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Toxicomics Report

Proteomic analysis of valproic acid-induced embryotoxicity in cultured post-implantation rat embryos

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ABSTRACT — Protein expression changes were examined in day 10.5 rat embryos cultured for 24 hr in the presence of valproic acid (VPA), using two-dimensional electrophoresis and mass spectrometry. Exposure to VPA at an embryotoxic concentration of 1.2 mM resulted in quantitative changes in many embryonic protein spots (22 decreased and 29 increased). For the increased protein spots, 10 proteins were identified, including alpha-fetoprotein, phosphorylated cofilin-1, and serum albumin. These proteins are candidate protein biomarkers that may be involved in embryotoxic mechanisms.

Key words: Valproic acid, Embryotoxicity, Proteomics, Rat

INTRODUCTION

Proteomic analysis is expected to provide mechanistic insights and protein biomarkers for the safety evaluation of developmental toxicants (Usami and Mitsunaga, 2011). To date, we have examined protein expression changes in cultured embryos exposed to certain developmental toxicants, i.e., selenium, indium, and ethanol, and found candidate protein biomarkers that may be involved in embryotoxic mechanisms (Usami *et al*., 2008; Usami *et al*., 2009; Usami *et al*., 2014). It is, however, still important to accumulate proteomic analysis data of developmental toxicants. In the present study, protein expression changes in day 10.5 rat embryos cultured for 24 hr in the presence of valproic acid (VPA), a well-known developmental toxicant in laboratory animals and humans (Schardein and Macina, 2007), were examined using two-dimensional electrophoresis (2-DE) and mass spectrometry (MS).

MATERIALS AND METHODS

Embryo culture and VPA treatment

Day 10.5 embryos (plug day = day 0.5) of Wistar rats (Crlj: WI, Charles River Laboratories Japan, Inc., Kanagawa, Japan) were cultured for 24 hr using the roller bottle method (Usami *et al*., 2008). VPA (sodium salt, CAS 1069-66-5, Calbiochem, Merck KGaA, Darmstadt, Germany) was dissolved in Hank's balanced salt solution (0.1 mL) and added to the culture medium (4.9 mL) composed of 100% rat serum at concentrations of 0, 0.3, 0.6, and 1.2 mM. All animal experiments were carried out according to the guidelines for animal use of the National Institute of Health Sciences.

2-DE and MS analyses of embryonic protein

Analysis of 2-DE gels (one embryo per gel; six gels per group) and identification of proteins using MS were carried out as previously reported (Usami *et al*., 2009), except that the gels were stained with a fluorescent dye (Flamingo gel stain, Bio-Rad, Hercules, CA, USA) and scanned using a laser scanner (FLA-5100, GE Healthcare UK Ltd., Amersham Place, Little Chalfont, UK) through the LPG filter at an excitation wavelength of 532 nm. Quantitative differences in protein spots of more than 1.5-fold, with statistical significance using Student's t-test at 5% probability level between the control and 1.2-

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mM VPA groups, were regarded as VPA-induced protein expression changes. NCBInr GI numbers of the identified proteins were mapped to UniProtKB AC, and gene ontology (GO) terms were assigned using the UniProt web site (http://www.uniprot.org/) (Jain *et al*., 2009; The UniProt Consortium, 2011).

RESULTS AND DISCUSSION

Effects of VPA on the development of cultured rat embryos

VPA caused significant abnormalities of the cultured embryos at 1.2 mM (Table 1). The abnormal organs included the neural tube (Fig. 1), which is a major target of VPA (Schardein and Macina, 2007). These effects were comparable to those observed in previous studies (Klug *et al*., 1990; Guest *et al*., 1994; Kao *et al*., 1981). Employing this embryotoxic concentration of VPA, all the cultured embryos in the control and 1.2-mM VPA groups (six embryos per group) were used for the subsequent proteomic analysis.

Effects of VPA on embryonic protein expression

Approximately 800 protein spots were matched through 12 2-DE gels between the control and 1.2-mM VPA groups (Fig. 2). Qualitative changes, i.e., appearance or disappearance, in the protein spots were not observed. VPA-induced quantitative changes were noted in 51 spots, i.e., 22 spots were decreased and 29 spots were increased by 1.5-fold or more (Fig. 3). These protein spots were

Table 1. Developmental features of day 10.5 rat embryos cultured in the presence of valproic acid (VPA).

