Identification of the responsible proteins for increased selenium bioavailability in the brain of transgenic rats overexpressing selenoprotein M

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Abstract. The present study was conducted to investigate whether the high antioxidant activity induced by selenium (Sel) treatment and selenoprotein M (SelM) overexpression affected the protein profile of the brain cortex. To accomplish this, the changes in global protein expression were measured in transgenic (Tg) rats expressing human SelM (CMV/hSelM) and non-Tg rats using two-dimensional electrophoresis (2-DE). The results revealed that: i) CMV/hSelM Tg rats showed a high level of enzyme activity for antioxidant protein in the brain cortex compared to non-Tg rats; ii) the high activity of these enzymes induced a decrease in total antioxidant concentration and γ-secretase activity in CMV/hSelM Tg rats; iii) five proteins were upregulated and three were downregulated by SelM overexpression; iv) among the five upregulated proteins, two associated with creatine kinase B-type (B-CK) and E3 ubiquitin-protein ligase RING1 (RING finger protein 1) were further increased in the two groups following Sel treatment, whereas synaptotagmin-15 (SytXV), eukaryotic translation initiation factor 4H (eIF-4H) and lactate dehydrogenase B (LDH-B) were increased or decreased under the same conditions; v) the three downregulated proteins did not induce a significant change in expression following Sel treatment; and vi) the protein expression level alterations of the two selected spots (B-CK and SytXV) identified by 2-DE were extremely similar to the results from western blot analysis. Overall, the results of the present study provide primary novel biological evidence that new functional protein groups and individual proteins in the brain cortex of CMV/hSelM Tg rats are associated with Sel biology, including the response to Sel treatment and SelM overexpression.

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Introduction

Selenium (Sel) is an ubiquitous trace element in nature that has been shown to be essential to various aspects of human health (1). This trace element has also been shown to be required for normal growth and reproduction during spermatogenesis (2). Furthermore, Sel deficiency induces multiple diseases associated with oxidative damage, such as fatal cardiomyopathy, which is endemic in Keshan (China) (3), and muscular dystrophy in patients subjected to long-term unsupplemented parenteral nutrition (4). It is also well known that vitamin E can partially replace Sel deficiency (5,6). Sel exists naturally in organic (such as selenomethionine and selenocysteine) and inorganic forms (such as selenite, selenate and selenide) (7). However, these compounds require catabolizing into inorganic precursors prior to insertion into proteins, and the rare amino acid selenocysteine (Sec) is essential for the catalytic function of selenoenzymes (8). Sec, which is the 21st proteinogenic amino acid, was not initially recognized in the

classical genetic code as it is encoded by the UGA 'STOP' codon. For Sec insertion at UGA codons in the translation process, a specific RNA stem-loop structure is required. In eukaryotes, this loop resides in the 3'-untranslated region of the mRNA, known as the Sec-insertion-sequence (9). Sel has also been shown to be an essential part of mammalian enzymes, such as glutathione peroxidase (GPx), thyroid hormone deiodinase and thioredoxin reductase. Thus far, 25 genes encoding selenoproteins in the sequenced human genome have been identified (10). Selenoprotein M (SelM) was first reported as a 0.7-kb cDNA gene that encoded a new selenoprotein identified from the mammalian EST database. This gene has a 145-amino acid open reading frame beginning with an ATG codon in a favorable Kozak context and contains an in-frame TGA as the Sec codon. Furthermore, homologous proteins have been identified in the rat, zebra fish and other vertebrates, and Sec was conserved in these homologs (11). There have also been several functional studies of SelM. For example, the study by Müller *et al* (12) showed that this protein plays a major role in spicule formation in the demosponge *Suberites domuncula*. In addition, Hwang *et al* (13) indicated that SelM plays a suppressive or protective role in the pathology of patients with Alzheimer's disease (AD). However, there have been no studies for whether SelM overexpression could affect the changes in global gene expression in CMV/hSelM transgenic (Tg) rats following Sel treatment.

Sel is maintained at high levels in the brain, even upon prolonged dietary Sel deficiency (14). Changes in Sel concentration in the brain and blood have been detected in AD, Parkinson's disease (PD), multiple sclerosis and brain tumors. Several studies have shown that Sel treatment leads to reduced seizures, improved electroencephalogram recordings (15), protection against the depletion of striatal dopamine (16) and a reduction in the progression of neurodegeneration (17). Furthermore, several selenoproteins have been expressed in the brain. Among these proteins, GPx has been localized in glial cells, and its expression level was significantly upregulated in damaged areas in PD (18). High expression of selenoprotein P (SelP) was also observed in the olfactory bulb, hippocampus and frontal cortex (19). Genetically-engineered models, including Tg and knock-out models, can provide a platform to define the *in vivo* function of genes and to also study the molecular events responding to environmental changes (20). Previous studies using a knock-out model of SelP showed that it had the important function of Sel delivery into the brain tissue (21,22). Other selenoproteins, including selenoprotein W, thioredoxin reductase, 15-kDa selenoprotein and SelP, have also been detected in the brain. However, numerous questions regarding the roles of these proteins in neuronal function remain.

In the present study, the global change of gene expression affected by SelM overexpression and Sel treatment in the brain cortex was investigated. Two-dimensional electrophoresis (2-DE) analysis showed that eight proteins were significantly changed in CMV/hSelM Tg rats. These results indicated that the information isolated from SelM overexpression and Sel treatment may be useful for studying the association between antioxidant conditions and brain disease, which shows a higher level of oxidative stress condition in specific tissues.

Materials and methods

Maintenance and identification of CMV/hSelM Tg rats. The CMV/hSelM Tg rats used in the study, showing high antioxidant status in various tissues, were developed by microinjection of the CMV/hSelM recombinant gene into fertilized rat eggs (13). The animal protocol was reviewed and approved based on the ethical and scientific care procedures of the Korea Food and Drug Administration (KFDA)-Institutional Animal Care and Use Committee. All rats were maintained in an accredited KFDA animal facility in accordance with AAALAC International Animal Care policies (Accredited Unit-KFDA; unit No. 000996). The rats were provided a standard irradiated chow diet (Purina Mills Inc., St. Louis, MO, USA) *ad libitum* and maintained in a specified pathogen-free state under a strict light cycle (lights on at 06:00 h and off at 18:00 h). All the pedigrees were hemizygous for their transgenes.

