의학박사 학위논문

망막질환에서 베타-아드레날린 수용체 항진제와 길항제가 혈관주위세포 생존에 미치는 영향에 관한 연구

Study of the effects of β-adrenergic receptor agonists and antagonists on pericyte survival in retinopathies

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Study of the effects of β-adrenergic receptor agonists and antagonists on pericyte survival in retinopathies

by

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A thesis submitted to the Department of Pharmacology in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medicine (Pharmacology) at Seoul National University College of Medicine

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ABSTRACT

Retinopathy is a disease caused by persistent or severe retinal damage. Diabetic retinopathy (DR) and retinopathy of prematurity (ROP) are representative diseases of retinopathy. DR and ROP are caused by the proliferation of abnormal new blood vessels or upregulation of vascular leakage. Increased expression of angiogenic factors, such as vascular endothelial growth factor (VEGF), or retinal pericyte loss has been demonstrated to lead to vascular leakage and neovascularization in DR or ROP. Thus, the prevention of pericyte loss is a potential target for the treatment of retinopathy. An increasing body of evidence suggests the importance of β-adrenergic receptor signaling in pericyte biology. For example, the loss of sympathetic neurotransmission leads to pericyte loss in the retinas of mouse and rat. In this study, I investigated the effects of β-adrenergic receptor agonists and antagonists on pericyte loss in retinal diseases. I demonstrated that β-adrenergic receptor agonists (β-agonists) inhibited pericyte apoptosis induced by high glucose, Ang2, and TNF-α, which are increased in diabetic retinas. β-Agonists also increased pericyte proliferation under high glucose conditions. Conversely, nonselective β-adrenergic receptor antagonists (nonselective β-antagonists), rather induced pericyte apoptosis and reduced pericyte proliferation under hypoxic conditions. β-agonists did not induce any changes in endothelial cell survival and proliferation and β-antagonists only induced a slight decrease of endothelial cell proliferation. Both β-agonists and β-antagonists were involved in the survival and proliferation of pericyte through the PI3K/AKT and MAPK/ERK

pathways. Interestingly, β_2-agonists were more effective in the induction of pericyte survival and proliferation than β_1-agonists; this resulted from higher expression of the β_2-adrenergic receptor than the β_1-adrenergic receptor in pericytes under high glucose conditions. Furthermore, β_2-agonists effectively prevented pericyte loss and vascular leakage in a mouse model of DR. On the other hand, β-antagonists aggravated vascular leakage and neovascularization by the promotion of pericyte loss in oxygen-induced retinopathy (OIR). Together, these results suggested that β_2-agonists effectively attenuated pericyte loss in DR by the promotion of pericyte survival and proliferation and may be a potential therapeutic agent for the prevention of retinal vascular leakage in DR. On the other hand, β-antagonists were not appropriate for the treatment of ROP by the induction of pericyte loss, and that administration to patients should be carefully monitored.

Keywords: Diabetic retinopathy, Retinopathy of prematurity, β-adrenergic receptor agonist, β-adrenergic receptor antagonist, Pericyte loss, Vascular leakage, Neovascularization

