European Journal of Pharmacology 615 (2009) 150–154

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Cardiovascular Pharmacology

Rosmarinic acid suppresses retinal neovascularization *via* cell cycle arrest with increase of p21^{WAF1} expression

Jeong Hun Kim [a,1], Byung Joo Lee [a,1], Jin Hyoung Kim [a], Young Suk Yu [a,*], Min Young Kim [b], Kyu-Won Kim [c]

[a] *Department of Ophthalmology, College of Medicine, Seoul National University & Seoul Artificial Eye Center Clinical Research Institute, Seoul National University Hospital, Seoul 110-744, Republic of Korea*
[b] *AngioLab, Inc. Bio-Diagnostic Fusion Center, Dae-Jeon 304-340, Republic of Korea*
[c] *NeuroVascular Coordination Research Center, College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul 151-742, Republic of Korea*

ARTICLE INFO

Article history:
Received 28 October 2008
Received in revised form 18 May 2009
Accepted 19 May 2009
Available online 24 May 2009

Keywords:
Anti-angiogenesis
Cell cycle arrest
p21^{WAF1}
Retinal neovascularization
Retinopathy of prematurity
Rosmarinic acid

ABSTRACT

Pathological angiogenesis is the most common cause of blindness at all ages including retinopathy of prematurity, diabetic retinopathy, and age-related macular degeneration. Despite advances in therapy, retinopathy of prematurity remains the most sight-threatening vaso-proliferative retinopathy in children. Herein, we demonstrated that rosmarinic acid has an anti-angiogenic activity to retinal neovascularization in a mouse model of retinopathy of prematurity, which is related to cell cycle arrest with increase of p21^{WAF1}. Rosmarinic acid significantly inhibited the proliferation of retinal endothelial cells in a dose-dependent manner, and inhibited *in vitro* angiogenesis of tube formation. Interestingly, the anti-proliferative activity of rosmarinic acid on retinal endothelial cells was related to G_2/M phase cell cycle arrest in a dose-dependent manner. With treatment of rosmarinic acid, retinal endothelial cells in G_2/M phase increased whereas those in G_0/G_1 and S phases decreased, which was accompanied by increase of p21^{WAF1} expression in a dose-dependent manner. Moreover, rosmarinic acid effectively suppressed retinal neovascularization in a mouse model of retinopathy of prematurity, and showed no retinal toxicity. These data suggest rosmarinic acid could be a potent inhibitor of retinal neovascularization and may be applied in the treatment of other vasoproliferative retinopathies.

1. Introduction

Physiological angiogenesis is tightly regulated by balancing between several pro-angiogenic and anti-angiogenic factors (Folkman, 2006). This normally occurs only during development and tissue repair; however, pathological angiogenesis exists, particularly within the eye. This is the most common cause of blindness at all ages including retinopathy of prematurity in children, diabetic retinopathy in young adults, and age-related macular degeneration in the elderly (Aiello et al., 1994). Retinopathy of prematurity is a leading cause of blindness in premature infant (Roth, 1977). Retinopathy of prematurity occurs through vaso-obliteration followed by the pathologic angiogenesis in developing retinal vasculature (Chen and Smith, 2007). Oxygen-induced retinopathy in the mouse is a highly reproducible animal model of retinopathy of prematurity, which is based on hyperoxia-induced vaso-obliteration of capillaries in mouse pups and their subsequent return to room air (Smith et al., 1994). This triggers retinal angiogenesis starting from the inner retina, characterized by growing into the vitreous. Therefore, the amelioration of pathological angiogenesis in retinopathy of prematurity is targeted for either blocking the vessel loss in order to control hypoxic condition or directly inhibiting vessel proliferation (Smith et al., 1994; Chen and Smith, 2007).

