Clinical studies have focused on type 2 diabetes mellitus and hypertension as risk factors for cognitive impairment. The detailed mechanisms of diabetes-induced cognitive impairment are not well understood, although it was recognized as early as the 1920s that diabetes may affect cognition. Diabetes and metabolic alterations also seem to be closely associated with consequent impairment of cognitive function in humans, such as in Alzheimer’s disease and vascular dementia. Moreover, an increased prevalence of insulin abnormalities and insulin resistance in Alzheimer’s disease may contribute to the disease pathophysiology and clinical symptoms. Recently, we demonstrated that KK-A’ mice, a genetic mouse model of type 2 diabetes mellitus and obesity, exhibited age-dependent impairment of cognitive function. This mouse model demonstrates age-dependent cognitive impairment and is thought to be suitable for analyzing the cognitive decline associated with diabetes.

Oxidative reactions have been implicated in the pathogenesis of diabetic complications involving changes in the blood-brain barrier and cognitive function. Nifedipine, a dihydropyridine calcium channel blocker (CCB), has been reported to exert pleiotropic effects on atherosclerosis mainly through its antioxidative and inflammatory properties. For example, nifedipine treatment decreased circulating plasma lipoperoxides and isoprostanes and increased plasma antioxidant capacity. We have also reported that administration of nifedipine improved vascular remodeling by inhibiting nuclear factor κB activation and increased endothelial NO bioavailability through antioxidative effects. Moreover, Berr et al. reported that increased levels of oxidative stress and/or antioxidant deficiency cause cognitive decline, indicating that nifedipine may have possible preventive effects on cognitive impairment by improving vascular remodeling by reducing oxidative stress. Furthermore, we reported previously that a CCB improved glucose intolerance mainly through inhibition of oxidative stress. Therefore, we could expect that administration of a CCB, nifedipine, could ameliorate diabetes-associated cognitive decline at least in part because of improvement of vascular remodeling and insulin resistance through its antioxidative effect. However, to date, the effect of nifedipine on diabetes mellitus–induced cognitive decline has never been studied.

Here, we examined the possibility that nifedipine could prevent cognitive decline in a type 2 diabetes mellitus mouse model, KK-A’, with a decrease in superoxide production, and could exert neuroprotective effects. Moreover, we examined
the effects of nifedipine on neural differentiation and its possible mechanisms using neurospheres.

Materials and Methods

This study was performed in accordance with the National Institutes of Health guidelines for the use of experimental animals. All of the animal studies were reviewed and approved by the animal studies committee of Ehime University.

Animals and Treatment

Adult male KK-A’ mice (CLEA, Tokyo, Japan) were used in this study. The animals were housed in a room where lighting was controlled (12 hours on and 12 hours off), and the temperature was kept at 25°C. Mice were fed a standard diet (MF, Oriental Yeast Co, Ltd) with or without a CCB, nifedipine (0.001% in laboratory chow), which was provided by Bayer AG, and water ad libitum. Nifedipine was administered to KK-A’ mice from 10 weeks of age for 7 weeks. We determined the dose of nifedipine (0.0010% in laboratory chow) as a nonhypotensive dose after pretreatment with various doses of nifedipine (0.0010%, 0.0005%, 0.0001%, and 0.00005% in laboratory chow). The final calculation of the daily dose of nifedipine administration, including food intake, was estimated to be 1 mg/kg per day.

Blood Pressure Measurement

Systolic blood pressure was monitored in conscious mice by the tail-cuff method (MK-1030, Muromachi Co, Ltd), as described in a previous report. Mice were held in a small plastic holder on a warming pad thermostatically controlled at 37°C. Measurements were performed before and after nifedipine treatment. Mean systolic blood pressure of 10 measurements in each group was determined by the tail-cuff method before nifedipine treatment and at the end of the experiment.

Passive Avoidance Test

Passive avoidance test was performed as described previously. Briefly, a shuttle avoidance cage (32 × 12.5 × 15 cm, Melqest) and an isolation cabinet (48 × 42 × 37 cm, Melqest) were used. The shuttle avoidance box was divided into equal-size chambers by a stainless steel divider. The floor of the shuttle box consisted of stainless steel rods. Scrambled shocks were delivered by a shock generator (SG-200, Melqest). Mice were individually placed in a chamber and given 20 inescapable electric shocks (0.3 mA) of 3 seconds’ duration, at intervals of 2 seconds. A tone signal was presented during the first 5 seconds of each trial. If there was no avoidance response within this period, the tone signal remained on, and a shock was delivered through the grid floor. In the case of no escape response within this period, both the tone and shock were automatically terminated. The number of escape failures, which was defined as a noncrossing response during shock delivery, was recorded. Avoidance rate was displayed as a comparison with the ratio of avoidance rate to that of 7 weeks of age in KK-A’ mice without nifedipine treatment (depicted as 1).