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LIST OF ABBREVIATIONS

Ang1 : angiopoietin 1

Ang2 : angiopoietin 2

Bax : bcl-2-like protein 4

Bcl-2 : B-cell lymphoma 2

Bcl-xL : B-cell lymphoma-extra large

BRB : blood-retinal barrier

BrdU : 5-bromo-2’-deoxyuridine

Con : control

COPD : chronic obstructive pulmonary disease

CREB : cAMP response element-binding protein

DM : diabetes mellitus

DR : diabetic retinopathy

ED50 : effective dose 50

HG : high glucose

HIF-1α: hypoxia-inducible factor-1 alpha

HM : high mannitol

HRMECs : human retinal microvascular endothelial cells

HUVECs : human umbilical vein endothelial cells

IH : infantile hemangioma

IL-1b : interleukin 1 beta

IL-6 : interleukin 6

IP : intraperitoneal

MAPK : mitogen-activated protein kinase

MTT : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NG : normal glucose

OIR : oxygen-induced retinopathy

PARP : poly(ADP-ribose) polymerase

PDGF-B : platelet-derived growth factor-B

PDGFRβ : platelet-derived growth factor receptor-beta

PI : propidium iodide

PI3K : phosphoinositide 3-kinase

ROP : retinopathy of prematurity

STZ : streptozotocin

TNF-α : tumor necrosis factor alpha

VEGF : vascular endothelial growth factor

VEGFA : vascular endothelial growth factor A

β-agonist : beta-adrenergic receptor agonist

β-antagonist : beta-adrenergic receptor antagonist

β_1-AR : beta1-adrenergic receptor

β_2-AR : beta2-adrenergic receptor

GENERAL INTRODUCTION

Retinopathy is caused by persistent or severe damage to the retina. Inflammation and vascular remodeling are the main events that induce retinopathy and cause blindness [1, 2]. There are many types of retinopathy: diabetic retinopathy (DR) caused by diabetes mellitus [1], retinopathy of prematurity (ROP) owing to the prematurity of the newborn [2], hypertensive retinopathy caused by arterial hypertension [3], radiation retinopathy resulting from exposure to ionizing radiation [4], and solar retinopathy resulting from exposure to direct sunlight [5]. Among these, ROP and DR are representative diseases of vascular remodeling [1, 2].

ROP and DR are leading causes of blindness in premature infants and working-aged people, respectively [1, 2]. In the case of premature infants, the premature development of blood vessels can induce ischemia. The resulting hypoxia increases the expression of angiogenic factors such as vascular endothelial growth factor (VEGF), which subsequently increases vascular leakage and retinal neovascularization [2]. In DR, the induction of ischemia and pericyte loss causes vascular leakage and neovascularization, which results in visual impairment in the retina [1]. Thus, the prevention of vascular leakage and retinal neovascularization is important in the treatment of ROP and DR.

Previous studies have shown that anti-VEGF drugs exert therapeutic effects in the treatment of retinopathy through the prevention of neovascularization and vascular leakage [1, 2]. However, VEGF inhibitors have

adverse effects such as hypertension, proteinuria [6, 7], and vitreoretinal fibrosis, which lead to retinal detachment [8-10]. Therefore, it is necessary to find alternative retinopathy treatments that are safe and effective.

Pericytes maintain the stability of blood vessels through interaction with endothelial cells (ECs) [11] and enhance the tight junction integrity of ECs through secretion of the protein Ang1 that acts on vascular ECs, which in turn prevent vascular leakage [12]. In addition, ECs secrete the protein platelet-derived growth factor B (PDGF-B), which activates the PDGF receptor β (PDGFRβ) in pericytes [11]. The activation of PDGFRβ in pericytes enhances pericyte survival and the recruitment of pericytes to ECs [11, 13], which indicates a close interaction between ECs and pericytes. However, as shown in PDGF-B knockout mice, the absence of pericytes results in an increase systemic vascular permeability [14], which indicates the participation of pericytes in the prevention of vascular leakage. Pericyte loss can also cause neovascularization through an increase in EC proliferation [15]. Therefore, the prevention of pericyte loss is important in the treatment of retinopathies.

β-Adrenergic receptor agonists (β-agonists) are widely used in the treatment of bradycardia, asthma, chronic obstructive pulmonary disease, and heart failure, whereas β-antagonists are used to manage cardiac arrhythmias and hypertension. β-Agonists are sympathomimetic agents that activate the β-adrenergic receptor, and β-antagonists are competitive inhibitors that obstruct the binding of epinephrine and norepinephrine to β-adrenergic receptors. There are three types of β-adrenergic receptors: β_1, β_2, and β_3. Among these, β_1- and

β_2-agonists are widely used against many clinical diseases. Previous studies have reported the therapeutic benefits of nonselective β-agonists in DR [16] and a nonselective β-antagonist in the OIR model [17, 18]. However, the precise mechanism by which both β-agonists and -antagonists attenuate the symptoms of the retinal diseases has not yet been fully elucidated. Therefore, I attempted to elucidate the effects of β-agonist and β-antagonist on pericytes and ECs in retinal diseases.

