[Full Paper]

Proteomic identification of oxidative-stress-reporting biomarkers differentially secreted from human neuroblastoma SH-SY5Y cells

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SUMMARY

The free-radical theory predicts that the oxidative stress accelerates the rate of aging and increases the onset of degenerative disorders in the elderly. Dopaminergic neurons are especially vulnerable to age-related neuronal disorders due to reactive oxygen species generated in the pathway of dopamine metabolism. Biochemical changes occurring in substantia nigra of Parkinson's disease patients suggest that the oxidative-stress-induced cell damages may be involved in the neurodegeneration. In our previous researches, we found that the dephosphorylation of elongation factor-2 and phosphorylation of nuclear lamin A/C might be neuronal cell specific response to oxidative stress. (Nakamura et al. BBA, 1763(9), 977–989, 2006)

The dephosphorylation and phosphorylation of those proteins are significant biomarkers for analyzing the molecular mechanisms of the stress response, however, such a phosphoproteome analysis is thought to be inappropriate for clinical investigation of neurodegeneration if it was not detectable in cerebrospinal fluid or serum of patients. Thus, we proceeded to the 2D-DIGE analysis of secretome, proteome of secreted proteins, using the culture system in which oxidative stress was applied to human SH-SY5Y neuroblastoma cells. As the result of our secretome analysis, we identified ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2 N, ubiquitin C-terminal hydrorase-L1, 14-3-3 protein isoforms, Rab GDP dissociation inhibitor β, Rho GDP-dissociation inhibitor 1, peroxiredoxin-2, glutathione S-transferase P, α enolase, LDH B chain as oxidative-stress-reporting biomarker candidates.

Key words: proteome, secretome, oxidative stress, neuroblastoma, SH-SY5Y.

INTRODUCTION

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are inevitably generated in all living cells as by-products of electron transport in mitochondria and redox enzyme reactions in the cytoplasm[1, 2]. Oxidative stress induced with ROS and RNS is suspected to be a major cause of chronic neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease. Dopamine neurons are especially vulnerable to age-related disorders due to exposure to high level ROS generated normally as part of dopamine metabolism[3]. Oxidative stress may cause neuronal cell dysfunctions through oxidative modification of macromolecules including proteins. The neuronal cells must have a protective system against oxidative stress to avoid inordinate cell death. However, little has been known about which proteins in dopamine neurons are involved in the response to oxidative stress.

In order to analyze proteins involved in the stress response, we established an in-vitro culture system in

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Abbreviations: 2D-DIGE, two-dimensional difference gel electrophoresis; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; 6-OHDA, 6-hydroxydopamine; DTT, dithiothreitol; MALDI-TOF MS, matrix-assisted laser-desorption time-of-flight mass spectrometry; CHCA, α-cyano-4-hydroxycinnamic acid; TFA, trifluoroacetic acid; PBS, phosphate-buffered saline; D-MEM, Dulbecco's minumum essential medium; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonicacid; DMSO, dimethyl sulfoxide.

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which cellular response to oxidative stress could be detected by proteomic analysis. In our previous study, we examined changes in whole proteins and phosphoproteins of human dopaminergic neuroblastoma cell line SH-SY5Y under oxidative stress induced with dopaminergic neurotoxin 6-OHDA[4, 5]. Total proteins of SH-SY5Y cells at various stages of oxidative stress were separated by 2-D PAGE and compared quantitatively each other. Increases in cellular levels of glutathione S-transferase P and heat shock cognate 71 kDa protein were detected by computer-aided image analysis. Stress-induced alterations in protein phosphorylation were also detected by Pro-Q Diamond staining. Elongation factor 2, lamin A/C, T-complex protein 1, and heterogeneous nuclear ribonucleoprotein H3 were identified by MALDI-TOF MS as stress-responsive phosphoproteins[6].

However, those intracellular phosphoproteins are not directly applicable to clinical investigations of the early stages of neurodegeneration. Thus in this paper, we report the result of proteomic identification of oxidative-stress-reporting biomarker proteins differentially secreted from human neuroblastoma SH-SY5Y cell under 6-OHDA-induced oxidative stress.