Measurement of Serum Glucose, Insulin, and Cholesterol Levels

Serum blood glucose level was measured by the glucose oxidase method (Glucose CII-test, WAKO Chemical Industries, Ltd). Insulin level was measured by ELISA (Ultra Sensitive Rat Insulin kit, Morinaga Institute of Biological Science, Inc). Serum cholesterol level was measured by the cholesterol oxidase method (Cholesterol E-test, WAKO Chemical Industries, Ltd).

Real-Time RT-PCR Method

mRNA was extracted from samples of the brain after homogenization in Sepazol (Nacalai Tesque Inc). Real-time quantitative RT-PCR was performed with a SYBR Green I kit (MJ Research, Inc). PCR primers for Id-1 were as follows: 5’-CAGAGGCAGCATG-TGGTCC-3’ (forward) and 5’-TCTGAAAAGAGGACGAC-3’ (reverse).

Detection of Superoxide Anion in Brain Sections

Detection of superoxide anion was carried out as described previously. In brief, frozen, enzymatically intact, 10-μm-thick sections were prepared from mouse brain and immediately incubated in dihydroethidium (10 μmol/L) in PBS for 30 minutes at 37°C in a humidified chamber protected from light. Dihydroethidium (Sigma-Aldrich Corp) is oxidized on reaction with superoxide to ethidium, which binds to DNA in the nucleus and fluoresces red. Samples were examined with an Axioskop microscope (Axioskop 2 Plus with AxiosCam, Carl Zeiss) equipped with a computer-based imaging system. Fluorescence of ethidium was detected with a 500- to 550-nm long-pass filter (Axioskop). The intensity of fluorescence was analyzed and quantified using computer-imaging software (Densitograph, ATTO Corp).

Measurement of Cerebral Blood Flow

Cerebral blood flow was determined by a laser speckle flowmetry (Omega-2000, laser speckle flow imager, Omegawave), which obtains high-resolution 2D images in a matter of seconds, as described previously. Briefly, mice were anesthetized with 65 mg/kg of Nembutal (Abbott Co) in saline IP. The skull was exposed and covered with plastic wrap. A 780-nm laser semiconductor laser illuminated the area of interest. We measured cerebral blood flow in the core region of the right middle cerebral artery territory. Light intensity was accumulated in a charge-coupled device camera and transferred to a computer for analysis. Image pixels were analyzed to produce average perfusion values.

Statistical Analysis

All of the data are expressed as means±SEs in the text and figures. Data were analyzed by 2-way ANOVA. When a statistically significant effect was found, posthoc analysis was performed to detect the difference between the groups. A value of P<0.05 was considered to be statistically significant.

Results

Nifedipine Improved Cognitive Function in KK-A’ Mice

KK-A’ mice exhibited significant failure of improvement in avoidance rate and worsening of its age-dependent decline after 12 weeks of age compared with control C57BL/6 mice. Administration of nifedipine (0.001% in laboratory chow) from 10 weeks of age not only inhibited age-dependent cognitive impairment but also improved cognitive decline to a level similar to that in age-matched C57BL/6 mice in a time-dependent manner (Figure 1). Treatment with nifedipine at this dose did not significantly change systolic blood pressure compared with that in KK-A’ mice without nifedipine treatment (Table 1). Moreover, no significant difference in average body weight was observed between KK-A’ mice treated with and without nifedipine (Table 2).

Serum Insulin Level Was Significantly Decreased by Nifedipine Treatment

Blood samples were obtained from 17-week-old mice at the end of the experiment. Administration of nifedipine markedly suppressed the increase in plasma insulin concentration (Figure 2A). However, serum glucose concentration showed no remarkable difference in KK-A’ mice with and without nifedipine treatment (Figure 2B). Moreover, serum cholesterol concentration also showed no difference in KK-A’ mice with and without nifedipine treatment (data not shown).
Treatment With Nifedipine Attenuated Superoxide Anion Production in the Brain

Next, we assessed superoxide anion production in the brain using dihydroethidium staining. In the cortex of the brain from KK-Ay mice, superoxide production was significantly increased compared with that in control C57BL/6 mice (Figure 3). Treatment with nifedipine significantly attenuated this increase in superoxide anion production. However, the levels of inflammatory cytokines, such as tumor necrosis factor-\(\alpha\) and monocyte chemotactic protein-1 in the brain were not significantly different in these groups (Figure 4).

