Iron and Ferritin Deficiencies in the Cerebral Cortex of Senescence-accelerated Mouse Prone 10

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Abstract

Previous studies have shown that iron (Fe) is essential for tyrosine hydroxylase (TH) activity and is a risk factor for cognitive decline and brain atrophy. The senescence-accelerated mouse prone 10 (SAMP10) strain exhibits a decline of catecholamines, brain atrophy, and behavioral deterioration, such as dysfunctions in learning and memory, with advancing age. The purpose of this study was to elucidate the contribution of iron to catecholamine metabolism in the brains of SAMP10 mice.

Iron concentration was determined using an inductively coupled plasma mass spectrometer. Expression of the metal binding proteins ferritin, divalent metal transporter-1 (DMT1), and hepcidin was determined by western blot analysis.

The present study demonstrated that iron and ferritin levels in SAMP10 mice were significantly lower than that in control animals. DMT1 and hepcidin levels, however, did not differ significantly between SAMP10 and control mice. Our previous study showed that the decline in dopamine (DA) and norepinephrine (NE) concentrations in the cerebral cortex of SAMP10 mice is caused by downregulation of TH activity. Given that Fe is necessary for TH activity, our results suggest that a decrease in iron causes the decline of TH activity in the cerebral cortex of SAMP10 mice.

Keywords: Iron, SAMP10, catecholamines, tyrosine hydroxylase, brain, ferritin

Introduction

Fe is a risk factors for cognitive decline and brain atrophy [1]. The human body has an average of 4–5 g of Fe, about 65% of which is in the form of hemoglobin and 4% in the form of myoglobin. Another 15-30% is stored mainly in the reticuloendothelial system and in the liver parenchymal cells, primarily in the form of ferritin [2]. The main transporters likely to be involved in brain Fe uptake include proteins that are involved in the transport of dietary Fe across the apical membrane of intestinal mucosal cells, as well as proteins that facilitate Fe export from the basolateral membrane into the blood stream. A major protein involved in the intestinal absorption of Fe from the diet is a divalent metal transporter known as DMT1 (also known as Nramp2 and DCT1) [3], which imports iron into intestinal epithelial cells (enterocytes) with the aid of a membrane-bound reductase, known as Dcytb, that reduces ferric iron (Fe³⁺) to the transportable ferrous form (Fe²⁺) [4]. It is not known how Fe traverses the polarized enterocytes, but an Fe exporter known as ferroportin (FPN) mediates Fe export from the enterocytes into the circulation. FPN is the only
mammalian Fe exporter that has thus far been identified, and its substrate is ferrous (Fe\(^{2+}\)) iron [5].

Intestinal Fe absorption is highly regulated by a hormone known as hepcidin [6], which is released mainly from the liver in response to Fe overload or inflammation. Hepcidin can bind to FPN and induce its internalization and degradation, thereby blocking Fe absorption in response to systemic Fe abundance. Intestinal Fe absorption is also regulated by hypoxia inducible factor 2 (HIF2), a transcription factor known mainly for its ability to mediate the response of cells to hypoxia, which regulates the transcription of DMT1 and Dcytb [7, 8]. Fe is stored intracellularly in the cytosolic protein ferritin, and the major repositories of Fe in the systemic circulation include the liver and the reticuloendothelial macrophages. All tissues in the body share a common system of Fe delivery and homeostasis, based on intestinal Fe absorption and circulating Tf, except for 2 regions that are separated from the systemic circulation by epithelial or endothelial barriers: the central nervous system (CNS) and the testes [9].

Catecholamines are a class of the neurotransmitters that includes dopamine (DA), norepinephrine (NE) and epinephrine. They are implicated in learning and memory. Studies indicate that catecholamines correlate with the aging of the brain. Tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of catecholamines, converts tyrosine to DOPA, which is further processed to DA by aromatic amino acid decarboxylase, Dopamine-β-hydroxylase (DβH) then converts DA into NE [10]. TH is a member of a family of enzymes that also includes the aromatic amino acid hydroxylases, such as phenylalanine hydroxylase and tryptophan hydroxylase; all are phosphorylated by cAMP-dependent protein kinase (PKA) [11]. When phosphorylated by PKA, TH is less susceptible to feedback inhibition by catecholamines [12]. The synthesis of DA is regulated by phosphorylation of the rate-limiting enzyme, TH, under physiological conditions, with the phosphorylation of Ser40 directly increasing TH activity [13]. The phosphorylation of TH at Ser40 is modulated by PKA and calmodulin kinase 2 (CaMKII) [10].

