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Age-related decrease in constructive activation of Akt/PKB in SAMP10 hippocampus

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ABSTRACT

Aging is the greatest risk factor for neurodegenerative diseases such as Alzheimer's disease (AD). Age-dependent alterations of cell signaling play an important role in the onset of AD. The serine/threonine kinase Akt is a critical cell signaling to neuronal survival. Using the senescence-accelerated mouse SAMP10, we investigated the effect of aging on AKT signaling in hippocampus tissue. During aging, the expression of Akt mRNA and protein remained stable. However, the constructive phosphorylation of Akt^{Ser473} displayed a continuous decrease after 6 months in SAMP10. When compared with the control SAMR1, aged SAMP10 mice showed significant reduced phosphorylation of Akt^{Ser473}. SAMP10 at the age of 6 months showed obvious deterioration in performance of learning and memory tasks. Thus, the data reported here suggested a potential link between the age-related alteration of Akt^{Ser473} and the deterioration in performance of learning and memory tasks in SAMP10 mouse.

Brain aging was accompanied by neuronal loss, synapses destruction, clear increases in inflammation and oxidative stress which may cause neurons to respond adaptively to overcome the stress or step into death [1,2]. Excessive neuronal loss and synapses destruction are common pathological changes observed in neurodegenerative diseases such as Alzheimer's disease, and often associated with the onset of impairments in learning and memory [3,4]. Age-dependent alterations of cell signaling are responsible for the generation of pathological changes in these neurodegenerative diseases. Recent evidences have revealed that aging-related abnormal activations of cell signaling pathways such as p38 and JNK were directly linked to tau phosphorylation and amyloid peptide Abeta deposits events in Alzheimer disease [5–7].

The serine/threonine kinase Akt, known as protein kinase B, is a signaling kinase downstream of PI3-kinase which is involved in cell survival. Akt was identified to regulate cellular survival by negatively regulating GSKβ activity [8]. Based on its role, Akt signaling is emerging as a major mediator of survival signals that protect cells from apoptosis and regulate cell proliferation in many cancer cells [9,10].

Akt signaling is also emerging as a central player in neuroprotection according to its anti-apoptosis properties. It has been reported that activated Akt exerts effective neuroprotection by reducing neuronal death following cerebral ischemia in rat CNS [11]. Several studies have demonstrated that activated Akt attenuated the apoptotic effect in PC12 cell which was induced by amyloid β-peptide [12,13]. Constitutively active Akt was also involved in myelination in the CNS [14]. From these studies, activated Akt was confirmed as a powerful agent to reduce neuronal death and maintain neuronal function.

Unlike cerebral ischemia, neurodegenerative diseases such as Alzheimer's disease result from the gradual and progressive loss of neural cells, lead to nervous system dysfunction. Akt activity was reported to be positively correlated with neurofibrillary changes in neurodegenerative diseases [15]. However, it remains unclear whether Akt signaling is causally linked with the hippocampal dysfunction and the neuronal death in neurodegenerative diseases such as Alzheimer's disease.

In the present study, we evaluated the age-related alteration of Akt expression and activation in hippocampus of the senescence-accelerated prone 10 (SAMP10), a mouse model of early onset of neurodegenerative dementia diseases. Our data are helpful to better understand the role of Akt signaling in the onset of neurodegenerative disease. SAMP10 exhibited age-dependent deficit in learning and memory and emotional disorder, senile amyloidosis as well as obvious age-dependent brain atrophy which are similar to that seen in patients suffering from neurodegenerative dementia diseases [16,17]. Therefore, this substrain of mouse has been used frequently to study the mechanisms underlying age-associated neurodegenerative dementia diseases, such as Alzheimer's disease and Pick's disease.

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Materials and methods

Experimental animals. Male SAMP10 and SAMR1 mice were used in this study and their phenotypes have been described in detail elsewhere [18]. SAMR1 mice were used for the control experiments. All animals used in this study were obtained from our breeding colony, maintained as an inbred strain, and originally obtained from Professor Takeda of Kyoto University, Japan. All procedures in the present experiment were handled in accordance with the requirements of the Provisions and General Recommendations of Chinese Experimental Animal Administration Legislation. The animals were bred under conventional conditions, housed at 23 ± 1 °C, and allowed free access to food and water. The light-dark cycle was set at 12 h. Six age groups (0, 2, 4, 6, 8, and 10 months) of mice and 10 mice in each group were used in this study.

