Food and Chemical Toxicology 48 (2010) 1528–1536

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

Alaternin attenuates delayed neuronal cell death induced by transient cerebral hypoperfusion in mice

Bum Young Shin [a,d], Dong Hyun Kim [c,d], Sook Kyung Hyun [e], Hyun Ah Jung [e], Jong Min Kim [c,d], Se Jin Park [c,d], Seong Yun Kim [f], Jae Hoon Cheong [g], Jae Sue Choi [e], Jong Hoon Ryu [b,c,d,*]

[a] Department of Pharmaceutical Science, College of Pharmacy, Kyung Hee University, Hoeki-dong, Dongdaemoon-Ku, Seoul 130-701, Republic of Korea
[b] Department of Oriental Pharmaceutical Science, College of Pharmacy, Kyung Hee University, Hoeki-dong, Dongdaemoon-Ku, Seoul 130-701, Republic of Korea
[c] Department of Life and Nanopharmaceutical Sciences, College of Pharmacy, Kyung Hee University, Hoeki-dong, Dongdaemoon-Ku, Seoul 130-701, Republic of Korea
[d] Kyung Hee East-West Pharmaceutical Research Institute, College of Pharmacy, Kyung Hee University, Hoeki-dong, Dongdaemoon-Ku, Seoul 130-701, Republic of Korea
[e] Division of Food Science and Biotechnology, Pukyong National University, Busan 608-737, Republic of Korea
[f] Department of Pharmacology College of Medicine, The Catholic University of Korea, 505 Banpo-dong, Socho-gu, Seoul 137-701, Republic of Korea
[g] Department of Pharmacy, Sahmyook University, Nowon-goo, Seoul 139-742, Republic of Korea

ARTICLE INFO

Article history:
Received 8 September 2009
Accepted 15 March 2010

Keywords:
Alaternin
Transient cerebral hypoperfusion
Nitrotyrosine
Inducible nitric oxide synthase
Microglia
Memory

ABSTRACT

The aim of this study was to determine whether alaternin exhibits neuroprotective activity after transient cerebral hypoperfusion induced by bilateral common carotid artery occlusion (BCCAO). Mice were subjected to BCCAO, and circulation was restored after 20 min. Alaternin (10 mg/kg, p.o) treatment significantly prevented nitrotyrosine and lipid peroxidation, as well as BCCAO induced-inducible nitric oxide synthase (iNOS) expression. Alaternin also significantly reduced microglial activation (a marker of inflammation). The number of viable neurons detected by Nissl staining increased with alaternin (10 mg/kg, p.o) treatment at 7 days post-BCCAO. In the passive avoidance task, alaternin significantly ameliorated BCCAO-induced cognitive impairments ($P < 0.05$). These results suggest that the neuroprotective effects of alaternin are mediated by its anti-inflammatory and radical scavenging activities.

1. Introduction

Numerous studies have examined the role of nitric oxide (NO) and nitric oxide synthase (NOS) in cardiac and cerebral ischemia (Murphy and Gibson, 2007; Rodrigo et al., 2005). These studies indicate that NO levels (Lei et al., 1999; Willmot et al., 2005) and NOS activity (Yoshida et al., 1995) are both increased after ischemia in the brain. Current therapeutic approaches to treating stroke achieve limited success (Adams et al., 2005), thus, there is a need to identify new targets and develop new drugs. NO and superoxide (O_2^-) are highly cytotoxic at high concentrations and are increased during inflammation. Furthermore, these molecules are involved in the pathologies of many neurodegenerative conditions, such as ischemic stroke and infectious diseases, as well as disorders associated with aging of the brain (Brown and Borutaite, 2006). The rapid reaction of NO with superoxide (O_2^-) results in peroxynitrite ($ONOO^-$) formation, detectable by assaying tyrosine nitration. Peroxynitrite is believed to mediate NO-triggered cell death in animal models of ischemic stroke (Takizawa et al., 2002). Accordingly, neuroprotective agents with radical scavenging or anti-inflammatory activity are of interest as possible lead compounds for the development of new drugs that target ischemic damage.

Previously, we reported that the ethanolic extract of the seed of *Cassia obtusifolia* (COE) has an ameliorating effect on scopolamine-induced memory impairment (Kim et al., 2007) and a neuroprotective effect on cerebral hypoperfusion-induced neuronal cell death in mice (Kim et al., 2009). In addition, we also observed that memory impairment induced by transient cerebral hypoperfusion was attenuated by the administration of COE (Kim et al., 2007). Thereafter, we attempted to isolate active compound(s) from COE that exhibit memory ameliorating or neuroprotective effects. We obtained several distinct compounds from the COE extract including

Abbreviations: COE, the ethanolic extract of the seeds of *Cassia obtusifolia*; GFAP, glial fibrillary acidic protein; NO, nitric oxide; iNOS, inducible nitric oxide synthase; NOS, nitric oxide synthase; PBS, phosphate-buffered saline; BCCAO, bilateral common carotid artery occlusion; ABC, abidin-biotin-peroxidase complex; PVDF, polyvinylidene fluoride; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; PMSF, phenylmethylsulphonyl fluoride; TTBS, Tween 20/Tris-buffered saline; TBARS, thiobarbituric acid reactive substances; ANOVA, analysis of variance; ROS, reactive oxygen species.

