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Antiangiogenic effect of betaine on pathologic retinal neovascularization via suppression of reactive oxygen species mediated vascular endothelial growth factor signaling

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ABSTRACT

Reactive oxygen species (ROS) as well as vascular endothelial growth factor (VEGF) play important roles in pathologic retinal neovascularization. We investigated whether betaine inhibits pathologic retinal neovascularization in a mouse model of oxygen induced retinopathy (OIR). Betaine was intravitreally injected in OIR mice at postnatal day (P) 14. At P17, the neovascular tufts area in OIR retina was analyzed. Intravitreal injection of betaine (200 μM) effectively reduced the neovascular tufts area in OIR retina (68.0 ± 6.7% of the control eyes, $P < 0.05$). Even in a high concentration (2 mM), betaine never induced any retinal toxicity or cytotoxicity. Betaine significantly inhibited VEGF-induced proliferation, migration, and tube formation in human retinal microvascular endothelial cells (HRMECs). Betaine suppressed VEGF-induced VEGFR-2, Akt and ERK phosphorylation in HRMECs. In human brain astrocytes, betaine reduced tBH-induced ROS production, and subsequently attenuated tBH-induced *VEGFA* mRNA transcription via suppression of ROS. Our data suggest that betaine has an anti-angiogenic effect on pathologic retinal neovascularization via suppression of ROS mediated VEGF signaling. Betaine could be a potent anti-angiogenic agent to treat pathologic retinal neovascularization.

1. Introduction

Pathologic retinal or choroidal neovascularization cause an angiogenesis-related vision impairment in retinopathy of prematurity (ROP), diabetic retinopathy (DR), and age-related macular degeneration, which are the most common cause of blindness in each age group [1,2]. Since its discovery [3], vascular endothelial growth factor (VEGF) has been known to play a critical role in angiogenesis mainly via vascular endothelial growth factor receptor-2 (VEGFR-2) signaling pathway [4]. Despite current successful treatment outcome of pathologic neovascularization with therapeutic agents which directly effect on VEGF [5–7], it is also required to develop new drugs which could regulate either upstream pathway of VEGF production or downstream VEGF signaling pathway [8].

Reactive oxygen species (ROS) as well as VEGF play an important role in angiogenesis [9,10]. Especially, ROS functions as a key signaling molecule in cell migration, proliferation, and gene expression [11,12]. In addition, ROS derived from nicotinamide adenine dinucleotide phosphate oxidase plays an important role in angiogenic process via VEGFR-2 signaling in endothelial cells [9,13]. In ROP, pathologic neovascularization occurs in developing retina when excessive oxygen supply generates ROS after vaso-obliteration [14,15]. In this oxidative stress condition, production of ROS increased with correlation of VEGF expression and angiogenesis [16]. Therefore, ROS modulation could be a

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potential target to intervene retinal neovascularization. We hypothesized that blocking ROS production during oxidative stress could regulate neovascularization via both VEGF transcription and VEGFR-2 downstream signaling.

Betaine (trimethylglycine) is an alkaloid and a component of *Fructus lycii* which is known to improve visual acuity [17,18]. The primary action of betaine is well known to be an osmolyte and a methyl donor with a betaine-homocysteine methyltransferase [19]. However, the effect of betaine on retinal neovascularization has remained to be elucidated. During our efforts on developing anti-angiogenic drugs [20–22], we recently reported that luteolin, a natural flavonoid, has an anti-angiogenic effect via blockade of ROS production [23]. Based on the anti-oxidative activity of betaine [24,25], we hypothesized that betaine could inhibit pathologic neovascularization via its anti-oxidant activity.

In the present study, we demonstrated that betaine effectively inhibited not only retinal neovascularization in OIR without toxicity but also in vitro angiogenesis in human retinal microvascular endothelial cells (HRMECs). With regard to the molecular mechanisms, we also demonstrated that betaine attenuated ROS production and subsequently suppressed both VEGFR-2 signaling pathway and VEGF production. Taken together, our results suggest that betaine could be a potential anti-angiogenic agent to treat pathologic retinal neovascularization in vasoproliferative retinopathies such as ROP.

2. Materials and methods

2.1. Animals

C57BL/6J mice, purchased from Central Lab. Animal (Seoul, Korea), were maintained under a standard 12-h dark-light cycle. The care, use, and treatment of all animals in this study were in strict agreement with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and the guideline established by the Seoul National University Institutional Animal Care and Use Committee.

