J. Appl. Biol. Chem. **54**(2), 101-107 (2011)

Article

Phosphoproteomic Analysis of the Brain of Ovariectomized Adult Rat

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Received April 30, 2011; Accepted May 24, 2011

Aging in females is associated with a reduced metabolic function, increased incidence of neurodegenerative diseases, and cognitive dysfunction, as a result of loss in gonadal function. The change can alter the states of phosphorylation on the proteins, which cause dramatic changes in the cellular location or activity of the proteins. In this study, the differential phosphorylation of the proteins responsible for the functions related to cognition was studied using the ovariectomized adult rats. Phosphoproteomic analysis using the cerebral and hippocampal tissues could identify 51 differentially phosphorylated proteins including 12 proteins for energy metabolism, 8 cytoskeletal proteins, 6 signaling proteins, and other functional proteins in the ovariectomized rats. Further, anti-oxidative enzymes, superoxide dismutase and peroxiredoxin-2, which are known to be inactivated by phosphorylation, were found to be differentially phosphorylated in the cerebellum and hippocampus of the ovariectomized rats, respectively. Many of the deactivated proteins by differential phosphorylation identified in this study were overlapped to those of Alzheimer's disease cases. These results will provide information for neurodegenerative learning and memory impairments in women as brought about by menopause.

Key words: 2-dimensional gel, learning and memory, ovariectomy, phosphoproteome

Protein phosphorylation is a post-translational modification occurring in 30% of all cellular proteins in eukaryotic organisms for the regulation of cell status [Kang *et al.*, 2007]. The information on the status of all phosphoproteins at a given time and condition of cell would be a requirement to further understand the physiological and/or pathological role of phosphoproteins. The study of phosphoproteome finds a wide application to understand the mechanism of certain neurological diseases. In particular, modifications in brain function have been described in aging viz. cognition, learning ability and memory retention which decline very frequently [Schmelzle and White, 2006].

One of the factors related to memory loss is the gradual decrease in the level of estrogen as a result of menopause. Brain areas such as hippocampus and cerebral cortex, which are involved in learning and memory processes have been shown to be extensively populated by estrogen receptors (ER). Low levels of estrogen have been linked to increased incidence of neurodegenerative diseases and deterioration of cognitive functions in women. To understand estrogen functions in the brain, basic fundamental research in cell signaling and biochemistry to identify the key proteins of the brain and nervous system, particularly, phosphoproteomic analysis offers an excellent potential for the identification of candidate regulatory proteins in various cellular states [Kanako *et al.*, 2007].

One suitable chemical method for monitoring the phosphorylation status of proteins from complex samples is through 2-dimensional (D) gel analysis and staining with Pro-Q Diamond dye (Molecular Probes, Eugene, OR), which is a fluorescent phosphosensor permitting quantitative detection of altered phosphoproteome patterns [Kanako *et al.*, 2007]. In this study, to mimic the memory loss in menopaused women, we designed to analyze the long term effect in the rat brain after ovariectomy. Differentially phosphorylated proteins involved in the onset of memory loss were identified by 2-D gel analysis, staining of phosphoproteins, and mass spectrometry (MS) analyses. We could show that the proteins involved in energy metabolism, cytoskeletal process, and protein folding are key player molecules in learning and memory. Interesting thing is that proteins quenching reactive oxidative species (ROS) are also differentially phosphorylated by memory loss in the hippocampus and cerebellum of the ovariectomized rat brain.

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doi:10.3839/jabc.2011.018

Materials and Methods

Animals. Twenty 7-month old Sprague-Dawley female rats

(SLC Inc., Hamamatsu, Japan), weighing 175-200 g were housed at 23°C and 60% humidity with *ad libitum* access to food and water at 12/12-h light/dark cycle. After two weeks, one half of the rats were ovariectomized (OVX) and the other half were sham operated (SHAM) to serve as control. The rats were further maintained up to 5 months. All animal procedures were followed by the Kyungpook National University (KNU) Animal Care and Use Committee.

Behavioral test. The T-maze was constructed according to the measures provided by Gerlai [1998]. After a variable inter-trial interval in the goal box (15 sec on average), the rat was placed in the start box for the next trial. The pre-training session continued until the rat made its first correct avoidance response. For three weeks, the rats were trained until they made five correct avoidance responses in six consecutive training trials (acquisition session). On the last week (fourth week), the actual test was conducted and after which a reversal session was performed with the correct goal box opposite to the one used during acquisition. The measures included the latency to reach the correct goal box and the number of trials prior to correct avoidance.

