[Full Paper]

Electrophoretic analyses of glutamate decarboxylase 67 kDa isoform and extracellular regulated kinase isozyme-2 in brains of senescence accelerated mice, SAM-P8

Takaho Tanaka

SUMMARY

Senescence accelerated mice (SAM-P8), which have been established as a model of early senescence, were used for analyses of glutamate decarboxylase 67 kDa isoform (GAD67) and extracellular regulated kinase-2 (ERK-2) which were altered by aging. The mice was characterized by neurological abnormal signs such as loss of memory, disorientation and weak response to stimuli, which appeared from 8 to 10 weeks-old. Those neurological disorders might be associated with changes in functions of brain proteins and enzymes regulating metabolic conversion of neurotransmitters and signal transduction. SDS-polyacrylamide gel electrophoresis of proteins in brains of male SAM-P8 showed that alterations in electrophoretic patterns of proteins, which would be involved in the development of brain, according to aging at 8 and 12 weeks in comparison with control mice (SAM-R1) although the alterations were not evidenced in female SAM-P8. Examinations of enzymes of GAD67 and ERK-2 by immunoblotting showed the decrease in their immunoreactivity in male SAM-P8 8 weeks-old, but not in female SAM-P8. These results suggest that the neurological disorder in male SAM-P8 is associated with the impairment of protein biosynthesis for the normal development of brain and with the decrease in enzymes for converting glutamate to gamma-aminobutyrate and for signal transduction of the response to stimuli. Furthermore, there are sex differences in the decrease of those enzymes by aging.

Key words: aging, extracellular regulated kinase, glutamate decarboxylase, SDS-PAGE, senescence accelerated mice.

INTRODUCTION

Studies of age-related changes in protein composition of mouse brain have taken many approaches.¹⁻⁴⁾ Recent studies have focused on neuronal cells and central nervous system whose functions are disordered by aging which leads to the onset of dementia. The molecular mechanisms of the disorder of neurological function have been extensively investigated, but still to be clarified. One of the characteristics of aging of neuronal cells and brain is the intra- and extra-cellular accumulation of altered proteins which are posttranslationally

modified to form disfunctioned protein aggregates such as senile plaques of Alzheimer's disease and amyloid proteins in brain vessel walls in which the degradation of such proteins may be impaired.⁵⁻⁷⁾ To investigate the alterations of brain proteins by aging, the animal model for aging studies has been established as senescence accelerated mice (SAM-P8) which have been inbred^{8,9)} and characterized by neuronal disorder such as loss of memory, disorientation and decreases in stress adaptation^{10,11)} which appear at age of 8 to 10 weeks much earlier than in normal senescence mice (SAM-R1).

A stimulatory neurotransmitter, L-glutamate is con-

田中隆穂; Department of Biochemistry and Biomolecular Sensing, Yamaguchi University School of Medicine.

Correspondence address: Takaho Tanaka, Department of Biochemistry and Biomolecular Sensing, Yamaguchi University School of Medicine, 1-1-1, Minami-kogushi, Ube 755-8505, Japan.

Abbreviations: ERK, extracellular regulated kinase; GAD, glutamate decarboxylase; GABA, gamma-amino butyric acid; MAPK, mitogen-activated protein kinase; PAGE, polyacrylamide gel electrophoresis; SAM, senescence accelerated mice; SDS, sodium dodecylsulfate.

(Received June 28, 2000, Accepted July 31, 2000, Published September 15, 2000)

verted into γ -aminobutylate (GABA) by an enzyme of glutamate decarboxylase (GAD) which has isoforms of 65 kDa (GAD65) and 67 kDa (GAD67).¹²⁾ Those isoforms are encoded by two independent genes,^{13,14)} and the rate of conversion of glutamate into GABA may be a critical factor for physiological behavior and response to neuronal stress.¹⁴⁾ Recently it was reported that mice lacking GAD65 maintained normal levels of GAD67 and GABA but are susceptible to seizure.¹⁵⁾ On the other hand, mice lacking GAD67 decreased the level of GABA and died of severe cleft palate during the first morning after birth.¹⁶⁾

