Neuroscience Letters 588 (2015) 18–23

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Ferulic acid attenuates the down-regulation of MEK/ERK/p90RSK signaling pathway in focal cerebral ischemic injury

Phil-Ok Koh*

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

HIGHLIGHTS

- Ferulic acid plays a neuroprotective role against neuronal cell injury.
- Ferulic acid prevents brain injury-induced decrease in Raf-1, MEK1/2, and ERK1/2 phosphorylation levels.
- Ferulic acid attenuates brain injury-induced decrease in p90RSK and Bad phosphorylation levels.

ARTICLE INFO

Article history:
Received 6 November 2014
Received in revised form
17 December 2014
Accepted 18 December 2014
Available online 24 December 2014

Keywords:
Brain ischemia
Ferulic acid
MAP kinase

ABSTRACT

Ferulic acid provides neuroprotective effects against a middle cerebral artery occlusion (MCAO)-induced cerebral ischemia. Mitogen-activated protein kinases can regulate extensive intracellular processes including cell differentiation, growth, and death. This study further investigated whether ferulic acid modulates a protective mechanism through the activation of Raf-MEK-ERK and its downstream targets, including 90 ribosomal S6 kinase (p90RSK) and Bad during cerebral ischemic injury. Male Sprague–Dawley rats were treated with ferulic acid (100 mg/kg) or vehicle after the onset of MCAO and brain tissues were collected 24 h after MCAO. These results indicated that ferulic acid decreases the volume of the infarct area and the number of cells positive in terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Although MCAO injury induces a decrease in the phosphorylation of Raf-1, MEK1/2, and ERK1/2, ferulic acid treatment prevents the injury-induced decrease in these phosphorylation levels. Ferulic acid also attenuates the injury-induced decrease in p90RSK and Bad phosphorylation levels. These findings suggest that ferulic acid prevents MCAO-induced neuronal cell death and that the MEK-ERK-p90RSK-Bad signaling pathway is involved in these neuroprotective effects.

Cerebral ischemia induces serious brain damage through the overproduction of oxidative stress and the destruction of energy metabolism [1,3]. Polyphenols, such as ferulic acid, exist ubiquitously in plants and play essential roles in cell growth and development as well as serve a protective function against neurodegenerative diseases such as Alzheimer's disease and stroke [5,6,23]. Ferulic acid acts as an antioxidant agent against radical oxidation in neuronal cell culture systems [12] and also exerts a neuroprotective effect through inhibition of apoptosis in focal cerebral ischemic injury [5,6]. Moreover, ferulic acid provides considerable protection against various oxidative stress related disorders [26].

Mitogen-activated protein (MAP) kinase is a family of serine/threonine protein kinases that modulate extensive cellular function such as cell proliferation, differentiation, and death [20]. The p44/42 MAP kinase (ERK1/2) signaling pathway, which interacts with extracellular stimuli such as growth factors and mitogens, is known as growth factor signaling [2,19,24]. The Raf protein is the most important up-stream activator of MAP kinase. Raf activates MEK1/2 through phosphorylation; phosphorylated MEK1/2 then phosphorylates and activates ERK1/2. Moreover, ERK1/2 regulates the down-stream targets of MAP kinase such as 90 kDa ribosomal S6 kinase (p90RSK) and transcription factor Elk-1 [7,14,18,22] . The phosphorylation of p90RSK by ERK1/2 leads to the phosphorylation of Bad which results in the inhibition of the apoptotic function of Bad [4,8,25]. Previous studies have been demonstrated the relation of ferulic acid and ERK kinase signaling [10,11,16,17]. Ferulic acid prevents cells death from radiation-induced oxidative stress and amyloid β-induced neurotoxicity through the activation of Akt and ERK signaling pathways [10,16]. Moreover, the ERK signaling pathway controls the anti-oxidation of ferulic acid by regulating