Rosmarinic acid is a water-soluble polyphenolic compound which is found in various plants including Lamiaceae species (Petersen and Simmonds, 2003). In addition to anti-oxidative (Frankel et al., 1996), anti-inflammatory (Youn et al., 2003), and anti-proliferative activity (Makino et al., 2000), rosmarinic acid was recently reported to have anti-angiogenic activity (Huang and Zheng, 2006). Although rosmarinic acid inhibited all angiogenic processes including proliferation, migration and tube formation of endothelial cells, which is mediated by suppression of H_2O_2-induced vascular endothelial growth factor (VEGF) expression and IL-8 release (Huang and Zheng, 2006), other molecular mechanisms responsible for anti-angiogenic activity remain to be delineated. Based on that rosmarinic acid could involve in cell cycle arrest in the G_0/G_1 and G_1/S phases (Makino et al., 2000), this anti-proliferative effect of rosmarinic acid suggests that proliferative vascular diseases including tumor, arthritis as well as retinopathy might be targets for the pharmacological application of rosmarinic acid. Especially, the effects of rosmarinic acid on retinal neovascularization have not been previously addressed.

In the present study, we demonstrated that rosmarinic acid inhibits proliferation of retinal endothelial cells and *in vitro* angiogenesis of tube formation. Moreover, retinal neovascularization in a mouse model of retinopathy of prematurity was significantly suppressed by rosmarinic

* Corresponding author. Tel.: +82 2 2072 3492; fax: +82 2 741 3187.
E-mail address: ysyu@snu.ac.kr (Y.S. Yu).
[1] These authors contributed equally to this work.

0014-2999/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2009.05.015

acid without retinal toxicity. And we also addressed that the anti-proliferative effect of rosmarinic acid is closely related to G_2/M phase cell cycle arrest which is accompanied by increase of $p21^{WAF1}$ expression.

2. Materials and methods

2.1. Animals

C57BL/6 mice were purchased from Samtako (Seoul, Korea). Care, use, and treatment of all animals in this study were in strict agreement with the association for research in vision and ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Cell culture

Human retina microvascular endothelial cells (HRMECs) were purchased from the Applied Cell Biology Research Institute and grown on attachment factor-coated plates in complete medium (Cell Systems, Kirkland, WA, USA) or in M199 medium supplemented with 20% fetal bovine serum, 3 ng/ml basic fibroblast growth factor (bFGF, Millipore, Bedford, MA, USA), and 10 U/ml heparin (Sigma, St. Louis, MO, USA). HRMECs used in this study were taken from passages 4 to 6.

2.3. Cell proliferation assay on retinal endothelial cells

Cell proliferation was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as in our previous description (Kim et al., 2008a). HRMECs (1×10^5 cells) were plated in 96 well plates and cultured overnight. Cells were treated with VEGF (20 ng/ml) (Sigma, St. Louis, MO, USA) or rosmarinic acid (10–100 μM) (Sigma, St. Louis, MO, USA) for 24 h. The medium was then replaced with fresh medium containing 0.5 mg/mL MTT for 4 h. After incubation, the medium was carefully removed from the plate and DMSO was added to solubilize formazan produced from MTT by the viable cells. Absorbance was measured at 540 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.4. Tube formation assay on retinal endothelial cells

Tube formation was assayed as in our previous description (Min et al., 2007). HRMECs (1×10^5 cells) were inoculated on the surface of the Matrigel, and treated with 50 μM rosmarinic acid or 20 ng/ml VEGF for 18 h. The morphological changes of the cells and tubes formed were observed under a microscope and photographed at a ×200 magnification. Tube formation was quantified by counting the number of connected cells in randomly selected fields at a ×200 magnification (Carl Zeiss, Chester, VA, USA), and dividing that number by the total number of cells in the same field.