Experimental design and Sel treatment. Sodium selenite ($NaSeO_3$) purchased from Sigma-Aldrich Co., (St. Louis, MO, USA) was dissolved in distilled water to a final concentration of 0.2 μmol/μl (23,24). Ten-week-old rats were randomly divided into two subgroups (n=6 per group). The first subgroup of the CMV/hSelM Tg and non-Tg rat groups each received a comparable volume of distilled water daily via intraperitoneal injection (vehicle-treated CMV/hSelM Tg and non-Tg groups), whereas rats in the second subgroup each received 5 μmol/kg body weight/day of sodium selenite via intraperitoneal injection for three weeks (Sel-treated CMV/hSelM Tg and non-Tg groups). At three weeks after Sel-solution injection, the animals were immediately euthanized using CO_2 gas, following which the cortex samples from their brains were prepared and stored in Eppendorf tubes at -70˚C until assayed.

Analysis of GPx and superoxide dismutase (SOD) activities and total oxidized products concentration. The levels of GPx and SOD in the brain cortex of CMV/hSelM Tg and non-Tg rats were detected by following the colorimetric assay procedure using Bioxytech SOD-525 and Bioxytech GPx-340 kits (OxisResearch™, Portland, OR, USA). The level of total oxidized products in the sera of CMV/hSelM Tg and non-Tg rats was detected using a Total Antioxidant Status kit (Randox Laboratories Ltd., Antrim, UK) as previously described (13).

γ-secretase activity analysis. The γ-secretase activity was detected with a γ-Secretase Activity kit (R&D System Inc., Minneapolis, MN, USA) using the manufacturer's instructions. Initially, cortex tissue was homogenized with a glass homogenizer in cold 1X extraction buffer to yield a final protein concentration of roughly 0.5-2.0 mg. These mixtures were incubated on ice for 10 min, after which they were centrifuged at 10,000 x g for 1 min to remove the unbroken fragments. The final supernatants collected from the centrifuged mixture were subsequently used for detection of secretase activity, and this process was carried out in the microplate provided by the manufacturer. To perform the enzyme reaction, 50 μl tissue lysate was added to each well, followed by 50 μl 2X reaction buffer and 5 μl substrate. The microplates were gently mixed and incubated in the dark at 37˚C for 1-2 h to induce an enzyme-substrate reaction. Final fluorescence produced by the

reaction was read on a fluorescent microplate reader using a filter that allowed EDANS excitation at between 335-355 nm.

Sample preparation for 2-DE. Analyses of global protein expression by 2-DE were performed according to the methods established by our laboratory in previous studies (25-27). The cortex samples isolated from the brain were homogenized in liquid nitrogen, following which the homogenized tissues were lysed in buffer [7 M urea, 2 M thiourea, 4% w/v CHAPS, 40 mM Tris and 100 mM dithioerythritol (DTE)]. The sample mixtures were subsequently centrifuged at 45,000 x g at 4˚C for 1 h, after which protein concentrations were determined by the Bradford protein assay (Bio-Rad, Hercules, CA, USA). In this process, a cortex sample was generated from a pool of the six animals in each group. The pooled samples were analyzed three times.

2-DE analysis. One-dimensional isoelectric focusing (IEF) was performed using 24 cm immobilized pH gradient (IPG) strips (GE Healthcare, Uppsala, Sweden) in a pH range of 3.0-10.0 (non-linear). Protein (1-mg) was loaded in a total volume of 450 μl, following which the samples were diluted with rehydration solution [7 M urea, 2 M thiourea, 4 % w/v CHAPS, 40 mM Tris, 100 mM DTE and 2% IPG buffer (pH 3.0-10.0)]. After rehydration for 13 h, the strips were focused at 30 V for 2 h, 100 V for 2 h, 200 V for 1 h, 500 V for 1 h, 1,000 V for 1 h and finally at 8,000 V for 22 h to obtain ~100,000 VHr (IPGphor; GE Healthcare). Once IEF was completed, the strips were equilibrated in 6 M urea containing 20% glycerol, 2% SDS and 0.01% bromophenol blue with 10 mM tributyl phosphine. Two-dimensional SDS-PAGE was performed using 8-18% linear gradient acrylamide gels on an EttanDalt system (GE Healthcare). Proteins were visualized by staining with Coomassie blue G-250 (Bio-Rad).

To analyze changes in protein expression between the types of rats according to SelM level, an average gel representing non-Tg rats was compared to an average gel representing the CMV/hSelM Tg rats. Only the filtered spots exceeding an intensity threshold of a 1.5 or 2-fold increase or decrease between non-Tg and CMV/hSleM Tg rats were studied further, whereas the threshold regulation factor for the significance level was set at $P \leq 0.05$. Furthermore, any spot showing a significant difference in expression between non-Tg and CMV/hSleM Tg rats was analyzed in all the rats to map changes in expression according to Sel-related factors. Finally, the spots showing significant changes in expression were subsequently identified by mass spectrometry.

Identification of protein spots. The stained gels were scanned with a GS800 densitometer (Bio-Rad) and analyzed using Image master™ (Swiss Institute of Bioinfomatics, Geneva, Switzerland). The spots were digested using trypsin, following which supernatant peptide mixtures were loaded onto a Poros R2 column (Applied Biosystems, Foster City, CA, USA) that had been washed with the following solutions: i) 70% acetonitrile in 5% formic acid; ii) 100% acetonitrile; and iii) 5% formic acid. Peptides were eluted using 5 μl of α-cyano-4-hydroxycinnamic acid and analyzed with a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Voyager DE-PRO; Applied Biosystems). For protein identification, masses of peptides determined by MALDI-TOF were matched with theoretical peptides in the NCBI (http://www.ncbi.nih.gov/) database using the MASCOT (http://www.matrixscience.com) and ProFound programs (http://prowl.rockefeller.edu).