* Tel.: +82 55 772 2354; fax: +82 55 772 2349.
E-mail address: pokoh@gnu.ac.kr

http://dx.doi.org/10.1016/j.neulet.2014.12.047
0304-3940/

the expression of the antioxidant enzyme, also play important roles in the protective effect of sodium ferulate against glutamate toxicity in cortical neurons [11,17]. I previously reported that ferulic acid exerts a neuroprotective effect by activation of the Akt signaling pathway in focal cerebral ischemia [13]. Although previous studies have been demonstrated the neuroprotective effect of ferulic acid, the precise neuroprotective mechanism by ferulic acid treatment in MCAO remains unclear. Moreover, little data is available regarding the regulation of ERK kinase by ferulic acid in a MCAO animal model. I propose that ferulic acid treatment regulates the MEK/ERK/p90RSK-signaling cascade in focal cerebral ischemia. Therefore, this study investigated whether ferulic acid modulates the activation of Raf-MEK-ERK and its downstream targets, p90RSK and Bad.

Sprague–Dawley rats (male, 220–230 g, $n = 40$) were obtained from Samtako Co. (Animal Breeding Center, Osan, Korea). Rats were kept under controlled temperature (25 °C) and lighting (12/12 h light/dark cycle) and were allowed free access to food. All experimental procedures for animal use were approved by the Institutional Animal Care and Use Committee at Gyeongsang National University. Animals were randomly divided into four groups: vehicle + sham group, ferulic acid + sham group, vehicle + middle cerebral artery occlusion (MCAO) group, and ferulic acid + MCAO group. A single dose of ferulic acid (100 mg/kg, Sigma Chemical Co., St. Louis, MO, USA) or vehicle was intravenously injected immediately after MCAO [5]. The vehicle was used the same volume of normal saline.

The MCAO model was induced as previously described [15]. Animals were anesthetized by an intraperitoneal injection of sodium pentobarbital (30 mg/kg). The right common carotid artery, external carotid artery, and internal carotid artery were exposed through a midline cervical incision. A 4/0 monofilament nylon suture with its tip slightly rounded by heat was inserted through the internal carotid artery until it blocked the origins of the middle cerebral artery. At 24 h after the onset of permanent occlusion, animals were sacrificed and brains were removed. Sham-operated animals underwent the same surgical procedures without insertion of a nylon filament.

Brains tissues were cut into coronal slices of 2 mm in thickness using a brain matrix, and these slices were stained with 2% triphenyltetrazolium chloride (TTC; Sigma) for 20 min at 37 °C. Brain slices were fixed by 10% formalin solution, photographed by a Nikon CoolPIX990 digital camera (Nikon, Tokyo, Japan) and measured for the ischemic lesion by Image-ProPlus 4.0 software (Media Cybernetics, Silver Spring, MD, USA). The ischemic lesion percentage of each brain slice was measured by the ratio of the infarction area to the whole slice area. The brain slices were embedded with paraffin and sectioned into slices of 4 μm thickness using a rotary microtome. The identification of apoptotic cells was performed using the DNA Fragmentation Detection Kit (Oncogene Research Products, Cambridge, MA, USA) following the manufacturer's protocol. The sections were reacted with equilibration buffer and terminal deoxynucleotidyl transferase (TdT) enzyme for 60 min at room temperature. The reaction was terminated by incubation in stop buffer for 5 min. The sections were washed in PBS three times for 10 min, incubated with horseradish peroxidase (HRP) conjugate, and visualized with diaminobenzidine tetrahydrochloride (DAB, Sigma) substrate. The sections were counterstained with hematoxylin and the slides were observed under microscope. Five fields for each section were selected from the right cerebral cortex. The total cell number and TUNEL-positive cell number were counted carefully in each field. The percentage of TUNEL-positive cells is described as the percentage of the number of TUNEL-positive cells to the total number of cells in each field.

