Introduction

The basal forebrain cholinergic systems, which consist of basal forebrain, medial septum, and horizontal and vertical diagonal bands of Broca providing major cholinergic projections to the cerebral cortex and hippocampus, play an important role in a variety of brain functions such as cognitive performance, learning and memory, cerebral cortical development, and cerebral blood flow regulation (1). Degeneration of this complex has been observed in a number of cognitive disorders including pathological aging such as Alzheimer’s disease (AD) (1). Particularly in AD, the cholinergic neurons in this complex have been assumed to undergo degenerative changes, resulting in cholinergic hypofunction that is related to the progression of learning and memory deficits (1, 2). Moreover, the correlation of dementia with reductions in a number of cortical cholinergic markers such as choline acetyltransferase (ChAT), muscarinic acetylcholine receptors (AChR), and nicotinic AChR, as well as levels of acetylcholine (ACh) (1 – 3) suggest an association of cholinergic hypofunction with cognitive deficits. In fact, we

Full Paper

Diabetes-Induced Central Cholinergic Neuronal Loss and Cognitive Deficit Are Attenuated by Tacrine and a Chinese Herbal Prescription, Kangen-Karyu: Elucidation in Type 2 Diabetes db/db Mice

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Abstract. We investigated the effect of kangen-karyu (KK), a Chinese herbal prescription, on cognitive deficits and central cholinergic systems of type 2 diabetic db/db mice. Seven-week-old db/db (Y-db/db) mice received daily administration of test drugs during an experimental period of 12 weeks. At 18 weeks of age (O-db/db), the animals underwent the water maze test. Compared with age-matched control strain mice (O-m/m), vehicle-treated O-db/db mice showed impaired learning and memory performance. KK (100 – 200 mg/kg per day) and the reference drug tacrine (THA: 2.5 mg/kg per day) ameliorated the performance of O-db/db mice without affecting their serum glucose level. O-db/db mice had lower levels of brain-derived neurotrophic factor (BDNF) mRNA and its protein in the brain than O-m/m mice. Expression levels of central cholinergic marker proteins in the hippocampus and the number of cholinergic cells in the medial septum and basal forebrain were also significantly lower in O-db/db than in O-m/m mice, whereas no significant differences in the expression levels of these factors and the cell number were found between Y-m/m and Y-db/db mice. KK and THA treatment significantly reversed the down-regulated levels of cholinergic markers, choline acetyltransferase–positive cell number, and BDNF expression in db/db mice. These findings suggest that KK as well as THA prevents diabetes-induced cognitive deficits by attenuating dysfunction of central cholinergic systems.

Keywords: kangen-karyu, diabetic, dementia, brain-derived neurotrophic factor (BDNF), cholinergic system
have previously reported that expression levels of ACh, ChAT, and some muscarinic receptor subtypes (M₁, M₃, and M₅) are down-regulated in animal models of cognitive deficits (4 – 7).

Cognitive deficits such as AD and vascular dementia (VD) have a variety of risk factors such as aging, vascular disorders, and diabetes. Several lines of evidence suggest an association between cognitive deficits such as AD and diabetes and demonstrate that diabetes increases the risk of suffering from AD several fold (8). In a clinical context, about 80% of AD patients appear to be diabetic or to have abnormal blood glucose levels and defects in insulin signaling that are associated with accumulation of the neurofibrillary tangles (NFTs) and senile plaques of AD (9). Similar learning and memory deficits have been reported by using db/db mice, an animal model of type 2 diabetes that fails to respond to leptin, a 16-kDa protein hormone with a key role in appetite, metabolism, and regulation of energy intake and energy expenditure (10). This animal strain exhibits not only hyperglycemia and hyperinsulinemia but also impaired hippocampus-dependent cognitive performance and long-term potentiation. These deficits have been reported to become evident in adulthood at 10 weeks of age and over. However, the mechanisms underlying cognitive dysfunction in diabetes have not been clearly understood (9).

Kangen-karyu (KK) is a Chinese herbal prescription consisting of six crude drugs: Paeoniae Radix, Cnidii Rhizoma, Carthami Flos, Cyperi Rhizoma, Saussureae Radix, and Salviae Miltiorrhizae Radix. This extract is used to treat symptoms related to blood circulation deficiencies and is well known to reduce blood and plasma viscosity and thereby improve microcirculation (11). Pharmacological evidence demonstrates that KK exhibits biological activities such as platelet aggregation inhibition (12), hypertension suppression (13), and anti-hyperlipidemia (14). Moreover, there is a report demonstrating that administration of KK ameliorates repeated brain ischemia-induced spatial cognitive performance and neuronal cell death (15). Recently, we have reported that KK administration is capable of ameliorating aging-induced cognitive deficits and dysfunction of signaling systems such as brain-derived neurotrophic factor (BDNF) production which are involved in neuroplasticity, a biochemical basis of learning and memory (16). Our findings have suggested therapeutic potential of KK for cognitive deficits related to aging and availability as an anti-dementia drug. However, although beneficial effects of KK have been elucidated in db/db mice with a diabetic insult, it remains unclear if KK is applicable for ameliorating cognitive dysfunction caused by diabetes.

In this study, we investigated the effects of KK and an acetylcholinesterase inhibitor tareine on water maze performance and expression levels of BDNF and central cholinergic marker proteins such as ChAT and muscarinic receptor subtypes (M₁, M₃, and M₅ receptors) of an animal model of db/db mice with a diabetic insult to clarify if KK can be used as an anti-dementia drug effective for diabetes-related cognitive deficits. Our findings in this study provide direct evidence that cholinergic neuron degeneration is associated with learning and memory deficits in db/db mice.