CHAPTER 1

Effects of β-adrenergic receptor agonists on pericyte survival in diabetic retinopathy

INTRODUCTION

DR is a complication of diabetes that leads to visual loss, and even blindness, in working-aged people. Despite efforts to attenuate the symptoms of DR via glycemic control and photocoagulation, it is the most common microvascular complication [1]. During the early stages of DR, the pericyte loss related to vascular leakage [19] is a characteristic feature of DR pathology [20].

Pericytes are contractile cells that surround ECs and support the integrity of the blood-retina barrier (BRB) [21]. In DR, pericyte loss weakens BRB structures and leads to increasing vascular leakage [22]. The suggested causes of pericyte loss in DR are pericyte apoptosis and migration [23, 24]. Pericyte apoptosis can be induced by high glucose, angiopoietin 2 (Ang2), and TNF-α, which are all increased in diabetic retinas [22, 23]. Thus, the prevention of pericyte loss may be a beneficial strategy for the treatment of DR.

β-Adrenergic receptor agonists are sympathomimetic agents that act upon the β-adrenoceptors. Among the three types of β-agonists, β_1- and β_2- agonists are widely used to treat diverse diseases such as asthma, bradycardia, and chronic obstructive pulmonary disease (COPD). Recent reports have shown that β-agonists activate the survival and proliferation pathways in many cell types, such as hemangioma-derived ECs, tumor cells, and eosinophils [25-27]. It has also been reported that a nonselective β-agonist attenuated retinal neurodegeneration and retinal capillary degeneration in diabetic retinas [16]. However, it is not well understood whether β-agonists affected vascular leakage

in DR. In this context, it is important to note that loss of sympathetic neurotransmission leads pericyte loss in mouse and rat retinas [28, 29]. Thus, I hypothesized that β-agonists, by sympathetic stimulation, may affect pericyte survival or proliferation in DR.

Diabetes mellitus (DM) and hypertension are common diseases that co-exist at a high frequency [30]. It has been reported that obesity is associated with DM and hypertension [30], however, it is not well understood why DM and hypertension co-exist frequently. Hypertension in the diabetic patients markedly increases the risk and accelerates the progression of cardiac disease and peripheral vascular disease [30-32]. Therefore, when hypertension occurs in patients with DM, hypertension treatment is performed along with diabetes treatment. However, since both β_1- and β_2- agonists among the β-agonists can induce hypertension, β-agonists may be regarded as inappropriate treatment for diabetic patients. Since there is a local treatment such as intravitreal injection or topical administration to eyes for patients with retinal disease, if β-agonists have therapeutic effects on DR, they can be used as a therapeutic agents for retinal diseases without causing hypertension. Thus, I thought that β-agonists could be used for DR even with side effects that could cause hypertension.

In this study, I demonstrated that β-agonists effectively attenuated pericyte loss in DR via the PI3K/AKT and MAPK/ERK pathways. Under high glucose conditions, β_2-agonists more effectively increased pericyte survival and proliferation than β_1-agonists, which occurred owing to higher expression of the β_2-adrenergic receptor than the β_1-adrenergic receptor in pericytes. β_2-

Agonists also effectively prevented vascular leakage in DR. Together, these results suggested that β_2-agonists effectively attenuated pericyte loss in DR by the promotion of pericyte survival and proliferation.

MATERIALS AND METHODS

1. Cell cultures

Human retinal microvascular endothelial cells (HRMECs, Applied Cell Biology Research Institute, Kirkland, WA, USA), and human pericytes (PromoCell, Heidelberg, Germany) were maintained in M199 medium with 20% fetal bovine serum (FBS) and pericyte media containing growth factors (PromoCell), respectively. Cells were cultured at 37 °C in an incubator with a humidified atmosphere of 5% CO_2. To generate the normal and high glucose conditions, cells were exposed to 5 mM glucose for the normal condition (NG) or 25 mM glucose for the high glucose condition (HG). A high mannitol concentration (20 mM mannitol plus 5 mM glucose, HM) was used as the osmotic control for high glucose exposure.