Senescence-accelerated mice (SAM) have been established as novel murine models to elucidate disease pathologies and the basic mechanisms of aging [14]. SAM comprises the senescence-accelerated mouse prone (SAMP) strains and the senescence-accelerated mouse resistant (SAMR) strains that have a normal aging process. SAMP10 mice exhibit brain atrophy and behavioral deterioration, such as dysfunctions of learning and memory, emotional disorders (reduced anxiety-like behavior and depressive behavior), and altered circadian rhythms with advancing age [15]. Our previous study suggests that the decline of catecholamines in the cerebral cortex of SAMP10 mice is caused by impairment of the catecholamine synthetic pathway, and not by enhancement of catabolic processes. The decrease of Ser40-phosphorylated TH levels is related to a decline in the protein levels of PKA; additionally, the decline of DA concentration is related to the decline of TH activity by Ser40 phosphorylation. There is no significant difference in the protein levels of TH [16]. As cofactors for TH activity in catecholamine synthesis, tetrahydrobiopterin (BH4) and Fe play a key role in the CNS. Moreover, TH uses diatomic oxygen and reduced biopterin, an oxidized degradation product of BH4 produced through the reaction with a bound Fe atom [13].

The role of Fe must be investigated further to elucidate the mechanism of abnormal catecholamine synthesis and the contribution of catecholamines to the age-related decline of learning and memory in SAMP10 mice. In order to understand how alterations in Fe concentration affect catecholamine metabolism in the brain, we investigated the fluctuation of Fe metabolism that is involved in TH activation in SAMP10 mice.

Materials and Methods

Animals

The experimental model for the present study comprised male SAMP10 and SAMR1 mice at 12 months of age; SAMP10 mice were used as the experimental group, while SAMR1 mice were used as the control group. SAMP10 and SAMR1 mice were bred in an animal room in which the light/dark cycle was set at 12 h and temperature and humidity were maintained at 22°C ± 1°C and 60% ± 5%, respectively. Free access to solid food (Crea, Japan) and tap water was provided. Animal studies were performed in accordance with the guidelines established by the Hokkaido University Committee on Animal Care and Use.

Brain preparation

SAMP10 and SAMR1 mice were killed under anesthetization with ether, and the brains were dissected into 7 discrete regions (cerebellum, medulla oblongata andpons, hypothalamus, midbrain and thalamus, striatum, hippocampus, and cerebral cortex) by the method of
Glowinski and Iversen [17] on an ice-cold plastic plate. The samples were then transferred to a desalted vessel and kept at -80°C prior to experimentation.

**Determination of Fe concentration in the cerebral cortex with an inductively coupled plasma mass spectrometer**

For trace element measurement, the brain sample was weighed and transferred to a Teflon decomposition vessel. After the addition of 1mL of >68% super pure grade nitric acid, the sample was decomposed for 1h in the oven at 110°C. After acid decomposition, the sample was diluted with distilled water. Fe concentration was determined with an inductively coupled plasma mass spectrometer (Seiko SPQ-6500, Japan) by the method previously described [18].