Materials and Methods

Animals

The present study was conducted according to the experimental protocols described in Fig. 1. Briefly, male 6-week-old C57BLKS/J-db/db mice and their age-matched non-diabetic m/m littermates were purchased from Japan SLC, Inc. (Hamamatsu). The animals were housed in a laboratory animal room maintained at 25°C ± 1°C with 65% ± 5% humidity on a 12-h light/dark cycle (lights on: 07:30 – 19:30) for at least 1 week before the start of the experiments. The animals were given food and water ad libitum. After one week of acclimatizing, blood samples were collected from the tail vein to measure glucose level in serum. The db/db mice were randomly divided into 4 groups and then daily administration of the test drugs was started and continued during the experimental period. The age-matched non-diabetic m/m mice were used as a normal control. All
experiments were conducted in accordance with the Guiding Principles (NIH publication #85-23, revised in 1985) for the Care and Use of Animals and had the approval of the Institutional Animal Use and Care Committee of the University of Toyama.

Drug treatment

Test drug administration was performed daily around 9 a.m. during the experimental period of 12 weeks according to our previous pharmacological studies on KK (14, 17). The m/m and db/db vehicle (control) group was orally given water, while the other groups were orally administered KK extracts dissolved in distilled water and administered orally daily at doses of 100 and 200 mg/kg body weight according to our previous reports (14, 17). Tacrine (THA; 9-amino-1,2,3,4-tetrahydro-acridine HCl; Sigma-Aldrich Co., St. Louis, MO, USA), a control, was dissolved in physiological saline and injected intraperitoneally (2.5 mg/kg per day). The doses of the drugs used in this study were selected based on our previous reports (4, 16).

Preparation and chemical analysis of KK extract

Kangenkaryu in the form of a dried powder extract was obtained from Iskra Industry Co., Ltd. (Tokyo). The composition of KK and the extraction procedure used in this study were the same as those reported in previous studies (16, 18). Briefly, the composition of KK used in this study was: 2.25 g Paeoniae Radix (Paeonia lactiflora Pall. L.), 2.25 g Cnidii Rhizoma (Cnidium officinale Makino) rhizome), 2.25 g Carthami Flos (Carthamus tinctorius L., petal), 1.125 g Cyperi Rhizoma (Cyperus rotundus L., rhizome), 1.125 g Aucklandiae Radix (Aucklandia lappa D.CNE., root), and 4.5 g Salviae Miltiorrhizae Radix (Salvia miltiorrhiza Bunge, root). This prescription was extracted with 25 volumes of water at 100°C for 1 h. After filtration, the solution was evaporated under reduced pressure to give an extract at a yield of 44%, by weight, of the starting materials.

To identify the chemical constituents of KK, liquid column chromatography–mass spectrometry (LC-MS) analyses were performed with a Shimadzu LC-IT-TOF mass spectrometer equipped with an ESI interface (Shimadzu, Kyoto) as previously described (5). The ESI parameters were as follows: source voltage, +4.5 kV; capillary temperature, 200°C; nebulizer gas, 1.5 l/min. The mass spectrometer was operated in positive ion mode scanning from m/z 200 to 2000. A Waters Atlantis T3 column (2.1 mm i.d. × 150 mm; Nihon Waters K.K., Tokyo) was used and the column temperature was maintained at 40°C. The mobile phase was a binary eluent of A) 5 mM ammonium acetate solution, B) CH3CN, under the following gradient conditions: 0 – 30 min linear gradient from 10% to 100% B, then 30 – 40 min isocratic at 100% B. The flow rate was 0.2 ml/min. Mass spectrometry data obtained from each extract have been deposited in MassBank database (19) and stored together with the pharmacological information on the extracts in the Wakan-Yaku DataBase system (http://wakandb.u-toyama.ac.jp/wiki/index.php/LCMS:Kangenkaryu), Institute of Natural Medicine, University of Toyama. The KK extract used in this study (voucher specimen #: 26,731) was deposited in the herbarium of the University of Toyama.

Water maze test

The water maze test was performed by slightly modifying the protocol adopted in previous studies (6, 20). Briefly, the water maze testing took place during the first half of the light phase. The test was performed in a circular pool (diameter 110 cm, height 30 cm), filled with water made opaque with semi-skimmed milk and maintained at 26°C ± 1°C, and the circular escape platform (11-cm diameter) was submerged 1-cm below the water surface. Cues were hung at four locations at the north, west, south, and east corners of the swimming pool wall. During the first days, the animals were subjected to a visible trial (Visible 1) of the water maze in which the platform was made visible 1-cm above the water surface. Training trials were performed daily for 4 days 1 day after the visible trial. Mice underwent 3 trials per day as training trials. Each trial lasted until the animal found the platform or for a maximum observation period of 120 s; animals that failed to find the platform within the maximum observation period were guided there by the experimenter. In each trial they were placed into the pool, facing the wall, with start locations varied pseudo-randomly. One day after the last acquisition training session, animals were tested in a single 120-s probe trial without the platform. For each trial, the latency to reach the platform (escape latency), moved distance, and mean swim speed were recorded via video capture and image analysis using the SMART® system (Panlab, S.L., Barcelona, Spain). In this study, we adopted this observation period for non-diabetic m/m and diabetic db/db mice because db/db mice showed significantly reduced activity in the swimming pool compared with the normal m/m mice.