Treatment With Nifedipine Decreased Id-1 mRNA Expression in the Hippocampus

The inhibitor of DNA binding (Id) proteins contribute to the regulation of the mammalian nervous system development.\(^{20}\) We focused on the hippocampus, which is important for learning ability and memory. In KK-A\(^{+}\) mice, treatment with nifedipine decreased the mRNA level of Id-1 in the brain hippocampus (Figure 5).

Treatment With Nifedipine Increased Cerebral Blood Flow

Finally, we assessed the effect of nifedipine treatment on cerebral blood flow using a laser speckle flowmetry. Mean blood flow in the brain was significantly increased in KK-A\(^{+}\) mice treated with nifedipine compared with KK-A\(^{+}\) mice without nifedipine treatment (Figure 6A and 6B).

Table 1. Systolic Blood Pressure (mm Hg) in KK-A\(^{+}\) Mice With or Without Nifedipine

<table>
<thead>
<tr>
<th>Groups</th>
<th>10 wk</th>
<th>17 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>KK-A(^{+})</td>
<td>96.9±3.4</td>
<td>98.9±1.7</td>
</tr>
<tr>
<td>KK-A(^{+}) with nifedipine</td>
<td>93.3±2.4</td>
<td>94.2±3.0</td>
</tr>
</tbody>
</table>

No significant change was observed between the 2 groups.

Table 2. Body Weight (g) in KK-A\(^{+}\) Mice With or Without Nifedipine

<table>
<thead>
<tr>
<th>Groups</th>
<th>10 wk</th>
<th>17 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>KK-A(^{+})</td>
<td>37.5±1.1</td>
<td>41.1±0.9</td>
</tr>
<tr>
<td>KK-A(^{+}) with nifedipine</td>
<td>37.4±1.3</td>
<td>40.8±1.0</td>
</tr>
</tbody>
</table>

No significant change was observed between the 2 groups.

Discussion

Metabolic syndrome–induced cognitive impairment has recently received much attention. In particular, type 2 diabetes mellitus–induced cognitive decline has been widely recognized clinically. However, its detailed pathogenesis is not well understood. Here, we demonstrated that nifedipine inhibits diabetes mellitus–associated cognitive impairment using KK-A\(^{+}\) mice through a reduction of serum insulin level, attenuation of superoxide production in the brain, and reduction of the inhibitor protein of neural differentiation, Id-1.

Clinical and experimental studies indeed show that altered glucose regulation impairs learning and memory.\(^{21}\) Moreover, neurologic recovery from closed head injury is impaired in diabetic rats.\(^{22}\) However, it is reported that memory is not enhanced during hyperglycemia when endogenous insulin release is suppressed.\(^{23}\) Nifedipine treatment decreased the serum insulin level to one fifth of that in KK-A\(^{+}\) mice without treatment, whereas the serum glucose level was not different in KK-A\(^{+}\) mice with and without nifedipine treatment, indicating that nifedipine improved hyperinsulinemia and could possibly attenuate insulin resistance in KK-A\(^{+}\) mice but with no apparent change in serum glucose level. Plasma glucose level is known to be influenced by not only by insulin but also by other factors, such as hepatic glucose uptake and the absorption of polysaccharides. Clinically, hyperinsulinemia is related to a significant decline in memory-related cognitive score.\(^{24}\)

Insulin transported across the blood-brain barrier by a saturable insulin receptor–mediated transport process is considered to facilitate memory; however, persistent hyperinsulinemia downregulates blood-brain barrier insulin receptors and reduces transport into the brain, thereby resulting in a cognitive decline.\(^{25,26}\) We did not examine insulin receptor expression in the blood-brain barrier and insulin level in the cerebrospinal fluid. However, it is possible that attenuation of hyperinsulinemia could con-
tribute to the beneficial effects of nifedipine treatment on cognitive impairment in KK-Ay mice.

Rat models of spontaneous type 1 and type 2 diabetes exhibit Alzheimer-like changes, such as accumulation of amyloid precursor protein and β-amyloid.27 These changes were more marked in a type 2 diabetes animal model than in a type 1 model. Moreover, diet-induced insulin resistance promoted amyloidosis in a transgenic mouse model of Alzheimer disease,28 and, thereby, insulin resistance promoted Alzheimer-like changes in the brain. Further investigation of amyloid deposition in the brain in KK-Ay mice may provide new insights into insulin-induced cognitive decline. Moreover, hyperinsulinemia and insulin resistance, the so-called metabolic syndrome, are also closely related to vascular injury. A population-based epidemiologic study conducted in the Chianti geographic area (Invecchiare in CHIANTI Study) indicated that insulin resistance might contribute to cognitive impairment through a vascular mechanism.29 Here, we have not assessed the effect of nifedipine on vascular function in KK-Ay mice; however, improvement in hyperinsulinemia by nifedipine treatment could prevent cerebrovascular damage, thereby resulting in protection of neurons. These possibilities need to be clarified in more detail.