Plasma estrogen (E_2) level. Blood samples (up to 0.5 mL) were withdrawn from the tail artery in heparinized syringes and centrifuged for 20 min at room temperature (RT). Plasma E_2 concentration was determined by using a commercial kit (Diagnostic Products, Los Angeles, CA). All samples and standards were measured in duplicate and repeated twice.

Protein sample preparation and 2-dimensional gel electrophoresis (2-DE). The brain tissues were extracted after perfusion with ice cold phosphate buffered saline (PBS) and immediately stored frozen at −80°C. Tissues were ground in liquid nitrogen and homogenized in lysis buffer containing 7 M urea, 2 M thiourea, 4% 3-[(3-Cholamidopropyl)dimethylammonio]-1-Propanesulfonate (CHAPS), 1 M dithiothreitol (DTT) and a mixture of protease inhibitors (Complete-Mini EDTA-Free, Roche, Indianapolis, IN). Samples were sonicated for 20 times. The protein samples in lysis buffer were directly applied to immobilized pH gradient (IPG) strips (pH 3-10 and 4-7, 17 cm; Bio-Rad, Herculus, CA), and separated on second dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Visualization of phosphoprotein by Pro-Q Diamond staining. The gels were stained using a Pro-Q Diamond phosphoprotein gel stain kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Images were acquired by Typhoon 9400 (GE Healthcare, Buckinghamshire, UK) and analyzed by an image analysis software (PDQuest, BioRad) to compare the phosphorylation levels of the equivalent spots on the gels. Some spots were selected by visual comparisons. After the scanning, the gels were stained again using a Coomassie Brilliant Blue R-250 method for visualization of the total protein profiles.

Mass spectrometry (MS) analysis and protein identification. The spots of interest were manually excised, washed with deionized water and destained using 50 mM ammonium bicarbonate/acetonitrile (6:4, v/v) with vigorous shaking. Digestion was carried out by adding sequencing grade modified trypsin (Promega, Madison, WI) onto the dried gel pieces and incubated overnight at 37°C. The peptides were extracted with extraction buffer (60% acetonitrile in water and 0.1% trifluoroacetic acid (TFA) after the digestion, and were dried with the aid of vacuum drier. From the concentrated peptide extract, 1 μL was taken and mixed with 1 μL of the matrix solution (10% α-cyano-4-hydroxycinnamic acid (CHCA) in 50% methanol and 0.1% TFA containing internal standards such as bradykinin, angiotensin and neurotensin) on a target matrix assisted laser desorption ionization (MALDI) plate. The acquired mass spectra were analyzed by Mascot from Matrix Science (http://www.matrixscience.com) and MS-Fit from Protein Prospector (http://prospector.ucsf.edu/).

Results

Behavioral test. Four months after the surgery, we checked the blood E2 level by a radioimmunoassay. The E2 level was significantly reduced in the OVX group (Fig. 1A). The fluctuation of the E2 level in the SHAM group might come from the hormonal cycle variations among individuals. The performance of the OVX and SHAM rats in T-maze tests are summarized in Fig. 1B and Table 1. Analysis of variance (ANOVA) revealed significant memory and cognition loss in the OVX group in comparison with SHAM group in terms of latency of reaching the goal box. Across training period, the OVX rats took longer time in reaching the goal box. The reversal of the goal box hand had shown a much wider time difference between the OVX rats from the normal ones. Number of trials before accomplishing five consecutive success of reaching the goal box showed significant difference during the reversal of the box position, as OVX rats took an average of two trials (Table 1).