Measurement of serum glucose

Serum samples were collected from the tail vein of each animal group before and after completing the behavioral studies. The serum glucose levels were measured using a commercially available kit (Glucose CII-Test; Wako Pure Chem., Osaka).
Quantitative real-time polymerase chain reaction (PCR)

To analyze changes in expression levels of BDNF mRNA in the brain, the animals were killed by decapitation after completing the behavioral studies. The brain was removed immediately and the cortex and hippocampus were dissected out and kept at -80°C until use. Quantitative PCR was conducted as previously described (16). Briefly, total RNA was extracted from the cortex and hippocampus using Sepazol® (Nacalai Tesque, Kyoto) according to the manufacturer’s instructions. First-strand cDNA synthesis was conducted using oligo (dT) primers and M-MLV Reverse Transcriptase® (Invitrogen, Rockville, MD, USA) in a total volume of 20 μl. The reaction was performed at 25°C for 10 min and heated at 37°C for 60 min and 98°C for 5 min before cooling to 4°C. DNA corresponding to the RNA was used as a template for real-time PCR. Quantitative real-time PCR was carried out using Fast SYBR Green Master Mix (Applied BioSystems, Foster City, CA, USA) in a StepOne Real-time PCR System (Applied BioSystems). The following primer sets used in this study were synthesized by Nippon EGT Co. (Toyama): BDNF mRNA (NM_007540): 5′-AGCTGAGCTGTGACAGT-3′ (forward) and 5′-TCCATAAGGCGCCGCAC-3′ (reverse) and β-actin mRNA (NM_007393): 5′-CATCCTAGATAGTAAGGGCCCGAAC-3′ (forward) and 5′-ATGGAGCCACCGATCCACA-3′ (reverse). Melting curve analysis of each gene was performed every time amplification was completed. Standard curves of the log concentration of each gene vs. cycle threshold were plotted to prove negative linear correlations. The correlation coefficients for standard curves of target genes were 0.9965 to 0.998.

Western blot analysis

Western blotting was performed by a slightly modified version of a method described previously (5, 16, 21). Briefly, tissue samples were taken from the hippocampus and proteins were extracted by homogenization in protein lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 0.5% sodium deoxycholate, 1% (v/v) NP-40, 0.1% (v/v) sodium dodecyl sulfate (SDS), 150 mM NaF, 8.12 μg/ml apro- tinin, 2 mM sodium orthovanadate, 10 μg/ml leupeptin, and 2 mM phenylmethylsulfonyl fluoride] using TissueLyser® (Qiagen, Osaka). Lysate samples were centrifuged at 10,000 rpm (4°C) for 5 min. The protein concentration was determined using a BCA™ protein assay kit (Thermo Scientific, Rockford, IL, USA) and a microplate reader (Sunrise Classic; TECAN Japan, Kawasaki). Total protein (15 μg) prepared from each sample was electrophoresed on a 7.5% – 15% SDS-polyacrylamide gel (SDS-PAGE) and then electro-blotted onto a polyvinylidene difluoride membrane (Clear Blot Membrane-p; ATTO, Tokyo). The membranes were incubated in a 5% non-fat milk–containing wash buffer (50 mM Tris (pH 7.5), 150 mM sodium chloride, and 0.1% Tween 20) for 1 h at room temperature. They were then probed with anti-ChAT goat polyclonal antibody (1:3000 dilution) (AB-144P; Millipore, Billerica, MA, USA), anti-muscarinic ACh receptor rabbit polyclonal antibodies (1:500 dilution) (M1: sc-9106; M2: sc-9108; M3: sc-9106; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-BDNF rabbit polyclonal antibody (1:500 dilution) (H-117, sc-20981; Santa Cruz Biotechnology, Inc.), and anti-β-actin mouse monoclonal antibody (1:10,000 dilution; Abcam®, Cambridge, UK) at 4°C for 24 h. After the membranes were rinsed in TBS-T, the blots were incubated with bovine anti-goat IgG secondary antibodies linked with horseradish peroxidase (Santa Cruz Biotechnology) or anti-rabbit or anti-mouse secondary antibodies linked with horseradish peroxidase (DakoCytomation EnVision + System-HRP labeled Polymer) (Dako Cytomation, Inc., Carpinteria, CA, USA) according to the manufacturer’s instructions. The immune complexes were detected by an enhanced chemiluminescence method (Immobilion™ Western Chemiluminescent HRP Substrate) (Millipore) and imaged using the Lumino Image Analyzer LAS-4000 (Fujifilm, Tokyo). Band images were analyzed by VH-H1A5 software (Keyence, Osaka). The quantity of immunoreactive bands of ChAT, muscarinic receptor subtypes (M1, M3, and M5), BDNF, and β-actin proteins was normalized by comparing with their expression levels in treatment-naïve control mice.

Immunohistochemistry

Analysis of cholinergic neurons in the middle septal and basal forebrain was conducted by the immunohistochemical technique with slight modification of the method described previously (19, 20). Mice were fixed by intracardiac perfusion with 4% paraformaldehyde in phosphate-buffered saline (PBS) under anesthesia. Brains were post-fixed with 4% paraformaldehyde overnight at 4°C. A series of 5-μm coronal brain sections including the prefrontal cortex and hippocampus, which have major roles in learning and memory, were obtained using a sliding microtome (IVS-410; SAKURA Finetek Japan, Tokyo). For immuno-labeling, the slices were probed with anti-ChAT (goat polyclonal antibody 1:400 dilution, AB-144P; Millipore), placed in special microwave equipment (MI-77; Azumaya, Tokyo) or anti-ChAT goat polyclonal antibody (1:400 dilution, AB-144P; Millipore), placed in special microwave equipment (MI-77; Azumaya, Tokyo), irradiated intermittently (250 W, 4 s on and 3 s off) avoiding an excessive temperature increase for 15 min, and then washed with distilled water. After washing the specimens with Tris-buffered saline, they were incubated with peroxi-
Inc., Richmond, CA, USA).