2. Reagents and antibodies

Salbutamol, formoterol, dobutamine, xamoterol, propranolol, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), wortmannin, streptozotocin, and FITC-dextran (70 kDa) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The human recombinant proteins Ang2 and TNF-α were purchased from R&D Systems (Minneapolis, MN, USA). The antibodies anti-phospho-AKT, anti-phospho-ERK, anti-phospho-CREB, anti-

AKT, anti-ERK, anti-CREB, anti-Bcl-2, anti-Bcl-xL, anti-Bax, anti-cleaved PARP antibodies, and PD98059 were purchased from Cell Signaling Technology, and anti-β-tubulin, anti-β_1-AR, anti-β_2-AR, and peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Measurement kits for the FITC-conjugated annexin V/PI assay (BD Biosciences, Franklin Lakes, NJ, USA) and BrdU cell proliferation ELISA (Roche, Indianapolis, IN, USA) were used.

3. Animals

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 (SNU-141008-2-2). Eight-week-old, pathogen-free male C57BL/6J mice were purchased from Central Laboratory Animal Inc. (Seoul, Korea). Diabetes was induced by a single intraperitoneal injection of freshly prepared streptozotocin (STZ; Sigma-Aldrich, MO, USA) at a concentration of 180 mg/kg body weight in 10 mM citrate buffer (pH 4.5). Age-matched controls were treated with citrate buffer only. The STZ-treated mice with blood glucose levels of at least 300-400 mg/dL were considered diabetic. After 3 months of STZ injection, the mice were sacrificed and retinas were harvested from the enucleated eyes under deep anesthesia and immediately frozen at -80 °C for quantitative RT-PCR or western blot assay, or fixed in 4% paraformaldehyde for retinal digestion. Glucose levels (Accu-Chek, Roche, Indianapolis, IN, USA)

and body weight also measured.

4. Retinal vascular leakage

Vascular leakage was assessed in mice after 3 months of STZ induction. Deeply anesthetized mice were intravenously injected with FITC-dextran dissolved in PBS. After perfusion for 1 h, the eyes were enucleated and fixed in 4% paraformaldehyde for 1 h. The retinas were dissected in PBS, flat-mounted, and viewed on a confocal microscope (FV1000, Olympus, Shinjuku, Tokyo, Japan). To quantify vascular leakage, four representative leakage sites in the mid-peripheral retina were selected in each mouse ($n = 4$), and the images were obtained using confocal microscopy. The vascular leakage area was determined using ImageJ software (NIH, MD, USA), and the average vascular leakage area was quantified by the percentage of the vascular leakage area of the total image area.

5. Retinal digest preparations

Vascular preparations of whole-mount retinas were performed using a trypsin digestion technique. The retinas were fixed for at least 24 h in 4% paraformaldehyde, incubated in water for 1 h, and then digested in 2.5% trypsin (Thermo Fisher, Waltham, MA, USA) at 37 °C for 1 h. After careful removal of the inner limiting membrane, the retinal vessels were isolated by careful

irrigation with filtered water. The samples were dried and stained with periodic acid-Schiff base for 15 min and hematoxylin.

6. Morphological quantification of pericytes

To determine the number of retinal pericytes, retinal digest preparations were analyzed. Pericytes were identified according to their morphologies and locations on capillaries. Total pericytes were counted in 10 randomly selected areas in the center third of the retinal capillary area. The number of pericytes was standardized to the capillary area (numbers of cells/mm^2 capillary area). The capillary area was calculated using the NIS-Elements AR 3.2 program (Nikon, Tokyo, Japan).

7. Cell viability assay

The Sigma-Aldrich MTT labeling kit was used to evaluate cell viability. In all experiments, 5×10^3 cells were seeded into 96-well plates. After 24 h, cells were treated with isoproterenol, salbutamol, and dobutamine (0-100 μM) under normal glucose conditions for 48 h. Then, cells were incubated with 100 μL of MTT (5 mg/mL) for 3 h. The blue formazan crystals were solubilized with acidified isopropanol, and the formazan levels were determined by measurement of the absorption intensity at 570 nm. Three independent experiments were performed for each experimental condition.