Morris water maze test. Note that a pilot study employing a cueing procedure demonstrated no significant differences in sensorimotor or motivational function between SAMR1 and SAMP10 mice (data not shown). Thus, to test hippocampal-dependent spatial cognition, SAMR1 and SAMP10 mice were trained in the standard Morris water maze (MWM) with a hidden platform. In this study we used a 5-day testing protocol, as previously described in detail [19,20]. Briefly, the mouse was gently placed in the water of the pool between quadrants, facing the wall of pool with drop location changing for each trial, and allowed 120 s to locate submerged platform. Then, it was allowed to stay on the platform for another 20 s. If it failed to find the platform within 120 s, it was guided gently onto platform and allowed to remain there for 20 s. Escape latency time (ELT) to locate the hidden platform in water maze was videotaped and analyzed with image tracking software (China Daheng Group, Inc., Beijing Image Vision Technology Branch, Beijing, China).

RNA extraction and reverse transcription. The hippocampus tissue samples were dissected from fresh brain and used immediately or stored at −80 °C until use. Total RNA was extracted from each sample using Trizol reagent (Invitrogen) according to the manufacture's instruction and dissolved in DEPC-treated water. The quality of the isolated RNA was checked by gel electrophoresis, and RNA concentration was determined by measuring OD at 260 nm 280 nm. Two micrograms of total RNA in a final volume of 40 μl was subjected to reverse transcription reaction. cDNA synthesis was carried out using random hexamer primers and MMLV-reverse transcriptase under the conditions recommended by the supplier (Invitrogen).

Primer design for real-time PCR. Real-time PCR primers for GAPDH were derived from the RTPrimerDB database (RTPrimerDB ID: 2921), which get rise to a 156 bps product. For three murine Akt mRNA variant (Akt2, and Akt3), specific primers were manually designed using Gene Runner software (Hastings Software, Inc.). The primer sequences were listed in Table 1. For Akt1 (NM_009652), Akt2 (NM_007434), and Akt3 (NM_011785), the specific primers should result in a 100, 103, and 128 bps product, respectively.

Table 1
SYBR primer for real-time PCR.

Gene	SYBR primer for real-time PCR
Akt1	5′-TGGACAAGGACGGGCACATCAAG-3′—sense
(NM_009652)	5′- TACTCCGGCGTTCCGCAGAATG-3′—antisense
Akt2	5′-TGTGGGCGACTTCATCCTTTGC-3′—sense
(NM_007434)	5′- TTCGGCAAGGTCATTCTGGTTCG-3′—antisense
Akt3	5′-TGAGTGAAAGGTGGTGAGGGTGAAG-3′—sense
(NM_011785)	5′- TCTGCAAGCGGACGGGAATAAG-3′—antisense
GAPDH	5′- TGGCAAAGTGGAGATTGTTGCC-3′—sense
(NM_001001303)	5′- AAGATGGTGATGGGCTTCCCG-3′—antisense

Relative quantification of Akt mRNA by real-time RT-PCR analysis. For the amplification of Akt, 1 μl cDNA was added to the SYBR Green preMix EX-Taq (TAKARA Bio) containing the specific primers. Real-time PCR was performed in a GenAmp 7500® (Applied Biosystems™) thermocycler. The same thermal settings was used for all genes in a total volume of 25 μl: 15 s of pre-incubation at 95 °C followed by 40 cycles of 5 s at 94 °C, 15 s at 60 °C, and 34 s at 72 °C. Calibrated and non-template controls were included in each assay. Each sample was run in triplicate. Melting curve analysis was always performed at the end of each PCR assay. Using the ABI prism 7500 SDS software, SYBR Green dye intensity was analyzed and Ct value was determined.

The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative mRNA expression [21]. According to this method, GAPDH gene was used as a specific endogenous control, and the gene expression of Akt mRNA was normalized to GAPDH mRNA. As calibrator, the control sample of 8-month-old SAMR1 was used and set to 100%.