	Valproic acid (mM)			
	0 (Control)	0.3	0.6	1.2
No. of embryos	6	6	6	6
No. of viable embryos	6 (100%)	6 (100%)	6 (100%)	6 (100%)
Crown-rump length (mm)	4.24 ± 0.27	4.19 ± 0.20	4.15 ± 0.21	3.98 ± 0.31
Head length (mm)	2.34 ± 0.16	2.29 ± 0.16	2.25 ± 0.16	2.10 ± 0.30
No. of somite pairs	27.3 ± 1.03	27.0 ± 0.63	27.2 ± 0.75	26.7 ± 0.82
No. of abnormal embryos	0	3 (50.0%)	3 (50.0%)	6 (100%)**
Deformed optic vesicle	0	0	0	6 (100%)**
Deformed otic vesicle	0	1 (16.7%)	1 (16.7%)	3 (50.0%)
Deformed tail	0	1 (16.7%)	0	0
Irregular somites	0	1 (16.7%)	2 (33.3%)	6 (100%)**
Open neural tube	0	0	3 (50.0%)	3 (50.0%)
Swollen neural tube	0	0	2 (33.3%)	6 (100%)**
Zigzag neural tube	0	2 (33.3%)	0	5 (83.3%)*

Embryos were cultured for 24 hr using the roller method. Asterisks indicate statistically significant differences compared to the control group identified using Fisher's exact test (* $p < 0.05$; ** $p < 0.01$).

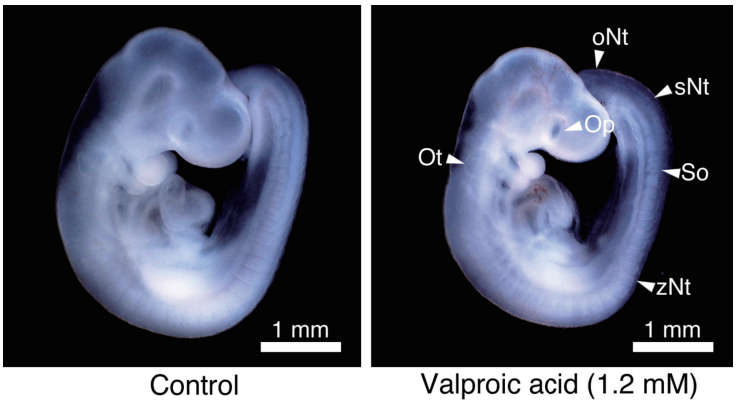

Fig. 1. Appearance of rat embryos cultured in the presence of valproic acid (VPA). Rat embryos at the end of a 24-hr culture are shown after removal of the embryonic membranes. Arrowheads indicate deformed organs. oNt, open neural tube; sNt, swollen neural tube; zNt, zigzag neural tube; Op, deformed optic vesicle; Ot, deformed otic vesicle; So, irregular somite.