MATERIALS AND METHODS

Materials

Immobiline DryStrips (pH 4–7, 18 cm) and Pharmalyte were purchased from GE Healthcare Bioscience (Tokyo, Japan). IC3-OSu and IC5-OSu were products of Dojindo Laboratories (Kumamoto, Japan). SYPRO Ruby Protein Gel Stain was from Invitrogen. CoolPhorStar 2-DE Apparatus System was from Anatech (Tokyo, Japan). Pharos FX, PDQuest system and RC DC protein assay kit were from Bio-Rad Laboratories Inc. (Tokyo, Japan). SDS, Tris, Tricine, DTT, TFA, CHCA, DMSO and protease inhibitor cocktail were from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals of HPLC grade were from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Trypsin was purchased from Promega (Madison, WI, USA).

Cell culture and induction of oxidative-stress response

The dopaminergic neuroblastoma cell line SH-SY5Y was obtained from the Human Science Research Resources Bank (Osaka, Japan). The cells were cultured in D-MEM (Gibco) supplemented with 10% heat-inactivated fetal bovine serum, streptomycin (0.1 mg/ml), and penicillin (100 units/ml) in a CO_2 incubator (5% CO_2/95% air) at 37°C.

Before inducing oxidative stress in the SH-SY5Y cells with 6-OHDA, attached cells and the culture dishes were rinsed twice with PBS to remove bovine serum proteins. The cells were then exposed to various concentrations of 6-OHDA (0, 10, 25 and 50 μM) in a serum-free D-MEM with N2-supplement. After 24-h incubation, the culture media were transferred to centrifuge tubes to remove cell debris by centrifugation.

Fluorescent labeling of proteins

Fluorescent labeling (FL) of proteins in the culture media was carried out according to the optimized procedure described in Fig. 1. In brief, each culture medium (5-ml per sample) was concentrated with Amicon Ultra 10,000 MWCO centrifugal filter device (Millipore) to be about 0.1 ml. Proteins were then precipitated with 10-fold volume of cold acetone (–20°C) and re-dissolved in 50 μl of FL Buffer (8 M urea, 2% (v/v) Triton-X100, 0.2% (w/v) SDS, 10 mM HEPES-NaOH, pH 8.6). The protein concentration was assayed by a modified Lowry's method using the RC DC Protein Assay Kit (Bio-Rad), and adjusted to 10 mg/ml by dilution. A 15-μl aliquot of the sample solution containing 0.15 mg protein was mixed with 1.5 μl of 0.4 mM IC3-OSu or IC5-OSu dissolved in DMSO. The FL was carried out at room temperature for 15 min in the dark, and then terminated by the addition of 10 mM ethanolamine-HCl, pH 8.6. The IC3-labeled samples were combined with their

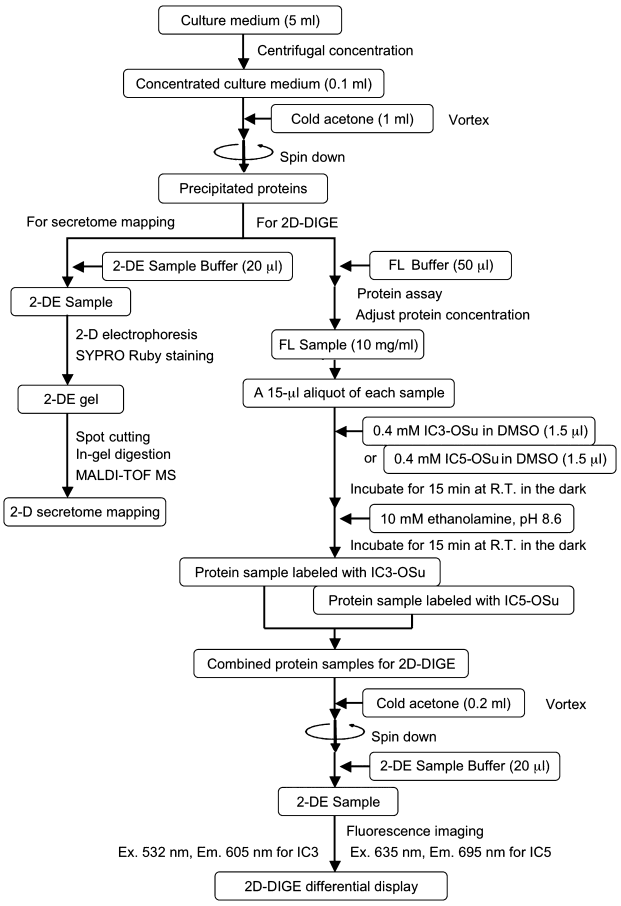

Fig. 1. The procedure for 2-D secretome mapping and 2D-DIGE analysis.