Western blotting analysis. AKT protein abundance and phosphate level of AKT in mouse hippocampus were determined by Western blotting analysis. Fresh hippocampus was dissected and used for preparation of whole-cell protein extraction described previously. Briefly, fresh hippocampus was homogenized in a glass homogenizer using 10 volume of the cold cell-lysis buffer composed of 50 mM Tris buffer, 250 mM NaCl, 0.1% NP-40, 1 mM NaF, 0.1 mM Na_3VO_4, 0.5 mM EDTA, 0.5 mM EGTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 100 μg/ml PMSF. The homogenate was centrifuged 12,500 rpm at 4 °C for 30 min, and the supernatant was taken for protein quantitation with a BCA protein assay kit (Pierce, Ltd.). One hundred micrograms of total protein in each lane was separated on a 12% SDS–PAGE. After electrophoresis, proteins were transferred to a 0.22 μm polyvinylidene difluoride (PVDF) membranes (Millipore) in transfer buffer containing 25 mM Tris (pH 8.3), 192 mM glycine, and 20% (v/v) methanol. Then, the membrane was subjected to Western blots. After incubated with the primary antibodies (Akt, Akt^{Ser473}, Akt^{Thr308}, from Cell Signaling Technology Inc., Beverly), the horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, USA) was used to bind the specific antibody. The bound antibody was detected using ECL reagents (Santa Cruz), and the bands were captured on X-ray film. The density of protein band was evaluated using Scion Image software.

Statistical analyses. All values were presented as the means + standard error of means (SEM) and analyzed using SPSS13.0 (SPSS Inc., Chicago, USA). Analysis of two-way ANOVA with repeated measures was conducted on the data obtained from the Morris water maze test. Comparisons between groups were made using one-way ANOVA followed by Bonferroni's test (multiple comparisons). *P*-values <0.05 were considered as statistically significant.

Results

Hidden platform trial

Fig. 1 shows the learning curves for escape latency from the Morris water maze over the course of the 5-day training period. In the Morris water maze, both 8-month-old SAMP10 and the control SAMR1 mice showed a significant reduction of the escape latency over the 5 days of training ($P < 0.01$). However, whereas the ability of aged SAMR1 to reach the hidden platform improved over the 5 days of training, aged SAMP10 mice did show an improvement in finding the hidden platform only during the first three days, but their escape latency differed significantly from SAMR1 in the last days. As a consequence, the slope of the escape latency curves for 8-month-old SAMP10 and the control SAMR1 mice significantly differed. Two-way ANOVA of escape latencies

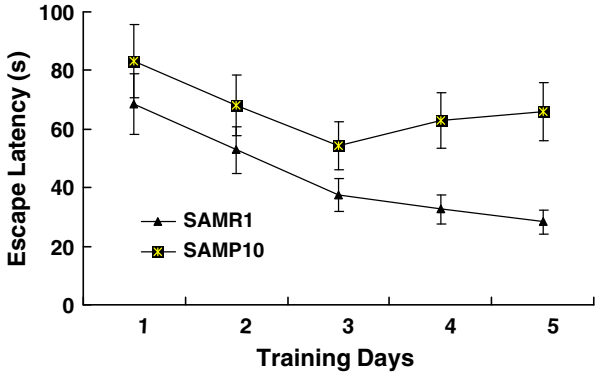

Fig. 1. Performance of the 8-month-old SAMP10 and the control SAMR1 mice during the five training days of a hidden platform. Two way ANOVA for the 8-month-old SAMR1 and SAMP10mice showed an effect of strain ($P < 0.0001$).

displayed by the 8-month-old SAMR1 and SAMP10 mice revealed an effect of strain ($P < 0.0001$).

Age-dependent Akt expressions in SAM P10 hippocampus

There were three highly conserved homologs of Akt1, Akt2, and Akt3. SYBR Green real-time PCR analysis was used, to determine the levels of Akt mRNA in SAMP10 hippocampus. The $2^{-\Delta\Delta Ct}$ analysis was adopted to quantify the relative changes of target gene expression. No significant difference in mRNA level of Akt transcripts was detected in SAMP10 from 0 to 10 months of age ($P > 0.05$, Data not shown). These results suggested that the level of all Akt transcripts remained stable with increasing age in hippocampus of SAMP10 mice.

Levels of the total Akt proteins in SAM P10 hippocampus were also measured using Western blot analysis. Normalized to the densitometry values of β-actin in each sample, the relative levels of Akt protein were presented in Fig. 2 as the 2-months-old sample was set to 100%. Data obtained here showed that no significant difference in Akt protein expressions was seen in SAMP10 mice from 2 to 10 months of age ($P > 0.05$, Fig. 2C and D). These results suggested that expression level of Akt protein remained stable with increasing age in hippocampus of SAMP10 mice.

