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# Amelioration of Cognitive Impairment in the Type-2 Diabetic Mouse by the Angiotensin II Type-1 Receptor Blocker Candesartan

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**Abstract**—Angiotensin II type-1 receptor blockers are widely used with the expectation of prevention of stroke, potential effects to ameliorate of type-2 diabetes, which seems to be closely associated with the impairment of cognitive function in humans. Recently, we have reported that an angiotensin II type-1 receptor blocker prevented cognitive impairment in mice after focal cerebral ischemia, at least partly through an angiotensin II type-2 receptor-mediated increase in a neuroprotective factor, methyl methanesulfonate sensitive-2. Here, we examined the possibility that an angiotensin II type-1 receptor blocker could improve cognitive function in a type-2 diabetic mouse model, KK-A<sup>y</sup>. KK-A<sup>y</sup> mice subjected to 20 trials of a passive avoidance task every week from 8 weeks exhibited a significantly impaired avoidance rate, and moreover, its age-dependent decline, especially after 14 weeks of age, compared with age-matched C57BL6 mice. Oral administration of candesartan at a nonhypotensive dose (0.005% in laboratory chow) in KK-A<sup>y</sup> mice improved cognitive function and inhibited the impairment of cognitive decline. Methyl methanesulfonate sensitive-2 expression in the brain was lower in KK-A<sup>y</sup> mice than in C57BL6 mice. Treatment with candesartan markedly increased mRNA expression of angiotensin II type-2 receptor and methyl methanesulfonate sensitive-2 in the brain in KK-A<sup>y</sup> mice, determined by quantitative RT-PCR. In KK-A<sup>y</sup> mice treated with candesartan, age-dependent increases in blood glucose and insulin were significantly suppressed. Our results suggest that candesartan ameliorates the impaired cognitive function in type-2 diabetes mice, at least because of an increased expression of methyl methanesulfonate sensitive-2, a neuroprotective factor, in addition to improvement of glucose intolerance. (*Hypertension*. 2007;50:1099-1105.)

**Key Words:** angiotensin II receptors ■ type-2 diabetes mellitus ■ cognitive impairment

Type 2 diabetes mellitus (T2DM) and hypertension have been highlighted as risk factors for cognitive decline in several clinical studies.<sup>1–3</sup> The increased risk of dementia associated with T2DM relates to both Alzheimer disease and vascular dementia.<sup>4</sup> Recently, Yaffe et al<sup>2</sup> reported that diabetic, as well as prediabetic, women have impaired cognitive function and a greater risk of developing cognitive impairment. Moreover, an emerging body of evidence suggests that an increased prevalence of insulin abnormalities and insulin resistance in Alzheimer disease may contribute to the disease pathophysiology and clinical symptoms.<sup>5</sup> Hyperinsulinemia observed in patients with impaired glucose tolerance (IGT) has also been focused on as an important risk factor for diminished cognition,<sup>6,7</sup> indicating that patients with T2DM or IGT would be highly exposed to the possibility of impaired cognitive performance. There is concern that the number of individuals diagnosed with T2DM are estimated to increase to 220 million by 2010,<sup>8</sup> and the number of people with undiagnosed diabetes or IGT will increase much

more, suggesting that T2DM- or IGT-induced cognitive decline will become a major worldwide clinical problem in the future. However, the detailed mechanism and therapeutic approach for cognitive impairment associated with T2DM or glucose intolerance, especially using a mouse model, have never been investigated.

Recent major clinical research, such as the Heart Outcomes Prevention Evaluation,<sup>9</sup> Losartan Intervention for Endpoint Reduction,<sup>10</sup> Acute Candesartan Cilxetil Therapy in Stroke Survivors,<sup>11</sup> Morbidity and Mortality After Stroke,<sup>12</sup> and Jikei Heart Study,<sup>13</sup> indicates that blockade of the renin-angiotensin system, especially by an angiotensin II type-1 receptor blocker (ARB), is effective to prevent a first or recurrent stroke or consequent cardiovascular events. ARBs are also widely used with the expectation of potential amelioration of the metabolic syndrome, which is defined as a cluster of obesity, high blood pressure, increase in glucose level including IGT, and dyslipidemia and appears to increase the incidence of cardiovascular diseases and stroke. Recently,