Briefly, the frozen cortex was weighed and homogenized in 10 volumes of 0.1 M phosphate buffer (pH 7.4) containing 1% Triton-X-100. After centrifugation at 15,000 × g at 4°C for 20 min, the clear supernatants were collected and served as the enzyme source. Cholinesterase activity was performed on the basis of the colorimetric method as previously described (13, 21). The amount of subspecies per tissue was determined in 50 μl aliquots of the homogenates (run as duplicates) in 96-well flat-bottom microplates. The reaction was started by adding 20 μl of 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid), 20 μl of 30 mM acetylthiocholine (Sigma-Aldrich Co.), and 160 μl of phosphate buffer. The spectrophotometric absorption at 405 nm during a 3-min incubation period at 25°C was quantitatively measured using a microplate reader (Sunrise Classic, TECAN Japan) and expressed as nmol ACh hydrolyzed/(min·mg tissue).

Statistical analysis

Statistical analysis in this study was conducted according to the guidelines reported by Curran-Evenett and Benos (22). All data are expressed as the mean ± S.D. The behavioral data obtained in the water maze test were analyzed using one-way ANOVA or two-way repeated measures ANOVA followed by the post hoc Student-Newman-Keuls test for multiple comparisons among different groups. The neurochemical data were analyzed by the unpaired Student’s t-test, one-way ANOVA, or two-way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons. Differences of P < 0.05 were considered significant. The analysis was conducted using SigmaStat® ver 3.5 (SYSTAT Software Inc., Richmond, CA, USA).

Results

Effect of KK on spatial learning and memory performance of diabetic db/db mice in water maze test

To test whether 18-week-old diabetic db/db mice show spatial learning and memory deficits, the Morris water maze test, a hippocampus-dependent spatial cognitive task in which mice are required to learn to locate an escape platform in a pool of water, was conducted. As shown in Fig. 2, the m/m and db/db control groups could learn the location of a submerged platform following repeated daily training [Ftraining(3,48) = 44.133, P < 0.001, two-way repeated measures ANOVA], but there was a significant difference in learning between the two animal groups [Fanimal group(1,16) = 54.247, P < 0.001, two-way repeated measures ANOVA]. In fact, escape latency in the control db/db group was significantly greater than that in the control m/m group [Fanimal-training(3,48) = 9.596, P < 0.001, two-way repeated measures ANOVA]. Moreover, swimming distance of the m/m and db/db mice was also significantly decreased by repeated training [Ftraining(3,48) = 11.055, P < 0.001, two-way repeated measures ANOVA], but the control db/db mouse group displayed significantly longer swimming distance to escape to the target platform [Fanimal group(1,16) = 12.037, P = 0.003, two-way repeated measures ANOVA]. On the other hand, repeated training had no effect on the swimming speed of these two animal groups [Ftraining(3,48) = 0.572, P = 0.636], but the swimming speed of the O-db/db group during training trials was significantly slower than that of the age-matched m/m group [Fanimal group(1,16) = 101.422, P < 0.001, two-way repeated measures ANOVA].

We also examined the effect of THA and KK on water maze performance of db/db mice in the water maze test. THA (2.5 mg/kg per day, i.p.) treatment significantly improved spatial learning performance observed in the db/db animal group in terms of escape latency [Fdrug(1,16) = 15.300, P = 0.001, Ftraining(3,48) = 15.474, P < 0.001; Fdrug-training(3,48) = 1.790, P = 0.162, two-way repeated measure ANOVA] and the swimming distance to reach the target platform in the training tests [Fdrug(1,16) = 11.500, P = 0.004, Ftraining(3,48) = 9.284, P < 0.001; Fdrug-training(3,48) = 1.092, P = 0.361, two-way repeated measures ANOVA]. However, this treatment had no effect on the swimming speed of this animal group [Fdrug(1,16) = 0.0106, P = 0.919, two-way repeated measures ANOVA] in the training test (Fig. 2: A, B, and C). Interestingly, daily treatment of db/db mice with 200 mg/kg KK also significantly and dose-dependently improved spatial learning performance of O-db/db animals in the training test [escape latency: Fdrug(1,16) = 12.410, P = 0.003, Ftraining(3,48) = 28.453, P < 0.001;
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\[ F_{\text{drug \times training}}(3,48) = 3.649, \quad P = 0.019, \]  
\text{two-way repeated measures ANOVA}. The swimming distance of O-db/db mice was also shortened by repeated training and drug treatment \[ F_{\text{drug}}(1,16) = 9.916, \quad P = 0.006, \quad F_{\text{training}}(3,48) = 11.148, \quad P < 0.001, \]  
\text{two-way repeated measures ANOVA} (Fig. 2: A and B) without affecting swimming speed of these animal groups \[ F_{\text{drug}}(1,16) = 1.292, \quad P = 0.272, \]  
\text{two-way repeated measure ANOVA} (Fig. 2C). In addition, administration of KK (100 mg/kg per day) led to significant changes in learning performance of db/db mice in the training test of the water maze task \[ F_{\text{drug}}(1,15) = 2.446, \quad P = 0.139, \quad F_{\text{training}}(3,45) = 12.122, \quad P < 0.001; \]  
\[ F_{\text{drug \times training}}(3,45) = 0.155, \quad P = 0.926, \]  
\text{two-way repeated measure ANOVA} and the swimming distance \[ F_{\text{drug}}(1,15) = 5.33, \quad P = 0.036, \quad F_{\text{training}}(3,45) = 7.478, \quad P < 0.001; \]  
\[ F_{\text{drug \times training}}(3,45) = 0.685, \quad P = 0.566, \]  
\text{two-way repeated measure ANOVA}, but not swimming speed \[ F_{\text{drug}}(1,15) = 0.0354, \quad P = 0.853, \]  
\text{two-way repeated measure ANOVA}.