Administration of nifedipine significantly attenuated superoxide anion production in the brain. Oxidative stress leads to the onset and subsequent complications of type 2 diabetes mellitus and enhances insulin resistance, neural damage, vascular dysfunction, and cognitive decline. For example, a dramatic loss of learning and memory function was observed in mice with large increases in brain oxidative stress, whereas an antioxidative treatment with low doses of superoxide dismutase/catalase mimetics almost completely reversed the behavioral changes.30 We also demonstrated here that basic helix-loop-helix transcription factors, such as Id-1, which is known as an inhibitor of differentiation genes, were downregulated after nifedipine treatment. Downregulation of this protein may be required for initiation of a regenerative response to axonal injury.31 We examined the effect of nifedipine on the Id-1 mRNA level in neurospheres generated from fetal mouse brain cortex (data not shown), but we did not observe significant changes, suggesting the decrease in

Figure 3. Nifedipine treatment attenuated superoxide anion production in the brain. A, Representative dihydroethidium staining of the brain. B, Histogram analysis of superoxide anion production. *P<0.05 vs C57BL6; †P<0.05 vs KK-Ay mice without nifedipine. n=4 for each group.

Figure 4. Comparison of mRNA levels of inflammatory cytokines, tumor necrosis factor (TNF)-α and monocyte chemoketic protein (MCP)-1. Expression of TNF-α (A) and of MCP-1 (B) in the brain were measured by quantitative RT-PCR. No significant change was observed in the 2 groups. n=5 for each group.

Figure 5. Treatment with nifedipine reduced expression of Id-1 in vivo. Expression of Id-1 in the brain was measured by quantitative RT-PCR. *P<0.05 vs KK-Ay mice without nifedipine. n=5 for each group.
Id-1 mRNA observed in vivo could not be induced by a direct effect of nifedipine on the neuron. For example, insulin-induced hypoglycemia increases mRNA expression of Id-1 in the cortex, indicating that Id-1 expression may be regulated by serum glucose level. Moreover, in cardiomyocytes, the expression of Id-1 leads to the induction of apoptosis through a redox-dependent mechanism. Interestingly, the expression of Id-1 in neonatal cardiac myocytes resulted in an increase in the level of intracellular reactive oxygen species, indicating that a decrease in Id-1 may attenuate oxidative stress in neural cells. Therefore, downregulation of Id-1 could further attenuate oxidative stress in the brain. These results suggest that nifedipine treatment caused a marked decrease in radical stress by multiple mechanisms, resulting in brain protection.

Calcium channel antagonists are reported to enhance learning and memory in schizophrenic patients with tardive dyskinesia. However, this clinical report was only a case report, and the basic mechanism has never been investigated. The calcium hypothesis of brain ageing and dementia has been put forward to account for a number of phenomena in the pathogenesis of dementia. Interestingly, 1-type channel blockers, such as nimodipine and nifedipine, are able to protect against the development of diabetic peripheral neuropathy in rats. CCBs also increase cerebral blood flow and may preserve microvascular integrity in the brain. Chronic treatment with nifedipine in stroke-prone spontaneously hypertensive rats can prevent the progression of microvascular damage by reducing the cerebral capillaries. Patients with Alzheimer’s disease exhibited microvascular alterations characterized by thinning and fragmentation of the basal lamina, indicating that alteration of microvascular circulation in the brain by nifedipine also affects cognitive decline. We have not yet investigated this, and further investigation is necessary to assess whether there is a change of cerebral blood flow in KK-Ay mice after nifedipine treatment.

**Perspectives**

Our results suggest that nifedipine ameliorates impaired cognitive function in type 2 diabetic mice, at least because of reduced superoxide production in the brain and differentiation inhibitor factor, Id-1, without lowering blood pressure. Therefore, nifedipine could be useful to improve the quality of life in persons who have insulin resistance through inhibition of cognitive decline. In addition, we reported recently that the angiotensin II type 1 receptor blocker candesartan ameliorates the impaired cognitive function in KK-Ay mice, at least because of an increased expression of MMS2, a neuroprotective factor, in addition to improvement of glucose intolerance. Therefore, we could expect that a combination of angiotensin II type 1 receptor blocker and CCBs in hypertensive patients with diabetes could exert additive and/or synergistic inhibitory effects on cognitive decline.

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**Disclosures**

None.

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