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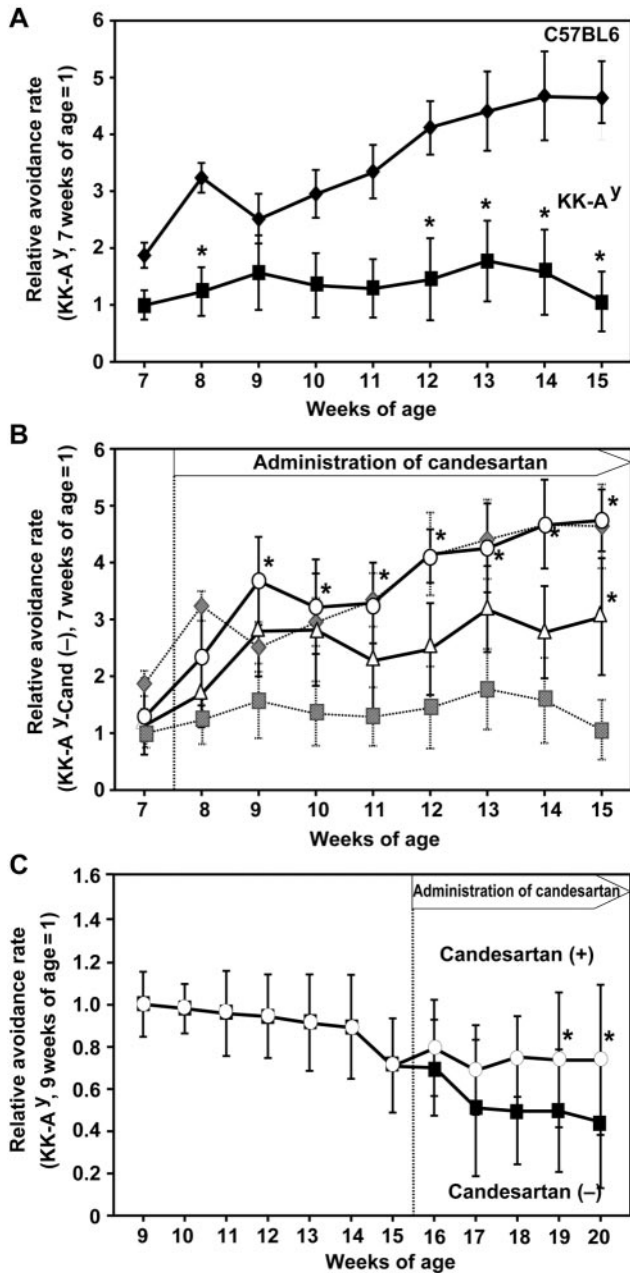
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**Figure 1.** KK-A<sup>y</sup> mice exhibited significant impairment of cognitive function after stroke. Passive avoidance tasks were performed weekly from 6 weeks old. Mice were given 20 inescapable scrambled shocks. Candesartan was administered from 8 weeks old (0.001% or 0.005% in laboratory chow) or 15 weeks old (0.005% in laboratory chow). A, Comparison of avoidance rate between C57BL6 and KK-A<sup>y</sup> mice. ◆, C57BL6 mice; ■, KK-A<sup>y</sup> mice. \*P<0.05 vs C57BL6 mice. n=10 for each group. Comparison with the ratio of avoidance rate to that of 7 weeks of age in KK-A<sup>y</sup> mice without candesartan treatment (depicted as 1). B, Improvement of avoidance rate by treatment with candesartan from 8 weeks old. ◆, C57BL6 mice; ■, KK-A<sup>y</sup> mice; △, KK-A<sup>y</sup> mice with candesartan (0.001% in laboratory chow); ○, KK-A<sup>y</sup> mice with candesartan (0.005% in laboratory chow). \*P<0.05 vs KK-A<sup>y</sup> mice without candesartan. n=7 to 10 for each group. Comparison with the ratio of avoidance rate to that of 7 weeks of age in KK-A<sup>y</sup> mice without candesartan treatment (depicted as 1). C, Beneficial inhibitory effect of treatment with candesartan from 15 weeks old on impairment of avoidance rate. ■, KK-A<sup>y</sup> mice; ○, KK-A<sup>y</sup> mice with candesartan (0.005% in laboratory chow). \*P<0.05 vs KK-A<sup>y</sup> mice without candesartan.