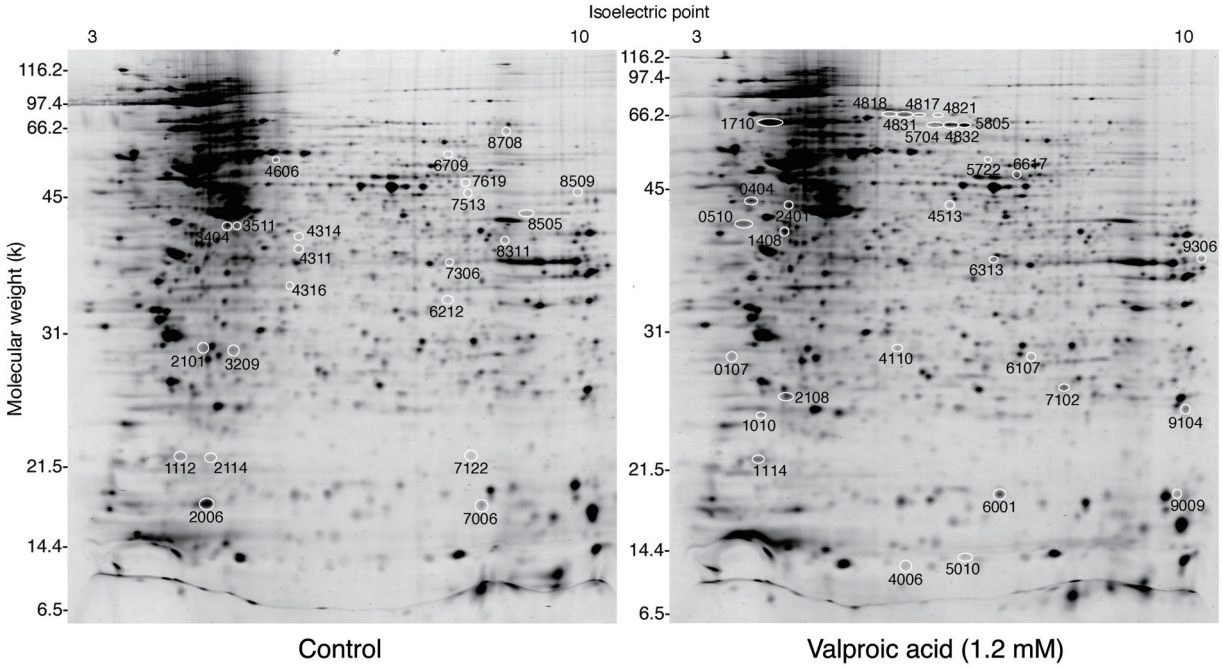

Fig. 2. Two-dimensional electrophoresis pattern of proteins from rat embryos cultured in the presence of valproic acid (VPA). Representative gels are shown for the control and VPA (1.2 mM) groups. Proteins with VPA-induced expression changes are indicated by circles with standard spot numbers (SSPs); decreased proteins are indicated in the "control" gel and increased ones in the "valproic acid" gel.

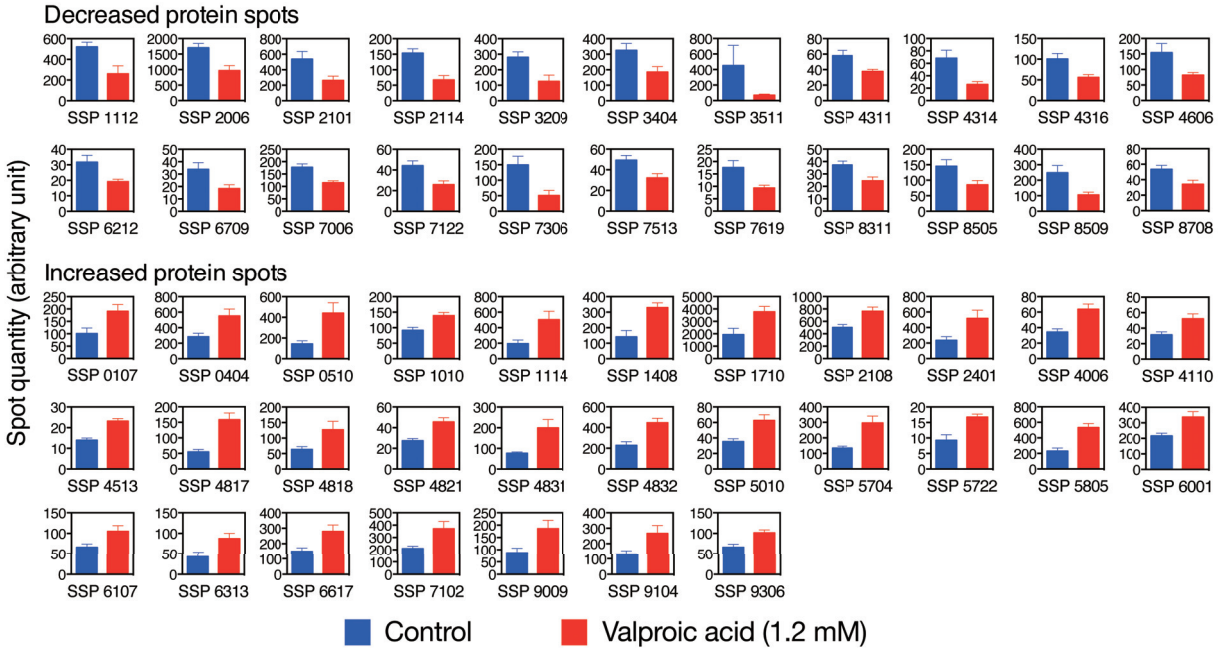

Fig. 3. Quantification of protein spots with expression changes in two-dimensional electrophoresis gels from rat embryos cultured in the presence of valproic acid (VPA). Intensities of protein spots with VPA-induced expression changes are shown (means of six gels per group). Error bars indicate the standard error of the mean.

