



Letter

## Focal Cerebral Ischemia Induces Decrease of Astrocytic Phosphoprotein PEA-15 in Brain Tissue and HT22 Cells

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PEA-15 is a small phosphoprotein (15 kDa) that is enriched in brain astrocytes. PEA-15 acts as an important modulator of cellular function including apoptosis and signal integration. This study investigated the expression of PEA-15 in focal cerebral ischemic injury. Cerebral ischemia was surgically induced in adult male rats by middle cerebral artery occlusion (MCAO), and brains were collected 24 hr after MCAO. A proteomic approach demonstrated decreases of PEA-15 protein spots in MCAO-operated animals in comparison to sham-operated animals. Western blot analysis clearly demonstrated that MCAO induces decreases in PEA-15 levels. We previously showed that glutamate toxicity induces cell death in a hippocampus-derived cell line (HT22). Glutamate exposure induces decreases of PEA-15 levels in HT22 cells. The results of this study suggest that focal cerebral ischemia induces cell death through down-regulation of PEA-15 protein.

**Key words:** HT22 cells, middle cerebral artery occlusion, PEA-15

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Stroke is a serious neurodegenerative disorder and is a major cause of morbidity and mortality. Middle cerebral artery occlusion (MCAO) is an important experimental model of stroke (Longa *et al.*, 1989; Ferrer and Planas, 2003; Koh *et al.*, 2010). The focal cerebral ischemia that is caused by MCAO results in the development of infarct lesions and leads to extensive neuronal damage in the cerebral cortex (Ferrer and Planas, 2003). We confirmed in a previous study that MCAO induces serious damage to the cerebral tissue (Koh, 2010). Cerebral ischemic injury induces down- and up-regulation of specific proteins that mediate neuronal cell death (Koh, 2010).

PEA-15 is a small acidic phosphoprotein (15 kDa) that is abundantly expressed in astrocytes (Araujo *et al.*, 1993; Danziger *et al.*, 1995). PEA-15 is a multifunctional protein that is involved in the regulation of the cell cycle and apoptosis (Danziger *et al.*, 1995; Renault *et al.*, 2003; Krueger *et al.*, 2005). Cerebral ischemia mediates apoptotic signaling and

survival signaling pathways and results in neuronal cell death (Ferrer and Planas, 2003). We previously reported that focal cerebral ischemia induces neuronal cell death by altering the expression levels of several proteins (Koh, 2010). However, the process of neuronal cell death in cerebral ischemia complex is poorly known. At present, little information is available regarding changes of protein expression that occur in damaged neurons. In this study, we detected decreases in PEA-15 proteins using a proteomic approach in an animal model of focal cerebral ischemia. We investigated the expressions of PEA-15 proteins in cerebral ischemic brain injury and hippocampal neuron culture conditions.

All animal experiments were performed according to a protocol approved by the Committee for Animal Experimentation at the Gyeongsang National University. Male Sprague-Dawley rats (225-250 g,  $n=20$ ) were purchased from Samtako Co. (Osan, Korea) and were randomly divided into two groups: sham-operated group and MCAO-operated group ( $n=10$  per group). Animals were maintained under controlled temperature (25°C) and lighting (14L:10D), and allowed free access to commercial food and purified water.

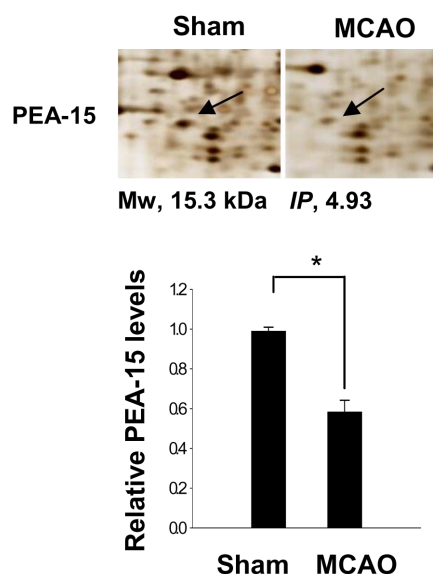
MCAO was performed to induce focal cerebral ischemic injury (Longa *et al.*, 1989). Animals were anesthetized with sodium pentobarbital (100 mg/kg). A piece of 4/0 mono-

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filament nylon suture with its tip slightly rounded by gentle heating was inserted through the right internal carotid artery to the base of the right middle cerebral artery. Animals were sacrificed 24 hr after MCAO for the investigation in late stage of apoptosis. The right cerebral cortex was removed. The tissues were homogenized in lysis buffer (8 M urea, 4% CHAPS, 0.5% ampholytes, and 40 mM Tris-HCl) and centrifuged at 16,000 g for 20 min at 4°C. The total protein concentration was determined using the Bradford method (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol.