**Table 1. Blood Pressure in KK-A<sup>y</sup> Mice After 7 Weeks of Treatment With or Without Candesartan From 8 Weeks of Age**

Groups	Systolic Blood Pressure, mm Hg
C57BL6	90.7±2.0
KK-A <sup>y</sup>	94.5±5.0
KK-A <sup>y</sup> Cand-1	89.4±3.6
KK-A <sup>y</sup> Cand-5	85.6±4.8

Cand-1 indicates candesartan (0.001%); Cand-5, candesartan (0.005%). No significant change was observed among these groups.

large clinical trials indicate that new onset of diabetes would be prevented by ARB<sup>14,15</sup> probably by an improvement of insulin sensitivity and glucose metabolism, although the detailed mechanisms are still waiting to be elucidated. We reported previously that an ARB increased insulin sensitivity with a decrease in plasma glucose concentration via an increase in insulin-mediated glucose transporter translocation to the plasma membrane in a T2DM mouse model, KK-A<sup>y</sup>,<sup>16</sup> indicating that ARB may have dual effects of brain protection and amelioration of the metabolic syndrome.

Recently, we have also reported that an ARB prevented cognitive impairment in mice after focal cerebral ischemia at least partly, with an angiotensin II type-2 (AT<sub>2</sub>) receptor-mediated increase in a neuroprotective factor, methyl methanesulfonate sensitive 2 (MMS2), which is one of the ubiquitin-conjugating enzyme variants,<sup>17</sup> indicating that treatment with an ARB could be therapeutically effective to prevent cognitive decline in T2DM patients. However, the effect of ARB on cognitive function in patients with T2DM is not well known. Here, we examined the possibility that an ARB could improve cognitive function in genetic T2DM model mice, KK-A<sup>y</sup>.

**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<sup>y</sup> mice and C57BL6 mice (CLEA, Tokyo, Japan) were used in this study. Mice were fed a powdered diet (Oriental Yeast Co Ltd) with or without a selective ARB, candesartan (provided by Takeda Pharmaceutical Co Ltd), and water ad libitum. We chose 2 different doses of candesartan (0.001% or 0.005% in laboratory chow) in terms of glucose metabolism as reported previously.<sup>18</sup> We performed 2 types of experiments as follows. In a short-term experiment, mice were treated with candesartan for 5 weeks from 15 weeks of age, at which point KK-A<sup>y</sup> mice showed a marked cognitive decline. In a long-term experiment, the mice were treated with candesartan for 7 weeks from 8 weeks of age. Plasma cholesterol level was measured by the cholesterol oxidase method (Cholesterol E-test, WAKO Chemical Industries, Ltd), and 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).

**Figure 1 (Continued).** n=5 for each group. Comparison with the ratio of avoidance rate to that of 9 weeks of age in KK-A<sup>y</sup> mice without candesartan treatment (depicted as 1).

**Table 2. Blood Pressure in KK-A<sup>y</sup> Mice After 5 Weeks of Treatment With or Without Candesartan From 15 Weeks of Age**

Groups	Systolic Blood Pressure, mm Hg
C57BL6	96.8±1.1
KK-A <sup>y</sup>	97.8±6.7
KK-A <sup>y</sup> with Cand	93.7±4.5

Cand indicates candesartan (0.005%). No significant change was observed among these groups.

### Blood Pressure Measurement

Systolic blood pressure was monitored in conscious mice by the tail-cuff method (MK-1030, Muromachi Co Ltd) twice before candesartan treatment and at the “end” of each experiment, as described in a previous report.<sup>19</sup> Mice were held in a small plastic holder on a warming pad that was thermostatically controlled at 37°C. Mean systolic blood pressure of 10 measurements in each group was determined.

### Passive Avoidance Test

A passive avoidance test was performed as described previously.<sup>17</sup> Briefly, a shuttle avoidance cage (32×12.5×15 cm; Melquest) and an isolation cabinet (48×42×37 cm; Melquest) were used. The shuttle avoidance box was divided into equal-size chambers by a stainless-steel divider. Scrambled shocks were delivered by a shock generator (SG-200, Melquest). 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.

### Real-Time RT-PCR Method

Real-time quantitative RT-PCR was performed with a SYBR green I kit (MJ Research, Inc). PCR primers were shown in Table S1 (available online at <http://hyper.ahajournals.org>).