It has been reported that the activation of mitogen-activated protein kinase (MAPK) is involved in cellular response to external signals such as hormonal stimuli with insulin¹⁷⁾ and estrogen,¹⁸⁾ neuronal stimuli,¹⁹⁾ and other physiological stress.²⁰⁾ Behavioral studies have implicated the hippocampus in the formation and storage of spatial memory. More recently it was reported that MAPK, also called extracellular regulated kinases (ERK) was activated in the dorsal, but not the ventral hippocampus of rats after training in a spatial memory task.¹⁹⁾ The activation of ERK was regulated by phosphorylation and dephosphorylation of the enzyme protein by serine/threonine kinases and dual specific tyrosine phosphatases.²¹⁾ Here, we show data obtained from analyses on alterations in brain proteins and in enzymes of GAD67 and ERK-2 which are involved in metabolic conversion a neurotransmitter, glutamate to GABA and in stress induced-signal transduction by means of sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblotting. The evidences for sex differences in those enzymes are also shown. The biological significance of the alterations in those enzyme for the appearance of neurological disorder by aging will be discussed.

MATERIALS AND METHODS

Animals

Two couples of inbred strains of mouse, senescence accelerated mice (SAM-P8) and normal senescence mice (SAM-R1) were gifted from Prof. T. Takeda (Chest Disease Research Institute, Kyoto University). The mice were bred with unlimited feeding under SP free condition according to the guidance of laboratory animal center of Yamaguchi University School of Medicine. Each group consisted of 3 female mice and 3 male mice at ages of 4 weeks-old, 8 weeks-old, 12 weeks-old and 6 months-old. Differences between SAM-P8 group and SAM-R1 group were tested by Student's *t*-test.

Brain samples

Each brain of mice was picked up by decapitation, weighed and immersed into liquid nitrogen for 30 min. The frozen brain was washed with 0.25 M sucrose solu-

tion, and then homogenized in 4 volume of 0.25 M sucrose solution with the glass homogenizer. The homogenate was centrifuged at $1,000 \times g$ for 10 min, and the supernatant was collected to be centrifuged at $1,700 \times g$ for 5 min. The supernatant was used as brain sample and the protein concentration was determined by Lowry's method.²²⁾

SDS-PAGE and immunoblotting

The brain was mixed with an equal volume of SDS sample solution of 125 mM Tris-HCl buffer, pH 6.8, containing 4% SDS, 20% glycerol and 10% mercaptoethanol and heated in boiling water for 3 min. SDS-PAGE using 10% T acrylamide slab gel with 3% T stacking gel was run according to the method of Laemmli.²³⁾ Ten micrograms of protein was loaded in each well of the gel. The proteins were then stained with Coomassie brilliant blue R-250 (CBBR) or electroblotted onto a polyvinylidene difluoride (PVDF) membrane in 10 mM CAPS, pH 9.0 containing 20% methanol. The membrane was treated with 5% skimmed milk for blocking, and then reacted with the first antibody of rabbit anti-GAD67 antisera (Chemicon International Inc., Temecular, CA), or rabbit anti-ERK-2 antisera (Biotechnology Inc., Lake Placid, NY). After washing with 20 mM Tris-HCl buffered saline, pH 7.5 containing 0.05% Tween 20 (TBS-T) the membrane was incubated with secondary antibody goat anti-rabbit IgG conjugated to horseradish peroxidase (Organon Technika Co., Durham, NC). Immunoreactive species were visualized by reaction with the substrates of 4-chloro-1-naphthol (Sigma Co., St. Louis, MI) and hydrogen peroxide.

The density of protein bands in the gel, which were stained with CBBR, was determined by a densitometer (Jookoo PAN-805, Tokyo) using a filter of 570 nm, and the density of immunoreactive bands on the PVDF membrane, which was translucent by immersing in silicon oil (Toray SH 2000, Tokyo), was determined by the densitometer at 570 nm. The density of stained band was expressed in arbitrary unit of peak area of the band.