Age-dependent Akt activation in SAMP10 hippocampus

We also analyzed the age-dependent Akt activation in hippocampus in SAMP10 by Western blot analysis with antibodies specific for the phosphorylated Akt^{Ser473} and Akt^{Thr308}. Typical results were shown in Fig. 2. Phosphorylation of Akt^{Thr308} was not detected in either SAMP10 or SAMR1 mice (data not show), but a constitutive phosphorylation of Akt^{Ser473} was observed in both SAM P10 and SAM R1 mice (Fig. 2A and B). The phosphorylation levels of Akt^{Ser473} were presented in Fig. 2C and D. One way ANOVA for the SAMP10 mice showed an effect of aging ($P < 0.05$), and a significant decrease ($P < 0.05$) in phosphorylation levels of Akt^{Ser473} was seen in both 8-month-old and 10-month-old SAMP10 mice. In control SAMR1, One way ANOVA also showed an effect of aging ($P < 0.05$), and a significant decrease ($P < 0.05$) in phosphorylation levels of Akt^{Ser473} was seen in 10-month-old SAMP10 mice. The results indicated that the phosphorylation level of Akt^{Ser473} remained stable in young SAM P10 mice and declined gradually in aged SAMP10 mice.

Difference in activation of Akt between aged SAMP10 and aged SAMR1

In contrast to SAMR1, aged SAMP10 mice exhibited obvious deficit in learning and memory. We compared differences in the

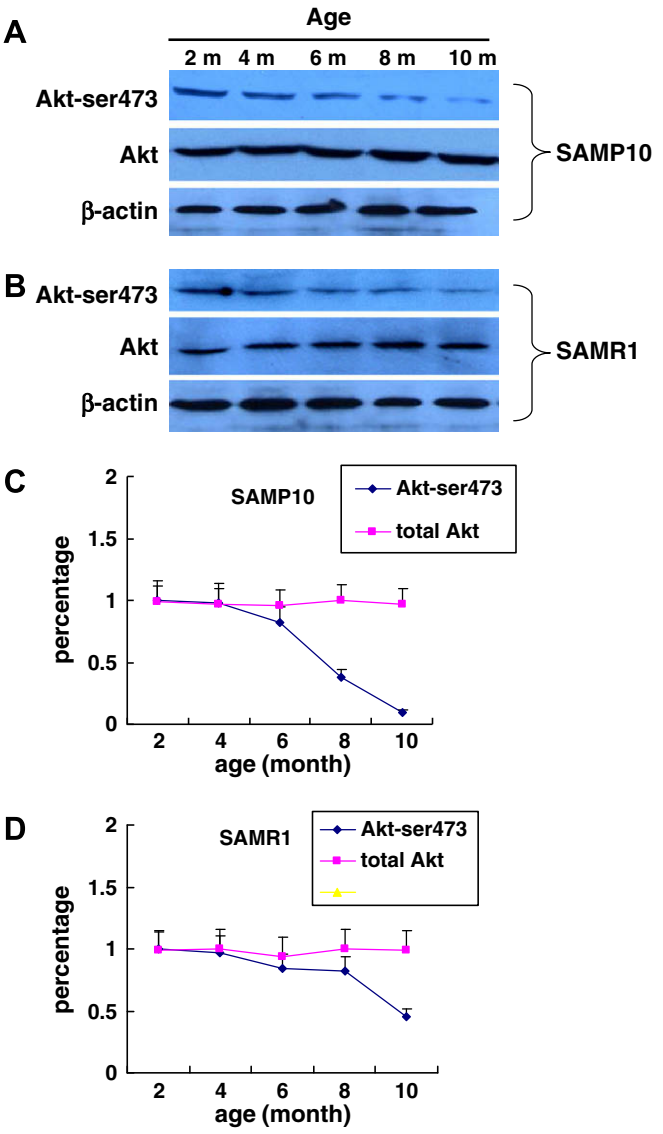

Fig. 2. Alteration of total Akt protein levels and phosphorylation level of Akt^{Ser473} with increasing age (2–10 months) in both SAMP10 and SAMR1 hippocampus by Western blots. Typical results by Western blots were shown in (B). The samples from 2-month-age SAM R1 or SAMP10 were used as calibrator and set to 100%, respectively. Relative levels of total Akt and phosphorylation Akt^{Ser473} were shown in (C,D). (A) Total Akt protein levels and phosphorylation of Akt^{Ser473} with increasing age in SAMP10 hippocampus. (B) Total Akt protein levels and Akt^{Ser473} phosphorylation with increasing age in SAMPR1 hippocampus. (C) Relative levels of total Akt protein levels and phosphorylation of Akt^{Ser473} with increasing age in SAMP10 hippocampus. Data are means + SEM, $n = 10$ mice in each group. Statistics: both 8- and 10-month-old SAMP10 mice were significantly different from other groups. (D) Relative levels of total Akt protein levels and Akt^{Ser473} phosphorylation with increasing age in SAMR1 hippocampus. Data are means + SEM, $n = 10$ mice in each group. Statistics: 10-month-old SAMP10 mice were significantly different from other groups.