### Statistical Analysis

All of the data are expressed as mean±SE in the text and figure. 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

### Candesartan Improved Cognitive Function and Inhibited Cognitive Decline in KK-A<sup>y</sup> Mice

KK-A<sup>y</sup> mice exhibited significant failure of improvement in the avoidance rate and its time-dependent decline after 14

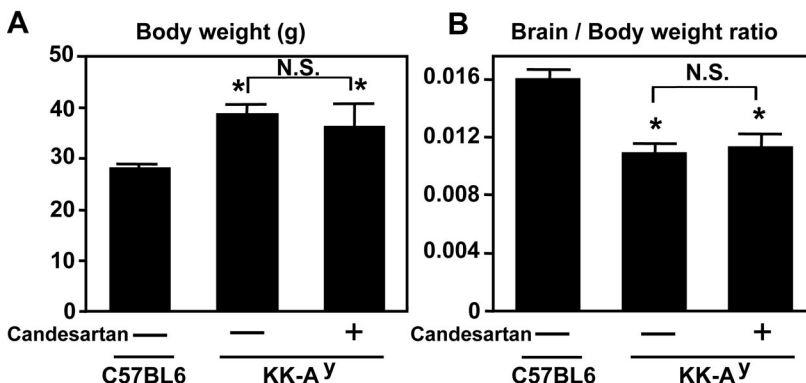
weeks of age compared with age-matched C57BL6 mice (Figure 1A). To examine the possibilities that candesartan could prevent and/or improve cognitive decline, we administered candesartan in 2 different ways, from 8 weeks of age and from 15 weeks of age. In KK-A<sup>y</sup> mice, administration of a higher dose of candesartan (0.005% in laboratory chow) from 8 weeks of age markedly improved cognitive function to a level similar to that in age-matched C57BL6 mice, and administration of a lower dose of candesartan (0.001% in laboratory chow) from 8 weeks of age also significantly restored cognitive function (Figure 1B). Blood pressure was not significantly changed in mice treated with candesartan from 8 weeks of age (Table 1). KK-A<sup>y</sup> mice showed severe cognitive decline at ≈15 weeks of age, and administration of candesartan (0.005% in laboratory chow) starting from 15 weeks of age inhibited this cognitive decline (Figure 1C), with no apparent change in blood pressure (Table 2). Body weight was higher in KK-A<sup>y</sup> mice, and brain:body weight ratio was lower in KK-A<sup>y</sup> mice at 21 weeks of age (Figure 2), with no apparent difference in brain weight between the 2 groups. Administration of candesartan (0.005% in laboratory chow) did not influence body weight or the brain:body weight ratio (Figure 2).

### Treatment With Candesartan Increased MMS2 Expression in KK-A<sup>y</sup> Mice

Next, we assessed the effect of candesartan on mRNA expression determined by quantitative RT-PCR in the brain of mice with or without administration of candesartan (0.005% in laboratory chow) starting from 15 weeks of age. Brain samples were obtained from 21-week-old mice at the end of the experiment in the late treatment group. In KK-A<sup>y</sup> mice, mRNA expression of the AT<sub>2</sub> receptor and MMS2 in the brain was lower compared with that in C57BL6 mice (Figure 3). Treatment with candesartan markedly increased mRNA expression of the AT<sub>2</sub> receptor (Figure 3A) and MMS2 (Figure 3B) in the brain of KK-A<sup>y</sup> mice. On the other hand, mRNA expression of the angiotensin II type-1 receptor in the brain was not significantly different between each group (data not shown).