The two-dimensional (2D) gel electrophoresis was performed by the following steps. The IPG strips (IPG, pH 4-7, 17 cm, Bio-Rad) were re-hydrated in rehydration buffer at room temperature (8 M urea, 2% CHAPS, 20 mM DTT, 0.5% IPG buffer, a trace of bromophenol blue) that contained lysed proteins for 13 hr. The protein samples were focused on Protean isoelectric focusing (IEF) Cell (Bio-Rad) at 20°C in three steps: 250 V (15 min), 10,000 V (3 hr), and then 10,000 V to 50,000 V. For the second dimension, gradient gels (7.5-17.5%) were prepared. After equilibration of the IEF strips, 2D gel electro-phoresis was carried out at 10 mA/gel using Protein-II XL electrophoresis equipment (Bio-Rad) when the dye reached end of the gels. For the silver stain, the gels were fixed in a solution (12% acetic acid, 50% methanol). The fixed gels stained with a silver solution (0.2% silver nitrate, 0.75 mL/L formaldehyde). The images of stained gels were visualized by Agfar ARCUS 1200™ (Agfar-Gevaert, Mortsel, BEL). The scanned gel images were analyzed using a standard protocol for PDQuest software (Bio-Rad). Differentially expressed proteins were identified in sham-operated and MCAO-operated animals. The spots of interest were cut and destained. The gel pieces were rehydrated with reduction solution (20 mM DTT in 0.1 M  $\text{NH}_4\text{HCO}_3$ ) and reacted in alkylation solution for 30 min. The gel particles were incubated with trypsin-containing digestion buffer (Promega, Madison, WI, USA). The mass analysis of extract peptides was performed on a Voyager-DE™ STR biospectrometry workstation (Applied Biosystem, Foster, CA, USA) for MALDI-TOF mass spectrometry. Proteins were identified using search programs MS-Fit and ProFound program for identification. SWISS-PROT and NCBI were used as the protein sequence databases.

A mouse hippocampal cell line (HT22) was propagated in Dulbecco's modified Eagle's medium (without L-glutamine), supplemented with 10% fetal bovine serum, streptomycin (100  $\mu\text{g}/\text{mL}$ ), and penicillin (100 unit/mL) (Gibco BRL, Gaithersburg, MD, USA). The cells were maintained at 37°C in a humidified chamber with 5%  $\text{CO}_2$  atmosphere. HT22 cells were seeded on 60-mm culture dishes at 100,000 cells per dish. Cell density was closely monitored to prevent

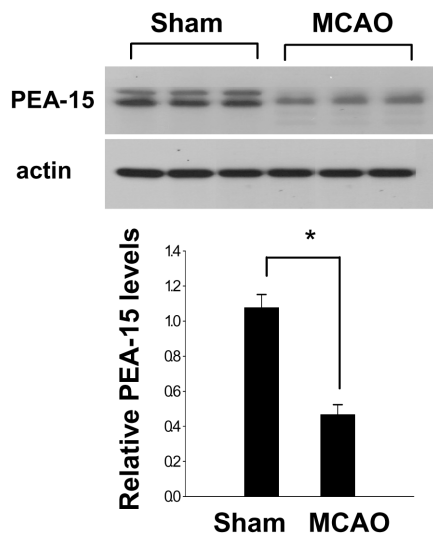


**Figure 1.** PEA-15 protein spots identified by MALDI-TOF. Arrows indicate the protein spots. The intensity of spots was measured using PDQuest software. The ratio of intensity is described as spots intensity of MCAO-operated animal to spots intensity of sham-operated animal. Data are shown as mean  $\pm$  S.E.M. \* $P < 0.05$  (vs. Sham). Mw and IP indicate molecular weight and isoelectrical point, respectively.

excessive growth and was maintained 70% or less confluence as described previously (Maher and Davis, 1996; Koh, 2007). Glutamate (Sigma, St. Louis, MO, USA) was diluted to a final concentration of 5 mM in culture medium and cells were exposed for 24 hr. Ethanol was used at a final concentration of 0.1% as a vehicle control. This concentration of ethanol had no effect on cell viability or glutamate toxicity.

Western blot analysis was performed as previously described method (Koh, 2008). Equal amounts of protein (30  $\mu\text{g}$ ) per lane were loaded to 10% SDS-polyacrylamide gels and separated by electrophoresis. Proteins were transferred to a poly-vinylidene fluoride membrane (Millipore, Billerica, MA, USA). Blots were washed in Tris-buffered saline containing 0.1% Tween-20 and then incubated with the following antibodies: anti-PEA-15 and anti-actin antibodies (diluted 1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) as primary antibodies. And the membrane was incubated with secondary antibody (1:5,000, Pierce, Rockford, IL, USA) and the ECL Western blot analysis system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The intensity analysis was carried out using SigmaGel 1.0 (Jandel Scientific, San Rafael, CA, USA) and SigmaPlot 4.0 (SPSS Inc., Point Richmond, CA, USA). All data are expressed as mean  $\pm$  S.E.M. The results in each group were compared by one-way analysis of variance (ANOVA) followed by Student's *t*-test.

