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A PROTEOMIC ANALYSIS OF THE EFFECT OF MAPK PATHWAY ACTIVATION ON L-GLUTAMATE-INDUCED NEURONAL CELL DEATH

SUNGHYUN KANG, EUN YOUNG KIM, YOUNG JAE BAHN, JIN WOONG CHUNG, DO HEE LEE, SUNG GOO PARK, TAE-SUNG YOON, BYOUNG CHUL PARK and KWANG-HEE BAE*
Korean Research Institute of Bioscience and Biotechnology (KRIBB),
52 Eoeun-Dong, Yusung-Gu, Daejeon, 305-333, Republic of Korea

Abstract: Oxidative stress has been implicated in the pathogenesis of neuronal degenerative diseases. It is also widely known that oxidative stress induces mitogen-activated protein kinase (MAPK) signaling cascades. In this study, we used proteomic analysis to investigate the role of the MAPK pathway in oxidative stress-induced neuronal cell death. The results demonstrated that several proteins, including eukaryotic translation elongation factor 2 (eEF2) and enolase I, showed a differential expression pattern during the neuronal cell death process, and this was MAPK pathway dependent. Several chaperone and cytoskeletal proteins including heat shock protein 70, calreticulin, vimentin, prolyl 4-hydroxylase β polypeptide, and transgelin 2 were up- or down-regulated, despite their expressions not depending on the MAPK pathway. These findings strongly suggest that the expressions of proteins which play protective roles are independent of the MAPK pathway. On the other hand, eEF2 and enolase I may be the downstream targets of the MAPK pathway.

Key words: Apoptosis, HT22, MAPK, Oxidative stress, Reactive oxygen species, U0126

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* Author for correspondence; e-mail: khbae@kribb.re.kr, tel: 82-42-860-4268

Abbreviations used: Cy2 – 3-(4-carboxymethyl) phenylmethyl-3'-ethyloxacarbocyanine halide; Cy3 – 1-(5-carboxypentile)-1'-propylindocarbocyanine halide; Cy5 – 1-(5-carboxypentile)-1'-methylindocarbocyanine halide; DIGE – differential in gel electrophoresis; GSH – glutathione; MAPK – mitogen-activated protein kinase; MEK – MAPK/ERK kinase; ROS – reactive oxygen species

INTRODUCTION

Reactive oxygen species (ROS), which are generated as by-products of many metabolic processes, may be the principal mediators of cell death in oxidatively stressed neuronal cells. These species may perturb the cells' natural antioxidant defense systems, resulting in damage to all of the major classes of biological macromolecules [1]. This process has been implicated in several biological and pathological processes like ischemia, trauma, and neurodegenerative diseases such as Parkinson's and Alzheimer's disease [2-4]. The signaling of neuronal cell death has been studied extensively, and its course has been found to differ depending upon the nature and the strength of the apoptotic stimuli, and on the types of neuronal cells.

L-glutamate induces neuronal cell death through two distinct pathways: ionotropic glutamatergic receptor-initiated excitotoxicity and non-receptor-mediated oxidative glutamate toxicity [5]. The HT22 mouse hippocampal cell line that phenotypically resembles neuronal precursor cells has been widely used for studies of L-glutamate-induced oxidative toxicity [5, 6]. Since this cell line lacks functional ionotropic glutamate receptors, the effect of L-glutamate on HT22 cells is not due to receptor-initiated excitotoxicity [7]. High levels of L-glutamate inhibit the activity of the glutamate/cystine antiporter, which is required for the uptake of cystine into neuronal cells. The inhibition of cystine uptake leads to the depletion of cellular glutathione (GSH), the activation of neuronal 12-lipoxygenase (12-LOX), the accumulation of ROS, and the elevation of intracellular Ca^{2+}. The increase in ROS production and high Ca^{2+} levels are important requirements for the initiation of the apoptotic pathway [5]. Therefore, L-glutamate toxicity in HT22 cells serves as a model to study the response of neuronal cells to oxidative stress. This pathway is quite distinct from the classic pathways, and is likely to be related to many forms of nerve cell degeneration [6].