2.2. OIR

OIR was induced in new-born mice as previously described [22,26]. Briefly, newborn mice were placed in hyperoxia (75 ± 0.5% O_2) from postnatal day (P) 7 to P12 and return to normoxia (21% O_2). Mice were randomly assigned to experimental groups and were deeply anesthetized using a mixture of Zoletil 50® (Virbac, Carros, France) and Rompun® (Bayer Korea, Seoul, Korea) (3:1 ratio, 1 ml/kg, i.p.). At P14, we intravitreally injected 1 μl of PBS or betaine (200 μM) into the right eyes of the mice ($n = 7$, respectively) using a Nanofil syringe with a 33G blunt needle (World Precision Instruments Inc., Worcester, MA) under an operating microscope (Leica Microsystems Ltd., Wetzlar, Germany) [22,27,28]. At P17, the enucleated eyes were processed for further analyses. For qualitative analysis, FITC-dextran (500 kDa, Sigma-Aldrich Ltd., St. Louis, MO, USA) was intracardially perfused 1 h before the sacrifice. The retinal flat mounts were observed with a fluorescence microscope (Eclipse 90i; Nikon, Tokyo, Japan). For quantitative analysis, the retinas were immunostained with Alexa Fluor® 594 isolectin GS-IB4 conjugate (5 μg/ml; Molecular Probes, Eugene, OR, USA). Then, the neovascular tufts area was marked with yellow and quantified with image processing software (NIS-Elements AR, v.3.2; Nikon, Tokyo, Japan) in a masked manner as previously described [27].

2.3. Histologic evaluation

To evaluate the retinal toxicity of betaine, we intravitreally injected 1 μl of 2 mM betaine or PBS to 6-week-old male C57BL/6J mice ($n = 6$, respectively). One week after the injection, the enucleated eyes were fixed in 4% paraformaldehyde and embedded in paraffin. Then, cross sections of eyes were processed with hematoxylin & eosin (H&E) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) stains. For the evaluation of changes in the retinal layers, the ratio of the retinal thickness from the internal limiting membrane (ILM) to the inner nuclear layer (INL, "A") to the retina thickness from the ILM to the outer nuclear layer (ONL, "B") was measured and compared with that of control mice, as previously described [29]. TUNEL staining was performed with an in situ cell death detection kit (Roche Diagnostics Corporation, Indianapolis, IN, USA) according to the manufacturer's instructions. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). TUNEL-positive cells were counted on five randomly selected fields in each slide (×200) under a fluorescein microscope.

2.4. Cell cultures

HRMECs (Applied Cell Biology Research Institute, Kirkland, WA, USA) were maintained in EGM-2MV (Lonza, Walkerville, MD, USA) containing all supplements on a gelatin-coated flask. Human brain astrocytes (Applied Cell Biology Research Institute) were maintained in DMEM (Hyclone, Logan, UT, USA) with 10% fetal bovine serum (Gibco BRL, Carlsbad, CA, USA). Cells were incubated at 37 °C in an incubator with a humidified atmosphere of 95% air and 5% CO_2. For the hypoxia treatment, cells were incubated in a hypoxic chamber with 1% of O_2.

2.5. Cell viability and proliferation assay

Cell viability and proliferation assay was assessed with a 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST)-1 assay using the EZ-CYTOX Cell Viability Assay kit (Daeillab, Seoul, Korea) [30]. For the cell viability, cells were seeded into each well of 96-well plates at a concentration of 8×10^3 cells/well. After an incubation for 24 h, cells were treated with either dimethyl sulfoxide as a control or various concentration (1, 10, 100, and 200 μM; 1 and 10 mM) of betaine for 48 h. The medium was exchanged with fresh media with WST-1 solution (10% v/v). After incubation at 37 °C for 2 h, absorption at 450 nm was measured using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). For the proliferation assay, HRMECs (3×10^3 cells/well) were incubated for 48 h in 1% FBS containing media with or without 200 μM betaine or 20 ng/ml VEGF (Cell Signaling Technology, Beverly, MA, USA). Three independent experiments were performed for each experimental condition.

2.6. Wound migration assay

Cell migration was evaluated with modified wound migration assay as our previous description [31]. HRMECs (4×10^4 cells) were placed on gelatin-coated culture dishes and cultured to 90% confluence. After overnight starvation with basal medium containing 0.1% FBS, the monolayers were wounded with a yellow pipette tip. Then, the medium was replaced with or without 20 ng/ml VEGF. The first image of the scratch was obtained under the light microscope (Leica) to set the reference line. Eight hours after wounding, the medium was removed, and cells were fixed with absolute methanol and stained with Giemsa's solution (BDH Laboratory Supplies, London, United Kingdom). Migration was quantified by counting the number of cells that moved beyond the reference line. Three independent experiments were performed for each experimental condition.

2.7. Tube formation assay

Tube formation was assayed as our previous description [32]. Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was coated on 48-well culture plates and polymerized for 30 min at 37 °C. HRMECs (5×10^4 cells) were seeded on the surface of matrigel and treated with 200 μM betaine or 20 ng/ml VEGF for 12 h. The morphologic changes of the cells and formed tubes were observed and photographed