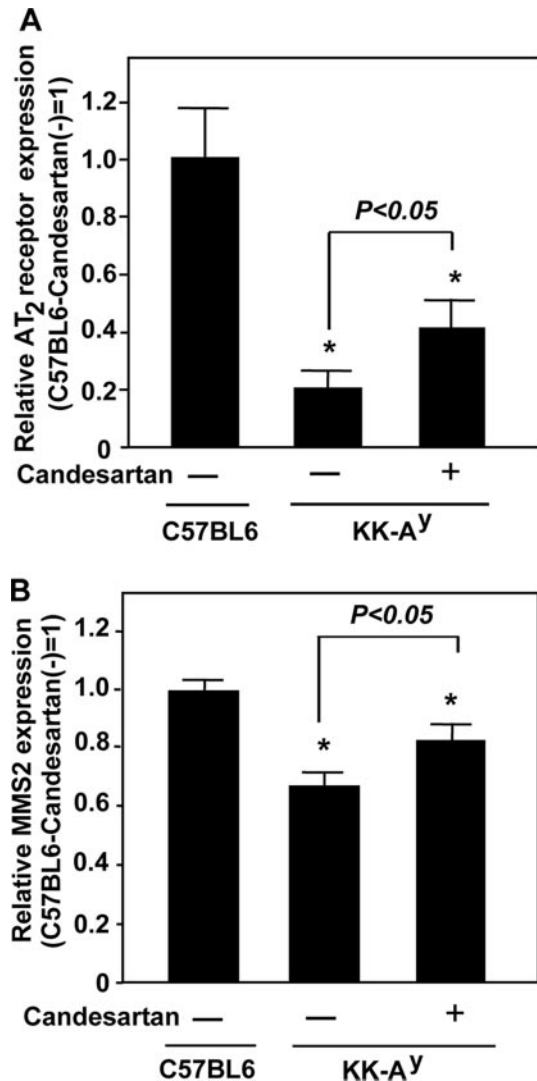
### Effect of Candesartan on Plasma Glucose and Insulin Concentrations in KK-A<sup>y</sup> Mice

In KK-A<sup>y</sup> mice, age-dependent increases in blood glucose and insulin levels were observed compared with C57BL6 mice. Blood samples were obtained from 21-week-old mice in the late-treatment group. Treatment with candesartan



**Figure 2.** Comparison of body weight (A) and brain:body weight ratio (B) between C57BL6 mice and KK-A<sup>y</sup> mice (21 weeks old) treated with or without candesartan (0.005% in laboratory chow). \* $P<0.05$  vs C57BL6 mice.  $n=5$  to 6 for each group.





**Figure 3.** Effect of candesartan on AT<sub>2</sub> receptor and MMS2 mRNA expression determined by the real-time PCR method. Comparison of AT<sub>2</sub> receptor (A) and MMS2 (B) between C57BL6 mice and KK-A<sup>y</sup> mice (21 weeks old) treated with or without candesartan (0.005% in laboratory chow). \**P*<0.05 vs C57BL6 mice. *n*=5 to 6 for each group.

sartan significantly suppressed such an increase in serum glucose and insulin (Figure 4A and 4B). We reported recently that treatment with candesartan suppressed the increase in the plasma glucose level in the oral glucose tolerance test without a significant change in the insulin concentration and glucose uptake, determined by 2-[<sup>3</sup>H] deoxyglucose uptake, in 10-week-old mice treated with candesartan for 2 weeks, an significantly increased in the brain of KK-A<sup>y</sup> mice and in adipose tissues, skeletal muscle, and heart,<sup>18</sup> indicating that treatment with candesartan improved the insulin resistance. Interestingly, KK-A<sup>y</sup> mice treated with candesartan showed a significant reduction of serum cholesterol level (Figure 4C).