In the probe test conducted 1 day after a 4-day training period, swimming time of the db/db control mice in the target quadrant where the platform had been located during training was significantly shorter than that of the m/m mice \[ t = 3.221, \quad df = 16, \quad P = 0.005, \quad t\text{-test}. \]  
The db/db groups treated with daily administration of KK (100 – 200 mg/kg per day) and THA (2.5 mg/kg per day) spent significantly more time in the target quadrant during the observation period than those of db/db treated with vehicle \[ F_{\text{drug}}(3,29) = 5.325, \quad P = 0.005, \]  
\text{one-way ANOVA} (Fig. 2D).

**Effects of KK and THA on serum glucose in diabetic db/db mice**

As summarized in Table 1, the body weights and serum glucose levels that were measured before and after completion of the behavioral experiments were significantly higher in the db/db mice than those in the age-matched m/m non-diabetic control mice. THA (2.5 mg/kg per day, i.p.) or KK (200 mg/kg per day, p.o.) had no effect on the glucose level of db/db mice.

**Effects of KK on the expression levels of BDNF mRNA and its protein in the brain**

Real-time PCR was performed to analyze changes in expression levels of BDNF mRNA in the cerebral cortex and hippocampus in 7-week-old (Y-m/m) and 19-week-old m/m (O-m/m) and db/db mice (Y- and O-db/db). No significant difference in the expression levels of BDNF mRNA were found between the Y-m/m and Y-db/db mice (Fig. 3-1: A, B). However, when the expression
levels were compared at the age of 19 weeks, the vehicle-treated 
O-\text{db/db} group showed significantly lower levels of BDNF mRNA in the cortex \([t = 5.572, \ df = 7, \ P < 0.001, \ t\text{-test}]) and hippocampus \([t = 6.274, \ df = 7, \ P < 0.001, \ t\text{-test}]) (Fig. 3). However, the expression levels of BDNF mRNA in the hippocampus and cortex were significantly up-regulated in O-\text{db/db} mice treated with KK and THA compared with those in the vehicle-treated O-\text{db/db} group (BDNF mRNA: \(F(3,14) = 26.416, \ P < 0.001, \ \text{one-way ANOVA}\), in the hippocampus and \(F(3,14) = 4.214, \ P = 0.026, \ \text{one-way ANOVA}\), in the cortex).

The vehicle-treated O-\text{db/db} group also showed significantly lower levels of BDNF protein in the hippocampus than the O-\text{m/m} group \((t = 3.289, \ df = 6, \ P = 0.017, \ t\text{-test})\), but the BDNF protein level in the hippocampi of O-\text{db/db} mice was significantly up-regulated by administration of KK and THA \((F(3,12) = 9.404, \ P = 0.002, \ \text{one-way ANOVA})\), whereas no significant difference in the expression levels of BDNF protein were found between the Y-\text{m/m} and Y-\text{db/db} mice \((t = -0.768, \ df = 4, \ P = 0.485, \ t\text{-test})\) (Fig. 3-2).

Table 1. Effect of KK and THA treatment on body weight and serum glucose level in \text{db/db} mice

<table>
<thead>
<tr>
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<th>7-week-old</th>
<th>19-week-old</th>
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<tbody>
<tr>
<td></td>
<td>\text{m/m}</td>
<td>\text{db/db}</td>
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<tr>
<td>Body weight (g)</td>
<td>20.7 ± 1.23</td>
<td>4.6 ± 1.9\text{***}</td>
</tr>
<tr>
<td>Serum glucose (mg/dl)</td>
<td>164.3 ± 28.4</td>
<td>318.9 ± 77.9\text{***}</td>
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Body weights and serum glucose levels were first measured at the age of 7-week-old just before starting drug administration. The \text{db/db} mice were randomly separated into 4 groups and underwent daily administration of vehicle, 100 and 200 mg/kg KK, or 2.5 mg/kg THA. After completion of the behavioral study, the body weight and serum glucose levels of each animal were again measured at the age of 19 weeks. KK and THA represent kangen-karyu extract and tacrine, respectively. Each datum represents the mean ± S.D. (n = 7 – 10). \text{***}\(P < 0.001\) vs. respective 7w-\text{m/m} or 19w-\text{m/m} group (t-test).

1) BDNF mRNA

2) BDNF and \(\beta\)-actin levels in the hippocampus

Fig. 3. Effects of THA and KK on BDNF mRNA and BDNF protein expression levels in the brain of \text{db/db} mice. After completion of the behavioral studies, the animals were decapitated and total RNA was extracted from the cortex and hippocampus for real-time PCR analysis (1). Protein from the hippocampus was extracted for western blotting analysis of BDNF and \(\beta\)-actin (2). Each data column represents the mean ± S.D. obtained from 4 to 5 brain samples each. \(*P < 0.05\) and \text{***}\(P < 0.001\) vs. vehicle-treated O-\text{m/m} (t-test). \(\#P < 0.05\), \text{**}P < 0.01, \text{***}P < 0.001\) vs. vehicle-treated O-\text{db/db} group (one-way ANOVA).
Effects of KK and THA on the expression levels of cholinergic marker proteins in the hippocampus of O-db/db mice