8. FACS analysis

In order to evaluate cell apoptosis, 5×10^5 cells were treated with isoproterenol, salbutamol, or dobutamine (25 μM) in normal glucose, high mannitol, and high glucose conditions at 37 °C for 48 h. To determine the survival effect of β-agonists, pericytes or HRMECs in high glucose conditions were treated with Ang2 (300 ng/mL) or TNF-α (100 ng/mL) and isoproterenol, salbutamol, or dobutamine. The cells were harvested, washed twice in phosphate-buffered saline (PBS), 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-PI negative cells were determined as apoptotic cells.

9. BrdU ELISA proliferation assay

To determine the proliferation of cells, the Cell Proliferation ELISA, BrdU assay (Roche) was used according to the manufacturer's protocol. Pericytes and HRMECs treated with isoproterenol, salbutamol, or dobutamine under normal glucose, high mannitol, and high glucose conditions at 37 °C for 48 h were analyzed. In addition, pericytes and HRMECs were treated with isoproterenol, salbutamol, or dobutamine in addition to Ang2 (300 ng/mL), TNF-α (100 ng/mL), wortmannin (1 μM), or PD98059 (20 μM) under high glucose conditions at 37 °C for 48 h. The cells were then labeled with 10 μM BrdU for

1 h and incubated for 30 min with FixDenat solution. The anti-BrdU peroxidase conjugated antibody was added to the cells and incubated for 90 min at room temperature. After washing, the bound peroxidase was detected by the substrate reaction, which was measured on an ELISA plate reader at 450 nm. Three independent experiments were performed for each experimental condition.

10. Quantitative RT-PCR

RNAs were collected and isolated from cells using the RNeasy Plus Mini kit (Qiagen, Valencia, CA, USA). cDNAs were prepared from RNAs (1 μg) using 2.5 μM oligo-dT primers, 1 mM dNTPs, and MuLV reverse transcriptase. Quantitative real-time PCR (qPCR) assays were performed with SYBR Green PCR Master MIX (Applied Biosystems, Life Technologies, Gaithersburg, MD, USA) using a 7900HT real-time PCR thermocycler (Applied Biosystems). The qPCR reaction conditions were 50 cycles of 95 °C for 5 s and 60 °C for 20 s. Samples containing 20 ng of cDNA were analyzed in triplicate. The values were normalized to those of the control gene expression levels. The sequences of the PCR primers are summarized in Table 1.

11. Western blot

The cells were harvested and lysed in radioimmunoprecipitation assay buffer with a protease inhibitor cocktail. Protein lysates were resolved by SDS-

PAGE and transferred onto a nitrocellulose membrane. The membranes were incubated with primary antibodies (1:1000) at 4 ℃ overnight and secondary antibodies (1:5000) at room temperature for 1 h. The membranes were incubated with an enhanced chemiluminescence substrate (Pierce, IL, USA) and exposed to film.

12. Statistical analysis

Statistical analyses were performed using a standard two-tailed Student's *t*-test assuming unequal variances. A value of $P < 0.05$ was considered statistically significant. Quantitative data and figures are presented as mean ± SD.

RESULTS

β-Agonists increase pericyte viability

I determined the effect of β-agonists on the cell viability of pericytes and HRMECs. All β-agonists, including isoproterenol, salbutamol, and dobutamine, increased pericyte viability in a dose-dependent manner (Figs 1A-C). Interestingly, the β_2-agonist salbutamol was more effective in increasing pericyte viability than the β_1-agonist dobutamine (Figs 1B, C). However, at most doses, no changes in HMREC viability were induced by β-agonists (Figs 1D-F).

β_2-Agonists more effectively prevent pericyte apoptosis induced by high glucose, Ang2, and TNF-α than β_1-agonists

Next, I performed annexin V/PI flow cytometric analysis to determine if β-agonists affected pericyte apoptosis induced by high glucose, Ang2, and TNF-α, which mimic the conditions in diabetic retinas. High glucose exposure, as well as Ang2 and TNF-α, synergistically increased pericyte apoptosis in high glucose conditions (Figs 2A-C). β-Agonists significantly inhibited pericyte apoptosis induced by high glucose, Ang2, and TNF-α (Figs 2A-C). The nonselective β-agonist isoproterenol and β_2-agonist salbutamol more effectively prevented pericyte apoptosis induced by high glucose, Ang2, and TNF-α than the selective β_1-selective agonist dobutamine (Figs 2A-C). However, β-agonists did not affect HRMEC survival in high glucose conditions

and after treatment with Ang2 or TNF-α (Figs 2D-F).

