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Propranolol increases vascular permeability through pericyte apoptosis and exacerbates oxygen-induced retinopathy

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ABSTRACT

Retinopathy of prematurity (ROP) is an eye disease that causes blindness due to delayed vascular growth, retinal ischemia, and resulting abnormal angiogenesis. Nonselective β-antagonist propranolol is in clinical trials for the treatment of ROP due to its effect of reducing VEGF expression and inhibiting retinal angiogenesis in oxygen-induced ROP models (OIR), but the mechanism by which propranolol acts on ROP vessels is still unclear. In the present study, we have focused on the effect of propranolol on pericyte survival and vascular permeability. We demonstrated that propranolol increases pericyte apoptosis more sensitively than endothelial cells (ECs), thereby weakening EC tight junctions to increase endothelial permeability in co-cultures of pericytes and ECs. Mechanistically, pericyte apoptosis by propranolol was due to the inhibition of Akt signaling pathway. We also demonstrated that propranolol increases pericyte loss and vascular permeability of retinal vessels in a mouse model of OIR. These results suggest that propranolol may be negative for blood vessels in retinas of OIR, and that the efficacy of propranolol for the treatment of ROP needs to be more thoroughly verified.

1. Introduction

Retinopathy of prematurity (ROP) is an eye disease that causes blindness in children [1]. ROP begins with delayed vascular growth and is followed by retinal ischemia. Hypoxia due to ischemia increases the stability of hypoxia-inducible factor-1 alpha (Hif-1α) proteins and accumulates them in cells, thereby increasing the expression of angiogenic factors [2]. Vascular endothelial growth factor (VEGF) is known to promote angiogenesis and plays an important role in abnormal angiogenesis and destruction of the blood-retinal barrier (BRB), which is characteristic of ROP [3]. Aberrant angiogenesis in retina and subsequent fibrosis may lead to retinal detachment and blindness [4]. Since VEGF plays a significant role in ROP lesions, anti-VEGF therapy is the basis for ROP treatment and has a therapeutic effect [5,6].

Nonselective β-antagonist propranolol is known to be effective in the treatment of infantile hemangiomas, benign vascular tumors [7]. Propranolol specifically suppresses β-adrenergic stimulation by blocking β1-and β2-adrenergic receptor (AR). Interestingly, propranolol has been reported to inhibit pathologic retinal neovascularization and BRB breakdown by reducing VEGF expression in oxygen-induced ROP models (OIR) [8,9]. However, the mechanism by which propranolol decrease VEGF levels is not clearly defined. Conflicting data have shown that propranolol has no effect on VEGF expression and does not prevent pathological neovascularization in OIR retina [10]. Despite these controversies, clinical trials are underway to evaluate propranolol therapy in patients with ROP [11]. Therefore, a precise understanding of the mechanism by which propranolol acts on ROP vessels is important in predicting the effects and side effects of propranolol, and this is the focus of this study.

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Pericytes play an important role in protection of the eyes, which maintains the integrity of the inner BRB by surrounding endothelial cells (ECs) [12]. Loss of pericytes may lead to capillary instability and vascular leakage leading to macular edema, and may also cause microaneurysms and angiogenesis due to EC proliferation at the site of pericyte loss [13]. Because pericytes also affect EC survival and apoptosis [14], it is important to maintain their survival during retinopathy. Many stimuli on the retinal vessels may cause pericyte loss. Recently, we have demonstrated that angiopoietin 2 (Ang2)-integrin signaling cause pericytes apoptosis leading to pericytes loss in diabetic retinopathy lesions [15]. Interestingly, it is reported that when sympathetic neurotransmission is impaired, pericyte loss is induced in mouse and rat retina [16,17]. Moreover, our previous findings show that β-adrenergic receptor agonists preferentially act on pericytes rather than ECs, increasing pericyte survival [18]. Considering that the abnormal EC proliferation observed in ROP and the ability of pericytes to inhibit EC proliferation, pericytes may play an important role in the pathogenesis of ROP. However, it is largely unclear whether and how β-adrenergic receptor antagonist propranolol affect pericyte survival and thereby affects ROP.

In the present study, we examined the effects of propranolol on pericyte survival and endothelial permeability in co-cultures of pericytes and ECs *in vitro*, and vascular permeability in OIR retinas of mice *in vivo*. We demonstrated that propranolol increases pericyte apoptosis that weakens the tight junction of ECs in co-cultures of pericytes and ECs, and increases vascular permeability *in vitro* and in OIR retinas of mice *in vivo*.