2.5. Cell cycle analysis by flow cytometry

As in our previous description (Kim et al., 2007), HRMECs were seeded in 60 mm dish (6×10^5 cells) and incubated for 24 h. For synchronization of HRMECs, the cells were starved for 12 h with 0.5% serum EGM-2. The cells were treated with or without rosmarinic acid (10 or 100 μM) for 24 h in the presence of 10% serum. Then, the cells were harvested and fixed in 70% ethanol. Before analysis, cells were washed with phosphate balanced solution (PBS) and resuspended in PBS (pH 7.4). RNase (80 μg/ml) and propidium iodide (50 μg/ml) were treated to suspended cells for 1 h. The DNA histograms were determined using a Beckton-Dickenson FACSVantage flow cytometer system (Beckton-Dickenson, San Jose, CA, USA) and the cell cycle distribution was analyzed using a Cell Quest software version 3.2 (Beckton-Dickenson, San Jose, CA, USA).

2.6. Western blot analysis

HRMECs were seeded in 100 mm dish (5×10^5 cells), and rosmarinic acid (10 or 100 μM) was treated for 24 h. The cell lysates were separated by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by transfer to polyvinylidene fluoride membranes (Milipore, Bedford, MA, USA) using standard electroblotting procedures. Blots were then blocked and immunolabeled overnight at 4 °C with primary antibodies, including anti-$p21^{WAF1}$ (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-ß-actin (Upstate Biotechnology, Lake Placid, NY, USA) antibodies. Immunolabeling was detected by an enhanced chemiluminescence kit (Amersham Life Science, Inc. Buckinghamshire, UK) according to the manufacturer's instructions.

2.7. Oxygen-induced retinopathy

Oxygen-induced retinopathy was induced as described by Smith et al. (1994) with some modifications (Kim et al., 2007; Kim et al., 2008b). Briefly, newborn mice were randomly assigned to experimental and control groups. At postnatal day (P) 7, pups (5 to 7 pups) in the experimental group were exposed to hyperoxia (75%±0.5% O_2) for 5 days (P7 to P11) and then returned to normoxia (room air) for 5 days. Neovascularization occurs upon return to normoxia and peaks at P17. To assess the anti-angiogenic activity of rosmarinic acid, the pups were injected intravitreously with 50 μM rosmarinic acid in 1 μl PBS on P14, when retinal neovascularization began. There were at least 10 animals in each group.

2.8. Qualitative assessment of retinal neovascularization by fluorescein angiography

As in our previous description (Kim et al., 2008b), at P17, deeply anesthetized mice were perfused through the tail vein with fluorescein conjugated dextran (molecular weight = 500,000; Sigma-Aldrich Ltd., St. Louis, MO, USA) dissolved in PBS. After 1 h perfusion, the eyes were enucleated and fixed in 4% paraformaldehyde for 2 h. The retinas were dissected, flat-mounted in Dako mounting medium (DakoCytomation, Glostrup, Denmark), and viewed by fluorescein microscopy (BX50, OLYMPUS, Japan) at a magnification of 4×.

2.9. Quantitative assessment of retinal neovascularization by counting vascular lumens

As in our previous description (Kim et al., 2008b), at P17, the eyes were removed, fixed in 4% paraformaldehyde for 24 h, and embedded in paraffin. Sagittal sections of 5 μm, each 30 μm apart, were cut through the cornea parallel to the optic nerve. The sections were stained with hematoxylin and eosin to assess retinal vasculature via light microscopy (Carl Zeiss, Chester, VA, USA). Any vascular lumens on the vitreal side of the inner limiting membrane were counted in at least 10 sections from each eye by two independent observers blind to the treatment (Jeo H Kim and BJ Lee). The average intravitreal vessels/section was calculated for each group.

2.10. Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay

Rosmarinic acid was intravitreously injected to 8-week-old female C57BL/6J mice. The mice were sacrificed at 3 days after intravitreal injection of 250 μM, five times over the effective therapeutic concentration, and enucleated. Enucleated globes were fixed in 4% paraformaldehyde for 24 h, and embedded in paraffin. TUNEL staining was performed with a kit (ApopTag Fluorescein Green; Intergen, Purchase, NY, USA), according to the manufacturer's instructions. TUNEL-positive cells were evaluated in randomly selected fields at a ×400 magnification viewed under fluorescein microscopy (BX50, OLYMPUS, Japan).