Western blot analysis. Total proteins prepared from a cortex sample of CMV/hSelM Tg and non-Tg rats were separated by electrophoresis on a 4-20% SDS-PAGE gel for 3 h and subsequently transferred to nitrocellulose membranes for 2 h at 40 V. Each membrane was incubated separately with anti-creatine kinase antibody (Abcam, Cambridge, UK), anti-synaptotagmin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and anti-actin (Sigma-Aldrich) antibodies overnight at 4˚C. The membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Zymed Laboratories, Inc., South San Francisco, CA, USA) at a 1:1,000 dilution at room temperature for 2 h. The membrane blots were developed using a Chemiluminescence Reagent Plus kit (ECL; Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA).

Statistical analysis. Tests for significance between vehicle- and Sel-treated rats were performed using a one-way analysis of variance test of variance (SPSS for Windows, Release 10.10, Standard Version; SPSS, Inc., Chicago, IL, USA). Tests for significance between CMV/hSelM Tg and non-Tg rats were performed using a post-hoc test (SPSS for Windows, Release 10.10, Standard Version) of variance, and significance levels are provided throughout. All the values are reported as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Enhancement of SOD and GPX activity in the brain cortex of CMV/hSelM Tg rats. To confirm whether or not alteration of antioxidative conditions is induced by Sel treatment and SelM overexpression in the cortex region of rat brains, the activity of SOD and GPX was measured in the brain tissue of CMV/hSelM Tg and non-Tg rats using a detection kit containing a specific substrate. In the vehicle-treated group, SOD/GPX activity in the CMV/hSelM Tg rats showed a higher level of enzyme activity than that of the non-Tg rats, although they showed differing rates of increase (Fig. 1A and C). Following Sel treatment, the activity of these enzymes increased significantly in the CMV/hSelM Tg and non-Tg rats. However, the rate of increase in the CMV/hSelM Tg rats was greater than that of the non-Tg rats (Fig. 1A and C). These results indicate that SelM overexpression and Sel treatment induced an increase of antioxidant protection in the brain cortex from CMV/hSelM Tg rats.

Change in total oxidized products concentration in the brain of CMV/hSelM Tg rats. To determine whether or not enhancement of SOD and GPX activity was accompanied by a decreased level of oxidized products, the concentration of total oxidized products was determined in the serum of CMV/hSelM Tg and non-Tg rats following Sel treatment using an ELISA kit. For the vehicle-treated group, the concentration of total oxidized products at the basal level was significantly lower in the

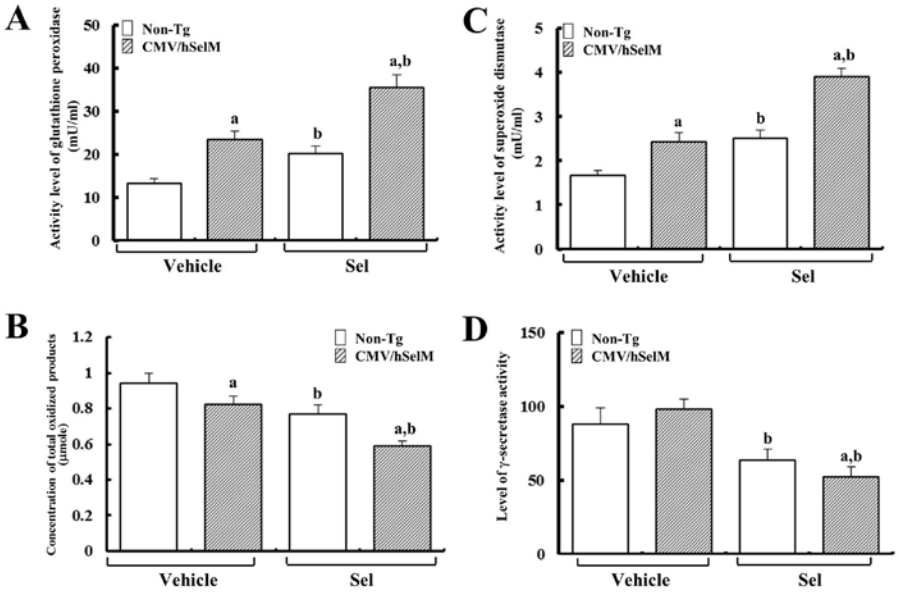

Figure 1. Effects of selenium (Sel) treatment and selenoprotein M (SelM)overexpression on (A) glutathione peroxidase (GPx) activity, (B) superoxide dismutase (SOD) activity, (C) concentration of total oxidized products and (D) γ-secretase activity in the brain cortex. The brains used in this assay were collected from CMV/hSelM Tg and Non-Tg rats following intraperitoneal injection of sodium selenite (5 μmole/kg body weight/day) for three weeks. Enzyme activity and total oxidized products were assayed in six rats per group by ELISA. The data represent the means ± standard deviation from three replicates. [a]Significant difference (P<0.05) when compared to non-Tg rats; [b]significant difference (P<0.05) when compared to the vehicle-treated group.

CMV/hSelM Tg rats compared to the non-Tg rats. Following Sel treatment, these levels decreased simultaneously in the CMV/hSelM Tg and non-Tg rats, although the concentration of total oxidized products in the CMV/hSelM Tg rats was consistently maintained at a low level compared to the non-Tg rats. In particular, the decreasing rate of total oxidized products concentration in the Sel-treated group was significantly greater compared to the vehicle-treated group (Fig. 1B). These results indicate that the overactivation of SOD and GPX induced by SelM overexpression and Sel treatment contributes to the increase in the functional activity for oxidized products protection in the brain cortex of CMV/hSelM rats.

Change of γ-secretase activity in the brain cortex of CMV/hSelM Tg rats. Furthermore, γ-secretase plays an important role in the production of Aβ-42 peptide in the pathogenesis of AD (28). To investigate the effects of Sel treatment and SelM overexpression on the physiological changes in neurodegenerative pathology, the activity of γ-secretase, a critical enzyme of Aβ-42 peptides production, was measured in the brains of CMV/hSelM Tg and non-Tg rats using a detection kit containing a specific substrate. For the vehicle-treated group, the activity of γ-secretase in the CMV/hSelM Tg rats was slightly higher compared to non-Tg rats, although this difference was not significant. However, following Sel treatment, these activities were significantly lower in the two groups of rats. Furthermore, the rate of decrease in γ-secretase activity in the CMV/hSelM Tg rats was higher compared to the non-Tg rats (Fig. 1D). These findings indicate that SelM overexpression and Sel treatment may lead to a decreased incidence of neurodegenerative disease in the brain cortex through the regulation of γ-secretase activity.

