin treatment significantly diminished diabetes-associated increased in the escape latency in a dose-dependent manner (P<0.01). Similarly, an obvious increase in the mean path length over four consecutive days of training was observed in the diabetic animals (P<0.01) as compared with that in the control group (Fig. 1B). However, treatment with baicalin dose-dependently reversed this phenomenon (P<0.01). Furthermore, the time of the animals remaining in the target quadrant and the number of times the animals crossed the former platform location on day 5 were markedly decreased in diabetic rats compared with those in the control group (P<0.01) (Fig. 1C and D). However, treatment with baicalin dose-dependently decreased these two indices (P<0.01). All of these results indicated that diabetes-associated decreases in learning and memory performance of the animals were attenuated by treatment with baicalin. As to the swimming speed, there was no statistical significance among all groups during the four training days (Fig. 1E).

Baicalin treatment normalizes the activities of AChE and ChAT in STZ-induced diabetic rats. As shown in Fig. 2A, the activity of AChE was found to be significantly elevated in the hippocampi of diabetic rats (P<0.01) compared with that in the control animals. However, administration of various doses of baicalin (50, 100 and 200 mg/kg) obviously prevented these increases (P<0.01). Furthermore, the hippocampi of diabetic rats exhibited decreased activity of ChAT, while baicalin treatment reversed this phenomenon in a dose-dependent manner (P<0.01) (Fig. 2B).

Baicalin attenuates diabetes-induced changes in the activation of MAPK proteins and BDNF expression. The activation of the MAPK proteins ERK, JNK and p38 were determined in the hippocampi of mice of different groups by western blot analysis (Fig. 3). Quantitative analysis revealed an evident decrease in the activities of these proteins in diabetic rats compared with those in the control group (P<0.01). However, administration of baicalin (50, 100 and 200 mg/kg) dose-dependently decreased these indices (P<0.01). Furthermore, the hippocampi of diabetic rats exhibited decreases in the activation of BDNF. However, this phenomenon was reversed by baicalin treatment in a dose-dependent manner (P<0.01).
of p-ERK, the activated form of ERK, in the hippocampi of diabetic rats (P<0.01) (Fig. 3A and B), while a marked elevation of p-JNK and p-p38 was observed (P<0.01) (Fig. 3C-F). Baicalin treatment of the diabetes-induced rats evidently augmented the expression levels of p-ERK protein (P<0.01) and diminished the protein levels of p-JNK (P<0.01) and p-p38 (P<0.01), compared with those in the vehicle-treated diabetic group. Furthermore, the protein levels of BDNF were significantly decreased in diabetic animals compared with those in the control group (P<0.01; Fig. 3G and H). However, treatment of the diabetes-induced rats with baicalin (50, 100 and 200 mg/kg) evidently increased the protein levels of BDNF (P<0.01).

Baicalin inhibits apoptosis-associated signaling in STZ-induced diabetic rats. In order to investigate whether baicalin affects the expression levels of regulator proteins of apoptosis in the hippocampi of rats with STZ-induced diabetes, western blot analysis was performed to detect the protein levels of caspase-3, Bax and Bcl-2 in the hippocampal samples from various experimental groups. As shown in Fig. 4A and B, caspase-3 protein levels in diabetic rats were significantly augmented compared with those in the control group (P<0.01), while baicalin treatment of the diabetic rats resulted in an obvious reduction of caspase-3 to almost basal levels (P<0.01). Furthermore, the diabetic rats exhibited elevated expression levels of Bax and decreased expression levels of Bcl-2 compared with those in the vehicle-treated group (P<0.01) (Fig. 4C and D), which was significantly attenuated by treatment of the diabetic animals with baicalin (P<0.01).

Baicalin inhibits caspase-3 activity in STZ-induced diabetic rats. In order to verify the anti-apoptotic effects of baicalin in the hippocampi of rats with diabetes, the activity of caspase-3 was further analyzed using a colorimetric assay. As shown in Fig. 5, caspase-3 was obviously activated in STZ-induced diabetic rats compared with that in the control (P<0.01). However, treatment...
with baicalin significantly attenuated this diabetes-induced caspase activation in a dose-dependent manner (P<0.01).

Discussion

The present study revealed a novel mechanism by which baicalin exerts protective effects on the brains of diabetic rats against cognitive deficits via altering MAPK cascades and suppressing BDNF protein levels. Body weight and plasma glucose levels of diabetic rats were normalized by baicalin treatment, suggesting a hypoglycemic effect of baicalin. Diabetic rats showed decreased learning and memory function in the water maze test, which was improved by treatment with baicalin. Furthermore, baicalin inhibited diabetes-induced increases in AChE activity and decreases in ChAT activity in rat hippocampi. In addition, baicalin attenuated diabetes-induced decreases of p-ERK and BDNF protein levels as well as increases of p-JNK and p-p38 protein levels. These findings indicated that the neuroprotective mechanisms of baicalin likely involved the modulation of MAPK cascades and inhibition of BDNF. Western blot analysis further revealed that baicalin treatment attenuated diabetes-associated upregulation of apoptosis-associated signaling, by diminishing increases in caspase-3 and Bax as well as decreases in Bcl-2. In line with these results, colorimetric analysis revealed a reduction of caspase-3 activity in baicalin-treated diabetic rats.