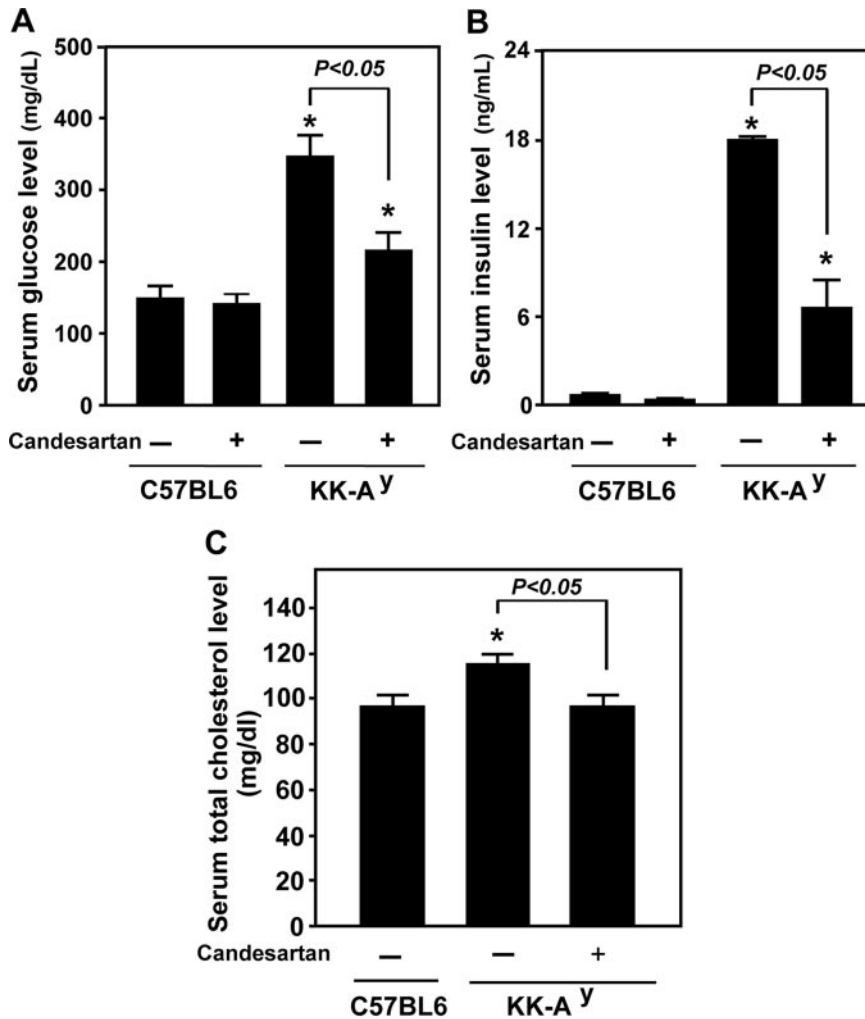
#### Effect of Candesartan on NADPH Oxidase Subunits in KK-A<sup>y</sup> Mice

Next, we assessed the involvement of oxidative stress in the cognitive decline in KK-A<sup>y</sup> mice. We measured mRNA levels

of reduced nicotinamide-adenine dinucleotide phosphate oxidase subunits, p47<sup>phox</sup> and Rac-1, in the brain by quantitative RT-PCR. In KK-A<sup>y</sup> mice, mRNA expressions of p47<sup>phox</sup> and Rac-1 have a tendency to increase compared with C57BL6 mice; however, no significant changes of these expressions were observed with candesartan treatment in KK-A<sup>y</sup> mice (Figure 5A and 5B).

#### Discussion

Our results demonstrated that cognitive function declined age dependently in T2DM mice, KK-A<sup>y</sup>, and administration of candesartan ameliorated the impaired cognitive function in T2DM mice, with no change in blood pressure, partly because of multitherapeutic efficacy, such as an improvement of glucose intolerance and an increase in the expression of a neural differentiation gene, MMS2. Metabolic syndrome-induced cognitive impairment has recently received attention. In particular, T2DM-induced cognitive decline has been widely recognized clinically. However, its mechanism is complicated and not well understood. Several mechanisms have been considered, such as the effects of prolonged hyperglycemia, hyperinsulinemia, associated hypertension, and cerebrovascular disease. Early findings suggest some beneficial effect on cognition to be gained through improving glycemic control, and the potential for preventing a downward spiral of cognitive impairment, decreased medication compliance, and worsening control should always be considered. Insulin resistance is a potentially modifiable midlife risk factor for cognitive decline and dementia.<sup>20</sup> Moreover, diabetes mellitus is associated with decreased cognitive function and greater cognitive decline, especially in women,<sup>2,21</sup> indicating that improvement of glucose metabolism could prevent cognitive decline. Microcirculation disorders and endothelial dysfunction are thought to be involved in cognitive impairment in T2DM patients. Treatment with candesartan is reported to improve tonic NO release and resulted in inhibition of endothelial dysfunction.<sup>22</sup> Moreover, treatment with candesartan decreased cerebral vascular inflammation,<sup>23</sup> decreased the macrophage infiltration, and increased inflammatory cytokines<sup>24</sup> in the spontaneously hypertensive rat. Oxidative stress is well known to induce brain aging and neurodegenerative disease.<sup>25</sup> An increase in advanced glycation end-product formation and aldose reductase-polyol pathway flux is reported to be among the main mechanisms induced by oxidative stress and involved in end-organ damage in diabetes mellitus.<sup>26,27</sup> However, we found no significant difference in the expressions of reduced nicotinamide-adenine dinucleotide phosphate oxidase subunits in the brain between C57BL6 and KK-A<sup>y</sup> mice, and candesartan did not affect these expressions, indicating that prevention of oxidative stress does not apparently play a major role in improving of cognitive decline in KK-A<sup>y</sup> mice by candesartan. However, more detailed analysis of oxidative stress, such as superoxide anion production, reduced nicotinamide-adenine dinucleotide phosphate oxidase activities, and scavenging system, etc, are necessary for further discussion.