Western blot analysis was performed as a previous described method [21]. The right cerebral cortex tissues ($n = 5$ per group) were removed and lysed in buffer [1% Triton X-100, 1 mM EDTA in 1 × PBS (pH 74)] containing 10 μM leupeptin and 200 μM phenylmethylsulfonyl fluoride. The lysates were centrifuged at 15,000 × g for 20 min at 4 °C and the supernatant was used for Western blotting. The protein concentration of each lysate was determined using the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA) according to the guidelines provided by the manufacturer. Total protein (30 μg) was applied to each lane on 10% SDS-polyacrylamide gels and was loaded using a Ready Gel (Bio-Rad Hercules, CA, USA). After electrophoresis, proteins were transferred from the gel to a poly-vinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membranes were incubated with skim milk solution, washed in Tris-buffered saline containing 0.1% Tween-20 (TBST), and then incubated with the following antibodies: anti-phospho-Raf-1(Ser^{338}), anti-Raf-1, anti-phospho-MEK1/2(Ser^{217}/Ser^{221}), anti-MEK1/2, anti-phospho-ERK1/2(Thr^{202}/Thr^{204}), anti-ERK1/2, anti-phospho-p90RSK(Ser^{383}), anti-p90RSK, anti-phospho-Bad(Ser^{112}), anti-Bad anti-cleaved PARP (diluted 1:1000, Cell Signaling Technology, Beverly, MA, USA), and anti-actin (diluted 1:1000, Milipore) as the primary antibody. The membrane was then incubated with the secondary antibody (1:5000, Pierce) and the ECL Western blot analysis system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was used according to the manufacturer's protocol. 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).

For the immunohistochemical study, brain tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4) solution, embedded with paraffin, and sectioned into 4 μm thick slices. The sections were deparaffined in xylene, blocked with 1% normal goat serum in PBS for 1 h and then incubated with anti-phospho-ERK1/2(Thr^{202}/Thr^{204}) antibody (diluted 1:200, Cell Signaling Technology) at 4 °C for 15 h in a humidified chamber. The sections were washed in PBS and were incubated with biotin-conjugated goat anti-rabbit IgG (1:200 in PBS) for 1 h. After washing with PBS, the sections were incubated with an avidin-biotin-peroxidase complex for 1 h from a Vector ABC *Elite* kit (Vector Laboratories Inc. Burlingame, CA, USA), and reacted with diaminobenzidine tetrahydrochloride (Sigma) solution with 0.03% hydrogen peroxidase. The sections were counterstained with hematoxylin, dehydrated in gradient ethyl alcohol series, and observed under a microscope.

All data are expressed as mean ± S.E.M. The results in each group were compared by two-way analysis of variance (ANOVA) followed by to *post-hoc* Scheffe's test. A $P < 0.05$ was considered to represent statistical significance.

Fig. 1 clearly shows confirmation the neuroprotective effects of ferulic acid against MCAO injury. MCAO injury induced brain infarction and ferulic acid treatment significantly decreased infarct regions (Fig. 1A). The infarct volume was 33.51 ± 3.90% and 15.66 ± 2.51% in vehicle- and ferulic acid-treated animals during MCAO, respectively (Fig. 1C). Moreover, there was a significant increase in the number of TUNEL-positive cells in the infarct region of vehicle-treated animals during MCAO, whereas ferulic acid treatment prevented a MCAO-induced increase in the number of TUNEL-positive cells (Fig. 1B). The proportions of TUNEL-positive cells were 78.1 ± 3.1% and 27.1 ± 3.5% in the cerebral cortex of vehicle- and ferulic acid-treated animals during MCAO, respectively (Fig. 1D).

Western blots showed that MCAO injury induced a reduction in phospho-Raf-1 and phospho-MEK1/2 levels while ferulic acid prevented injury-induced down-regulation of phospho-Raf-1 and phospho-MEK1/2 levels. The levels of phospho-Raf-1 were 0.41 ± 0.03 and 0.78 ± 0.02 in the cerebral cortex of vehicle- and ferulic acid-treated animals during MCAO, respectively (Fig. 2A). Moreover, the levels of phospho-MEK1/2 were 0.28 ± 0.02 and