Proteome analysis of total proteins in brain cortex from of CMV/hSelM Tg rats. To characterize the changes in global protein expression in the brain cortex of CMV/hSelM Tg rats in response to the induced bioavailability of Sel treatment and SelM overexpression, whole proteins extracted from the brain cortex of 10-week old CMV/hSelM Tg and non-Tg rats were analyzed on analytic 2-DE gels. Computer analysis of gel images showed good matching in four analytical replicates, including vehicle-treated non-Tg, Sel-treated non-Tg, vehicle-treated CMV/hSelM Tg and Sel-treated CMV/hSelM Tg rats. The 2-DE protein maps of the samples from the four groups are shown in Fig. 2. Approximately 270 spots were detected in one gel from brain cortex. The spots that showed significantly different expression were selected for further analysis. Eight spots were identified as key proteins differentially expressed in four experimental groups. Furthermore, in the vehicle-treated group, eight spots were classified into two groups, including upregulated spots (five spots) and downregulated spots (three spots), according to their expression level in CMV/hSelM Tg rats. As shown in Table I, the five upregulated proteins included creatine kinase B-type (B-CK), synaptotagmin-15 (SytXV), E3 ubiquitin-protein ligase RING1 (RING1 finger protein 1), lactate dehydrogenase B (LDH-B) and eukaryotic translation initiation factor 4H (eIF-4H), whereas the three downregulated included centromere protein N (CENP-N), proteasome subunit K and dihydropyrimidinase-related protein 2 (DRP-2).

Following Sel treatment, the eight spots showed different patterns. The five-upregulated spots were classified into three groups according to their expression pattern. Two neighboring spots in the first group showed a marked increase in response to Sel treatment and were identified as B-CK and RING1 finger protein 1 (Fig. 3). The volume ratio of these two spots was significantly higher in the CMV/hSelM Tg rats compared to non-Tg rats. Following Sel treatment, these volumes were markedly increased in the two groups relative to the vehicle-treated group. Even though the volume of these

Table I. List of the differentially expressed proteins in the four experimental groups.

Spot no.	Protein name	Gene name	Accession no.	Sequence coverage, %	Mw, Da/pI	Mascot score	Vehicle		Selenium	
							Non-Tg	Tg[a]	Non-Tg[a]	Tg[a]
1	Creatine kinase B-type (EC 2.7.3.2) (Creatine kinase B chain) (B-CK)	Ckb	P07335	53	42698/5.39	124	1	3.07±0.31	2.19±0.18	3.87±0.25
2	E3 ubiquitin-protein ligase RING1 (EC 6.3.2.-) (Polycomb complex protein RING1) (RING finger protein 1)	Ring1	Q6MGB6	23	42634/5.54	30	1	2.72±0.18	2.98±0.21	3.62±0.28
3	Synaptotagmin-15 (Synaptotagmin XV) (SytXV)	Syt15	P59926	21	47562/8.45	32	1	1.66±0.12	2.37±0.18	1.25±0.11
4	L-lactate dehydrogenase B chain (EC 1.1.1.27) (LDH-B) (LDH heart subunit) (LDH-H)	Ldhb	P42123	14	36589/5.70	76	1	2.24±0.15	3.19±0.25	1.57±0.19
5	Eukaryotic translation initiation factor 4H (eIF-4H) (Williams-Beuren syndrome chromosomal region 1 protein homolog)	Eif4h	Q5XI72	51	27307/6.67	26	1	1.78±0.22	1.10±0.29	1.09±0.13
6	Proteasome subunit alpha type-3 (EC 3.4.25.1) (Proteasome component C8) (Proteasome subunit K) (Macropain subunit C8) (Multicatalytic endopeptidase complex subunit C8)	Psma3	P18422	58	28401/5.29	25	1	0.71±0.05	0.86±0.07	0.70±0.06
7	Centromere protein N (CENP-N)	Cenpn	Q5U2W4	31	39434/8.09	37	1	0.53±0.04	1.14±0.25	0.49±0.05
8	Dihydropyrimidinase-related protein 2 (DRP-2) (Turned on after division, 64 kDa protein) (TOAD-64) (Collapsin response mediator protein 2) (CRMP-2)	Dpysl2	P47942	32	62239/5.95	126	1	0.34±0.03	1.17±0.23	0.36±0.03

[a]Values in the last three columns on the right hand side are expressed as the relative value of the spot volume for the non-Tg vehicle group, which was defined as 1.