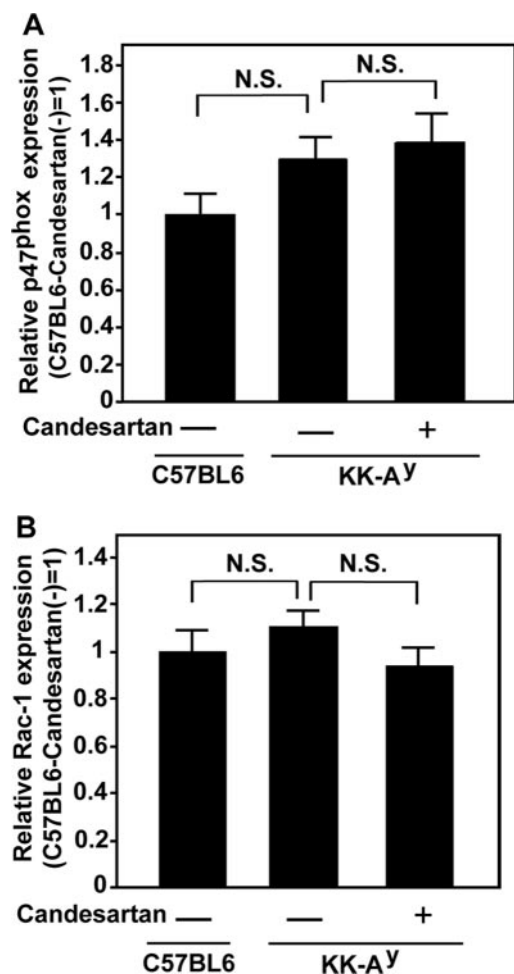


**Figure 4.** Effect of candesartan on serum glucose, insulin, and total cholesterol levels. Comparison of serum levels of glucose (A), insulin (B), and total cholesterol (C) between C57BL6 mice and KK-A<sup>y</sup> mice (21 weeks old) treated with or without candesartan (0.005% in laboratory chow). \**P*<0.05 vs C57BL6 mice. n=5 to 6 for each group.

Our findings also showed a reduction of serum cholesterol level in KK-A<sup>y</sup> mice treated with candesartan. Hypercholesterolemia was independently correlated with memory dysfunction in a stroke-free cohort.<sup>28</sup> An ARB, olmesartan, has been reported to reduce the plasma cholesterol level in angiotensin II-infused, fructose-rich, chow-fed rats.<sup>29</sup> On the other hand, an AT<sub>2</sub> receptor agonist, CGP42112A, significantly reduced total cholesterol and non-high-density lipoprotein cholesterol in similarly treated rats,<sup>29</sup> indicating that angiotensin II receptor signaling partly works as a determinant of cholesterol levels. Moreover, many reports support that blockade of the renin-angiotensin system improves the metabolic syndrome.<sup>30</sup> We reported that treatment with candesartan increased the expression of adiponectin and decreased tumor necrosis factor- $\alpha$  expression in adipose tissue in KK-A<sup>y</sup> mice.<sup>18</sup> Similarly, it has been reported that candesartan treatment decreased serum leptin levels and tumor necrosis factor- $\alpha$  mRNA expression and increased serum adiponectin levels in Wistar Kyoto rats.<sup>31</sup> In contrast, Erbe et al<sup>32</sup> demonstrated that oral administration of candesartan for 4 days did not affect either glucose or insulin levels in *ob/ob* mice; however, this apparent discrepancy could be mainly because of the duration of candesartan treatment and probably because of the differences in mouse models. These

results indicate that candesartan could improve lipid metabolism and adipocyte differentiation and result in improvement of cognitive function in KK-A<sup>y</sup> mice.