Analysis of phosphorylated proteins in the brain of OVX and SHAM rats. From an initial step of analyzing the phosphorylated proteins of SHAM and OVX using wide range IPG strips (pH ranges 3-10), we realized most phosphoproteins were found on the acidic side of the gel corresponding to pI values lower than 7 (Fig. 2). Therefore, the first dimensional isoelectric focusing was performed on pH 4-7 IPG strips in order to obtain a better resolution (Fig. 3). Approximately 266 different phosphoprotein spots were detected in Pro-Q Diamond stained 2-DE gel. Among them, a total of 30 proteins were

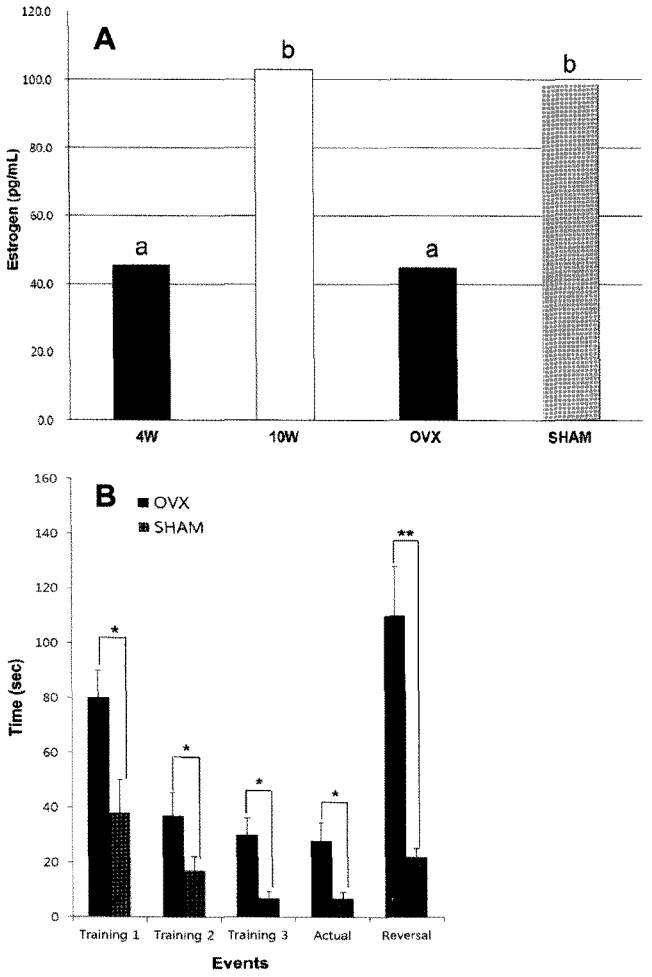

Fig. 1. Blood E2 concentration in OVX and SHAM rats. The E2 levels were compared with that of 4-week (4W) old rats for negative and 10-week (10W) old rats for positive controls (A). The rats were enforced to find a safe goal box in the T-maze by an electric shock from the grid floor. Latency period to reach the safe goal box was determined for SHAM and OVX rats (B). Statistically different groups are captioned with different letters (a and b), while insignificant groups are designated with the same letter (a or b). All statistical significance of the tests are designated with a significant level ($p<0.05$, *; $p<0.001$, **).

visualized as specific for the hippocampus from the OVX rats (OVX-H), 9 for the hippocampus from the SHAM rats (SHAM-H), 25 for the cerebral cortex from the OVX rats (OVX-H) and 13 for the cerebral cortex from the SHAM rats (SHAM-C). All these specific spots were excised and analyzed by MALDI time of flight (TOF)-MS or MALDI-TOF-TOF (MS/MS) from which 21 proteins were identified for OVX-H, 17 for OVX-C, 6 for SHAM-H and 7 for SHAM-C as shown in Table 2 to 4. These proteins are classified into distinct functional groups of metabolic enzymes (24%), cytoskeleton (16%), signaling proteins (10%), chaperone proteins (6%), cell cycle (6%), transport protein (4%), other functional proteins (20%), and proteins of unknown function (14%).

Table 1. Average number of trials made by 8 rats per group prior to entrance to correct goal box in the T-maze

Group	Average No. of Errors		
	Training	Actual	Reversal
SHAM	1±0.5	0	1±0
OVX	1.6±0.74	1±0.5	2±0.5

Discussion

E2 binds and functions through both nuclear and membrane bound E2 receptor found widely distributed in the brain [reviewed in Moura and Petersen, 2010]. E2 deprivation in postmenopausal women not only affects the neural control of fertility but also affects osteogenesis, as well as learning and memory [Neal-Perry *et al.*, 2010]. In this study, the cognitive loss in the OVX rats was correlated with the phosphoproteomic changes in the brain compared to SHAM group.