2. Materials and methods

2.1. Cell cultures

Human umbilical vein endothelial cells (HUVECs, Lonza, Rockland, ME, USA), and human pericytes from placenta (PromoCell, Heidelberg, Germany) were in M199 medium (HyClone, Logan, UT, USA) with 20% fetal bovine serum (FBS) and DMEM medium (PromoCell), respectively. Cells were grown in a humidified 5% CO_2 atmosphere in an incubator, with oxygen tension at either 140 mmHg (20% O_2 v/v, normoxic conditions) or 7 mmHg (1% O_2 v/v, hypoxic conditions).

2.2. Reagents and antibodies

Propranolol, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Ceramide C6, and FITC-dextran (70 kDa) were purchased from Millipore-Sigma (St. Louis, MO, USA), and SC79 from Selleck Chemicals (Houston, TX, USA). Other reagents and antibodies used were: human phospho-kinase array kit (R&D systems, Minneapolis, MN, USA); FITC-conjugated annexin V/PI assay kit (BD Biosciences, Franklin Lakes, NJ, USA); anti-phospho-Akt, anti-phospho-Erk1/2, anti-Akt, anti-Erk1/2, anti-Bcl-2, anti-Bcl-xL, anti-Bax, anti-cleaved PARP antibodies (Cell Signaling Technology); anti-β-tubulin, and peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA); anti-TER119 (BD Biosciences); anti-CD31 and anti-NG2 antibodies (Millipore); FITC- or Cy3 conjugated species specific IgGs (Jackson ImmunoResearch, West Grove, PA, USA).

2.3. Cell viability assay

Cell viability was measured by the MTT labeling kit (Millipore-Sigma). 5×10^3 cells were seeded into 96-well plates for 24 h and treated with propranolol. The cells were then incubated with 100 μL of MTT (5 mg/mL) for 3 h. The formazan levels were calculated following measurement of the absorbance intensity at 570 nm.

2.4. Cell cycle analysis

Cells were incubated with vehicle or propranolol for 48 h, harvested, and fixed in 75% ethanol for 30 min. The cells were washed with PBS, labeled with propidium iodide (0.05 mg/mL) in the presence of RNase A (0.5 mg/mL), and incubated at room temperature in the dark for 30 min. DNA content was analyzed using flow cytometry by excitation of the propidium iodide incorporated into DNA at 488 nm and detection at 650 nm.

2.5. FACS analysis

In order to evaluate apoptosis, 5×10^5 cells were treated with propranolol under normoxic and hypoxic conditions at 37 °C for 48 h. The cells were harvested and washed twice in PBS. The cells were stained with FITC annexin V and PI for 15 min, and analyzed by flow cytometry on the FACS Calibur (BD Biosciences) and data analyzed with Flowjo software. Annexin V positive cells were determined to be apoptotic cells.

2.6. Western blot analysis

Western blot analysis was performed as previously described [18]. Briefly, the appropriately treated cells were harvested, lysed in a lysis buffer, and subjected to SDS-PAGE. The blots were incubated with the indicated primary antibodies. Horseradish peroxidase-conjugated species-specific IgGs were used as secondary antibodies. The blots were incubated with enhanced chemiluminescence substrate (Pierce, IL, USA) and exposed to film.

2.7. Phosphokinase array

Cell lysates were incubated with human phosphokinase array membranes in which antibodies against 43 different kinases are spotted and processed according to the manufacturer's protocol (R&D systems).

2.8. Endothelial permeability assay

Endothelial permeability was measured by spectrophotometric assay of the flux of Evans blue (Millipore-Sigma)-labeled bovine serum albumin (BSA; Millipore-Sigma) across cell monolayers using a transwell plate as described previously [18]. Briefly, cells were seeded onto both sides of transwell filter (Costar) and cultured in normoxic and hypoxic conditions, with or without propranolol for an additional 48 h. Endothelial permeability was evaluated by Evans blue dye in the culture medium. The optical density of the medium in the bottom chamber was spectrophotometrically measured at 650 nm (Tecan, Infinite M200PRO).

2.9. Oxygen-induced retinopathy

The animal experiments in this study were approved by and in strict agreement with the guidelines of the Seoul National University Animal Care and Use Committee. C57BL/6J mice were purchased from Central Laboratory Animal Inc. (Seoul, Korea). An oxygen-induced retinopathy (OIR) mouse model was developed as in a previous study [19]. Briefly, mice pups were exposed to hyperoxia (75% oxygen) for 5 days from P7 to P12 and then returned to normoxic conditions (room air; 21% oxygen). Mice were intraperitoneally injected with propranolol at the indicated doses daily from P12 to P16, and retinal vascularization was evaluated at P17.

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