**Western blotting of ferritin, DMT1 and hepcidin**

For western blots, cerebral cortex tissues were homogenized, as described by Palmiter [19]. The brain sample was added to 1 mL of homogenate buffer, containing 55 mM Tris pH 7.4, 2.2% sodium dodecyl sulfate (SDS), 5.5% β-mercaptoethanol (β-ME), 11% glycerol, and 55 µM PMSF, in an Eppendorf tube and homogenized with a Polytron (PCU Drehzahlregler, Kinematica, Switzerland) twice for 30 s on ice. The homogenate was then centrifuged at 15,000 × g for 15 min at 4°C, and the supernatant was transferred to a fresh tube. Tubes were heated for 5 min at 100°C, and 0.1% bromophenol blue (BPB)-glycerol was added. Proteins in the tissue supernatants (20-30 µg) were separated by SDS-PAGE (12.5% e-PAGEL, ATTO, Japan). Biotinylated protein molecular weight markers (M&S TechnoSystems, Japan) were used as protein standards. Proteins were then electrophoretically transferred onto a nitrocellulose membrane (Bio-Rad, USA) with blotting buffer that contained 48 mM Tris buffer, 39 mM glycin, 0.02% SDS, and 10% methanol, or by using the iBlot transfer system (Invitrogen, USA). The nitrocellulose membrane was incubated overnight at 4°C, in a 5% blocking solution, containing 40 mM Tris-HCl buffer (pH 7.4), 0.9% NaCl, 0.3% Tween 20, and 1% blocking reagent or Odyssey Blocking Buffer. Again, the membrane was washed 3 times for 3 min in 40 mM Tris-HCl buffer (pH 7.4), 0.9% NaCl, and 0.3% Tween20, and then exposed to the secondary antibody: anti-rabbit IgG IRDye 680 or anti-mouse IgG IRDye 800 (M&S Techno Systems, Japan), diluted 1500 times in 1% blocking solution. Finally, the membrane was washed 3 times for 3 min in 40 mM Tris-HCl buffer (pH 7.4), 0.9% NaCl, and 0.3% Tween 20. Protein bands were quantitated with an Odyssey Infrared Imaging System (M&S Techno Systems, Japan). The protein concentration of the obtained homogenate was measured by Bradford assay (Protein Assay, Bio-Rad, USA) [20].

**Statistics**

Each value is expressed as mean ± SEM. Statistical differences between the 2 mouse strains were assessed using the two-tailed Student’s t-test. Statistical significance was assessed for p < 0.05.

**Results**

**Determination of Fe concentration in the cerebral cortex of SAMP10 and SAMR1 strains**

In this study, we first investigated the concentrations of Fe in the cerebral cortex of the SAMP10 and SAMR1 strains. Fe concentration was determined with an inductively coupled plasma mass spectrometer. Fe concentrations in the cerebral cortex of SAMP10 mice at 12 months of age were significantly lower than those of SAMR1 mice (p < 0.05) (Fig. 1).

**The protein level of ferritin in the cerebral cortex of SAMP10**

To elucidate the contribution of Fe storage protein to Fe deficiency in the cerebral cortex of SAMP10 mice, the protein level of ferritin was determined by western blot analysis. As shown in Fig.2, the level of ferritin in the cerebral cortex of SAMP10 mice was significantly lower than that of SAMR1 mice (p < 0.05) (Fig. 1).

**The protein level of hepcidin in the cerebral cortex of SAMP10**

To elucidate the contribution of Fe regulatory hormone to Fe deficiency in the cerebral cortex of SAMP10 mice, the protein level of hepcidin was determined by western blot analysis. As shown in Fig.3, no significant difference
was detected in the protein levels of hepcidin in the cerebral cortex of SAMP10 mice.

**The protein level of DMT1 in the cerebral cortex of SAMP10**

To elucidate the contribution of Fe transporter to Fe deficiency in the cerebral cortex of SAMP10 mice, the protein level of DMT1 was determined by western blot analysis. As shown in Fig.4, no significant difference was detected in the protein levels of DMT1 in the cerebral cortex of SAMP10 mice, as compared with SAMR1 mice.

![Western blot analysis of ferritin](image1)

*Fig. 1* Fe concentrations in the cerebral cortex of SAMR1 and SAMP10 mice at 12 months of age. 12M R1 (left column) indicates SAMR1 mice at 12 months of age. 12M P10 (right column) indicates SAMP10 mice at 12 months of age. The values are expressed as mean ± SEM (n = 6 - 7); * p < 0.05, as compared with SAMR1.

![Western blot analysis of hepcidin](image2)

*Fig. 2* Western blot analysis of ferritin

The graph shows the mean staining intensity of western blots for ferritin in the cerebral cortex of SAMR1 and SAMP10 mice at 12 months of age. 12M R1 (left column) indicates SAMR1 mice at 12 months of age. 12M P10 (right column) indicates SAMP10 mice at 12 months of age. The values are expressed as mean ± SEM (n = 7); ** p < 0.01, as compared with SAMR1 mice.