Chronic hyperglycemia was reported to be closely associated with the pathogenesis of cognitive dysfunction (16). Indeed, patients with type 1 diabetes showed cognitive impairment according to neurological cognitive tests, including visuospatial deficits (17) as well as impairment of learning and memory (18). These findings suggested that glycemic control...
MA et al: BAICALIN ALLEVIATES DIABETES-ASSOCIATED COGNITIVE DEFICITS

is essential to ameliorate DACD. Previous studies demonstrated the protective effects of several medicinal herbs against cognitive decline in diabetic animals on the basis of their hypoglycemic properties (19,20). The present study illustrated that baicalin significantly decreased blood glucose levels and had neuroprotective effects against DACD in rats. Another previous study reported the anti-hyperglycemic effects of baicalin in a STZ-nicotinamide induced rat model of diabetes, which was in part consistent with the results of the present study (13). Furthermore, Waisundara et al (12) also reported that baicalin decreased hyperglycemia-induced mitochondrial damage in diabetic rats, which further evidenced the hypoglycemic properties of baicalin. In conclusion, the present and previous studies indicated that the hypoglycemic properties of baicalin were in parallel with its amelioration of cognitive impairment in diabetes.

AChE and ChAT are considered as two specific markers of cholinergic dysfunction, which has a critical role in the pathogenesis of cognitive deficits induced by diabetes (21). Under normal circumstances, acetylcholine, an index positively correlated with memory function, is decomposed by AChE and synthesized by ChAT in the hippocampus and cerebral cortex (21). In fact, the selective AChE inhibitors donepezil and huperzine A have been shown to improve the learning and memory performance in a gerbil model of ischemia (22) and a rat model of diabetes (23). The results of the present study demonstrated that treatment with baicalin markedly attenuated diabetes-associated increases in AChE activity and decreases in ChAT activity. Furthermore, preliminary experiments by our group identified synergic effects of the AChE inhibitor donepezil and baicalin on the reduction of plasma glucose levels, suggesting that baicalin exerts its anti-hyperglycemic effects in an AChE-dependent manner.

It is known that neuronal apoptosis is involved in the occurrence of diabetes-induced learning and memory dysfunction (24). The present study revealed an evident attenuation of diabetes-associated increases of caspase-3 (an executioner molecule in the apoptotic cascades) and Bax (a pro-apoptotic molecule) as well as decreases of Bcl-2 (a cell survival molecule) in the hippocampi of rats by administration of baicalin. These findings suggested that baicalin exerted its neuroprotective effects against cognitive deficits in diabetes-induced rats via suppressing the apoptotic signaling pathway.

Emerging evidence supports that MAPK is a key regulator of long-term synaptic plasticity and long-term memory (25,26). Furthermore, the ERK cascade has been reported to contribute to synaptic plasticity that underlies learning and memory (27). Activation of ERK by MAPK/ERK kinase resulted in the phosphorylation of cyclic adenosine monophosphate response element-binding protein and subsequently improved long-term memory in young rats (27). In addition, Xuan et al (28) found that the inhibition of p-p38 by fenofibrate facilitated the functional
recovery of memory deficits in rat hippocampi following global cerebral ischemia. In fact, suppression of p38 and JNK phosphorylation were shown to prevent high-glucose-induced neurotoxicity in PC12 cells (29). The present study demonstrated that baicalin attenuated DACD-associated decreases in ERK1/2 phosphorylation and increases in p-JNK and p-p38 phosphorylation, which was partly consistent with the results of previous studies (28). This also implied that the modulation of the MAPK signaling pathway is involved in the neuroprotective effects of baicalin against DACD.

BDNF is the major component of the neurotrophin family and reduces the risk of mild cognitive impairments in patients (30). It was also reported that decreased levels of BDNF in hippocampi led to serious cognitive dysfunction in a STZ-induced rat model of diabetes (21). Consistent with these findings, the present study revealed that baicalin rescued diabetes-associated decreases of BDNF in the hippocampi of rats, suggesting that baicalin protects against DACD via elevating the BDNF content in the hippocampus.

In conclusion, the present study illustrated that baicalin has normalized blood glucose levels, improves learning and memory function and reduces neuronal damage in diabetic rats. Modulation of MAPK cascades and elevation of BDNF expression may be linked with the neuroprotective effects of baicalin in diabetic rats. According to the results of the present study, baicalin may serve as a useful drug for the treatment of patients with DACD; however, it is essential to further explore whether other modulators are involved in neuroprotective effects of baicalin against DACD.

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References