Mitogen-activated protein kinase (MAPK) family members, including extracellular signal-regulated kinases (ERK1/2), p38 kinase, and c-Jun N-terminal kinase (JNK), are serine-threonine protein kinases. They are activated by a wide spectrum of stimuli and cellular stresses and act on a diverse range of cellular targets [8, 9]. The ERK1/2 are two closely related members of the MAPK family that are predominantly activated by growth factors [10, 11]. In neurons, ERK can function to either support cell survival or promote cell death [12, 13]. Especially in HT22 cells, it was reported that a robust activation of ERK is essential for neuronal cell death during L-glutamate-induced oxidative toxicity [9, 14, 15]. In our study, we investigated the effects of MAPK pathways on L-glutamate-induced neuronal death using proteomic studies of HT22 cells. The MEK-specific inhibitor U0126 was used to inhibit the activation of MAPK/ERK kinase (MEK).

MATERIALS AND METHODS

Chemicals, strains and media

U0126 was purchased from Promega (Madison, WI, USA). Cy dyes were from GE Health Care and anti-ERK antibody from Santa Cruz Biotechnology. The HT22 cell line was derived from the HT4 cell line [16], and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Atlantic Biological, Norcross, GA).

Sample preparation and subcellular fractionation

Cells were seeded into plates at a density of 5×10^4 cells per ml (5×10^5 cells per 100-mm dish). Twenty-four hours after seeding, the cells were exposed to 5 mM L-glutamate to induce cell death. U0126 was added to a final concentration of 10 μM when it was necessary. The viability was measured using the MTT assay [9]. For western blot analysis, lysates were sonicated in SDS-PAGE sample buffer and twenty micrograms of proteins were separated by SDS-polyacrylamide gel electrophoresis, and transferred onto a PVDF membrane. Western blotting was carried out using a phosphor-ERK1/2 specific antibody (1:1000) to detect the phosphorylated form of ERK1/2. To determine the total ERK level, the immunoblots were stripped and re-probed with anti-ERK1/2 antibody. HT22 cells treated w/o L-glutamate for 8 h were fractionated using the differential centrifugation method [17]. Western blot analysis of cytosol fractionation samples was also carried out using antibodies against organelle-specific proteins.

Differential in gel electrophoresis (DIGE) and protein identification by mass spectrometry

The fractionated samples were first solubilized in a lysis buffer (30 mM Tris, pH 8.5, 7 M urea, 2 M thiourea, 4% CHAPS). CyDyes (Cy2, 3 and 5 from GE Healthcare) were then added to an each protein lysate and incubated on ice in the dark for 30 min. The protein samples that separated on the same gel were pooled and 3 volumes of acetone were added. After centrifugation, the precipitate was rehydrated using a rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% ampholyte, and 20 mM DTT), and isoelectic focusing was carried out using a Multiphor II (GE Healthcare) apparatus. After focusing, each strip (24-cm immobilized pH gradient strips, pH 4-7) was equilibrated twice, each time for 15 min in 2.5 ml equilibrium buffer. The equilibrium buffer contained 50 mM Tris, pH 8.8, 6 M urea, and 30% glycerol. During the second equilibrium step, 260 mM iodoacetamide was added to the equilibrium buffer. The IPG strips were then loaded and separated on a 12% acrylamide SDS-PAGE gel using a DALT12 (GE Healthcare) apparatus. Images were acquired with a Typhoon 9410 scanner (GE Healthcare) using 520BP40 (Cy2), 580BP30 (Cy3), and 670BP30 (Cy5) emission filters. The resolution was set to 100 μm. Images were analyzed using the DeCyder Differential Analysis Software v5.0 (GE Healthcare) in both the DIA module and BVA module. Only spots with

1.5-fold changes in volume after normalization in at least three separate experiments (p-value <0.05) were defined as altered.