expression and activation of Akt between aged SAMP10 and aged SAMR1 mice. Eight and 10 months old SAM mice were defined as aged individual. No significant difference ($P < 0.05$) in the level of Akt protein was detected between aged SAMP10 and aged SAMR1 mice (Fig. 3C and D). However, phosphorylation level of Akt^{Ser473} was significant lower ($P < 0.05$) in both 8- and 10-month-old SAMP10 than in the control SAMR1 mice (Fig. 3A and B). The phosphorylation level of Akt^{Ser473} in 8-months-old SAMP10 is about 50% of that in the 8-months-old SAMR1control. The phosphorylation level of Akt^{Ser473} in 10-months-old SAMP10 became about 36% of that in the 10-months-old SAMR1 control (Fig. 3A and B). These

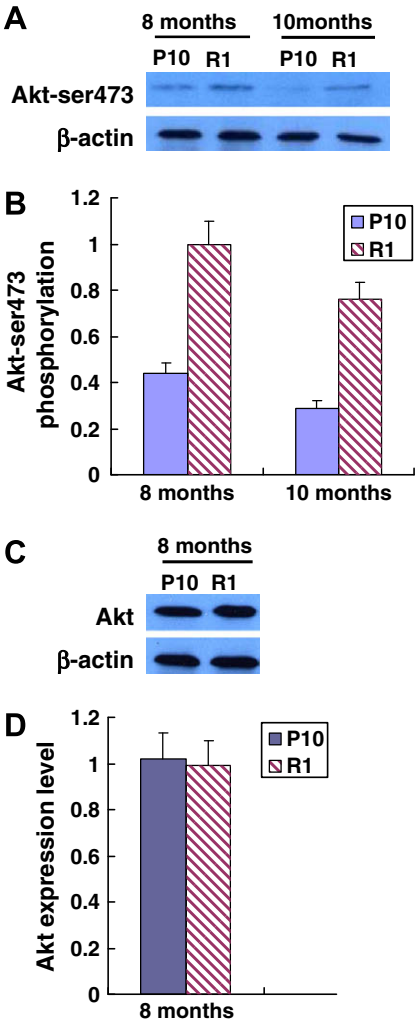

Fig. 3. Difference in total Akt protein levels and the phosphorylation of Akt^{Ser473} in hippocampus between aged SAMP10 and aged SAMR1 by Western blots. Typical results by Western blots were shown in (A,C). The sample of 8-months-old SAM R1 was used as calibrator and set to 100%. Relative expression of total Akt and phosphorylation Akt^{Ser473} were shown in (B,D). (B) Relative level of phosphorylation Akt^{Ser473} in hippocampus of both aged SAMP10. Statistics: significantly different from the control SAMR1 mice $P < 0.05$. (D) Relative levels of total Akt protein hippocampus of both aged SAMP10 and SAMR1. Statistics: there were no significant differences between SAMP10 and SAMR1 ($P > 0.05$).

results suggested that there was greater decrease in activation of Akt signaling in aged SAMP10 mice than that in the SAMR1 control.

Discussions

We evaluated the age-dependent alteration in Akt expression and activation in the hippocampus of SAMP10 mice. Results from RT-PCR and Western blots analysis showed that Akt expression remains stable with increasing age in hippocampus of both SAMP10 and SAMR1 mice, and a constitutive activation of Akt^{Ser473} was also observed in the hippocampus of both SAMP10 and SAMR1 mice. Furthermore, Western blots analysis showed that aging led to greater decrease of phosphorylation of Akt^{Ser473} in the hippocampus of aged SAMP10 mice than the SAMR1 control. Aged SAMP10 mice showed obvious deficit in learning and memory due to dysfunction of the hippocampus. Thus, our findings indicate potential links between the age-related decrease of phosphorylation of Akt^{Ser473} and the dysfunction of the hippocampus in SAMP10 mice.