* Corresponding author at: Department of Oriental Pharmaceutical Science, College of Pharmacy, Kyung Hee University, #1 Hoeki-dong, Dongdaemoon-Ku, Seoul 130-701, Republic of Korea. Tel.: +82 2 961 9230; fax: +82 2 966 3885.
E-mail address: jhryu63@khu.ac.kr (J.H. Ryu).

0278-6915/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fct.2010.03.020

alaternin. Park et al. (2004) reported that alaternin inhibits peroxynitrite-mediated nitration, and suggested that alaternin is a peroxynitrite scavenging agent and/or antioxidant (Park et al., 2004). Thus, we reasoned that alaternin attenuates neuronal cell death via peroxynitrite or other radicals, and that this prevention of cell death is responsible for its amelioration of memory dysfunction induced by cerebral hypoperfusion in mice.

The purpose of this study was to determine whether alaternin has neuroprotective activity in transient cerebral hypoperfusion-induced delayed neuronal cell death in mice. Cognitive dysfunctions resulting from delayed neuronal cell death and the functional neuroprotective effects of alaternin were evaluated using the passive avoidance task. In addition, morphological changes in a transient cerebrally hypoperfused mouse hippocampus were investigated with or without alaternin treatment using histochemical, immunohistochemical, and western blot methods.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice (23–25 g, 8 weeks) were purchased from the Orient Co., Ltd., a branch of Charles River Laboratories (Seoul). Animals were housed five per cage and allowed access to water and food *ad libitum*. The cages were maintained at a constant temperature (23 ± 1 °C) and relative humidity (60 ± 10%) under a 12 h light/ dark cycle (lights on from 07:30 to 19:30). Animal treatment and maintenance conformed to the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and the Animal Care and Use Guidelines issued by Kyung Hee University, Korea.

2.2. Materials

Alaternin was donated by one of the authors (J.S. Choi), and was suspended in a 10% Tween 80 solution. Dizoclipine (MK-801) was purchased from Sigma Chemical Co. (St. Louis, MO), and rat anti-mouse CD11b (OX-42) from Serotec Ltd. (Oxford, UK). Rabbit anti-inducible NOS (iNOS), rabbit anti-β-actin, and rabbit anti-nitrotyrosine antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). Cy3-conjugated donkey anti-rat antibody and FITC-conjugated donkey anti-mouse antibody were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Abidin-biotin-peroxidase complex (ABC) kits were purchased from Vector Laboratories (Burlingame, CA). All other materials were of the highest grade available and were obtained from normal commercial sources.

2.3. Surgery, measurement of rCBF and drug administration

C57BL/6 mice were anesthetized with 2.0% isoflurane and 70% nitrous oxide in oxygen. Mice were subjected to transient cerebral hypoperfusion, as previously described (Kim et al., 2007). Transient cerebral hypoperfusion was induced by bilateral common carotid artery occlusion (BCCAO) with aneurysm clips for 20 min, and circulation was restored by removing the clips. Mice that received the same surgical operation without carotid artery clipping served as sham-operated controls. During the surgical procedure, rectal temperature was maintained at 36 ± 0.5 °C with heating pad (Biomed S.L., Spain). Regional cerebral blood flow (rCBF) was monitored using laser Doppler flowmetry (LDF; Perimed, PF5010, JarFalla, Sweden) from the time of anesthetic induction to 10 min after reperfusion. Cyanoacrylate adhesives were used to attach a flexible probe (model 407, Perimed, Jarfalla, Sweden) to the intact skull 3.5 mm to the right of the bregma. The change in rCBF was measured for 1 min immediately after BCCAO and expressed as a percentage of the baseline value (Cho et al., 2007). After reperfusion, the animals were placed in a warm incubator (32–33 °C). There was no significant difference between vehicle-treated control group and alaternin-treated group (Fig. 1).

Mice were treated orally (p.o) with 1 or 10 mg/kg of alaternin immediately after reperfusion. The sham-operated vehicle treatment group and the untreated BCCAO-control group were administered 10% Tween 80 solution of the same volume at the same time. MK-801 (3 mg/kg) as a positive control was administered intraperitoneally (i.p.).