The separated proteins in SDS-PAGE gels were visualized by silver staining. The stained gel images were compared with the original DeCyder analysis experiments and matched. The spots of interest were either manually excised or automatically detected and excised using the Xcise™ apparatus (Shimadzu Biotech, Japan). Gel pieces were washed twice with 150 μl of 100 mM ammonium bicarbonate (pH 8.2) and 70% v/v acetylnitrile (ACN), and dried at 37ºC for 20 min. Trypsin in 50 mM ammonium bicarbonate (20 μg/μl) was added to each gel piece and incubated at 37ºC for 2 h. Peptides were extracted in 20 μl of 0.1% v/v trifluoroacetic acid (TFA), 70% ACN. The peptide solution was either manually or automatically desalted and concentrated using ZipTips™ from Millipore (Bedford, MA, USA), and spotted onto Axima MALDI target plate. Peptide mass spectra of tryptic peptides were generated via Axima CFR+ matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS, Shimadzu Biotech, Japan). The obtained peptide masses were matched with the theoretical peptide masses of all the proteins from the mouse database of the NCBInr using MASCOT with the automated MASCOT Daemon (Matrix Sciences v.2.0, London, UK).

RESULTS AND DISCUSSION

First, we assessed the level of cell death in HT22 after treatment with 5 mM L-glutamate. The viability of HT22 cells began to decrease after the addition of L-glutamate, and the most of cells had died after 24 h of exposure (Fig. 1A). However, when there was co-treatment with U0126 and L-glutamate, cell death was suppressed even at 24 h. This protective effect of U0126 was dose-dependent, and near-complete suppression was shown at 10 μM (Fig.1B). We investigated the activation of ERK1/2 in HT22 by L-glutamate treatment. A basal level of phospho-ERK was observed, but a dramatic increase was detected after L-glutamate treatment. Co-incubation with U0126 dramatically blocked phosphorylation of ERK1/2. The total ERK1/2 level did not change (Fig. 1C). These results demonstrate that U0126 protects HT22 cells from L-glutamate-induced oxidative stress, probably inhibiting ERK1/2 activation.

Next, using 2D-DIGE, we compared the protein expression profiles of L-glutamate-treated HT22 cell fractions with or without U0126 co-treatment. We analyzed the cytosol fraction. Fig. 2 shows 2D-DIGE images of the cytosol fractions of L-glutamate-treated HT22 with or without U0126 treatment. The spots that exhibited significant changes were identified by MALDI-TOF Mass spectrometry (Tab. 1).

Several proteins, including heat shock protein 70, calreticulin, vimentin, prolyl 4-hydroxylase β polypeptide, and transgelin 2, were up-regulated by L-glutamate treatment. Although U0126 treatment completely blocks the activation of MAPK,