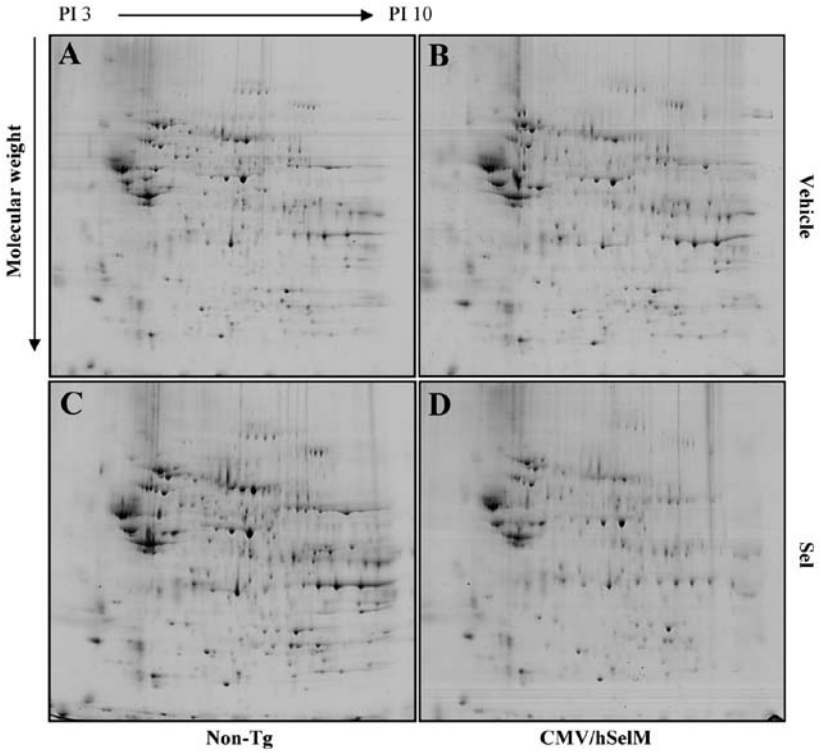

Figure 2. Two-dimensional electrophoresis (2-DE) protein patterns in the brain tissues from CMV/hSelM Tg and non-Tg rats. Analyses was of the brains cortex proteins by 2-DE. Cortex lysates (1 mg) from the rat brains of four groups; (A) vehicle-treated non-Tg, (B) selenium (Sel)-treated non-Tg, (C) vehicle-treated CMV/hSelM Tg and (D) Sel-treated CMV/hSelM Tg; were separated by one-dimensional isoelectric focusing (IEF) using 24 cm immobilized pH gradient strips with the pH range, 3.0-10.0 (non-linear). Second dimensional SDS-PAGE was then performed using 8-18% linear gradient acrylamide gels in an EttanDalt system. Protein spots were visualized by Coomassie blue G-250 staining.

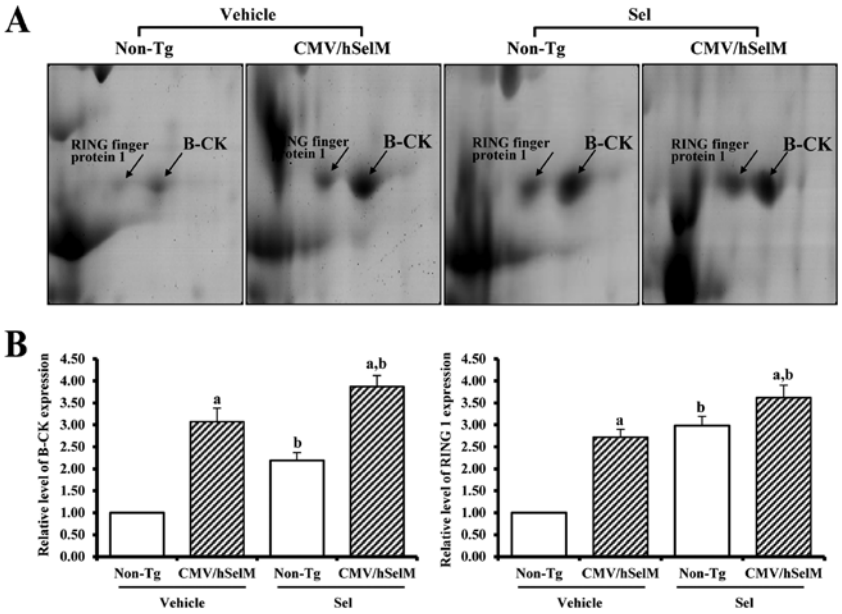

Figure 3. (A) Gel enlargement image and (B) relative expression level of RING finger protein 1 and creatine kinase B-type (B-CK) showing differential upregulation between cortex extracts from four experimental groups. Upregulated protein spots of RING finger protein 1 and B-CK were detected in the brain extracts from the four experimental groups. The spots differentially expressed on two-dimensional electrophoresis (2-DE) were further analyzed by a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer. The data represent the means ± standard deviation of three replicates. [a]Significant difference ($P<0.05$) when compared to non-Tg rats; [b]significant difference ($P<0.05$) when compared to the vehicle-treated group.

two spots increased in the Sel-treated group, they remained at a higher level in the CMV/hSelM Tg rats compared to the non-Tg rats (Fig. 3). The next group consisted of two spots that showed moderate changes in SytXV and LDH-B (Table I). The spot identified as SytXV was expressed at higher levels in the CMV/hSelM Tg rats than non-Tg rats that had received

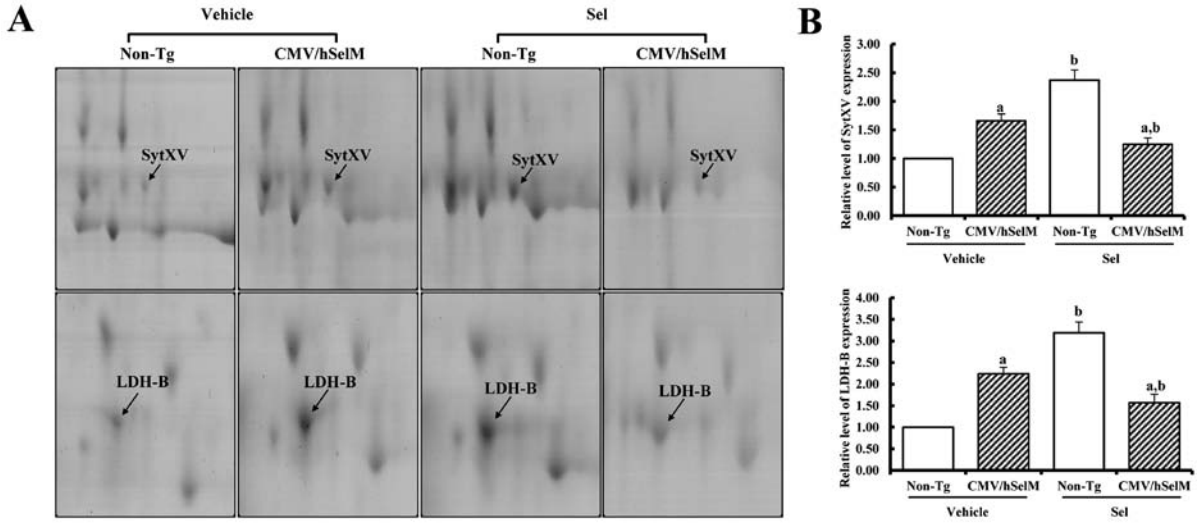

Figure 4. (A) Gel enlargement image and (B) relative expression level of synaptotagmin-15 (SytXV) and lactate dehydrogenase B (LDH-B) showing differential upregulation between cortex extracts from four experimental groups. Upregulated protein spots of SytXV and LDH-B were detected in the brain extracts from the four experimental groups. The spots differentially expressed on two-dimensional electrophoresis (2-DE) were further analyzed using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer. The data represent the means ± standard deviation from three replicates. [a]Significant difference (P<0.05) when compared to non-Tg rats; [b]significant difference (P<0.05) when compared to the vehicle-treated group.