For secretome mapping, non-labeled proteins were separated by 2-DE and stained with SYPRO Ruby. For 2D-DIGE analysis, proteins were labeled with IC3-OSu or IC5-OSu, combined each other and separated by 2-DE.

IC5-labeled counterparts for differential analysis. Proteins in the coupled samples were precipitated again with 10-fold volume of cold acetone, and dissolved in 20 μl of 2-DE Sample Buffer (8.5 M urea, 2% (v/v) Triton X-100, 0.2% (w/v) SDS, 10 mg/ml DTT, 2% (v/v) Pharmalyte 3–10).

2-D PAGE and protein staining

2-DE for secretome mapping and 2D-DIGE was carried out according to our standard method[7], of which the updated version was made public on our web site (http://www.proteome.jp/2D/2D_method.html). In the first dimensional separation, immobilized pH-gradient isoelectric focusing was carried out using Immobiline DryStrip (pH 4–7, 18-cm long) in the CoolPhoreStar horizontal electrophoresis system (Anatech). Tricine-SDS-PAGE was run on a 7.5%T slab gel in the second-dimensional separation.

Fluorescence imaging of IC3/5-labeled proteins was performed using the 532-nm laser and the 605-nm band-pass filter for IC3-labeled proteins, the 635-nm laser and the 695-nm band-pass filter for IC5-labeled proteins, respectively on the Pharos FX system (Bio-Rad). Differential display and quantitative spot analysis were done using the PDQuest software. Non-labeled proteins for secretome mapping were visualized by staining with SYPRO Ruby after 2-DE.

Spot picking and in-gel digestion

Spot picking was performed using EXQuest Spot Cutter (Bio-Rad). In-gel tryptic digestion was carried according to our optimized procedure shown in our web site.

MALDI-TOF MS and protein identification

MALDI-TOF MS was performed using AXIMA-CFR (Shimadzu), and protein identification was done with the help of the Mascot search engine (Matrix Science).

RESULTS

Secretome mapping of the human SH-SY5Y neuroblastoma cells under oxidative stress

Proteins, secreted from human SH-SY5Y neuroblastoma cells into the serum-free medium containing 25 μM 6-OHDA, were separated by 2-DE and visualized by staining with SYPRO Ruby (Fig. 2). The protein spots numbered in the figure were excised from the slab gel. Protein identification was performed by in-gel tryptic digestion and peptide mass fingerprinting. The identified proteins were listed in Table 1. The major spots pointed with arrowheads were serotransferrin supplemented to the serum-free culture medium as N2-supplement. Fifty-five spots on the map were identified as secreted proteins from the SH-SY5Y cells.

Differential display of oxidative-stress-reporting biomarker proteins by 2D-DIGE

The IC3/5-labeled proteins were simultaneously separated on a single gel, and the differential gel image was acquired and displayed using the PDQuest 2-D Gel Analysis Software. The result of the differential display by 2D-DIGE is shown in Fig. 3, in which the red color indicates increased proteins in the medium under the oxidative stress induced with 25 μM 6-OHDA for 24 h. The green color indicates decreased proteins. The yellow color of

Fig. 2. 2-D gel secretome map of SH-SY5Y cells under oxidative stress.

The major spots pointed with arrowheads were serotransferrin supplemented to the serum-free culture medium as N2-supplement.

