Nicotinamide Attenuates the Decrease of Astrocytic Phosphoprotein PEA-15 in Focal Cerebral Ischemic Injury

Phil-Ok KOH1)*

1)Department of Anatomy, College of Veterinary Medicine, Research Institute of Life Science, Gyeongsang National University, 900 Gajwa-dong, Jinju 660–701, South Korea

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ABSTRACT. Nicotinamide exerts neuroprotective effects against focal cerebral ischemic injury. Phosphoprotein enriched in astrocytes 15 (PEA-15) is prominently expressed in astrocytes that exert broad anti-apoptotic functions. This study investigated whether nicotinamide modulates PEA-15 and levels of two phosphorylated PEA-15 (Serine 104 and 116) in an animal model of middle cerebral artery occlusion (MCAO)-induced injury. Adult male rats were treated with vehicle or nicotinamide (500 mg/kg) 2 hr after the onset of MCAO and cerebral cortices were collected at 24 hr after MCAO. In a proteomic approach, MCAO induced decreases of PEA-15 levels, while nicotinamide treatment attenuated the injury-induced decrease in PEA-15. The results of Western blot analysis suggest that nicotinamide prevented injury-induced reduction in phospho-PEA-15 (Serine 104) and phospho-PEA-15 (Serine 116) levels. The phosphorylation of PEA-15 exerts anti-apoptotic functions, and reduction of PEA-15 phosphorylation leads to apoptotic cell death. These results suggest that nicotinamide exerts a neuroprotective effect by attenuating the injury-induced decreases of PEA-15 and phospho-PEA-15 (Ser 104 and Ser 116) proteins.

KEY WORDS: neuroprotection, nicotinamide, PEA-15.

Nicotinamide is a precursor of nicotinamide adenine dinucleotide (NAD+) that exerts neuroprotective effects against neurodegenerative conditions such as Alzheimer’s disease and trauma [2, 8]. Nicotinamide reduces infarct volumes after focal cerebral ischemic injury and attenuates neuronal cell death by inhibiting apoptotic signaling pathways [18, 20].

PEA-15 is a small phosphoprotein that is prominently expressed in astrocytes. PEA-15 modulates essential cellular functions including apoptosis and cell proliferation [1, 9, 16] and contains a death effector domain (DED). PEA-15 binds the DED of both the Fas-associated death domain (FADD) and caspase-8, and inhibits apoptosis initiated by the Fas ligand [6]. PEA-15 exerts anti-apoptotic effects by inhibiting formation of the death-inducing signaling complex (DISC) and preventing activation of the caspase cascade [6, 7]. PEA-15 is phosphorylated on Serine 104 by protein kinase C (PKC), and on Serine 116 by protein kinase B/Akt and calcium-calmodulin kinase 2 [1, 11]. The phosphorylation of PEA-15 modulates its anti-apoptotic functions [18]. Nicotinamide exerts neuroprotective effects by preventing the ischemic brain injury-induced decrease of the Akt signaling pathway [3, 5]. Although previous studies have demonstrated the neuroprotective effects of nicotinamide, there is little information available regarding the expression levels of PEA-15 and two phosphorylated PEA-15 forms (Serine 104 and 116) in MCAO-induced brain injury. Thus, we investigated PEA-15, phospho-PEA-15 (Serine 104) and phospho-PEA-15 (Serine 116) levels in the presence of nicotinamide in an animal model of MCAO-induced ischemic brain injury.

All experimental procedures were followed in accordance with the Guide for the care and use of laboratory animals, published by the U.S. National Institutes of Health. Sprague–Dawley rats (male, 220–230 g, n=30) were purchased from Samtako Co. (Animal Breeding Center, Korea) and were randomly divided into 3 groups, sham-operated, vehicle-treated, and nicotinamide-treated (n=10 per group). Animals were allowed to have free access to food and water before surgery. Animals were anesthetized with sodium pentobarbital (30 mg/kg) and were performed middle cerebral artery occlusion (MCAO) to induce focal cerebral ischemia. MCAO were carried out as a previously described method [12]. Briefly, the right common carotid artery, external carotid artery, and internal carotid artery were exposed through a midline cervical incision. A 4/0 nylon suture with its tip slightly rounded by heating over a flame was inserted the external carotid artery into the internal carotid artery until tip occluded the origin of the MCA. Nicotinamide (Sigma, St. Louis, MO) was dissolved in normal saline as vehicle. A single dose of vehicle or nicotinamide (500 mg/kg) was treated via an intraperitoneal injection at 2 hr after the onset of MCAO [20]. A dose of nicotinamide (500 mg/kg) exerts a neuroprotective effect during MCAO [9, 20]. At 24 hr after the onset of permanent occlusion, animals were decapitated and brains were removed.

