Naringin ameliorates cognitive deficits via oxidative stress, proinflammatory factors and the PPARγ signaling pathway in a type 2 diabetic rat model

ZHONGHUA QI1, YINGHUI XU2, ZHANHUA LIANG1, SHENG LI1, JIE WANG2, YI WEI2 and BIN DONG2

Departments of Neurology and Neurosurgery, The First Affiliated Hospital of Dalian Medical University, Dalian, Liaoning 116011, P.R. China

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Abstract. Naringenin is a flavonoid polyphenolic compound, which facilitates the removal of free radicals, oxidative stress and inflammation. The present study aimed to obtain a better understanding of the effects of Naringenin on the regulation of diabetes-associated cognitive decline, and its underlying mechanisms. An experimental diabetes mellitus (DM) rat model was induced by streptozoticin (50 mg/kg). Following treatment with naringin (100 and 200 mg/kg) for 16 weeks, the body weight and blood glucose levels of the DM rats were measured. A Morris water maze test was used to analyze the effects of naringin on the cognitive deficit of the DM rats. The results demonstrated that naringin increased the body weight, blood glucose levels, and cognitive deficits of the DM rats. The levels of oxidative stress and proinflammatory factors in the naringin-treated rats were significantly lower, compared with those of the DM rats. In addition, naringin activated the protein expression of PPARγ, and administration of the PPARγ inhibitor decreased the protein expression of PPARγ, and attenuated the effects of naringin on cognitive deficit. These results suggested that naringenin ameliorated cognitive deficits via oxidative stress, proinflammatory factors and the PPARγ signaling pathway in the type 2 diabetic rat model. Furthermore, oxidative stress, proinflammatory factors and PPARγ signaling may be involved in mediating these effects.

Introduction

With the increase in population age, senile dementia has become a social problem worldwide (1). The central nervous system damage caused by type 2 diabetes mellitus (T2DM) is attracting increasing attention (2). Several studies have demonstrated that chronic treatment with oxymatrine alleviates DACD, which is associated with oxidative stress, inflammation and apoptosis in rats. Previous evidence has demonstrated that inflammation is involved in pathological damage via multiple mechanisms, which damages vascular function integrity in DACD (9). In treating the pathological mechanisms, reducing the inflammatory reaction in the brain can significantly improves neuronal damage and nerve fiber degeneration, thereby improving learning and memory (10). Mao et al (11) reported that Huperzine A alleviates DACD via oxidative stress, inflammation and apoptosis. In addition, Li et al (9) reported that chrysin markedly alleviates DACD via oxidative stress, inflammation and apoptosis (9).

Key words: naringenin, diabetes-associated cognitive decline, oxidative stress, peroxisome proliferator-activated receptor γ, inflammation
Peroxisome proliferator-activated receptor γ (PPARγ) belongs to the superfamily of nuclear receptors and is a ligand-dependent transcription factor (12). PPARs regulate the gene expression of various start regions containing PPAR response elements, intracellular transcription levels and fatty acid and glucose metabolism, and inhibit the inflammatory response (12). Tharakeshwari et al (13) reported that trigonelline and diosgenin attenuate endoplasmic reticulum stress and enhance adipose tissue PPARγ activity in T2DM rats. Capobianco et al (14) demonstrated that PPAR activation regulates nitric oxide production, lipid concentration and lipoxygenation in the placenta of patients with T2DM. In addition, pioglitazone ameliorates memory deficits in diabetic mice via the activation of PPARγ (15).

Naringin is a natural dihydro flavonoid, which is widely distributed in grapefruit and other citrus fruits and may also be chemically synthesized (16). Previous studies have demonstrated that naringin exhibits various pharmacological effects, including anti-tumor, anti-mutagenic and anti-atherosclerotic effects (17-19). Studies have suggested that naringin may reverse the liver damage caused by drugs or toxic chemical compounds, including alcohol, cadmium, carbon tetrachloride, oxytetracycline and dimethyl nitrosamine (20-23). The present study aimed to investigate the effects of naringin on DACD. In addition, the correlation between nuclear oxidative stress, pro-inflammatory factors, PPARγ and DACD was investigated.

Materials and methods

Animals. A total of 32 male 6-week-old Sprague-Dawley rats (weight, 270±20 g) obtained from the experimental center of the Dalian Medical University (Dalian, China) were selected for the experimental procedures in the present study. All animal procedures were performed in accordance with the guidelines of the First Affiliated Hospital of the Dalian Medical University and were approved by the Ethics Committee of Dalian Medical University (Dalian, China). Prior to experimentation, the rats were provided with ad libitum access to food and water, and were housed in a laboratory animal room at 23±1°C in 50-70% humidity on a 12 h light/dark cycle.