SAMP10 mice were assessed for their spatial memory performance using Morris water maze task. The water maze is a particularly useful tool for assessment of spatial memory ability in aged rodents because it is reliably sensitive to age-related impairments in spatial memory. In the present study, there were significant differences on the performance in the escape latency between the 8-month-old SAMR1 and SAMP10 mice. Moreover, the SAMP10 mice, unlike the controls, failed to improve the performance in finding the hidden platform over the first 3 days of training. These observations suggested that aged SAMP10 mice exhibited obvious deterioration in performance of learning and memory tasks.

Brain aging is accompanied by neuronal loss, synapses destruction, clear increases in inflammation and oxidative stress. Selective regional neuronal loss was reported to be partially responsible for age-dependent dementia in neurodegenerative diseases such as Alzheimer's disease. In this study, hidden platform trial suggested that aged SAMP10 mice exhibited obvious deterioration in performance of learning and memory tasks which are similar to that in neurodegenerative diseases, suggesting potential neuronal loss in aged SAMP10 mice. There were increases in inflammation, oxidative stress, deposit of senile amyloidosis in aged SAMP10 [22]. Moreover, SAMP10 mice showed obvious age-dependent brain atrophy which was typical pathological changes of neuronal death. Although hardly any evidences of neuronal death in hippocampus of SAMP10 mice were presented, we speculated that neuronal death may be, at least partially, responsible for deterioration in performance of learning and memory tasks in aged SAMP10 mice.

The Akt protein contains a central kinase domain with specificity for serine or threonine residues in its substrate proteins. It also contains two regulatory phosphorylation sites, Thr308 in the activation loop within the kinase domain and Ser473 in the C-terminal regulatory domain [23]. Full activation of Akt depends on the phosphorylation at these main phosphorylation sites. PI3-K kinase contributes to phosphorylation of both Thr308 and Ser473 site of Akt. PI3-K was activated by various stimuli such as growth factors, insulin and insulin-like growth factor-I (IGF-I) [23]. Conditional activation of Akt^{Ser473} was well known in numerous cells, whereas the constitutive activation of Akt^{Ser473} has only been reported in cancer cells. Few studies have focused on the constitutive activation of Akt^{Ser473} in neurons. In the last decade, evidence from different studies demonstrated that there was a constitutive activation of Akt^{Ser473} in human malignancies such as prostate cancer, mantle cell lymphoma (MCL), urothelial carcinoma, Hodgkin's lymphoma and breast cancer. Several studies had shown that loss of PTEN in melanoma cell was in line with the constitutive activation of Akt^{Ser473} and the constitutive activation of Akt^{Ser473} played a critical role of anti-apoptosis, immune activation and cell proliferation in cancer progress [16,24]. Constitutive activation of Akt^{Ser473} also has important functions in the regulation of cellular metabolism besides its roles in cell survival and anti-apoptosis. Interestingly, we observed the constitutive activation of Akt^{Ser473} in hippocampus. Based on the critical role of Akt signaling, constitutive activation of Akt^{Ser473} in hippocampus similar to that in cancer cells suggests that it should have played an important role in neurons of SAMP10 hippocampus.

Two facts in this study suggested potential links between the age-related decrease of phosphorylation of Akt^{Ser473} and the dysfunction of the hippocampus in SAMP10 mice. One was that aging led to greater decline in constitutive activation of Akt^{Ser473} in SAMP10 than that in SAMR1 mice. Based on the roles of Akt in cell survival and anti-apoptosis, this fact suggested that accelerated aging may result in greater neuronal death in hippocampus of SAMP10 mice than in SAMR1 mice. The other one was that the phosphorylation level of Akt^{Ser473} declined gradually in SAMP10 mice after 8 months, which was consistent with the age when SAMP10 mice exhibited obvious deterioration in performance of

learning and memory tasks. This consistence suggested that greater decline in constitutive activation of Akt^{Ser473} may be partially responsible for greater neuronal death in hippocampus of SAMP10 mice.

Akt signaling has important functions in the regulation of cellular metabolism besides its roles in cell survival and anti-apoptosis. In the present study, constitutive activation of Akt^{Ser473} in SAM hippocampus similar to that in cancer cells suggests that it should have played an important role in neurons of SAMP10 hippocampus. Moreover, aging led to greater decline in Akt^{Ser473} of hippocampus in aged SAMP10. Considering its roles in cell survival and anti-apoptosis, we presume that age-dependent decline in activation of Akt may partially be responsible for deterioration in performance of learning and memory tasks in aged SAMP10 mice. However, further investigations are needed to confirm this conclusion.

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