RESULTS AND DISCUSSION

As shown in Fig. 1, brain proteins of SAM-P8 and SAM-R1 were clearly separated by SDS-PAGE into more than 20 bands which were numbered as band-1 to -21 from the bottom of gel and band-22 at the top of gel. In both groups of female and male mice the relative mobility of the main protein bands was not changed by aging. The mass of some proteins was seemed to be varied although the protein amount of samples was fixed to be 10 μ g. The sum of density of those protein bands was also varied among samples. This may be due to the differences in the mass of proteins and peptides having molecular weights smaller than 20 kDa which could not be separated using 10% polyacrylamide gels for SDS-PAGE. In the case of male mice the protein bands

Fig. 1. Patterns of brain proteins in senescence accelerated mice (SAM) separated by SDS-PAGE. The patterns show the pooled protein samples from male (left panel) and female (right panel) of early senescent mice (P8) and its control mice (R1) at ages of 4 weeks-old (4 wks), 8 weeks-old (8 wks), 12 weeks-old (12 wks), and 24 weeks-old (24 wks). The numerals indicate molecular weights of standard proteins (left side) and the numbers of protein bands (right side) separated in the gel. SDS-PAGE was carried out as described in MATERIALS AND METHODS.

Fig. 2. The immunoblotting patterns of glutamate decarboxylase 67 kDa isoform (GAD67) in brains of senescence accelerated mice. The panels show the patterns of GAD 67 from male (left panel) and female (right panel) early senescent mice (P8) and control mice (R1). The open triangles indicate the GAD bands. The reaction times for peroxidase with 4-chloro-1-naphthol were 60 min for male samples and 30 min for female samples because of low immunoreactivity of male P8 samples.

having higher molecular weights than 67 K significantly decreased their density in SAM-P8 as compared with SAM-R1. This was not the case of female mice even though the density of the bands of No. 20 having Mr of 220 K and No. 21 having Mr 290 K was decreased in SAM-P8. In SAM-R1 those protein bands increased their density in the mice of 8 and 12 weeks-old, and then decreased the density at the age of 6 months. In SAM-

P8 those protein bands did not increase their density at the level of 6 months-old. This may be due to decrease in gene expression of those proteins not due to the increase of protein degradation in the brain of SAM-P8 because the density of protein bands of lower molecular weights was not increased in SAM-P8 and comparable to SAM-R1. It has been reported that ubiquitination of proteins regulates the protein degradation^{24,25)} and the

ubiquitinated proteins are accumulated in brains of aged mice.⁵⁾ The accumulation of ubiquitinated proteins in brains of male mice of SAM-P8 could not be evidenced by immunoblotting which was combined with highly sensitive detection system of enhanced chemiluminescence (ECL) assay (data not shown).

GAD67, which is an isoform of glutamate decarboxylase converting glutamate into GABA, plays important roles not only for the metabolic regulation of the neurotransmitters, but also the loss of the enzymic function leads to seizure and early death in mice. The mass of GAD67 immunoreacting with anti-GAD67 antisera was increased in brains of male mice of SAM-R1 and kept the high level until 6 months-old (Fig. 2). On the other hand, the mass of GAD67 in male mice of SAM-P8 was

much lower and gradually increased by aging up to 12 weeks-old and kept the level until 6 months-old ($p < 0.05$) at which the level was a half of male mice of SAM-R1 (Fig. 3). In the case of female mice the level of GAD67 in SAM-P8 was not different from the level in SAM-R1 significantly. It has been reported that mRNA levels of GAD67 in rat brain are modulated by estrogen.²⁶⁾ Ovariectomized rats, which are treated with estradiol, significantly increase the levels of mRNA of GAD65 but decrease the level of mRNA of GAD67. In the dorsal medial nucleus of the hypothalamus, the rats decrease GAD65-mRNA but increase GAD67-mRNA. In the midbrain central gray, there is a significant increase in the number of cells which could be labeled for mRNAs of both GAD65 and GAD67. These indicate that

Fig. 3. Alterations in immunoreactivity of GAD67 in brains of senescence accelerated mice.

The panels show the changing of densities of immunoreactive bands of GAD67 in male (left panel) and female (right panel) early senescent mice (P8) in the solid line with closed triangles and control mice (R1) in the dotted line with open circles. The bars indicate standard deviations.

Fig. 4. The immunoblotting patterns of extracellular regulated kinase-2 (ERK-2) in brains of senescence accelerated mice.

The panels show the patterns ERK-2 of 42 kDa in pooled protein samples from male (left panel) and female (right panel) early senescent mice (P8) and control mice (R1). The open triangles indicate the ERK-2 bands. The reaction time for peroxidase with 4-chloro-1-naphthol was 30 min.

Fig. 5. Alterations in immunoreactivity of ERK-2 in brains of senescence accelerated mice.