Drugs and chemicals. Naringin (purity, ≥98%) and STZ were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glutathione peroxidase (GSH-Px; S0056), glutathione (GSH; S0053), superoxide dismutase (SOD; S0038), malondialdehyde (MDA; S0131), ELISA kits, bicinchoninic acid (BCA; P0006) protein assay kits and caspase-3 (C1115) and caspase-9 (C1157) activity test kits were purchased from Beyotime Institute of Biotechnology (Haimen, China), and tumor necrosis factor α (TNF-α; RT029) and interleukin (IL)-6 (R1001) were purchased from Shanghai Gefan Biological Technology Co., Ltd. (Shanghai, China).

Establishment of the diabetic rat model. At the onset of the experiment, the body weights of the experimental rats were measured. Plasma glucose levels were then detected using an enzymatic glucose oxidase peroxidase diagnostic kit. Fasting blood glucose levels >250 mg/dl were considered diabetic and were used for further experimentation. The chemical structure of naringin is shown in Fig. 1. After 1 week of acclimatization, the rats received a single intraperitoneal injection of STZ (50 mg/kg) to induce T2DM, with the exception of the normal healthy controls, as previously described (24). At the end of the experiment (48 h following injection), the body weight and blood glucose levels of the experimental rats were measured.

Experimental design. In total, eight normal rats were injected with physiological saline, defined as the control group; eight diabetic rats were injected with physiological saline, defined as the DM group; 24 diabetic rats were randomly divided into three groups, and were treated with naringin at doses of 100 mg/kg and 200 mg/kg into the caudal vein, as previously described (25). Rats from each group (4/group) were immediately sacrificed by cervical dislocation under 30 mg/kg pentobarbital (Sigma-Aldrich), and brain tissue and blood samples were collected under anesthesia (300 mg/kg intraperitoneally). The samples were stored at -80°C for further experimentation.

Morris water maze (MWM) assessment. Following treatment with naringin (100 and 200 mg/kg) for 16 weeks, MWM assessments were performed, as previously described (26,27). Rats from each group (4/group) were trained to swim freely with a circular Plexiglas platform (14 cm diameter) submerged 1.5 cm beneath the surface of the water prior to performing the MWM test. The platform was located in a fixed position, equidistant from the center and the wall of the tank. The rats were subjected to four training trials per day. The rats were placed into the tank at one of the four designated start points per day, in a pseudorandom order, and trained for as many days as required to reach the criterion of 25 sec to reach the platform. If the rats failed to find the platform within 60 sec, they were manually guided to the platform and allowed to remain there for 5 sec. Following the final training session, a probe trial was performed after 24 h, consisting of a 60 sec free swim in the pool in the absence of the platform. The MWM assessments were recorded via video capture and analyzed using a SMARTW system (PanLab, Barcelona, Spain).

Oxidative stress assessment. Tissue sections (~5 mg) of the cerebral cortex and hippocampus were added to 100 µl tissue lysis buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) on ice, and incubated for 10 min. The homogenates were then centrifuged at 20,000 x g for 15 min
at 4°C. The clear supernatant was collected and analyzed for oxidative stress. This was achieved by quantifying the levels of GSH-Px, GSH, SOD and MDA in the cerebral cortex and hippocampus, using commercially-available kits (Beyotime Institute of Biotechnology), according to the manufacturer's instructions.

**TNF-α and IL-6 assessment.** Tissue sections (~5 mg) of the cerebral cortex and hippocampal samples were added to 100 µl tissue lysis buffer on ice, and incubated for 10 min. The homogenates were then centrifuged at 20,000 x g for 15 min at 4°C. TNF-α and IL-6 ELISA kits (Beyotime Institute of Biotechnology) were used to quantify the protein levels, according to the manufacturer's instructions, and the samples were analyzed spectrophotometrically (Model 550; Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 450 nm absorbance.