Next we investigated the pathophysiological states of cholinergic systems using western blotting analysis to measure the expression levels of cholinergic marker proteins, ChAT and muscarinic M₁, M₃, and M₅ receptors, in the hippocampus, which receives cholinergic projections from the medial septum. As summarized in Fig. 4, no significant difference in the expression levels of these marker proteins was found between Y-m/m and Y-db/db groups. However, the O-db/db group treated with the vehicle water had significantly lower levels of these marker proteins than the O-m/m group (ChAT: $t = 3.281$, df = 8, $P = 0.011$, t-test; M₁ receptor: $t = 3.735$, df = 8, $P = 0.006$, t-test; M₃ receptor: $t = 4.167$, df = 8, $P = 0.003$, t-test; M₅ receptor: $t = 3.724$, df = 8, $P = 0.006$, t-test). Interestingly, O-db/db mice treated with daily administrations of KK and THA had significantly increased levels of ChAT, M₅ receptor, and M₅ receptor, but not M₁ receptor (ChAT: $F(3,16) = 9.207$, $P < 0.001$, one-way ANOVA; M₁ receptor: $F(3,16) = 2.880$, $P = 0.068$, one-way ANOVA; M₃ receptor: $F(3,16) = 6.810$, $P = 0.004$, one-way ANOVA; M₅ receptor: $F(3,16) = 4.820$, $P = 0.014$, one-way ANOVA) (Fig. 4B).

KK and THA prevent diabetes-induced degeneration of central cholinergic systems

To investigate the mechanism underlying diabetes-induced decrease of cholinergic marker proteins in the hippocampus and its reversal by KK and THA treatment, we immunohistochemically analyzed ChAT-immunopositive cells located in the medial septum and basal forebrain, major cholinergic nuclei projecting to the hippocampus and cortex. Both the medial septum and basal forebrain area showed an age-related decrease in ChAT-immunopositive cells in young and old animals (Y- and O-m/m, and Y- and O-db/db mice) [$F_{age(1,10)} = 61.113$, A)}

**Fig. 4.** Effects of KK on ChAT and M₁-, M₃-, and M₅-receptor protein expression in the hippocampus. After completion of the behavioral studies, the animals were decapitated and proteins were extracted from the hippocampus of each animal group. A) Typical photos indicating the expression levels of each factor in the hippocampus. The young m/m control (Y-m/m) (lane a), young db/db control (lane b), old m/m control (O-m/m) (lane c), old vehicle-treated db/db (O-db/db) (lane d), KK (100 mg/kg per day)-treated db/db (lane e), KK (200 mg/kg per day)-treated db/db (lane f), and THA (2.5 mg/kg per day) group (lane g). B) Quantitative comparisons of each factor among different animal groups were conducted as described in the Material and Methods. The data are expressed as the percentage of the value obtained from vehicle-treated naïve control mice. Each data column represents the mean ± S.D. obtained from 4 – 5 brain samples. *$P < 0.05$, **$P < 0.01$ vs. vehicle-treated O-m/m group (t-test). *$P < 0.05$ and **$P < 0.01$ vs. respective vehicle-treated O-db/db group (one-way ANOVA).
$P < 0.001, \ F_{\text{adj}}(1,8) = 21.547, \ P = 0.002$, two-way ANOVA], whereas no significant difference in the number of ChAT-immunopositive cells in the basal forebrain [$t = 0.422, \ df = 4, \ P = 0.695$, t-test] or medial septum [$t = 0.591, \ df = 6, \ P = 0.576$, t-test] was found between Y-$m/m$ and Y-$db/db$ mice (Fig. 5: A, B). However, significant differences in the number of ChAT-immunopositive cells in the basal forebrain [$t = 6.724, \ df = 4, \ P = 0.003$, t-test] and medial septum [$t = 5.108, \ df = 4, \ P = 0.007$, t-test] were found between O-$m/m$ and O-$db/db$ mice. On the other hand, the number of ChAT-immunopositive cells in these brain portions of O-$db/db$ mice was significantly reversed to the levels in the O-$m/m$ group by daily administrations of KK (200 mg/kg per day) and THA (2.5 mg/kg per day) (basal forebrain: $F(2,7) = 6.338, \ P = 0.027$, one-way ANOVA; medial septum: $F(2,8) = 7.142, \ P = 0.017$, one-way ANOVA) (Fig. 5).

Effects of THA and KK administration on the ex vivo acetylcholinesterase activity in the brain

On the basis of similar pharmacological effects of THA and KK, we tested the possibility that KK, as well as THA, can inhibit the activity of acetylcholinesterase in the brain, an enzyme implicated in degradation of acetylcholine in the central nervous system. The enzyme activities in the brain with treatments with vehicle, THA (2.5 mg/kg per day, i.p.), and KK (200 mg/kg per day, 238 Q Zhao et al