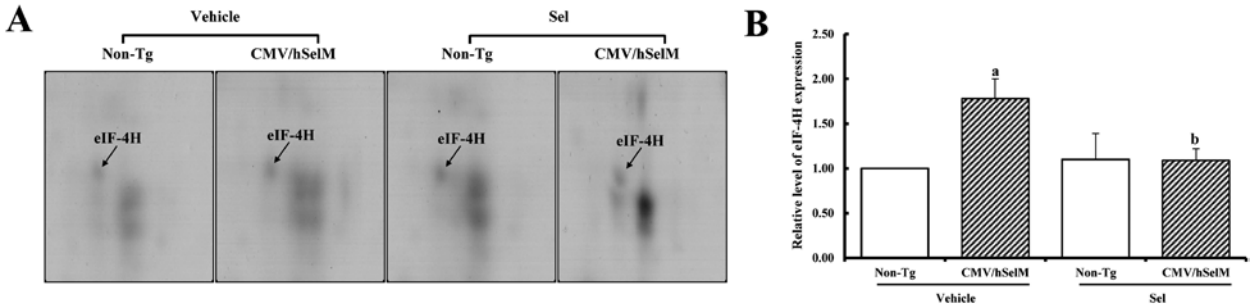

Figure 5. (A) Gel enlargement image and (B) relative expression level of eukaryotic translation initiation factor 4H (eIF-4H) showing differential up- or downregulation between cortex extracts from the four experimental groups. Up- or downregulated protein spots of eIF-4H were detected in the brain extracts from the four experimental groups. The spots differentially expressed on two-dimensional electrophoresis (2-DE) were further analyzed by a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer. The data represent the means ± standard deviation from three replicates. [a]Significant difference (P<0.05) when compared to non-Tg rats; [b]significant difference (P<0.05) when compared to the vehicle-treated group.

vehicle treatment. Sel treatment induced an increase in spot volume in non-Tg rats, whereas it was significantly decreased in CMV/hSelM Tg rats (Fig. 4). The spot isolated as LDH-B showed a change similar to the spot of SytXV. However, the basic level of these spots was higher in the CMV/hSelM Tg rats compared to the non-Tg rats (Fig. 4). The third group was not affected by Sel treatment and was only induced by SelM overexpression. Spot eIF-4H showed a significantly higher level in CMV/hSelM Tg rats, but was significantly downregulated following Sel treatment, whereas it remained unchanged in the non-Tg rats (Fig. 5).

The group that showed downregulated SelM was further classified into two subgroups. One large spot known as proteasome subunit K belonged to the first subgroup. The volume of this spot was lower in CMV/hSelM Tg rats than non-Tg rats under vehicle-treated conditions. However, Sel treatment induced a decrease in the proteasome subunit K level in non-Tg rats, whereas it remained at a constant level in the CMV/hSelM Tg rats (Fig. 6). In the second subgroup, two spots identified as CENP-N and DRP-2 showed similar patterns under the two conditions (Table I), with a lower level being observed for CMV/hSelM Tg rats than non-Tg rats. These levels were not changed following Sel treatment in either group (Fig. 7).

Confirmation of B-CK and SytXV expression. Western blot analysis was conducted to validate the changes in the protein expression levels of the two selected spots (B-CK and SytXV) identified by 2-DE. As shown in Fig. 8, the expression of B-CK was significantly higher in the CMV/hSelM Tg rats than non-Tg rats. Following Sel treatment, its level increased further in the two groups, although its total expression pattern was maintained. In the case of SytXV, its expression level was higher in CMV/hSelM Tg rats compared to non-Tg rats under vehicle treatment conditions. However, expression of this protein increased significantly in response to Sel treatment in non-Tg rats, whereas CMV/hSelM rats showed no significant difference in expression. The expression pattern of the above proteins observed upon western blot analysis was extremely similar to that in the 2-DE gel image, indicating that the alteration of protein spots detected by 2-DE reflects changes

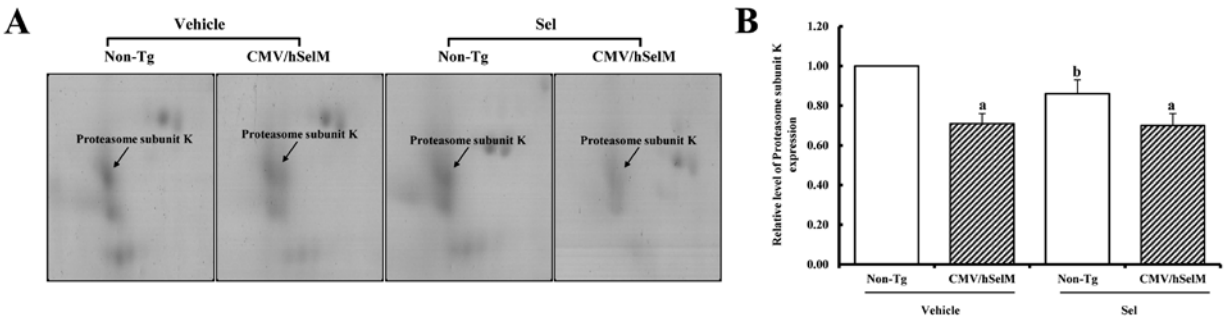

Figure 6. (A) Gel enlargement image and (B) relative expression level of proteasome subunit K showing differential downregulation between cortex extracts from four experimental groups. Downregulated protein spots of proteasome subunit K were detected in the brain extracts from the four experimental groups. The spots differentially expressed on two-dimensional electrophoresis (2-DE) were further analyzed by a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer. The data represent the means ± standard deviation from three replicates. [a]Significant difference ($P<0.05$) when compared to non-Tg rats; [b]significant difference ($P<0.05$) when compared to the vehicle-treated group.