Figure 1 illustrates PEA-15 protein spots identified in the

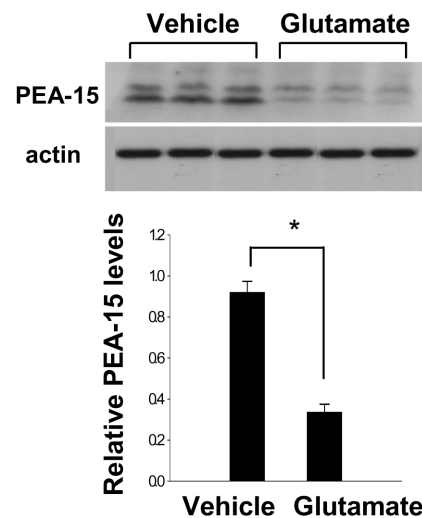


**Figure 2.** Western blot analysis of PEA-15 in the cerebral cortex from sham-operated and MCAO-operated animals. Each lane represents an individual experimental animal. Densitometric analysis of PEA-15 levels is represented as intensity of PEA-15 to intensity of actin. Data are shown as mean ± S.E.M. \* $P < 0.05$  (vs. Sham).

cerebral cortexes of MCAO-operated and sham-operated rats. The peptide mass of PEA-15 is 5/36 and the sequence of this protein is 37%. These protein levels decreased in MCAO-operated animals compared to sham-operated animals. Western blot analysis clearly demonstrated that MCAO induces decreases in PEA-15 levels (Figure 2). The levels of PEA-15 were  $1.09 \pm 0.04$  and  $0.48 \pm 0.03$  in the cerebral cortexes of sham-operated and MCAO-operated animals, respectively. We previously showed that glutamate toxicity induces cell death in HT22 cells (Koh, 2007). Figure 3 indicates that glutamate exposure induces decreases of PEA-15 levels in HT22 cells. The levels of PEA-15 were  $0.91 \pm 0.03$  in the vehicle-treated group, and  $0.35 \pm 0.03$  in the glutamate-treated group, respectively (Figure 3).

Previous studies have established that MCAO leads to focal cerebral cortex ischemia and causes neuronal cell death through the apoptotic signaling pathway (Li *et al.*, 1997; Ferrer and Planas, 2003). We previously identified differentially expressed proteins following ischemic brain injury using a proteomic approach (Koh, 2010). These proteins include 60 kDa heat shock protein, dehydroypyrimidinase-related protein 2, thioredoxin, peroxiredoxin-2, stathmin, and ubiquitin carboxy-terminal hydrolase L1. Cerebral ischemic injury induces down- and up-regulation of these proteins. In the present study, we additionally identified the down-regulation of PEA-15 protein after MCAO-induced brain injury in an animal model.

PEA-15 is an abundant phosphoprotein in brain astrocytes



**Figure 3.** Western blot analysis of PEA-15 in HT22 cells. Vehicle or glutamate (5 mM) was exposed to HT22 cells for 24 hr. Each lane represents an individual experimental animal. Densitometric analysis of PEA-15 levels is represented as intensity of PEA-15 to intensity of actin. Data are shown as mean ± S.E.M. \* $P < 0.05$  (vs. Vehicle).

and plays a major role in modulating signal pathways that regulate apoptosis and cell proliferation (Araujo *et al.*, 1993; Danziger *et al.*, 1995; Renault *et al.*, 2003; Krueger *et al.*, 2005). PEA-15 inhibits tumor-necrosis factor- $\alpha$  (TNF- $\alpha$ )-induced apoptosis, blocks activation of caspase, and regulates apoptosis (Renault *et al.*, 2003; Sharif *et al.*, 2003). Focal cerebral ischemia induces TNF- $\alpha$  expression, while inhibition of TNF- $\alpha$  mediates neuroprotection (Dawson *et al.*, 1996). PEA-15 binds to the Fas associated death domain and mediates apoptosis pathway (Krueger *et al.*, 2005). Cerebral ischemia induces Fas-mediated apoptosis and leads to neuronal cell death (Wetzel *et al.*, 2008; Jia *et al.*, 2009). Moreover, PEA-15 binds extracellular signal-regulated kinase and regulates mitogen-activated protein kinase signaling (Krueger *et al.*, 2005). We identified decreases in PEA-15 protein spots in MCAO-induced brain injury. Western blot analysis confirmed that ischemic brain injury significantly decreases PEA-15 levels. It is accepted that glutamate induces oxidative stress and causes neuronal cell death. Oxidative toxicity induces the disruption of redox homeostasis and results in cell death. Thus, glutamate has been used as the inducer of ischemic condition *in vitro* study. Glutamate exposure induces a significant reduction in PEA-15 levels in neurons. Focal cerebral ischemia induces decreases in PEA-15 and leads to neuronal cell death. The results of this study demonstrate that PEA-15 decreases in MCAO-induced injury and glutamate-exposed HT22 cells. PEA-15 mediates anti-apoptotic function and cell proliferation. However, decrease of PEA-15 in cerebral

ischemia is not yet reported. Although further studies are required to explain the mechanism of PEA-15 in ischemic brain injury, this study can demonstrate that decrease of PEA-15 in cerebral ischemia inhibits anti-apoptotic function and induces neuronal cell death. Thus, the results of this study suggest that decrease in PEA-15 mediates neuronal cell death in brain injury.

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