Fig. 5. Effects of KK and THA treatment on diabetes-induced cholinergic neuron degeneration in the basal forebrain (A-1 and -2) and medial septum (B-1 and -2). A-1 and B-1: Representative brain sections used to observe the changes of cholinergic neurons. Cholinergic neurons were identified by ChAT immunostaining in Y-$m/m$ (a), Y-$db/db$ (b), O-$m/m$ (c), and O-$db/db$ (d), treated with KK (200 mg/kg per day) (e), and THA (2.5 mg/kg per day) (f). Confocal laser scanning images of cholinergic neurons were stained with anti-ChAT antibody, showing that predominant ChAT expression occurred in the cell body of cholinergic neurons. Scale bar = 100 or 200 $\mu$m. A-2 and B-2: Quantitative analyses of the number of ChAT-immunopositive cells in the basal forebrain and medial septum. Each bar represents the mean ± S.D. obtained from 3 – 4 mice in each group. **$P < 0.01$ vs. vehicle-treated O-$m/m$ group (t-test). *$P < 0.05$ vs. vehicle-treated O-$db/db$ group (one-way ANOVA) followed by the Newman-Keuls test. $$$P < 0.001$ vs. Y-$m/m$ and Y-$db/db$ group (two-way ANOVA).
p.o.) were 20.3 ± 2.8, 11.3 ± 2.7, and 17.7 ± 2.6 nmol/ (mg tissue-min) (mean ± S.D., n = 4), respectively. The ex vivo activity of cholinesterase in the cerebral cortex was significantly lower in the THA (2.5 mg/kg per day)-but not KK (200 mg/kg per day)-treated db/db group compared to that in the vehicle-treated db/db group [F_{group}(2,9) = 11.324, P = 0.003, one-way ANOVA; vehicle vs. THA: P < 0.05; vehicle-group vs. KK-group: P ≥ 0.05, post hoc Dunnett’s test].

Discussion

This study aims to clarify if KK has an ameliorative effect on cognitive deficits relevant to diabetes. The results demonstrate that a type 2 diabetic model of db/db exhibits severe cognitive deficits and degeneration of the basal forebrain cholinergic complexes and that KK, as well as tacrine, attenuates diabetes-related cognitive deficits and cholinergic dysfunction. Our findings suggest that KK has a beneficial effect in the treatment of diabetes-induced cognitive deficits.

In this study, we found that the swimming speed of O-db/db mice analyzed during the observation period was almost two times slower than that of the age-matched control strain m/m (Fig. 2C), indicating that O-db/db mice have a reduced ability to swim compared with the control strain. The reduced swimming ability of O-db/db mice observed in this study is probably due to the elevated body weight of this animal group compared with the control strain. This observation is supported by the findings reported by Sharma et al. (23) that adult (10 – 11-week-old) db/db mice are hypolocomotive as evident by a decrease of basic and fine movements. On the basis of this fact, we adopted a 120-s observation period instead of a conventional 60-s observation period (6, 20) for O-db/db mice and age-matched control m/m mice, in the training and probe tests of the water maze task in order to reduce contribution of different swimming abilities to their cognitive performance. This protocol clearly indicated that vehicle-treated O-db/db mice exhibit spatial cognitive deficits compared with age-matched m/m control strain mice. Although there is a conflicting report on spatial cognitive deficits of adult db/db mice (23), the spatial cognitive deficits of diabetic animals revealed in this study are consistent with previous findings (24 – 27). Moreover, it should be noted that administrations of KK and THA ameliorated learning performance in the training test and memory retrieval performance of O-db/db mice in the probe test without affecting swimming speed of this animal group. These data suggest that O-db/db mice treated with THA and KK acquired a strategy to efficiently escape to the platform located in the target quadrant during the training sessions. Considering the present data that neither KK nor THA treatment affects the serum glucose level of db/db mice, which was significantly elevated compared with the level in the age-matched m/m group, our findings obtained using the water maze task raise the possibility that KK as well as the anti-dementia drug THA is beneficial for prevention of diabetes-induced cognitive deficits and that the effects of KK and THA are independent of serum glucose level in db/db mice.

Next we analyzed whether administration of KK and THA to db/db mice affects the expression levels of BDNF mRNA and BDNF protein in the hippocampus, a key brain region implicated in spatial learning and memory performance, since studies including our previous reports (16, 28 – 30) have demonstrated that BDNF plays an important role in cognitive and emotional function in the brain and can be a novel marker of cognitive function. In agreement with the data obtained from other animal models of cognitive deficits (16, 29), vehicle-treated O-db/db mice with severely impaired spatial cognitive performance had significantly lower expression levels of BDNF mRNA and its protein in the hippocampus than the control m/m strain mice. Considering the insignificant difference in these expression levels between Y-db/db and Y-m/m groups, it seemed very likely that the decreases in BDNF mRNA and protein occur in an age-dependent manner. Moreover, it was found that O-db/db mice, the cognitive performance of which had been ameliorated by administrations of THA and KK during an experimental period, had normal expression levels of BDNF mRNA and its protein in the hippocampus. Taken together with the cognitive functional role of BDNF in the hippocampus, the present findings provide molecular biological evidence for the preventive effects of THA and KK on diabetic-induced cognitive deficits.

To have a better understanding of a putative mechanism underlying the action of KK and THA in a diabetic animal model, we next elucidated the effect of THA and KK on the central cholinergic systems since these systems play an important role in cognitive performances and learning and memory processes (16, 29) and the basal forebrain cholinergic complex has been described to undergo degenerative changes during aging and development of dementia such as AD, resulting in cholinergic hypofunction that has been closely related to the progression of memory deficits (1, 2, 31). Indeed, there are several lines of evidence indicating that muscarinic receptors such as M_1, M_3, and M_5 subtypes have an important role in cognitive function in rodents (4, 32 – 35). The present study revealed that there were no significant differences in the expression levels of cholinergic marker proteins in the hippocampus, namely, ChAT and muscarinic M_1, M_3, and M_5 receptors, between Y-m/m and
Y-db/db groups, whereas the expression levels of these marker proteins were down-regulated in O-db/db mice compared with those in the age-matched m/m mice. Consistent with previous findings that diabetic symptoms, such as hyperglycemia and hyperlipidemia (14), appear in a manner dependent on aging, the down-regulation of cholinergic marker expression in the hippocampus was also considered to occur in an aging-dependent manner (1), indicating dysfunction of central cholinergic systems in db/db mice.