To test the specificity of the agonist to β-receptors, I determined pericyte apoptosis following treatment with a different β_1- or β_2-agonist. The selective β_2-agonist formoterol also effectively reduced pericyte apoptosis induced by high glucose (Fig. 2G). However, the β_1 selective adrenergic agonist xamoterol did not prevent high glucose-induced pericyte apoptosis (Fig. 2G).

Next, I examined pericyte apoptosis using poly (ADP-ribose) polymerase (PARP) cleavage using western blot analysis. High glucose-induced cleaved PARP expression was reduced by β-agonists (Fig. 2H). Isoproterenol and salbutamol more effectively decreased the levels of high glucose-induced cleaved PARP than dobutamine (Fig. 2H). When I examined the effect of β-agonists on the expression of pro- and anti-apoptotic proteins, β-agonists increased BCL-2 expression in pericytes, which is known to promote cell survival (Fig. 2I). Isoproterenol and salbutamol also more effectively increased BCL-2 expression in pericytes than dobutamine (Fig. 2I). I confirmed the β-agonist-induced increase in *BCL2* expression in pericyte mRNA (Fig. 2J). These findings suggested that β_2-agonists are more effective in the prevention of pericyte apoptosis than β_1-agonists.

β_2-Agonists are more effective than β_1 agonists on proliferation increase in pericytes

To determine the effect of β-agonists on the proliferation of pericytes and HRMECs, I performed the BrdU ELISA proliferation assay. Although high

mannitol, high glucose, and Ang2 did not affect pericyte proliferation, β-agonists increased pericyte proliferation (Figs 3A, B). In addition, isoproterenol and salbutamol more effectively increased pericyte proliferation than dobutamine (Figs 3A, B). Although TNF-α decreased pericyte proliferation, β-agonists increased pericyte proliferation even after TNF-α treatment (Fig. 3C). However, β-agonists did not induce any change in HRMEC proliferation (Figs 3D-F). The β_2-agonist formoterol more effectively increased pericyte proliferation than the β_1-agonist xamoterol (Fig. 3G). When I determined cell proliferation using counting methods, isoproterenol and salbutamol more effectively increased pericyte cell number than dobutamine (Fig. 3H). These findings suggested that β_2-agonists are more effective for the induction of pericyte proliferation than β_1-agonists.

β-Agonists increase pericyte survival and proliferation via the PI3K/AKT and MAPK/ERK pathways

Next, I tried to identify the mechanism that mediates the β-agonist-induced increase in pericyte survival and proliferation in high glucose conditions. During my research, I found that propranolol, a nonselective β-adrenergic receptor antagonist, reduced pericyte survival and proliferation by inhibition of the AKT and ERK signaling pathways. Thus, I aimed to identify that β-agonists were able to activate the AKT and ERK signaling pathways. Isoproterenol induced AKT and ERK phosphorylation at 15, 30, and 60 min in pericytes (Fig. 4A). All β-agonists, including isoproterenol, salbutamol, and dobutamine, also

induced AKT and ERK phosphorylation at 30 min in both normal glucose and high glucose conditions (Figs 4B, C). Salbutamol was consistently able to induce AKT and ERK phosphorylation more effectively than dobutamine in pericytes (Figs 4B, C).

Next, I determined whether β-agonists induced pericyte survival and proliferation by activation of the PI3K/AKT and MAPK/ERK signaling pathways in high glucose condition. β-Agonist-induced AKT phosphorylation was attenuated by the PI3K inhibitor wortmannin but not by the MAPK kinase inhibitor PD98059 (Fig. 4D). Similarly, β-agonist-induced pericyte survival was abolished by wortmannin, but not by PD98059 (Fig. 4E), and β-agonist-induced upregulation of BCL-2 expression was also attenuated by wortmannin (Fig. 4F), which indicated that β-agonists increased pericyte survival through PI3K/AKT signaling activation and the subsequent increase in BCL-2 expression. In contrast, β-agonist-induced ERK phosphorylation was attenuated by PD98059 but not by wortmannin (Fig. 4D). Similarly, β-agonist-induced pericyte proliferation was also attenuated by PD98059 but not by wortmannin (Fig. 4G), which indicated that β-agonists increased pericyte proliferation via the MAPK/ERK signaling pathway. Moreover, the β-agonist-induced increase in pericyte survival and proliferation was attenuated by the nonselective β-antagonist propranolol (Fig. 4H). These findings suggested that the activation of β-adrenergic receptors by β-agonists increased pericyte survival and proliferation through the PI3K/AKT and MAPK/ERK signaling pathways, respectively.

