Neurobiology of Aging 36 (2015) 993–1006

Contents lists available at ScienceDirect

Neurobiology of Aging

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

# Estrogen receptor-mediated resveratrol actions on blood-brain barrier of ovariectomized mice

Jin A. Shin [a], Seikwan Oh [b], Jung-Hyuck Ahn [c], Eun-Mi Park [a,*]

[a] Department of Pharmacology, Tissue Injury Defense Research Center, School of Medicine, Ewha Womans University, Seoul, Republic of Korea
[b] Department of Neuroscience, Tissue Injury Defense Research Center, School of Medicine, Ewha Womans University, Seoul, Republic of Korea
[c] Department of Biochemistry, Tissue Injury Defense Research Center, School of Medicine, Ewha Womans University, Seoul, Republic of Korea

ARTICLE INFO

*Article history:*
Received 10 April 2014
Received in revised form 5 September 2014
Accepted 25 September 2014
Available online 13 October 2014

*Keywords:*
Blood-brain barrier permeability
Estrogen receptors
Ischemic stroke
Ovariectomy
Resveratrol
Tight junction protein

ABSTRACT

To test whether resveratrol provides benefits via estrogen receptors (ERs) in the blood-brain barrier of estrogen-deficient females, ovariectomized mice were treated with resveratrol then were subjected to transient middle cerebral artery occlusion (MCAO). Compared with vehicle treatment, resveratrol reduced infarct volume and neurologic deficits after MCAO. Basal tight junction (TJ) protein levels in the brain were increased by resveratrol. After MCAO, blood-brain barrier breakdown reduced levels of TJ proteins, and induction of HIF-1α and VEGF were attenuated by resveratrol. These effects were reversed by the ERs antagonist, ICI182,780. In mouse brain, endothelial cells (bEnd.3) exposed to hypoxia, resveratrol treatment protected the cells against cytotoxicity, increases of paracellular permeability and changes in levels of TJ protein and HIF-1α/VEGF proteins. These effects were reversed by ICI182,780 but not by specific ERα or ERβ antagonists, indicating nonspecific ER mediated effects. Altogether, these results showed that neuroprotective effects of resveratrol in ovariectomized mice were mediated by ERs and associated with tightening of blood-brain barrier, suggesting that resveratrol can be an alternative to estrogens to protect the brains of estrogen-deficient females against ischemic insult.


## 1. Introduction

Epidemiologic studies have shown that the incidence of stroke is lower in women during their reproductive years, but after menopause, stroke occurs more often in women than men of comparable age (Haast et al., 2012). These studies imply that endogenous estrogen is responsible for the resistance of female brains to ischemic stroke. From early observational studies of hormone replacement therapy and several rodent models of ischemic stroke, it was suggested that exogenous estrogen administration provides neuroprotection in postmenopausal women (Behl, 2002). However, there are concerns about exogenous estrogen use because the Women's Health Initiative reported increased risks of coronary heart disease and ischemic stroke in women with hormone replacement therapy (Rossouw et al., 2002). As the postmenopausal period for women has come to be longer because of increased longevity, the risk of degenerative brain diseases is becoming an important issue for estrogen-deficient aged women. The discovery of safe estrogen substitutes is crucial for preventing or ameliorating brain diseases in postmenopausal women.

Resveratrol, a polyphenolic phytoestrogen found in grapes and wine, has protective effects against neurodegenerative diseases and ischemic insults in animal models (Sun et al., 2010). As resveratrol binds to estrogen receptors (ERs) and activate gene transcription, ERs mediate some of resveratrol's actions in the brain (Di Liberto et al., 2012; Saleh et al., 2010; Singh et al., 2007). Therefore, we postulate that resveratrol could be a potential alternative to estrogen for prevention of brain damage against ischemic stroke in estrogen-deficient females.