Energy metabolism is especially important in the brain. Enzymes involved in energy metabolism including pyruvate kinase (PK) and aldose reductase in the cerebral cortex of SHAM group (Fig. 3C as spot 6 and 7, respectively, and Table 4), L-Lactate dehydrogenase (LDH) in the hippocampus of OVX rats (spot 8 in Fig. 2B and Table 2), and creatine kinase from the hippocampus of OVX rats (Fig. 3B as spot 6 and Table 3) were found to be elevated phosphorylation. Impaired glycolytic function directly relates to less ATP available to the cells and various cellular processes that require ATP may be impaired, which is consistent with findings of altered glucose metabolism and tolerance in Alzheimer's Disease (AD) patients [Messier and Gagnon, 2000].

ATP synthase, identified as preferentially phosphorylated proteins in the hippocampus of OVX (Table 3, spot 3 and 5 from Fig. 3B) and SHAM rats (Table 4, spot 2 from Fig. 3A), and in the cerebral cortex of OVX (Table 2, spot 10 from Fig. 2D), is a mitochondrial regulating subunit of complex V that plays a key role in energy production. Inactivity of ATP synthase could contribute to a decrease in the activity of the entire electron transport chain and impaired ATP production, resulting in possible electron leakage from their carrier molecules to generate ROS, suggesting an alternative rationalization for the well documented existence of oxidative stress in AD and mild cognitive impairment (MCI) [Butterfield *et al.*, 2002]. Phosphorylation of ATP synthase in a mouse AD model was identified by a phosphosensor dye [Takano *et al.*, 2009].

Estrogen-mediated neuro-protection for Alzheimer's disease and Parkinson's disease in brain are known to be related to regulation of electron transport and energy production [Chen *et al.*, 2009]. Voltage-dependent anion channel-protein (VDAC) is

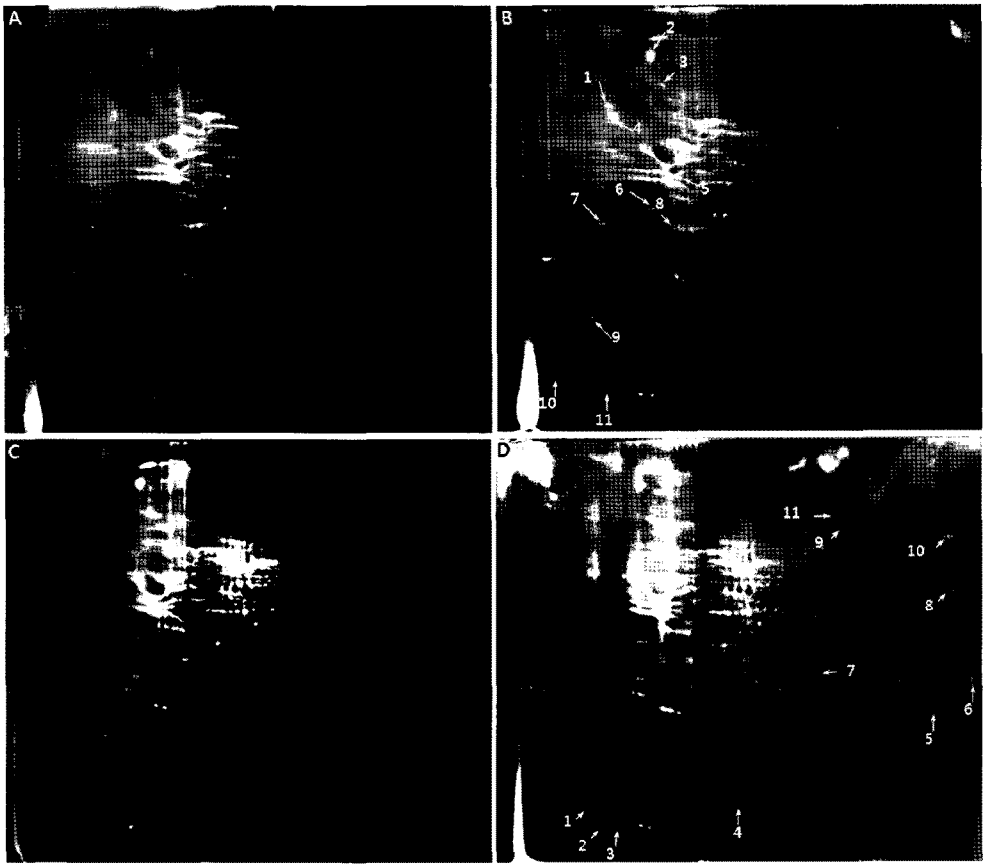

Fig. 2. Phosphoprotein map of hippocampus-SHAM (A), hippocampus-OVX (B), cerebral cortex-SHAM (C), and cerebral cortex-OVX (D) separated on 2-DE with pH 3-10. The spots indicated by arrows are group-specific phosphoproteins identified by MS/MS.