2.4. Tissue preparation

Mice were anesthetized with pentobarbital sodium (60 mg/kg, i.p.) at 1 day (for nitrotyrosine), 4 days (for iNOS and OX-42), or 7 days (for Nissl staining) after BCCAO and perfused transcardially with 0.1 M phosphate buffer (pH 7.4) followed by ice-cold 4% paraformaldehyde. We observed that the immunoreactivities of nitrotyrosine, OX-42 and GFAP, and cell death peaked at 1, 4, and 7 days after BCCAO, respectively. Brains were removed and postfixed in phosphate buffer (50 mM, pH 7.4) containing 4% paraformaldehyde overnight and then immersed in 30% sucrose solution (in 50 mM phosphate-buffered saline, PBS), and stored at 4 °C until sectioned. Frozen brains were coronally sectioned into 30 μm slices on a cryostat and then stored in storage solution at 4 °C.

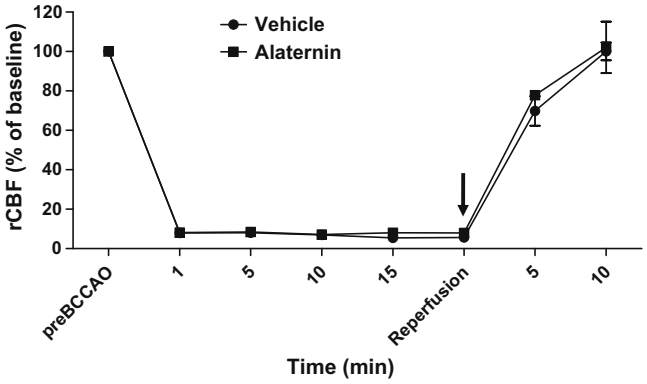

Fig. 1. Cortical microperfusion of vehicle and alaternin-treated mice subjected to transient bilateral common carotid artery occlusion (BCCAO). No significant difference in regional cerebral blood flow (rCBF) was found between the vehicle and alaternin-treated group. Arrow indicates vehicle or alaternin (10 mg/kg, p.o) administration time. Values are mean ± SEM (n = 4/group).

2.5. Cresyl violet staining

After mounting sections onto gelatin-coated slides, they were stained with 0.5% cresyl violet, dehydrated through graded alcohols (70%, 80%, 90%, and 100% × 2 times), placed in xylene, and coverslipped using Histomount medium.

2.6. Immunohistochemistry and double immunofluorescence labeling

Immunohistochemistry was conducted, as previously described (Kim et al., 2007). Free floating sections were incubated for 24 h in PBS (4 °C) containing a rabbit anti-nitrotyrosine antibody (1:500 dilution), a rabbit anti-iNOS antibody (1:200 dilution), or a rat anti-CD11b antibody (OX-42, 1:500 dilution) and 0.3% Triton X-100, 0.5 mg/ml bovine serum albumin, and 1.5% rabbit serum (for CD11b) or goat serum (for nitrotyrosine and iNOS) from ABC kit. Sections were then incubated for 90 min with a biotinylated secondary antibody (1:200 dilution), treated with ABC solution (1:100 dilution) for 1 h at room temperature, and reacted with 0.02% 3,3′-diaminobenzidine and 0.01% H_2O_2 for about 3 min. After each incubation step, sections were washed 3 times with PBS for 5 min. Finally, sections were mounted on gelatin-coated slides, dehydrated in an ascending alcohol series, and cleared in xylene.

For double immunofluorescence labeling, sections obtained from vehicle-treated control group at 4 days after BCCAO were blocked in 20% normal donkey serum in PBS, followed by an overnight incubation with rat anti-OX-42 antibody (1:1000) and rabbit anti-iNOS antibody (1:200) in 1% BSA diluted in PBS. After several washes, sections were incubated with secondary antibodies conjugated with Cy3 or FITC for 1 h at room temperature, then mounted on glass slides with Vecta-shield mounting medium. Image analysis was conducted by confocal microscopy (Zeiss Axiovert LSM 510 META, Germany).

2.7. Western blot analysis

To investigate the effect of alaternin on nitrotyrosine formation and iNOS expression, isolated hippocampal tissues were homogenized in ice-chilled Tris–HCl buffer (20 mM, pH 7.4) containing 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 μg/ml aprotinin, 15 μg/ml leupeptin, 10 μg/ml bacitracin, 10 μg/ml pepstatin, 15 μg/ml trypsin inhibitor, 50 mM NaF, and 1 mM sodium orthovanadate. Samples of homogenates (15 μg of protein) were then subjected to SDS–PAGE (6% gel for iNOS or 8% gel for nitrotyrosine gels) under reducing conditions. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes in transfer buffer [25 mM Tris–HCl buffer (pH 7.4) containing 192 mM glycine and 20% v/v methanol] and further separated at 400 mA for 2 h at 4 °C to determine nitrotyrosine or iNOS levels. Western blots were incubated overnight in blocking solution (5% skimmed milk) at 4 °C, followed by a 4 h incubation in 1:1000 dilution of anti-iNOS or anti-nitrotyrosine antibodies, ten washes with Tween 20/Tris-buffered saline (TTBS), a 1 h incubation in a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody at room temperature, and twelve washes with TTBS. Western blots were then developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL). Blots were then stripped and incubated with a