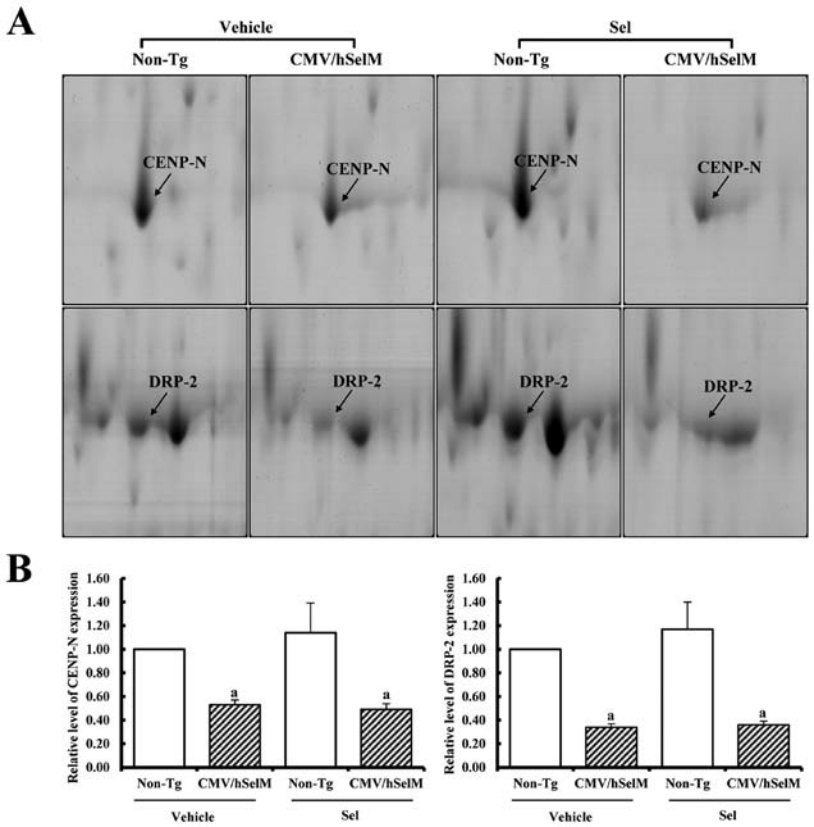

Figure 7. (A) Gel enlargement image and (B) relative expression level of CENP-N and DRP-2 showing differential downregulation between cortex extracts from four experimental groups. Downregulated protein spots of CENP-N and DRP-2 were detected in the brain extracts from the four experimental groups. The spots differentially expressed on two-dimensional electrophoresis (2-DE) were further analyzed by a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer. The data represent the means ± standard deviation from three replicates. [a]Significant difference ($P<0.05$) when compared to non-Tg rats. CENP-N, centromere protein N; DRP-2, dihydropyrimidinase-related protein 2.

in protein expression in the brains of the CMV/hSelM Tg and non-Tg rats.

Discussion

Sel deficiency is associated with a variety of serious diseases, including infectious disease, cardiovascular disease, cancer and neurodegenerative disorders (7). Savaskan *et al* (29) provided direct evidence that Sel plays a pivotal role in neuronal susceptibility to excitotoxic lesions. The results of this study indicated that the neuroprotective effects of Sel are not directly mediated via the antioxidative effects of selenite, but require *de novo* protein synthesis. Therefore, Sel deficiency in the brain tissue leads to increased oxidative stress with subsequent NK-κB activation and neuronal cell death. Several studies reported that intracellular ETKs were activated by either sodium selenite, an inorganic salt known to activate ERK (30), or transfected with a constitutively active mutant of MEK1, an immediate upstream activator of ERKR (31,32). The present study investigated whether the high level of

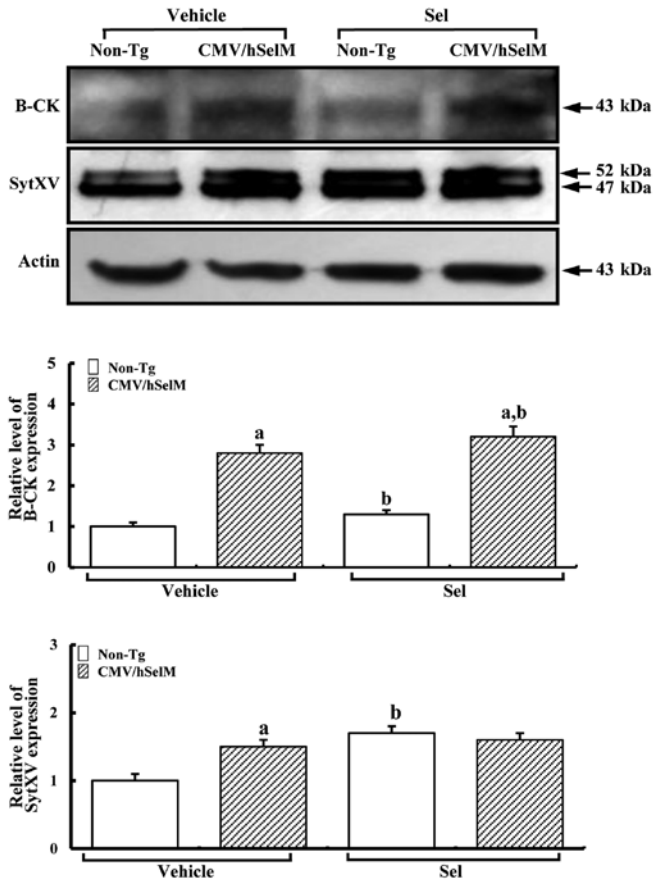

Figure 8. Conformation of creatine kinase B-type (B-CK) and synaptotagmin-15 (SytXV) expression in the brain cortex using western blot analysis. Tissue lysates were prepared from the brain tissue of vehicle-treated and selenium (Sel)-treated rats. Protein (50 μg per sample) were immunoblotted with the antibody for each protein. The expression level of B-CK and SytXV was detected with specific primary antibodies and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G antibodies. The intensity of each protein was calculated using an imaging densitometer. The data represents the mean ± standard deviation from three replicates. [a]Significant difference ($P<0.05$) when compared to non-Tg rats; [b]significant difference ($P<0.05$) when compared to the vehicle-treated group.

antioxidants induced by Sel treatment and SelM overexpression affected the change in the cortex proteins of CMV/hSelM Tg and non-Tg rats. The CMV/hSelM Tg rats used in the study showed a higher level of antioxidant enzymes, such as SOD and GPX, compared to non-Tg rats. Additionally, the concentration of total oxidized products regulated by these enzymes was significantly decreased in the brain cortex of the CMV/hSelM Tg rats compared to non-Tg rats. These results indicate that the antioxidant condition was induced by Sel treatment and SelM overexpression in CMV/hSelM Tg rats. Furthermore, these findings are concordant with those of our previous study (13).