Blood-brain barrier (BBB) maintenance is important for brain homeostasis and function; thus, dysregulated BBB permeability is implicated in many brain diseases (Stanimirovic and Satoh, 2000). Transmembrane tight junction (TJ) proteins are components of BBB and regulate paracellular permeability between adjacent endothelial cells (Coisne and Engelhardt, 2011). Because reduced TJ protein levels results in BBB breakdown and brain edema (Jiao et al., 2011; Yang et al., 2007), their changes in protein levels are associated with the development and progression of brain diseases. In addition, age-dependent BBB dysfunctions and alteration of TJ integrity in the absence of disease have been demonstrated (Bake et al., 2009; Farrall and Wardlaw, 2009; Hafezi-Moghadam et al., 2007), implying that

* Corresponding author at: Department of Pharmacology, Tissue Injury Defense Research Center, School of Medicine, Ewha Womans University, 1071 Anyangcheon-ro, Yangcheon-gu, Seoul, 158-710, Republic of Korea. Tel.: +82 2 2650 5743; fax: +82 2 2653 8891.
*E-mail address:* empark@ewha.ac.kr (E.-M. Park).

0197-4580/$ – see front matter © 2015 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.neurobiolaging.2014.09.024

aged brains are susceptible to develop brain diseases. Recently, it has been reported that resveratrol protects brain endothelial cells against oxidized low-density lipoprotein (Lin et al., 2010), recurrent ischemic stroke (Clark et al., 2012), and mutations in the palmitoyl-protein thioesterase-1 (*Ppt1*) gene (Saha et al., 2012), and its effects are associated with prevention of reduction of TJ protein levels induced by such insults (Lin et al., 2010; Saha et al., 2012). These results suggest that resveratrol provides BBB protection via modulation of TJ protein level against noxious stimuli.

The purpose of the present study was to investigate the effects of resveratrol on ischemic brain injury in female ovariectomized (OVX) mice, a postmenopausal model, and whether its effects are mediated by ERs. Then, to characterize the effects of resveratrol with respect to BBB permeability, changes in TJ protein levels were evaluated in the brain of OVX mice. In addition, we examined the effects of resveratrol on in vitro brain endothelial cells before and after insults.

## 2. Methods

### 2.1. Experimental animals

Female C57BL/6 mice, aged 10–11 weeks, were used (Orient Bio Inc, Seongnam, Republic of Korea). All procedures were approved by the Institutional Animal Care and Use Committee at the Medical School of Ewha Womans University and conformed to international guidelines for the ethical use of experimental animals. The number of animals used was minimized to reduce animal suffering.

### 2.2. Ovariectomy and drug administration

Two weeks before middle cerebral artery occlusion (MCAO), female mice were subjected to aseptic bilateral surgical ovariectomy via a dorsal incision under anesthesia. In the first part, OVX mice were divided randomly into vehicle and resveratrol treatment groups. The dose of resveratrol (Sigma-Aldrich, St. Louis, MO, USA) was determined as 0.1 mg/kg to minimize systemic effects of ethanol in the vehicle because of its poor solubility in water. The final concentration of ethanol in distilled water (0.1 mL) was 0.2% (vol/vol). Vehicle or resveratrol was administered orally by gavage for 10 days starting 7 days before MCAO. In the second part of the experiments, resveratrol-treated OVX mice were divided randomly into sesame oil (0.1 mL per mouse) and the pure nuclear ER antagonist ICI182,780 (100 μg in 0.1 mL of sesame oil; Tocris Biosciences, Bristol, UK) treatment groups. Sesame oil or ICI182,780 was given to resveratrol-treated mice subcutaneously for 10 days starting 7 days before MCAO. The dose of ICI182,780 was based on the previous study (Shin et al., 2011). To measure estrogen activity, plasma estradiol levels were measured with an ELISA assay kit (Cayman Chemical, Ann Arbor, MI, USA), and uterine weight was assessed as a biological assay of peripheral ER activation.

### 2.3. Transient MCAO

Procedures for transient MCAO and cerebral blood flow (CBF) monitoring were previously described (Shin et al., 2010). After 30 minutes MCAO, only animals that exhibited a greater than 85% reduction in CBF during MCAO that recovered by more than 80% after 10 minutes of reperfusion were included. Rectal temperature was maintained at 37.0 °C ± 0.5 °C during surgery and recovery until mice regained consciousness. Control animals did not undergo surgery.