A proteomic analysis was carried out as a previously
described method [17]. The right cerebral cortices including both ischemic area and penumbra area were suspended in lysis buffer (8 M urea, 4% CHAPS, ampholytes, and 40 mM Tris-HCl) and centrifuged at 16,000 g for 20 min at 4°C. The protein concentration was determined using the Bradford method (Bio-Rad, Hercules, CA, U.S.A.) according to the manufacturer’s protocol. For the 2-dimensional (2D) gel electrophoresis, the immobilized pH gradients (IPG, pH 4–7, pH 6–9, 17 cm, Bio-Rad) gel strips were incubated in rehydration buffer (8 M urea, 2% CHAPS, 20 mM DTT, 0.5% IPG buffer, bromophenol blue) that contained sample proteins for 13 hr. The protein samples were focused on Protean 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. After IEF focusing, 2D gel electrophoresis was performed using gradient gels (7.5–17.5%) with Protein- XI electrophoresis equipment (Bio-Rad) at 10°C. Current conditions were 5 mA per gel for 2 hr and followed by 10 mA per gel for 10 hr. The silver stain was carried out by the following steps. The gels were fixed in a solution (12% acetic acid, 50% methanol) for 2 hr, washed in 50% ethanol for 20 min, and then treated in 0.2% sodium thiosulfate for 1 min. The gels were washed with deionized water, impregnated in a silver solution (0.2% silver nitrate, 0.75 ml/l formaldehyde) for 20 min, and washed with deionized water. The gel was developed in a solution (0.2% sodium carbonate, 0.5 ml/l formaldehyde) and the reaction was stopped by stop solution (1% acetic acid). The silver stained gels were scanned using Agfar ARCUS 1200™ (Agfar-Gevaert, Mortsel, BEL). The scanned gel images were analyzed using a standard protocol for PDQuest software (Bio-Rad). The gel pieces containing the desired protein spots were excised and destained. The gel particles were incubated with reduction solution and alkylation solution for 30 min, and followed by trypsin-containing digestion buffer. The extract peptides were analyzed in a Voyager-DE™ STR biospectrometry workstation (Applied Biosystem, Forster city, CA, U.S.A.) for MALDI-TOF mass spectrometry. Proteins were identified using search programs MS-Fit and ProFound program. SWISS-PROT and NCBI were used as the protein sequence databases.

For the Western blot analyses, total protein (30 μg) was applied to each lane onto 10% SDS-polyacrylamide gels. After electrophoresis and immunoblotting, the polyvinylidene fluoride membranes (Millipore, Billerica, MA) were washed in Tris-buffered saline containing 0.1% Tween-20 (TBST) and then incubated with the following antibodies: anti-total PEA-15, anti-phospho-PEA-15 (Serine 104), anti-phospho-PEA-15 (Serine 116) (diluted 1:1,000, Cell Signaling Technology, Beverly, MA.), anti-actin (Millipore) as primary antibody. And the membrane

Fig. 1. PEA-15 protein spots identified by MALDI-TOF (A) and Western blot analysis of PEA-15 (B) in the cerebral cortices from vehicle + MCAO, nicotinamide + MCAO, vehicle + sham, nicotinamide + sham animals. Rats were treated with vehicle or nicotinamide at 2 hr after the onset of MCAO. Arrows indicate the protein spots. Mw and IP indicate molecular weight and isoelectrical point, respectively. 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 (n=5) are represented as mean ± S.E.M. * P<0.05.
was incubated with secondary antibody (1:5,000, Pierce, Rockford, IL) and the ECL Western blot analysis system (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer’s protocol was used for detection. The intensity analysis was carried out using SigmaGel 1.0 (Jandel Scientific, San Rafael, CA) and SigmaPlot 4.0 (SPSS Inc., Point Richmond, CA). The results are the mean of 5 independent experiments. All data are expressed as mean ± S.E.M. The results in each group were compared by one-way analysis of variance (ANOVA) followed by Student’s t-test. The difference for comparison was considered significant at $P<0.05$.