Table 1. Identified proteines excreted by SH-SY5Y under oxidative stress

Spot Number	Swiss-Prot Protein ID	Accession Number	Protein name	Synonums
101	CALM_HUMAN	P62158	Calmodulin	CaM
310	C1QBP_HUMAN	Q07021	Complement component 1 Q subcomponent-binding protein	Mitochondrial matrix protein p32
311	1433E_HUMAN	P62258	14-3-3 protein epsilon	14-3-3E
313	HS90B_HUMAN	P08238	Heat shock protein HSP 90-beta	HSP 84, HSP 90
1216	1433G_HUMAN	P61981	14-3-3 protein gamma	Protein kinase C inhibitor protein 1
1217	GDIR_HUMAN	P52565	Rho GDP-dissociation inhibitor 1	Rho-GDI alpha
1218	1433Z_HUMAN	P63104	14-3-3 protein zeta/delta	14-3-3Z
1219	1433E_HUMAN	P62258	14-3-3 protein epsilon	14-3-3E
1220	1433B_HUMAN	P31946	14-3-3 protein beta/alpha	Protein kinase C inhibitor protein 1
1404	RSSA_HUMAN	P08865	40S ribosomal protein SA	34/67 kDa laminin receptor
1512	TBB5_HUMAN	P07437	Tubulin beta chain	
1513	TBB3_HUMAN	Q13509	Tubulin beta-3 chain	
1705	GRP78_HUMAN	P11021	78 kDa glucose-regulated protein	Heat shock 70 kDa protein 5
1706	MMP2_HUMAN	P08253	72 kDa type IV collagenase	Matrix metalloproteinase-2
1809	HS90A_HUMAN	P07900	Heat shock protein HSP 90-alpha	Renal carcinoma antigen NY-REN-38
2213	TMM31_HUMAN	Q5JXX7	Transmembrane protein 31	
2214	LMNB1_HUMAN	P20700	Lamin-B1	
2412	ACTB_HUMAN	P60709	Actin, cytoplasmic 1	Beta-actin
2610	TBA1A_HUMAN	Q71U36	Tubulin alpha-1A chain	
2611	TBA1A_HUMAN	Q71U36	Tubulin alpha-1A chain	
2816	TERA_HUMAN	P55072	Transitional endoplasmic reticulum ATPase	
2817	DDB1_HUMAN	Q16531	DNA damage-binding protein 1	UV-damaged DNA-binding factor
3207	UCHL1_HUMAN	P09936	Ubiquitin carboxyl-terminal hydrolase isozyme L1	UCH-L1
3409	ACTB_HUMAN	P60709	Actin, cytoplasmic 1	Beta-actin
3410	ACTB_HUMAN	P60709	Actin, cytoplasmic 1	Beta-actin
3411	IF4A2_HUMAN	Q14240	Eukaryotic initiation factor 4A-II	ATP-dependent RNA helicase eIF4A-2
3412	ILF2_HUMAN	Q12905	Interleukin enhancer-binding factor 2	Nuclear factor of activated T-cells 45 kDa
3508	TRFE_HUMAN	P02787	Serotransferrin	Transferrin
3613	TCPE_HUMAN	P48643	T-complex protein 1 subunit epsilon	TCP-1-epsilon
3614	FKBP4_HUMAN	Q02790	FK506-binding protein 4	Peptidyl-prolyl cis-trans isomerase
3615	FKBP4_HUMAN	Q02790	FK506-binding protein 4	Peptidyl-prolyl cis-trans isomerase
3711	HSP7C_HUMAN	P11142	Heat shock cognate 71 kDa protein	Heat shock 70 kDa protein 8
3712	HSP74_HUMAN	P34932	Heat shock 70 kDa protein 4	Heat shock 70-related protein APG-2
3821	UBE1_HUMAN	P22314	Ubiquitin-activating enzyme E1	A1S9 protein
4108	PRDX2_HUMAN	P32119	Peroxiredoxin-2	Thioredoxin peroxidase 1
4206	GSTP1_HUMAN	P09211	Glutathione S-transferase P	GST class-pi
4413	CSN4_HUMAN	Q9BT78	COP9 signalosome complex subunit 4	Signalosome subunit 4
4615	HSP71_HUMAN	P08107	Heat shock 70 kDa protein 1	HSP70.1, HSP70-1/HSP70-2
4705	GRP75_HUMAN	P38646	Stress-70 protein, mitochondrial	Mortalin, Heat shock 70 kDa protein 9
5107	STMN1_HUMAN	P16949	Stathmin	Phosphoprotein p19
5108	NDKA_HUMAN	P15531	Nucleoside diphosphate kinase A	NDP kinase A
5209	PARK7_HUMAN	Q99497	Protein DJ-1	Parkinson disease protein 7
5312	LDHB_HUMAN	P07195	L-lactate dehydrogenase B chain	
5510	PRS7_HUMAN	P35998	26S protease regulatory subunit 7	Proteasome 26S subunit ATPase 2
5611	TCPA_HUMAN	P17987	T-complex protein 1 subunit alpha	TCP-1-alpha
6007	UBE2N_HUMAN	P61088	Ubiquitin-conjugating enzyme E2 N	Ubiquitin-protein ligase N
6209	GRB2_HUMAN	P62993	Growth factor receptor-bound protein 2	SH2/SH3 adapter GRB2
6412	DCUP_HUMAN	P06132	Uroporphyrinogen decarboxylase	URO-D
6513	TRFE_HUMAN	P02787	Serotransferrin	Transferrin
6514	GDIB_HUMAN	P50395	Rab GDP dissociation inhibitor beta	Rab GDI beta
7309	GSTO1_HUMAN	P78417	Glutathione transferase omega-1	GSTO 1-1
7508	GDIB_HUMAN	P50395	Rab GDP dissociation inhibitor beta	Rab GDI beta
8508	TCPB_HUMAN	P78371	T-complex protein 1 subunit beta	TCP-1-beta
8509	ENOA_HUMAN	P06733	Alpha-enolase	Plasminogen-binding protein
8510	ENOA_HUMAN	P06733	Alpha-enolase	Plasminogen-binding protein
8711	TRFE_HUMAN	P02787	Serotransferrin	Transferrin