### 2.4. Behavioral tests

Neurologic scores and performance in the wire suspension test were examined before and 3 days after MCAO. A 4-point graded scoring system for neurologic scores and a 5-point graded scoring system for the wire suspension test were used (Shin et al., 2013a).

### 2.5. Measurements of infarct volume

Three days after MCAO, brains of mice were removed, frozen, and sectioned (30 μm thick) with a cryostat. Serially collected brain sections (600-μm intervals) were stained with cresyl violet. Infarction volume was determined using an image analyzer (Axiovision LE 4.1, Carl Zeiss, Jena, Germany). Values were reported after correction for postischemic swelling as previously described (Shin et al., 2010).

### 2.6. IgG extravasation and Western blot analysis

To measure BBB permeability, IgG extravasation was examined in the ipsilateral cortex 6 hours after MCAO as described previously (Shin et al., 2013b). Immobilon-P membranes (Millipore Corporation, Billerica, MA, USA) loaded with 50 μg of protein were incubated overnight with primary antibody against biotinylated anti-mouse IgG (1:250; Vector Laboratories, Burlingame, CA, USA), and then incubated with an avidin peroxidase kit (Vectastain Elite; Vector Laboratories) for 1 hour. For quantification, total chain densities (heavy chain + light chain) of each protein were normalized to the density of actin (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) bands on corresponding blots using the Image J 1.37v program (National Institutes of Health, Bethesda, MD, USA).

Cortical tissues from each hemisphere and cultured brain endothelial cells were used for Western blotting. Protein was isolated from cells and tissues, and 80 μg of protein for brain tissue and 50 μg for bEnd.3 cells were loaded, electrophresed, and transferred to Immobilon-P membranes as previously described (Shin et al., 2013b). Membranes were incubated overnight with antibodies against occludin (1:1000; Invitrogen Corporation, Carlsbad, CA, USA), claudin-5 (1:1000; Invitrogen Corporation), VEGF (1:500; Santa Cruz Biotechnology), HIF-1α (1:500; BD Biosciences, San Jose, CA, USA), ERα (1:500), ERβ (1:500; Santa Cruz Biotechnology) or actin (1:1000). After 1 hour incubation with horseradish peroxidase-conjugated secondary antibodies, protein bands were visualized with Western Blotting Luminol Reagent (Santa Cruz Biotechnology). For quantification, densities of each band were normalized to the density of actin using the Image J 1.37v program.

### 2.7. Cell culture and treatments

Immortalized mouse brain endothelial cells (bEnd.3) were purchased from ATCC (Manassas, VA, USA). Cells were cultured on 60 mm dishes (for Western blotting), 24-well plates (for cytotoxicity assay and immunofluorescence staining), 96-well plates (for cell viability assay), Transwell-membrane inserts (for paracelluar permeability) or Millicell cell culture inserts in 12-well plates (for TEER assay, Millipore Corporation) at a seeding density of $5.0 \times 10^4$–$5.0 \times 10^5$ cells per well. Cells were maintained in Dulbeccos Modified Eagle Medium (ATCC) at 37 °C in a humidified incubator under 5% $CO_2$ and 95% air. Cobalt chloride ($CoCl_2$, 50–200 μM; Wako Pure Chemical Industries, Japan) dissolved in phosphate-buffered saline and resveratrol (10–1000 nM) in ethanol and ICI182,780 (0.1–10 μM), 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole (MPP, 1 μM; Tocris Biosciences) and 4-[2-phenyl-5,7-bis(trifluoromethyl) pyrazolo[1.5-a]pyrimidine-3-yl]phenol (PHTPP, 1 μM; Tocris Biosciences) in dimethyl sulfoxide (DMSO) were added to cultures. For cotreatment, resveratrol was added to cells 48 hours before $CoCl_2$ exposure, and ICI182,780, MPP, or PHTPP was added 1 hour before