In a proteomic approach, PEA-15 protein spots were decreased in the cerebral cortex of rats with MCAO-induced brain injury, whereas decreases of these proteins were attenuated in nicotinamide-treated animals (Fig. 1A). Western blot analysis confirmed that PEA-15 levels were significantly decreased in vehicle-treated animals, whereas nicotinamide treatment prevented injury-induced decreases in PEA-15. In MCAO-operated group, the levels of PEA-15 were 0.61 ± 0.03 and 0.92 ± 0.02 in the cerebral cortices of vehicle- and nicotinamide-treated animals, respectively (Fig. 1B). The phosphorylation of PEA-15 is a critical step in the anti-apoptotic function of PEA-15. The levels of phospho-PEA-15 (Serine 104) and phospho-PEA-15 (Serine 116) were decreased in vehicle + MCAO animals, whereas nicotinamide treatment attenuated decreases of these proteins. The levels of phospho-PEA-15 (Serine 104) were 0.32 ± 0.02 and 0.67 ± 0.03 in vehicle + MCAO and nicotinamide + MCAO animals, respectively (Fig. 2). The levels of phospho-PEA-15 (Serine 116) were 0.18 ± 0.02 and 0.63 ± 0.02 in vehicle + MCAO and nicotinamide + MCAO animals, respectively (Fig. 3).

Nicotinamide reduces infarct volumes and decreases apoptotic cell death after focal cerebral ischemia [14]. Nicotinamide prevents apoptosis in the brain by regulating pro- and anti-apoptotic proteins [15]. We previously reported that nicotinamide mediates neuroprotective effects by up- and down-regulation of specific proteins [17]. A proteomics approach demonstrated that ischemic brain injury decreases PEA-15 protein levels, while nicotinamide prevents decreases in these proteins [17].
The phosphorylation of PEA-15 influences anti-apoptotic function [18]. PEA-15 is phosphorylated on two serine residues, Ser 104 and Ser 116. Ser 104 is regulated by PKC, whereas Ser 116 is mediated by Akt and calcium-calmodulin kinase [1, 11]. Nicotinamide exerts cytoprotective effects by activating Akt via phosphorylation [13]. This study showed that MCAO-induced injury reduces PEA-15 levels, whereas nicotinamide prevents the injury-induced decrease of PEA-15. Levels of two phosphorylated forms, phospho-PEA-15 (Ser 104) and phospho-PEA-15 (Ser 116), are also decreased in ischemic brain injury, while nicotinamide attenuates decreases of these phospho-proteins. A previous study demonstrated that nicotinamide modulates PKC activity [4]. PKC can phosphorylate PEA-15 at Ser 104. This study showed that nicotinamide mediates the expression of phospho-PEA-15 (Ser 104). These results indicate that nicotinamide regulates the Akt signaling pathway and consequently modulates phosphorylation of PEA-15. Moreover, nicotinamide exerts neuroprotective effects through the mitogen-activated protein kinase (ERK) signaling pathway. Phosphorylation of PEA-15 at serine 104 can transfer ERK into the nucleus and modulate ERK-dependent transcription. Taken together, the decline of PEA-15 phosphorylation inhibits anti-apoptotic function, and causes apoptotic cell death in focal cerebral ischemia. Nicotinamide prevents declines in these proteins and reduces neuronal cell death in focal cerebral ischemia. Although further studies are needed to elucidate the relationships between nicotinamide and PEA-15 in ischemic brain injury, this study demonstrates that inhibition of decrease in PEA-15 and phospho-PEA-15 (Ser 104 and Ser 116) proteins by nicotinamide in MCAO-induced injury may inhibit apoptotic cell death. In conclusion, these results suggest that nicotinamide attenuates neuronal cell death in focal cerebral ischemia through the modulation of PEA-15 and phospho-PEA-15 (Ser 104 and Ser 116) proteins.

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