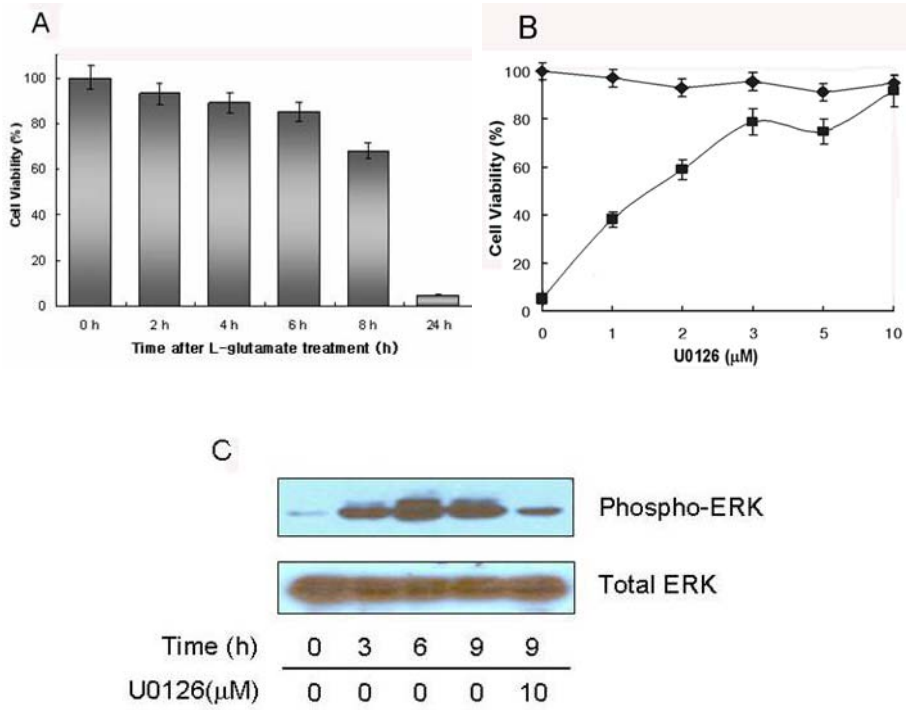

Fig. 1. The viability assay, U0126 effect, and ERK activation test during L-glutamate-induced cell death. A – The effect of L-glutamate on HT22 cell viability. After L-glutamate treatment, cell viability was measured using the MTT assay at several time points. The data is the means ± S.D. from at least three independent determinations. B – The dose-dependent protective effect of U0126 against L-glutamate induced oxidative stress. The cells were exposed to various concentrations of U0126 for 24 h and then assessed by the MTT assay. The data is the means ± S.D. from at least three independent determinations. ◆, with L-glutamate; ■, without L-glutamate. C – The activation of ERK by L-glutamate-induced oxidative stress and the inhibition of ERK activation by U0126.

these proteins also up-regulated in U0126 co-treated cells. From this result, it is suggested that these proteins may be not regulated by the MAPK signaling pathway. These proteins function in protein folding (as molecular chaperones) or cytoskeletal remodeling. Recently, the DeFranco group reported that ERK inhibition by U0126 treatment did not alter the rate or the extent of ROS productions in primary immature cortical cells during L-glutamate-induced oxidative toxicity [18]. This report and our results imply that these proteins may work against stress in L-glutamate-treated HT22 cells. These responses are not related to the MAPK pathway. Vimentin was detected at several positions. It was reported that vimentin was rapidly proteolyzed by multiple caspases into similar-sized fragments during apoptosis induced by various stimuli [19]. The caspase cleavage of vimentin disrupts its cytoplasmic network of intermediate

filaments and generates pro-apoptotic N-terminal cleavage product. In HT22 cells, it is unclear whether caspase pathways are involved in L-glutamate-induced cell death. Furthermore, interaction between caspase pathways and MAPK pathways is unclear in L-glutamate-induced neuronal cell death.

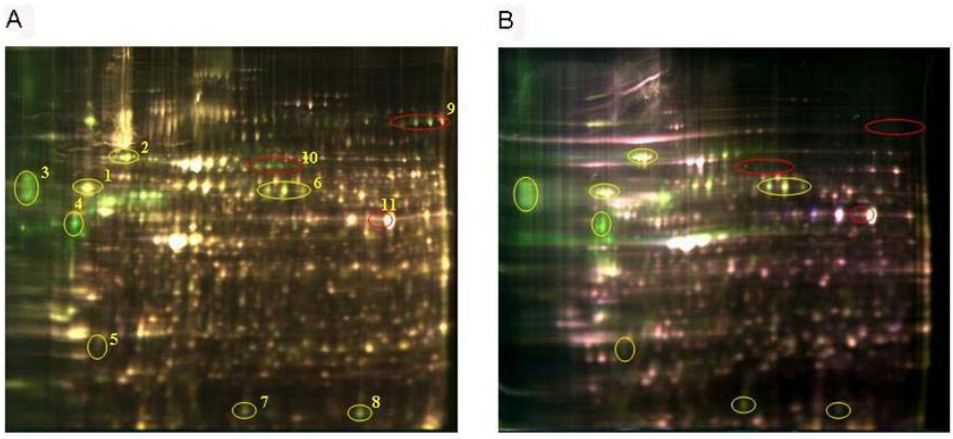

Fig. 2. Representative 2D-DIGE images of L-glutamate-treated HT22 cytosol fraction with or without U0126 co-treatment. The samples were prepared as described in the text. A – Pooled standard, L-glutamate-treated (8 h), and untreated samples (8 h) were labeled with Cy2 (blue), Cy3 (green), and Cy5 (red), respectively. B – Pooled standard, L-glutamate-treated (8 h), and L-glutamate and U0126 co-treated (8 h) samples were labeled with Cy2 (blue), Cy3 (green), and Cy5 (red), respectively. The experiments were repeated at least three times. The yellow circles mark spots which show similar protein levels in L-glutamate-treated and L-glutamate and U0126 co-treated samples. Red circles mark the spots which showed different expression levels.