Interestingly, the administrations of THA and KK significantly reversed the aging-dependent downregulation of ChAT and M1 and M5 muscarinic receptor subtypes in db/db mice. The administration also showed a tendency to reverse the down-regulated expression of M1 muscarinic receptor but the effect was not significant. The reason for different susceptibilities of these muscarinic receptor subtypes to THA and KK administration is unclear but our findings suggest that dysfunction of central cholinergic systems is accelerated in diabetic animals and that KK and THA treatments may reduce diabetes-induced acceleration of cholinergic dysfunction. This idea is supported by the present immunohistochemical study analyzing ChAT-immunopositive cells in the basal forebrain and medial septum, major cholinergic systems, which provide cholinergic projections to the cerebral cortex and medial septum, respectively. The results demonstrated that O- but not Y-db/db mice had significantly reduced numbers of ChAT-immunopositive cells in these brain regions compared with the age-matched control m/m mice. It has been generally assumed that the cholinergic neurons in the basal forebrain and medial septum undergo moderate degenerative alterations during aging and thereby induce cholinergic hypofunction related to the progression of memory deficit with aging (1). Taken together, a plausible explanation for the present results is that that aging-induced degeneration of central cholinergic systems may be accelerated in diabetic db/db mice and that the acceleration of cholinergic dysfunction at least in part underlies spatial cognitive deficits of db/db mice.

It should be noted that the O-db/db mice, which had received daily administrations of THA and KK from the age of 7 weeks during the experimental period, had significantly larger numbers of ChAT-immunopositive cells in the basal forebrain and medial septum than vehicle-treated db/db mice. These immuno-histochemical findings suggest that THA and KK treatments can negatively modulate mechanisms underlying the acceleration and thereby attenuate aging-induced degeneration of cholinergic neurons in the brain. The present results also seem to explain the mechanism of the anti-dementia effects of THA and KK observed in db/db animals.

The mechanisms underlying the effects of KK and THA on the central cholinergic dysfunction in db/db mice are unclear. The pharmacological profiles of THA as an inhibitor of cholinesterase activity allow us to infer that facilitation of the cholinergic system itself plays a key role in the prevention of diabetes-induced cholinergic dysfunction in the brain. This idea is supported by several in vivo studies. Firstly, Tanaka et al. demonstrated that the stimulation of muscarinic ACh receptors provides neuroprotection against transient ischemia–induced neuronal cell death, even when reperfusion has been carried out in an animal model of transient forebrain ischemia (36, 37). Secondly, we previously found that THA administration reverses chronic cerebral hypoperfusion-induced cognitive deficits and downregulation of genes encoding ChAT and M1 and M5 muscarinic receptors in the brain (4). Moreover, Yang et al. (38) reported the anti-apoptotic ability of the M2-muscarinic receptor and indicated that the poly-basic region in the short C-terminal tail conveys the cytoprotective property of the M2-muscarinic receptor.

In this study, in contrast to the effect of THA, KK administration failed to affect the ex vivo cortical cholinesterase activity in db/db mice, suggesting that the principal target(s) of KK to facilitate central cholinergic systems may differ from that of THA. In fact, there are some reports that support this idea. In our previous study (16), we suggested that the ameliorative effect of KK on aging-induced cognitive deficits is most likely to involve vascular endothelial growth factor (VEGF) systems in the brain, which reportedly play a protective role not only for endothelial cells but also for neuronal cells (39, 40). Therefore, it is likely that such trophic factors also play an important role in the ameliorative effects of KK on diabetes-induced cognitive deficits and neurodegeneration of the central cholinergic system. Possible involvement of such trophic factors in the action of KK in db/db mice is currently under investigation in our laboratory.

It is still unclear which crude drug(s) and/or chemical component(s) included in KK are responsible for the effects on the learning and memory performance and cholinergic neurodegeneration in db/db mice. Previous studies in our laboratory demonstrated that the extract from Paeoniae radix and its major chemical component, paeoniflorin, ameliorate spatial cognitive deficits caused by dysfunction of central cholinergic systems and aging in rodents (41, 42). In fact, the present chemical profiling using LC-MS analysis (see: http://wakandb.u-toyama.ac.jp/wiki/index.php/LCMS:Kangenkaryu) revealed paeoniflorin as one of the major chemical constituents of KK. There are also reports demonstrating that some chemical constituents from Salviae Miltiorrhizae Radix,
tanshinone, and its congeners (43, 44), such as rosmarinic acid (45, 46) and lithospermate B (47), improve cognitive deficits caused by scopolamine, β-amyloid, or aging in rodents. However, the contribution of tanshinone and its congeners to the effect of KK seems to be limited or nonexistent, since their amounts included in the KK extract used in this study were low compared with those of paconiflorin and other water-soluble constituents of KK. Our speculative explanation of the action of KK is that such water-soluble constituents or their metabolites or both are involved in the effects of KK observed in this study. Nevertheless, further investigations are needed to clarify the active chemical constituent(s) involved in the KK-induced amelioration of diabetes-induced impairment of cognitive deficits and central cholinergic dysfunction.

In conclusion, this study demonstrates that KK as well as THA attenuates diabetes-induced cognitive deficits. Neurochemical and immunohistochemical data suggest that the effects of KK involve suppression of accelerated neurodegeneration of central cholinergic systems in db/db mice. Our findings provide further evidence for the anti-dementia effect of KK.

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