**Western blot analysis.** Tissue sections (~5 mg) of the cerebral cortex and hippocampal tissue samples were added to 100 µl tissue lysis buffer on ice and incubated for 10 min. The homogenates were then centrifuged at 20,000 x g for 15 min at 4°C. The supernatant was collected and the protein concentration was measured using a BCA protein assay kit. The protein was separated by 8-12% SDS-PAGE (Sigma-Aldrich), and electrotransferred to polyvinylene difluoride membranes (0.22 mm; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China). The membranes were blocked with tris-buffered saline (TBS; Wuhan Boster Biological Technology, Ltd., Wuhan, China) containing 5% non-fat milk for 2 h. The membranes were subsequently incubated with monoclonal mouse anti-human PPARγ (sc-7273; 1:2,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and polyclonal mouse anti-human β-actin (bs-0061R; 1:500; BIOSS, Beijing, China) primary antibodies, overnight at 4°C. The membranes were washed three times with TBS with Tween 20 (1%; Sigma-Aldrich) for 2 h, and then incubated with anti-mouse horseradish peroxidase-conjugated IgG (sc-2370; 1:3,000; Santa Cruz Biotechnology, Inc.), for 2 h. The bands were visualized using an ECL kit (Bio-Rad Laboratories, Inc.) and quantified using densitometry (Image Quant LAS 4000 software; GE Healthcare Life Sciences, Chalfont, UK).

**Caspase-3 and caspase-9 activity level quantification.** Tissue sections (~5 mg) of the cerebral cortex and hippocampal tissue samples were added to 100 µl tissue lysis buffer on ice and incubated for 10 min. The homogenates were then centrifuged at 20,000 x g for 15 min at 4°C. The activity levels of caspase-3 and caspase-9 were measured using a caspase-3 and caspase-9 activity test kit, according to the manufacturer's instructions, and incubated at 37°C for 120 min. The levels of caspase-3 and caspase-9 were measured using the Model 550 spectrophotometer at 405 nm.

**Statistical analysis.** Statistical analyses were performed using one-way analysis of variance followed by Dunnett's test. Statistical analyses were performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA), and the data are expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

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Figure 2. Effects of naringin on the (A) body weight and (B) blood glucose levels of DM rats. Data are expressed as the mean ± standard deviation. *P<0.01, vs. the Con group; **P<0.01, vs. the DM group. Con, control group; DM, diabetes group; NAR (100), naringin (100 mg/kg)-treated group; NAR (200), naringin (200 mg/kg)-treated group.

**Results**

**Effects of naringin on body weight and blood glucose levels.** The chemical structure of naringin is shown in Fig. 1. The DM rats exhibited a significant decrease in body weight and an increase in blood glucose levels, compared with the normal control group. Following treatment with naringin (100 and 200 mg/kg) for 16 weeks, the body weights and blood glucose levels of the DM rats were significantly increased and decreased, respectively, compared with those of the untreated DM group (Fig. 2A-B).

**Effects of naringin on cognitive deficit.** Following 16 weeks treatment with naringin (100 and 200 mg/kg), a MWM test was used to assess cognitive function. The escape latency markedly increased in the DM group, compared with the control group (Fig. 3A). Following treatment with naringin, the escape latency was reduced, compared with that of the DM group (Fig. 3A). The mean path length significantly increased in the DM rats, compared with the control group. The mean path length following treatment with naringin was significantly reduced, compared with that of the DM group (Fig. 3B). The duration spent in the target quadrant and the number of times the rats crossed the former platform location were significantly reduced in the DM group, compared with those observed in the control group (Fig. 3C-D). Treatment with naringin markedly reversed these effects in the DM rats (Fig. 3C-D). However, the swimming speed of the rats in the control group was similar to those observed in the other groups (Fig. 3E).

**Effects of naringin on diabetes-induced changes in oxidative stress.** To examine the effects of naringin on oxidative stress in the brain tissue, the expression levels of GSH-Px, GSH, SOD
and MDA were measured in the cerebral cortex and hippocampus tissue samples. As shown in Fig. 4A-C, the expression levels of GSH-Px, GSH and SOD were significantly decreased in the cerebral cortex and hippocampus of the DM group, compared with those of the control group. Treatment of the STZ-induced diabetic rats with naringin (100 and 200 mg/kg) significantly increased the expression levels of GSH-Px, GSH and SOD in the cerebral cortex and hippocampus (Fig. 4A-C). In addition, the expression levels of MDA in the DM group were significantly increased, compared with those of the control group (Fig. 4D). Following treatment with naringin (100 and 200 mg/kg) for 16 weeks, these expression levels were significantly reduced (Fig. 4D).

Effects of naringin on DM-induced changes in proinflammatory cytokines. To determine the effects of naringin on the brain proinflammatory cytokines in the DM rat, the expression levels of TNF-α and IL-6 were measured in the cerebral cortex and hippocampus. Compared with those of the control group, the expression levels of TNF-α and IL-6 were significantly increased in the cerebral cortex and hippocampus tissues of the STZ-induced DM rats (Fig. 5A and B). Treatment with naringin (100 and 200 mg/kg) significantly reversed the elevated expression levels of TNF-α and IL-6 in the cerebral cortex and hippocampus, compared with the DM group (Fig. 5A and B).