![Western blot analysis of DMT-1](image3)

*Fig. 3* Western blot analysis of hepcidin.

The graph shows the mean staining intensity of western blots for hepcidin in the cerebral cortex of SAMR1 and SAMP10 mice at 12 months of age. 12M R1 (left column) indicates SAMR1 mice at 12 months of age. 12M P10 (right column) indicates SAMP10 mice at 12 months of age. The values are expressed as mean ± SEM (n = 4).

![Western blot analysis of DMT-1](image4)

*Fig. 4* Western blot analysis of DMT-1.

The graph shows the mean staining intensity of western blots for DMT-1 in the cerebral cortex of SAMR1 and SAMP10 mice at 12 months of age. 12M R1 (left column) indicates SAMR1 mice at 12 months of age. 12M P10 (right column) indicates SAMP10 mice at 12 months of age. The values are expressed as mean ± SEM (n = 3).
Discussion

The purpose of this study was to link alterations in Fe metabolism to changes in catecholamine metabolism in the brains of SAMP10 mice. Therefore, we investigated fluctuations in the metabolism of Fe, which is essential for the activity of the rate-limiting enzyme TH in the synthesis of catecholamines.

We focused on the synthetic pathway of the catecholamines. TH is the key, specific enzyme in the synthesis of catecholamines, regulating the levels of DA, NE, and epinephrine [10]. The phosphorylation of TH is necessary for its enzymatic activity [21]. Insufficient phosphorylation of TH with aging may lead to a deficiency in TH activity and catecholamine concentrations [22]. Even in the absence of a decrease in TH protein levels with aging, a decrease in the affinity of TH for both substrate and coenzymes (such as BH4 and Fe) may result in a decrease in TH activity, and thus cause the levels of DA to decrease [23].

Our previous study showed that the decrease of catecholamines in the cerebral cortex of SAMP10 mice was caused by impairment of the catecholamine synthetic pathway, and suggested that the decline of catecholamines was closely related to the dysfunction of learning and memory in SAMP10 mice that occurs with aging. Moreover, the decline of DA and NE concentrations may be closely related to the decline in TH activity caused by downregulation of TH phosphorylation due to PKA deficiency [16]. As cofactors for TH activity in catecholamine synthesis, BH4 and Fe play a key role in the CNS. Moreover, TH uses diatomic oxygen and reduced bipterin, an oxidized degradation product of BH4 produced through a reaction with the bound Fe atom [13]. The binding affinity of TH for BH4 and the role of Fe must be investigated to elucidate the mechanism of abnormal catecholamine synthesis and the contribution of catecholamines to the age-related decline of learning and memory in SAMP10 mice.

Fe deficiency has a significant negative impact on brain development and cognition, and recent studies indicate that both the striatal dopaminergic-opiate system and the cholinergic systems are adversely affected by Fe deficiency [24]. Many human studies have demonstrated the negative effects of Fe deficiency on learning, memory, and affective and social behaviors [25]. These studies suggest that the decline of Fe levels may be related to the dysfunction in learning and memory that occurs in SAMP10 mice with aging.

Next, we investigated changes in the Fe regulatory proteins – the Fe transporter, DMT1, the Fe storage protein, ferritin and the iron regulatory hormone, hepcidin – to elucidate the mechanism of Fe deficiency in the cerebral cortex of SAMP10 mice. The level of ferritin in the cerebral cortex of SAMP10 mice was significantly lower than that of SAMR1 mice. No significant differences, however, were detected in the protein levels of hepcidin and DMT1 in the cerebral cortex of SAMP10 mice, as compared with SAMR1 mice. These results suggest that Fe deficiency in the cerebral cortex of SAMP10 mice is closely related to the decline of the Fe storage protein, ferritin, but not to Fe uptake.

The present study indicates that the reduction of TH activity in the brains of SAMP10 mice may be caused by a decline in the Fe concentration, which correlates with a decrease in ferritin storage. As a cofactor for TH activity in catecholamine synthesis, BH4 plays a key role in the CNS. The binding affinity of BH4 must be investigated further to elucidate the mechanism of abnormal catecholamine synthesis and the contribution of catecholamines to the age-related decline of learning and memory in SAMP10 mice.

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