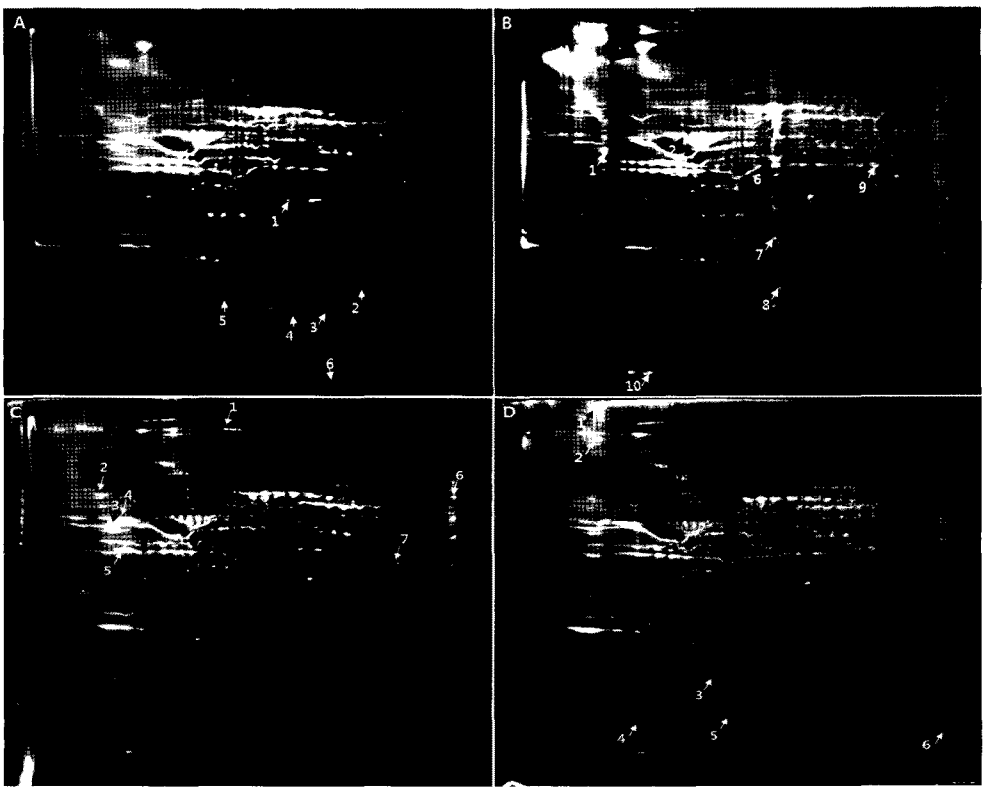

Fig. 3. Phosphoprotein map of (A) hippocampus-SHAM, (B) hippocampus-OVX, (C) cerebral cortex-SHAM, and (D) cerebral cortex-OVX separated on 2-DE with pH 4-7. The spots indicated by arrows are group-specific phosphoproteins identified by MALDI-TOF.

Table 2. List of identified differentially phosphorylated proteins in the brain of OXV rats separated on 2-DE with pH 3-10 from MS/MS analysis