Effects of naringin on the expression levels of PPARγ. To investigate whether naringin exerts its effects through upregulation of the expression of PPARγ, the expression levels of PPARγ were measured in the cerebral cortex and hippocampus tissues using western blotting. As shown in Fig. 6A and B, the expression levels of PPARγ were significantly decreased in the cerebral cortex and hippocampus of the DM group, compared with those of the control group. Treatment of the STZ-induced DM rats with naringin (100 and 200 mg/kg) significantly increased the protein expression levels of PPARγ in the cerebral cortex and hippocampus, compared with the DM rats without naringin (Fig. 6A-B).

PPARγ inhibitor regulates the effects of naringin on DACD. To confirm the observed results that the PPARγ GW9662 inhibitor (0.3 mg/kg) regulated the effects of naringin on DACD, a MWM test was performed. The PPARγ inhibitor significantly decreased the protein expression levels of PPARγ (Fig. 7A-B). In addition, PPARγ inhibitor reversed the effects of naringin on DACD following treatment with naringin (100 and 200 mg/kg) for 16 weeks (Fig. 7C-G).

Effects of naringin on the expression levels of caspase-3 and caspase-9. To examine whether naringin affected the levels of caspase-3 and caspase-9, the expression levels of caspase-3...
and caspase-9 were measured in the cerebral cortex and hippocampus tissues using ELISA assays. As shown in Fig. 8A and B, naringin notably increased the expression levels of caspase-3 and caspase-9 in the cerebral cortex and hippocampus of the DM group, compared with the control group. Treatment with naringin (100 and 200 mg/kg) decreased the expression levels of caspase-3 and caspase-9 in the cerebral cortex and hippocampus, compared with the control group (Fig. 8A and B).
Figure 7. PPARγ inhibitor regulates the effects of naringin on DACD. (A) Representative western blots and (B) quantitative results of the protein expression of PPARγ in the cerebral cortex and hippocampus. Effects of naringin on the (C) escape latency, (D) mean path length, (E) mean percentage of time spent in the target quadrant, (F) number of times of platform crossing, and (G) swimming speed in the rats. Data are expressed as the mean ± standard deviation. **P<0.01, vs. the Con group; ##P<0.01, vs. the DM group; and ###P>0.05, vs. the DM group. Con, control group; DM, diabetes group; NAR (200), naringin (200 mg/kg)-treated group; NAR+GW (naringin, 200 mg/kg+GW9662, 0.3 mg/kg)-treated group. PPARγ, peroxisome proliferator-activated receptor γ.

Figure 8. Effects of naringin on the expression levels of caspase-3 and caspase-9. (A and B) The effects of naringin on the activity levels of caspase-3 and caspase-9 in rats. Data are expressed as the mean ± standard deviation. **P<0.01, vs. the Con group; ***P<0.01, vs. the DM group. Con, control group; DM, diabetes group; NAR (100), naringin (100 mg/kg)-treated group; NAR (200), naringin (200 mg/kg)-treated group.
Discussion

With changes in lifestyles and aging of the population, T2DM incidence is increasing year by year (28). As a systemic disease, T2DM can cause a variety of organizational, structural and functional changes in organs, and the lesions can affect the whole body. Therefore, early recognition and treatment of DACD can delay and reduce the incidence of dementia, and improve the life quality of patients with T2DM (29). In the present study, naringin significantly increased the body weight and reduced the levels of blood glucose in DM rats. Oršolić et al (30) reported that the DNA-protective effects of naringenin increased the body weight of alloxan-induced diabetic mice. Naringenin has also been observed to markedly normalize the body weights of albino mice (31). Priscilla et al (32) demonstrated that naringenin reduces the postprandial glycemic response in DM rats. The results of the present study demonstrated that naringin effectively improved the cognitive deficit of DM rats.