The panels show the changing of densities of immunoreactive bands of ERK-2 of 42 kDa in male (left panel) and female (right panel) early senescent mice (P8) in the solid line with closed squares and control mice (R1) in the dotted line with open rhombi. The bars indicate standard deviations.

the decrease of GAD67 protein in male mice of SAM-P8, which is associated with the appearance of neurological abnormal signs from 8 weeks-old, may be one of factors for the cause of neuronal disorder in the mice.

ERK-2, which is an isoform of mitogen-activated protein kinase, which is activated for stress response¹⁷⁾ and one of major substrates for stresses to nerve cells.^{19,20)} The mass of ERK-2 immunoreacting with rabbit anti-ERK-2 antisera was increased in male mice of SAM-R1 at age of 8 weeks-old and then decreased gradually to the level of 6 months-old which was lower than the level of 4 weeks-old (Fig. 4). On the other hand, the mass of ERK-2 in male mice of SAM-P8 at age of 4 weeks-old was a half of the male mice of SAM-R1 but gradually increased to the level of 12 weeks-old (Fig. 5). In the case of female mice of SAM-P8 the level of ERK-2 was kept at the comparable level of SAM-R1 until 6 months-old. The decrease of ERK-2 in male mice of SAM-P8 was associated with the appearance of the neurological abnormal signs as in the case of GAD67. But ERK-2 level was increased to the level of SAM-R1 at age of 6 months-old. These findings suggest that the decrease in enzymes of GAD67 and ERK-2 is involved in the early onset of neuronal disorder of SAM-P8 male mice at ages from 8 weeks-old to 12 weeks-old.

It is conclusive that the decrease of GAD67 and ERK-2 is associated with the early onset of neuronal disorder in male mice of SAM-P8 but not in female mice. Further examination will be necessary to clarify the mechanism of impairment of gene expression of those enzymes in SAM-P8 which is associated with neuronal aging, the localization of those enzymes in aged mice, and dietary effects on aging²⁷⁾ to understand the senescence. The accumulation of database of murine brain proteins by two-dimensional gel electrophoresis²⁸⁾ will be urgent task for understanding the molecular mecha-

nism of aging of brain.

I wish to acknowledge Professor Kazuyuki Nakamura (Department of Biochemistry and Biomolecular Sensing) for his keen insight and guidance in this investigation. I am also grateful to Dr. K. Takeda in Kyoto University and Dr. M. Miyamoto in Takeda Pharmaceutical Company for their kind gift of senescent accelerated mice (SAM-P8/SAM-R1) and to Dr. M. Fujimoto (Department of Biochemistry and Biomolecular Sensing) for his technical assistance.

REFERENCES

- 1) Grossfeld RM, Shooter EM. A study of the changes in protein composition of mouse brain during ontogenetic development. *J Neurochem* 1971; 18: 2265-77.
- 2) Wilson PD. Enzyme changes in aging mammals. *Gerontologia* 1973; 19: 79-125.
- 3) Vaughan WJ, Calvin M. Electrophoretic analysis of brain proteins from young adult and aged mice. *Gerontology* 1977; 23: 110-26.
- 4) Richardson A, Cheung HT. The relationship between age-related changes in gene expression, protein turnover, and the responsiveness of an organism to stimuli. *Life Sci* 1982; 31: 605-13.
- 5) Ohtsuka H, Takahashi R, Goto S. Age-related accumulation of high-molecular weight ubiquitin protein conjugates in mouse brains. *J Gerontol* 1995; 50 A: B 277-81.
- 6) Mori H, Kondo J, Ihara Y. Ubiquitin is a component of paired helical filaments in Alzheimer's disease. *Science* 1987; 235: 1641-4.
- 7) Stadtman ER, Berlett BS. Reactive oxygen-mediated protein oxidation in aging and disease. *Chem Res Toxicol* 1997; 10: 486-94.
- 8) Arai H, Kashiwagi S, Nagasaka Y, Uchida K, Hoshii Y, Nakamura K. Oxidative modification of apolipoprotein E in human very-low-density lipo-