Eukaryotic translation elongation factor 2 (eEF2) was found at several spots dispersed horizontally on the 2D gels. These spots were up-regulated upon L-glutamate treatment. This implies that eEF2 is modified at several positions after translation during oxidative stress (Fig 2A). However, in U0126-treated cells eEF2 showed no evidence of post-translational modification (PTM) (Fig. 2B). eEF2 is phosphorylated and inactivated by cellular stress [20, 21]. It is a member of the GTP-binding translation elongation factor family and essential for protein synthesis. It is implied that the expression or PTMs on these proteins may be directly or indirectly regulated by the MAPK signaling pathways. It was reported that CD40-mediated ERK activation is involved in the regulation of protein synthesis in carcinoma cells via a pathway involving the activation of p90 ribosomal S6 kinase (p90Rsk) and p70S6 kinases, which are upstream of the eEF2 [20]. In addition, Wang and Proud reported that the phosphorylation of eEF2 was regulated by MEK-dependent signaling in adult rat cardiomyocytes [21]. From these reports and our results, it is suggested that eEF2 may be the downstream target of the MAPK pathway.

Tab. 1. A list of the proteins identified on the 2D DIGE map as differentially regulated spots.

Spot No.	Protein name	*M*r/p*I*	Accession No.	Mowse score	Sequence coverage (%)	Up/Dn#
1	Prolyl 4-hydro-xylase β-subunit	57/4.79	gi|54777	128	27	Up (+2.7/+2.7)
2	Heat shock protein 70 (HSP70)	72.5/5.0	gi|29748016	190	41	Up (+1.6/+1.5)
3	Calreticulin	48/4.33	gi|6680836	112	42	Up (+2.3/+1.9)
4	Vimentin	51.5/4.9	gi|2078001	196	55	Up (+3.9/+3.1)
5	Vimentin	51.5/4.9	gi|2078001	218	61	Up (+3.1/+3.4)
6	Glucose regulated protein (ERp57)	57/5.88	gi|23958822	198	39	Up (+3.6,+3.4,+2.8 /+3.1,+3.2,+2.8)
7	Transgelin 2	22/8.39	gi|30519911	116	46	Up (+3.4/+3.0)
8	Cofilin, non-muscle	19/8.22	gi|55777182	64	46	Up (+4.2/+4.1)
9	Eukaryotic elon-gation factor 2	96/6.41	gi|33859482	142	25	Up (+2.6,+2.9,+3.8 /+1.1,+1.0,+1.1)
10	N.D.	-	-	-	-	-
11	Enolase I, a non-neuron	47/6.37	gi|54673814	139	42	Dn (-1.2/-6.3)

Up- or down-regulation (L-glutamate/L-glutamate+U0126). The values are the means from three independent determinations

Oxidative L-glutamate-induced HT22 cell death is quite distinct from classical apoptotic pathways [6], and it is likely to be involved in many forms of CNS nerve cell degeneration. In this process, persistent nuclear retention of activated ERK1/2 was reported to be a critical factor in eliciting proapoptotic effects [9]. However, the detailed mechanism of action of ERK1/2 on L-glutamate-induced neuronal cell death is as yet unknown at the proteomic level. Here, we reported on the candidate target proteins of the MAPK pathway during L-glutamate-induced neuronal cell death. In addition, our results demonstrated the decoupling phenomena between the MAPK pathway and the ROS defense response. Our proteomic studies may provide useful clues to understanding the effects of the MAPK pathway on oxidative stress-induced neuronal cell death.

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