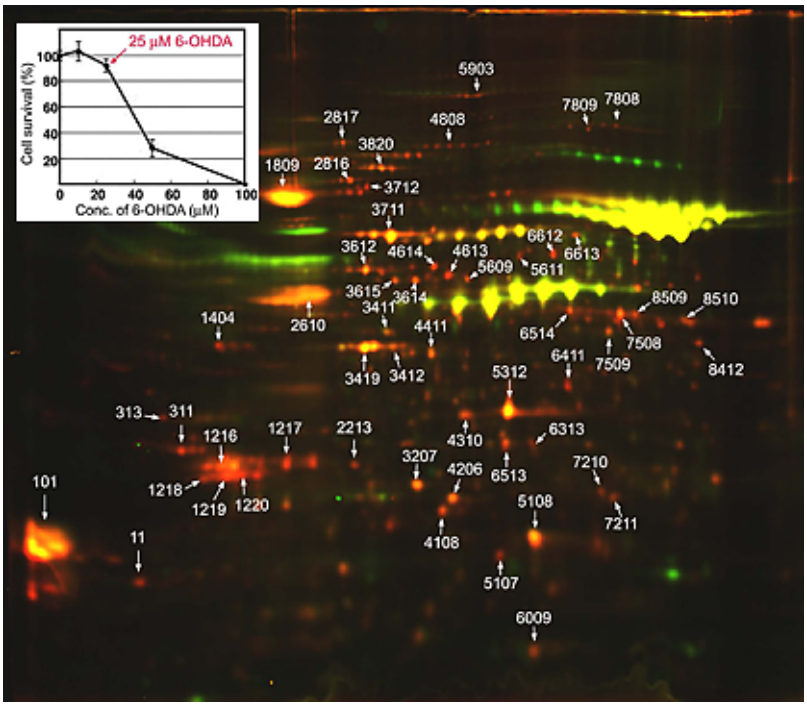

Fig. 3. Differential display of oxidative-stress-reporting biomarker proteins by 2D-DIGE.

The pseudo-colored image was generated using the "Multi-Channel Viewer" function of PDQuest software. The red color indicates increased proteins in the culture medium of SH-SY5Y cells under the oxidative stress induced with 25 μM 6-OHDA for 24 h. The insert indicates the survival of SH-SY5Y cells under various concentrations of 6-OHDA in the serum-free medium.

serotransferrin spots means the abundance of the protein in culture medium is conserved during the 24-h incubation.

DISCUSSION

As the results of our in-vitro research, Parkinson's disease protein 7 (DJ-1), ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2 N, 26S protease regulatory subunit 7, 14-3-3 protein isoforms (beta/alpha, gamma, epsilon), Rab GDP dissociation inhibitor beta, Rho GDP-dissociation inhibitor 1, COP9 signalosome complex subunit 4, peroxiredoxin-2, glutathione S-transferase P, heat shock cognate 71 kDa protein, heat shock protein HSP 90-alpha, 78 kDa glucose-regulated protein were identified as oxidative-stress-reporting biomarker candidates.

We expect that these proteins will be a potent molecular biomarkers for monitoring early stage of neurodegeneration occurring under oxidative stress in elderly people of advanced age.

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