- protein and its inhibition by glycosaminoglycan. *Arch Biochem Biophys* 1999; 367: 1-8.
- 9) Takeda K, Hosokawa M, Takeshita S, Irino M, Higuchi K, Matsushita T, Tomita Y, Yasuhira K, Hamamoto H, Shimizu K, Ishii M, Yamamoto T. A new murine model of accelerated senescence. *Mech Ageing Dev* 1981; 17: 183-94.
- 10) Miyamoto M, Kiyota Y, Nishiyama M, Nagaoka A. Senescence-accelerated mouse (SAM): Age-related reduced anxiety-like behavior in SAM-P/8 strain. *Physiol Behav* 1992; 51: 979-85.
- 11) Takeda T, Hosokawa M, Higuchi K. Senescence-accelerated mouse (SAM): A novel model of accelerated senescence. *J Am Geriatr Soc* 1991; 39: 911-9.
- 12) Kaufman DL, Houser CR, Tobin AJ. Two forms of the γ -aminobutyric acid synthetic enzyme glutamate decarboxylase have distinct intraneuronal distributions and cofactor interactions. *J Neurochem* 1991; 56: 720-3.
- 13) Erlander MG, Tillalkaratne NJK, Feldblum S, Patel N, Tobin AJ. Two genes encode distinct glutamate decarboxylases. *Neuron* 1991; 7: 91-100.
- 14) Erlander MG, Tobin AJ. The structural and functional heterogeneity of glutamic acid decarboxylase: A review. *Neurochem Res* 1991; 16: 215-26.
- 15) Asada H, Kawamura Y, Maruyama K, Kume H, Ding R-G, Ji FY, Kanbara N, Kuzume H, Sanbo M, Yagi T, Obata K. Mice lacking the 65 kDa isoform of glutamic acid decarboxylase (GAD65) maintain normal levels of GAD67 and GABA in their brains but are susceptible to seizures. *Biochem Biophys Res Commun* 1996; 229: 891-5.
- 16) Asada H, Kawamura Y, Maruyama K, Kume H, Ding R-G, Ji FY, Kanbara N, Kuzume H, Sanbo M, Yagi T, Obata K. Cleft palate and decreased brain γ -aminobutylic acid in mice lacking the 67 kDa isoform of glutamic acid decarboxylase. *Proc Natl Acad Sci USA* 1997; 94: 6496-9.
- 17) Sturgill TW, Wu J. Recent progress in characterization of protein kinase cascades for phosphorylation of ribosomal protein S6. *Biochim Biophys Acta* 1991; 1092: 350-7.
- 18) Improt-Brears T, Whorton AR, Codazzi F, York JD, Meyer T, McDonnell DP. Estrogen-induced activation of mitogen-activated protein kinase requires mobilization of intracellular calcium. *Proc Natl Acad Sci USA* 1997; 96: 4686-91.
- 19) Blum S, Moore AN, Adams F, Dash PK. A mitogen-activated protein kinase cascade in the CA1/CA2 subfield of the dorsal hippocampus is essential for long-term spatial memory. *J Neurosci* 1999; 19: 3535-44.
- 20) Korneyev AY. Stress-induced Tau phosphorylation in mouse strains with different brain Erk 1+2 immunoreactivity. *Neurochem Res* 1998; 23: 1539-43.
- 21) Todd JL, Tanner KG, Denu JM. Extracellular regulated kinases (ERK) 1 and ERK 2 are authentic substrates for the dual-specificity protein-tyrosine phosphatase VHR. *J Biol Chem* 1999; 274: 13271-80.
- 22) Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193: 265-75.
- 23) Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680-5.
- 24) Hersko A, Ciechanover A. The ubiquitin system for protein degradation. *Ann Rev Biochem* 1992; 61: 761-807.
- 25) Gregori L, Poosch M, Cousins G, Chau V. A uniform isopeptide-linked multiubiquitination insufficient to target substrate for degradation in ubiquitin-mediated proteolysis. *J Biol Chem* 1990; 265: 8354-7.
- 26) McCarthy MM, Kaufman LC, Brook PJ, Pfaff DW, Schwarz-Giblin S. Estrogen modulation of mRNA levels for the two forms of glutamic acid decarboxylase (GAD) in female rat brain. *J Comp Neurol* 1995; 360: 685-97.
- 27) Lee C-K, Klopp RG, Weindruch R, Prolla TA. Gene expression profile of aging and its retardation of caloric restriction. *Science* 1999; 285: 1390-4.
- 28) Gauss C, Kalkum M, Lowe M, Lehrach H, Klose J. Analysis of the mouse proteome. (1) Brain proteins: Separation by two-dimensional electrophoresis and identification by mass spectrometry and genetic variation. *Electrophoresis* 1999; 20: 575-600.