Recently, we reported the contribution of AT<sub>2</sub> receptor signaling to neural differentiation, with upregulation of MMS2.<sup>17</sup> MMS2 is one of the ubiquitin-conjugating enzyme variants and is related to neural differentiation<sup>33</sup> and DNA repair.<sup>34</sup> MMS2 promotes error-free bypass in the RAD6 pathway of DNA damage tolerance.<sup>35</sup> In patients with T2DM, urinary 8-hydroxy-2'-deoxyguanosine, a sensitive biomarker of oxidative DNA damage, has been reported to be increased.<sup>36</sup> Our recent report demonstrated that MMS2 expression is increased in pathological conditions such as stroke and that knocking down of the MMS2 gene using small-interference RNA in vivo enhanced the decline in cognitive function after brain damage,<sup>17</sup> indicating that MMS2 plays a pivotal role in cognition under critical conditions. The MMS2 mRNA level was increased by candesartan treatment in the brain of KK-A<sup>y</sup> mice. Therefore, upregulation of MMS2 may protect diabetes-associated brain damage with preventive effects on DNA damage. We speculate on the possibility that AT<sub>2</sub> receptor-induced MMS2 upregulation may contribute to the improvement of cognitive function in KK-A<sup>y</sup> mice. However, the exact localizations of the AT<sub>2</sub> receptor and



**Figure 5.** Comparison of mRNA levels of p47<sup>phox</sup> (A) and Rac-1 (B) in the brain measured by quantitative RT-PCR. No significant change was observed in 3 groups. n=5 for each group.

MMS2 have to be elucidated in KK-A<sup>y</sup> mice, and the effects of AT<sub>2</sub> receptor stimulation in the brain in KK-A<sup>y</sup> mice on insulin sensitivity have to be elucidated. Therefore, it is still immature to conclude that an increase in the AT<sub>2</sub> receptor in KK-A<sup>y</sup> mouse brain could contribute to the improvement of cognitive function by candesartan. In the future, more detailed studies are necessary to discuss the roles of AT<sub>2</sub> receptor stimulation in cognition in diabetes.

We could not observe the significant change in blood pressure measured by the tail-cuff method between KK-A<sup>y</sup> mice with or without candesartan. However, ≈10% reduction in blood pressure was observed in 0.005% of candesartan-treated KK-A<sup>y</sup> mice. Therefore, it is possible that this reduction of blood pressure in our experiments could contribute to the inhibitory effect of candesartan on cognitive impairment in diabetic mice. Moreover, clinically, poor blood pressure control may affect cognitive function in patients with diabetes. This animal model did not show hypertension during the experiment. Therefore, this study design using KK-A<sup>y</sup> mice is different from clinical cases and includes experimental limitation. Moreover, KK-A<sup>y</sup> mice show obesity. Therefore, more clinically relevant experiments to assess the clinical importance of

our observation related more to T2DM-associated cognitive decline need to be designed in the future.

Clinically, recent the large clinical trial in the Jikei Heart Study demonstrated that the addition of the ARB valsartan reduced 40% in stroke onset compared with supplementary conventional treatment without ARB independent of blood pressure reduction.<sup>13</sup> Furthermore, a double-blind clinical trial<sup>37</sup> proved that an ARB, losartan, has an additional therapeutic effect on impaired cognitive function beyond its antihypertensive effect, compared with another antihypertensive drug, hydrochlorothiazide, indicating a contribution of ARB to brain protection involving the prevention of cognitive impairment. The present study demonstrated T2DM-associated cognitive impairment and an inhibitory effect of an ARB on this disorder using a mouse model. Further clinical investigations are necessary to confirm this beneficial effect of candesartan in the future.

### Perspectives

Our results demonstrated that candesartan ameliorates the impaired cognitive function induced by T2DM, with multiple beneficial effects. Hundreds of millions of people would be faced with T2DM in the future. Moreover, cognitive decline associated with T2DM would impair the quality of life in patients with T2DM, indicating that a preventive approach for T2DM-induced cognitive impairment will be the worldwide critical issue. Therefore, an interventional approach by ARB is expected to contribute to the inhibition of T2DM-associated cognitive impairment and to improve the quality of life for elderly persons.

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### Disclosures

None.

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