No.	Fold Difference	Protein Name	Accession No.	Score	Function
c1*	VS	RIKEN cDNA 0610037D15, isoform CRA	gi|148698644	50	unknown
c2	VS	Protein kinase C, epsilon type	gi|148672835	36	kinase
c3	48	Chain A, N-Terminal Domain of Sialoadhesin In Complex with 3'sialyllactose	gi|4930109	35	unknown
c4	VS	Superoxide dismutase [Cu-Zn]	gi|1213217	36	anti-oxidation
c5	VS	phytoceramidase, alkaline, isoform CRA_e	gi|148684393	36	metabolic enzyme
c6	VS	unnamed protein product	gi|14148850	43	unknown
c7	VS	PREDICTED: hypothetical protein LOC74366	gi|149254560	41	unknown
c8	VS	Glial fibrillary acidic protein (GFAP)	gi|387163	91	cytoskeleton
c9	VS	Pyruvate kinase	gi|31981562	72	metabolic enzyme
c10	47	ATP synthase alpha subunit precursor	gi|203055	78	metabolic enzyme
c11	VS	Dihydropyrimidinase-like 2	gi|40254595	48	signaling
h1*	14	Neurofilament triplet L protein	gi|149025721	38	cytoskeleton
h2	5.9	Neuronal cell adhesion molecule precursor	gi|27695749	40	differentiation
h3	4	84 kDa heat shock protein	gi|91234898	38	molecular chaperone
h4	18	Zinc finger protein 334	gi|162287397	40	nucleic acid binding
h5	VS	Bone marrow macrophage cDNA	Q36804	77	protein binding
h6	NP	PREDICTED: similar to Tekt5 protein	gi|149270419	45	cytoskeleton
h7	19	olfactory receptor Olr35	gi|47577201	17	signaling
h8	17	L-lactate dehydrogenase B chain	gi|77020279	72	metabolic enzyme
h9	VS	Peroxiredoxin-2	gi|16758348	83	anti-oxidation
h10	107	RIKEN cDNA 0610037D15	gi|148698644	50	unknown
h11	17	Spinocerebellar ataxia type 1 protein	gi|29150141	34	RNA metabolism

*, the spot numbers of the identified proteins are prefixed with c and h to designate cerebral cortex and hippocampus, respectively.
VS, visual selection; NP, newly phosphorylated

Table 3. List of identified differentially phosphorylated proteins in the brain of OVX rats separated on 2-DE with pH 4-7 from MALDI-TOF analysis

No.	Fold Difference	MOWSE	Coverage (%)	Accession No.	Protein Name	Function
c1*	4.9	16459	20.3	P12839	Neurofilament medium polypeptide	cytoskeleton
c2	2.6	313	15.3	P18598	Potassium-transporting ATPase subunit beta	metabolic enzyme
c3	VS	355	20.8	Q9JM13	Rab5 GDP/GTP exchange factor	neuronal signaling
c4	NP	16.6	10.3	Q8K2R5	Zinc finger protein 668	transcription factor
c5	2.4	299	25.4	Q9CQI6	Coactosin-like protein	cytoskeleton
c6	2.1	740843	45.1	P10111	Peptidyl-prolyl cis-trans isomerase A	chaperone
h1*	VS	455	21.3	P97355	Spermine synthase	Growth
h2	2.7	7.06E+08	36.3	P68373	Tubulin alpha-1C chain	cytoskeleton
h3	VS	1.03E+07	25	P50516	Vacuolar ATP synthase catalytic subunit A	metabolic enzyme
h4	VS	23560	17.5	P63038	60 kDa heat shock protein, mitochondrial precursor	chaperone
h5	3.5	6613	15.3	P62814	Vacuolar ATP synthase subunit B, brain isoform	metabolic enzyme
h6	5.2	6.53E+07	34.1	Q04447	Creatine kinase B-type	metabolic enzyme
h7	2.1	34936	22.4	P67778	Prohibitin	Cell cycle
h8	VS	5984	11.2	Q8BMD7	MORC family CW-type zinc finger protein 4	transcription
h9	VS	375	11.4	Q08274	Dystrophia myotonica WD repeat-containing protein	regulate meiosis
h10	2.8	6763	12.5	Q9Z2Q6	Septin-5	cell cycle

*, the spot numbers of the identified proteins are prefixed with c and h to designate cerebral cortex and hippocampus, respectively.
VS, visual selection; NP, newly phosphorylated

the outer component of the mitochondrial permeability transition pore (MPTP). In the presence of E2, ER at the cell surface might indirectly regulate some potential modulators such as VDAC in the hippocampus [Marin *et al.*, 2007] by phosphorylation (Table 7 and spot 5 from Fig. 8A).