The biological basis of DACD in cognitive impairment may be associated with abnormal brain development, in which a large number of reactive oxygen species produced by oxidative stress and secondary cell injury are important (33). Under normal circumstances, the body has an effective antioxidant defense mechanism, and SOD is one of the most important antioxidant enzymes (34). An increase in the levels of reactive oxygen species can cause lipid peroxidation, DNA damage and cell-associated molecule or gene regulation, thereby inducing the nerve cell damage characteristic of certain cognitive deficits (35). The compensatory activity of antioxidant enzyme SOD is then increased. Therefore, the possible biological basis of DACD may be associated with SOD. Oxidized low density lipoprotein is highly cytotoxic, which can lead to vascular endothelial cell necrosis, and it closely associated with the occurrence of atherosclerosis and other cardiovascular and cerebrovascular diseases (36). MDA is a major metabolite involved in the biological membrane damage by free radicals, and can cause disorders of protein synthesis, which results in memory and mental decline (36). The levels of MDA reflect the degree of lipid peroxidation in the body (36). The results of the present study further determined that the effects of naringin markedly increased the expression levels of GSH-Px, GSH and SOD in the cerebral cortex and hippocampus of the DM rats. In addition, the expression levels of MDA were significantly reduced following treatment with naringin in the DM rats. Previous studies have demonstrated that naringenin has a similar protective effects to L-arginine in monocrotaline-induced pulmonary hypertension through oxidative stress, inflammation and nitric oxide in rats (37). Jeon et al (38) reported that naringenin increases the expression levels of GSH-Px, GSH and SOD in rats fed a high-cholesterol diet. Hermenean et al (39) demonstrated that naringenin increases the expression of MDA and decreases the expression level of SOD, catalase, GSH and GSH-Px in the mouse kidney (39).

TNF-α is a type of cytokine produced by activated monocytes, which exhibits a wide range of activities (40). TNF-α may be involved in nerve damage, and a previous study on the brain neuroinflammatory response of patients with dementia demonstrated that the primary deposit of TNF-α is β-amyloid, which is one of the causes of neurodegenerative dementia (41). IL-6 is produced by monocytes or macrophages, and is a stimulating factor produced by several cells in vivo; IL-6 exhibits a wide range of biological activities, and is an important member in the complex network of cytokines in the body, which are involved in various pathophysiological processes (42). An increase in the expression levels of IL-6 in the plasma of elderly patients is closely associated with cognitive impairment, which is a risk factor for cognitive dysfunction, and the rise of inflammatory factors in the plasma of patients occurs prior to the clinical diagnosis of dementia, suggesting that inflammatory cytokines of the peripheral blood may be involved in the pathophysiology of dementia (43). The results of the present study demonstrated that naringin decreased the expression levels of TNF-α and IL-6 in the cerebral cortex and hippocampus of DM rats. A previous study demonstrated that naringin significantly decreased the production and expression levels of IL-1β and IL-6 in diabetic mice (44). The inhibition of TNF-α and IL-6 by naringenin may contribute to its anti-inflammatory activity in rats with ethanol-induced liver injury (45). Bodet et al (46) also reported that naringin exhibits anti-inflammatory properties in macrophages.

PPARs are a type of ligand-activated nuclear transcription factor that regulate the expression of several key genes, including those involved in glucose and lipid metabolism (47). There are three subtypes of PPARs: PPAR-α, PPAR-γ and PPAR-δ. PPAR-γ is an important transcription factor in lipogenesis, and promotes adipocyte differentiation, increased insulin sensitivity and lower blood sugar levels (48). PPAR-γ reduces the levels of blood fat and increases insulin sensitivity. Therefore, PPAR-γ has become the focus of investigations on anti-DM drugs (49). In the cerebral cortex and hippocampus, naringin activated the protein expression of PPARγ in DM rats. Sharma et al (50) provided evidence that naringin ameliorates insulin resistance, hepatic steatosis and kidney damage in T2DM rats by regulating oxidative stress, inflammation and upregulation of PPARγ. Naringin decreases ethanol intake and ethanol-conditioned place preference in mice (51). The present study demonstrated that the PPARα inhibitor, GW9662, decreased the protein expression of PPARγ, as well as the effects of naringin on the cognitive deficit of the DM rats. These results suggested that naringin enhanced the cognitive deficit of DM rats via the upregulation of PPARγ.

The results of the present study demonstrated that naringin reduced the expression levels of caspase-3 and caspase-9 in the cerebral cortex and hippocampus of DM rats, and decreased cell apoptosis in the brain tissues of the DM rats. Treatment with naringin improves functional recovery via the inhibition of caspase-3 following spinal cord injury in rats (52). In addition, naringin reduces the levels of 3-nitropropionic acid-induced apoptosis through decreased caspase-3 activation (53).

In conclusion, the present study demonstrated that naringin significantly ameliorated cognitive deficits via oxidative stress, and the proinflammatory and PPARγ signaling pathways in T2DM rats. Future research will focus on the characterization of naringin, and aim to investigate the therapeutic effects of naringin on DACD in vitro and in vivo.
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