Aβ-42 peptide in the cortex of the brain is closely correlated with deposition at neuritic plaques in the pathogenesis of AD (28,33). The production of these peptides is tightly regulated by γ-secretase, which is composed of presenilin and four additional cofactors, nicastrin, Aph-1, Pen-2 and TMP-21, to create a multimeric protease complex (34,35). Thus far, limited studies investigating the correlation of Sel and γ-secretase have been conducted. The present study investigated whether the level of γ-secretase activity could be changed by Sel treatment and SelM overexpression in the brain of CMV/hSelM Tg rats. The results showed that the γ-secretase activity significantly decreased in response to Sel treatment. Therefore, the protein spot detected in the study may be correlated with the production of Aβ-42 peptides in CMV/hSelM Tg rats.

The study identified eight spots that were differentially expressed among four groups, which were vehicle-treated non-Tg, vehicle-treated CMV/hSelM Tg, Sel-treated non-Tg and Sel-treated CMV/hSelM Tg rats. Among these spots, creatine kinase was significantly upregulated in response to Sel treatment and SelM overexpression. B-CK, one of the creatine kinases, catalyzed the conversion of creatine to phosphocreatine, which was expressed in various tissue types (36). Generally, this enzyme is known as a buffering system of cellular ATP concentration and used as a marker of several diseases, including myocardial infarction, rhabdomyolysis, muscular dystrophy and renal failure (37). Decreased creatine kinase activity was recently reported to be tightly associated with neurodegenerative pathways in neurodegenerative diseases (38), ischemia (39) and other diseases (40,41). Creatine kinase and pyruvate kinase are crucial for energy homeostasis and antioxidant defense through inhibition of two enzyme activities (42). In the present study, the expression of B-CK was higher in CMV/hSelM Tg rats overexpressing SelM protein than non-Tg rats under vehicle-treated conditions. In addition, Sel treatment induced an increase of this enzyme expression in the two groups. Furthermore, this change pattern was observed in the spot identified as RING1. This enzyme, which is known as a Parkin, appeared to be part of the cell's defense against damage caused by environmental insults (43). This enzyme ubiquitinates itself and promotes its own degradation, and the impairment of ubiquitin ligase function may cause familial autosomal recessive PD. Furthermore, oxidative and nitrosative stress were closely associated with the ubiquitin ligase function of PD and the associated α-synucleinopathy (44).

SytXV and LDH-B showed similar protein change patterns on the 2-DE gels. Syt constitutes a family of membrane-trafficking proteins that are characterized by an N-terminal transmembrane region, a variable linker and two C-terminal domains (45,46). This protein is known as a Ca^{2+} sensor that regulates Ca^{2+}-dependent membrane trafficking, including endocrine exocytosis (47,48), synaptic vesicle exocytosis (49,50), plasma membrane repair (51,52) and acrosomal reaction (53). Several studies have also documented the correlation between neurotransmitter release and Syt function (54,55). However, limited studies of whether selenoprotein and Sel treatment were affected by SytXV expression have been conducted thus far. In the present study, the expression of this protein was significantly increased in response to SelM overexpression compared to non-Tg rats. However, in the CMV/hSelM Tg rats, Sel treatment did not induce a significant change in SytXV expression in the brain cortex. These results indicate that SelM overexpression could be affected by Ca^{2+}-dependent membrane trafficking through the regulation of Syt expression. Another spot was identified as LDH-B, which catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD^+ (56). Nazam Ansari *et al* (57) showed that serum lactate dehydrogenase and GPX were significantly lower in rats with ischemia. However, in the present study, LDH-B was increased

by SelM overexpression in CMV/hSelM Tg rats, but increased by Sel treatment in non-Tg rats, indicating that selenoprotein and Sel treatment are extremely important for normal brain function.

eIF-4H was the last member in the groups showing upregulated expression in response to SelM overexpression. This protein regulates protein synthesis through facilitating the binding of initiator tRNA to ribosomes (58). Several stress-responding genes induced by ultraviolet light, ER stress and reactive oxygen species were tightly regulated by eIF2. Furthermore, an abnormally high concentration of NO in the nervous system increases the eIF2α level of phosphorylated forms, thus suppressing protein synthesis in neurons (59). The eIF2α level of the phosphorylated form was also increased in hippocampal CA1 neurons following ischemia (60). In the present study, SelM overexpression induced increased eIF2α expression, but Sel treatment did not induce any change in either group.

The expression of CENP-N was extremely similar to that of DRP-2 upon 2-DE analysis. CENP-N was first reported as a member of three new human centromere proteins (CENP-M, CENP-N and CENP-T), which comprised a CENP-A nucleosome-associated complex (CENP-A NAC) (61). Additionally, disruption of CENP-A NAC by reduction of these three proteins induced a mitotic defect or significant mitotic delay in cells. However, there have been no studies of the correlation between CENP-N and antioxidant status. Therefore, to the best of our knowledge, the present study is the first to indicate that SelM overexpression led to a significant decrease in the expression of CENP-N.

DRP-2 is turned on following division of the 64-kDa protein or collapsing response mediator protein 2 (CRMP-2). This protein is highly expressed in the adult brain, is an important molecule in neurite outgrowth and axonal guidance, and plays a role in several neurological diseases, including AD, epilepsy and ischemia (62,63). Furthermore, a dramatic decrease of intact CRMP-2 was observed following maitotoxin treatment, *N*-methyl-D-asparatate treatment and ischemia induction, accompanied by the appearance of distinct breakdown products of CRMP-2 (63). The present study showed that SelM overexpression led to significant induction of the decrease of DRP-2 in CMV/hSelM Tg rats compared to non-Tg rats. These results indicate that SelM could be referred to as a regulator of DRP-2, although further study of the degradation process is necessary.

All the aforementioned results showed that SelM overexpression and Sel treatment play a crucial role in the regulation of brain function via modulation of eight associated proteins. In addition, our proteomic analysis allowed the verification of the change in protein patterns of brain antioxidant regulation. However, intensive study is required to define the role and detailed mechanism of SelM and Sel in the cortex region of the brain.

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