Antioxidant enzymes, peroxiredoxin-2 (spot h9 in Table 2, Fig. 2B) and superoxide dismutase (SOD) (Fig. 2D, spot c4 in Table 2) are increased in phosphorylation in the OVX rats. Phosphorylation can alter their functions as antioxidants [Wilcox *et al.*, 2009] Phosphorylation on SOD-1 facilitates monomerization of the enzyme, which is a necessary initiating step in SOD-1 aggregation-inactivation [Hu *et al.*, 2006]. The inactivation of peroxiredoxin-2 by phosphorylation is also reported in immune cells [Woo *et al.*, 2010]. It seems like that the neuroprotective

Table 4. List of identified differentially phosphorylated proteins in the brain of SHAM rats separated on 2-DE with pH 4-7 from MALDI-TOF analysis

No.	Fold Difference	MOWSE	Coverage (%)	Accession No.	Protein Name	Function
c1*	VS	73154	20.9	Q01853	Transitional endoplasmic reticulum ATPase	metabolic enzyme
c2	2.6	383	23.6	Q6RUG5	Islet cell autoantigen 1-like protein	unknown
c3	4.4	245	22.6	Q80W22	Threonine synthase-like 2	lyase
c4	VS	22926	14.4	Q6P9T8	Tubulin beta-2C chain	cytoskeleton
c5	VS	249621	28.3	P60710	Actin, cytoplasmic 1	ctyoskeleton
c6	VS	98875	18.6	P11980	Pyruvate kinase isozymes M1/M2	metabolic enzyme
c7	VS	3316	16.1	P07943	Aldose reductase	metabolic enzyme
h1*	3.9	3983	20	Q9CWS0	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	hydrolase
h2	20	2162	31.7	P31399	ATP synthase subunit d, mitochondrial	chaperone
h3	VS	874936	49.2	P02793	Ferritin light chain 1	iron storage
h4	99	116	11.8	P15431	Gamma-aminobutyric acid receptor subunit beta-1 precursor	transport
h5	8	948	10.3	Q9Z351	Potassium voltage-gated channel subfamily KQT member 2	transport
h6	NP	2288	18.8	Q5SP85	Coiled-coil domain-containing protein 85A	unknown

*, the spot numbers of the identified proteins are prefixed with c and h to designate cerebral cortex and hippocampus, respectively.
VS, visual selection; NP, newly phosphorylated

role of E2 is mediated through the preventing the inactivation of the anti-oxidant enzymes by phosphorylation.

Cytoskeletal proteins are crucial for maintenance of cell shape, cell migration, cell adhesion, mitosis, and intracellular transport of organelles [Kashem *et al.*, 2008]. Maintaining cytoskeletal integrity is important in neuronal activities, because shortened dendritic length can be associated with or AD pathology [Spires *et al.*, 2005]. Phosphorylation-mediated conformational changes in neurofilaments NF-L and NF-M in the brain from the OVX rats (spot h1 in Table 2, Fig. 2D; spot c1 in Table 3 and Fig. 3B) are important in the maintenance of axonal caliber [Stevenson *et al.*, 2011]. Glial fibrillary acidic protein (GFAP), an astrocyte-specific marker, which was found to be increased in phosphorylation in the cortex of OVX (spot 8 from Fig. 2D, Table 2), is the major intermediate filament protein related to memory retention in rats. Higher levels of GFAP were observed in the AD brain [Fountalakis and Tsangaris, 2005].

The elevated phosphorylation of HSP60 (Table 3, spot 4 from Fig. 3D) and peptidyl prolyl *cis-trans* isomerase in the hippocampus of OVX rats (Table 3, spot 6 from Fig. 3B) suggests that similar to the cases in AD and Parkinson's disease [Salminen *et al.*, 2011] lowered activities of the chaperones by phosphorylation could cause cognitive dysfunction in OVX rats by the inefficient synapse formation [Hall *et al.*, 2010; Jinwal *et al.*, 2010].

In summary, the low level of E2 in female adult rats, which was induced through ovariectomy, resulted to cognitive dysfunctions which may be attributed to the energy metabolism, cytoskeleton, protein-folding, signaling, and anti-oxidation. This study shows that there is a functional overlap of brain proteins between cognitive dysfunction in OVX rats and in the progression of mild cognitive impairment and AD.

These identified proteins may be considered as candidate biomarkers for the study of cognitive dysfunction during menopause. Further studies on these candidate phosphoproteins could help in delineating the mechanism behind the pathology of cognitive dysfunction and in developing effective therapeutics to slow or prevent the conversion and/or progression to higher level of memory impairment, such as mild cognitive impairment and AD.

Acknowledgment. This work was supported by the grant (No. 313-2007-2-F00138) from National Research Foundation, s-Korea.

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