Table 2. Identified proteins exhibiting significant elevation in rat embryos cultured in the presence of valproic acid (VPA).

Standard SPot number (SSP)	Protein Name	UniProt KB AC	Gene Ontology (GO) term for Biological Process
0107*	Protein Pbdc1	G3V6C3	Not available
	OR		
	RGD1562502 protein	B2RYV5	Not available
4110	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial	Q9DCT2	GO:0030308 negative regulation of cell growth GO:0055114 oxidation-reduction process GO:0072593 reactive oxygen species metabolic process GO:2001243 negative regulation of intrinsic apoptotic signaling pathway
4818 4831	Alpha-fetoprotein	P02773	GO:0001542 ovulation from ovarian follicle GO:0001889 liver development GO:0006810 transport GO:0010033 response to organic substance GO:0019953 sexual reproduction GO:0031016 pancreas development GO:0031100 organ regeneration GO:0042448 progesterone metabolic process GO:0060395 SMAD protein signal transduction
4832 5805	Serum albumin	P02770	GO:0006810 transport GO:0006950 response to stress GO:0007584 response to nutrient GO:0009267 cellular response to starvation GO:0010033 response to organic substance GO:0019836 hemolysis by symbiont of host erythrocytes GO:0042311 vasodilation GO:0043066 negative regulation of apoptotic process GO:0046010 positive regulation of circadian sleep/wake cycle, non-REM sleep GO:0046689 response to mercury ion GO:0051659 maintenance of mitochondrion location GO:0070541 response to platinum ion
6001	Cofilin-1 (phosphorylated)	P45592	GO:0006606 protein import into nucleus GO:0007010 cytoskeleton organization GO:0022604 regulation of cell morphogenesis GO:0030030 cell projection organization GO:0045792 negative regulation of cell size
6313*	m7GpppX diphosphatase	Q8K4F7	GO:0006397 mRNA processing GO:0008380 RNA splicing GO:0036245 cellular response to menadione GO:0043069 negative regulation of programmed cell death GO:0045292 mRNA cis splicing, via spliceosome
	AND		
	Serine/threonine-protein phosphatase PP1-gamma catalytic subunit	P63088	GO:0005975 carbohydrate metabolic process GO:0005977 glycogen metabolic process GO:0006470 protein dephosphorylation GO:0007049 cell cycle GO:0030182 neuron differentiation GO:0032922 circadian regulation of gene expression GO:0042752 regulation of circadian rhythm GO:0043153 entrainment of circadian clock by photoperiod GO:0048511 rhythmic process GO:0051301 cell division
	AND		
	Serine/threonine-protein kinase PRP4 homolog4	Q61136	GO:0006397 mRNA processing GO:0006468 protein phosphorylation GO:0008380 RNA splicing GO:0016310 phosphorylation
7102	Glutathione S-transferase alpha-4	P14942	GO:0006805 xenobiotic metabolic process GO:0008152 metabolic process GO:0009635 response to herbicide GO:0010043 response to zinc ion GO:0035094 response to nicotine GO:0071285 cellular response to lithium ion

Asterisks indicate inconclusive protein identification by mass spectrometry; “OR” indicates multiple proteins with the same peptides, and “AND” indicates multiple different proteins present in single protein spots.

mapped to our 2-DE map of embryonic proteins, resulting in the identification of no proteins for the decreased spots but 10 proteins for 9 of the increased spots (Table 2). No further proteins were newly identified by MS analysis in this experiment. GO term classification and functional mapping were not carried out because of the small number of identified proteins. It was noted, however, that the identified proteins included alpha-fetoprotein [standard spot numbers (SSPs 4818, 4831)], phosphorylated cofilin-1 (SSP 6001), and serum albumin (SSPs 4832, 5805). These three proteins have been considered candidate protein biomarkers that may be involved in embryotoxic mechanisms, as reported in our previous studies (Usami *et al*., 2014; Usami *et al*., 2009; Usami *et al*., 2008).

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Conflict of interest---- The authors declare that there is no conflict of interest.

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