β_2-Adrenergic receptor expression is higher than β_1-adrenergic receptor expression in pericytes in high glucose conditions.

As shown in Figs 2 and 3, β_2-agonists more effectively increased pericyte survival and proliferation than β_1-agonists. I hypothesized that expression of the β_2-adrenergic receptor was higher than that of the β_1-adrenergic receptor in pericytes in high glucose conditions. To determine the levels of the β_1- and β_2-adrenergic receptors (ARs) in pericytes in high glucose conditions, I performed quantitative RT-PCR and western blot analyses. I confirmed that β_2-AR (*ADRB2*) mRNA expression was higher than β_1-AR (*ADRB1*) mRNA expression in pericytes (Fig. 5A). High glucose decreased both β_1-AR mRNA and protein level in pericytes (Figs 5A, B), but did not induce any change in β_2-AR levels in pericytes (Figs 5A, B). Therefore, the enhanced effect of β_2-agonists on pericyte survival and proliferation in high glucose conditions resulted from the increased ratio of β_2-AR to β_1-AR expression in pericytes in high glucose conditions.

β_2-Agonists reduce vascular leakage by prevention of pericyte loss in diabetic retinas

Next, I examined whether β_2-agonists prevented pericyte loss and vascular leakage in diabetic retinas *in vivo*. To determine the activity of β_2-agonists in mouse retinas, I examined the phosphorylation of CREB, AKT, and ERK in mouse retinas after a daily intraperitoneal injection of salbutamol, for 15 consecutive days, in streptozotocin (STZ)-induced diabetic mice.

Intraperitoneal injection of salbutamol at doses above 5 mg/kg/day increased the phosphorylation of CREB, AKT, and ERK in the retinas of STZ-induced diabetic mice (Fig. 6A). The intraperitoneal injection of salbutamol at 5 mg/kg/day did not affect STZ mice survival (Fig. 6B). In the retinal digest preparations of salbutamol-treated STZ mice for 3 months, I found that retinal pericyte loss was attenuated in the salbutamol-treated STZ mice compared with the STZ mice (Figs 6C, D). Salbutamol also effectively reduced vascular leakage in the retinas of STZ mice (Figs 6C, E). However, salbutamol did not induce any changes in the mRNA expression of angiogenic factors and cytokines, including *Vegfa*, *Angpt1*, *Angpt2*, *Tnfa*, *Il6*, and *Il1b* (Figs 6F, G).

Table 1.

Nucleotide sequences of primers used in experiments

Targets of PCR	**Forward primers (5′ to 3′)**	**Reverse primers (5′ to 3′)**
human *BCL2*	GGTCATGTGTGTGGAGAGCG	GGTGCCGGTTCAGGTACTCA
human *BCL2L1*	AATGTCTCAGAGCAACCGGG	CATCCAAACTGCTGCTGTGC
human *BAX*	GACGAACTGGACAGTAACATGG	TCCCAAAGTAGGAGAGGAGGC
human *ADRB1*	GGGGCTGGGCAGATCTTAAA	CACCAGCAAATTCACGTGGG
human *ADRB2*	TGATCGCAGTGGATCGCTAC	GGACACGATGGAAGAGGCAA
mouse *Vegfa*	CCAAGATCCGCAGACGTGTA	GCGTGGTGGTGACATGGTTA
mouse *Angpt1*	GATCCGGCCCTTGGACTTTT	GCTCCAAACCCAGGTGACAT
mouse *Angpt2*	CCAACTCCAAGAGCTCGGTT	CGGTGTTGGATGACTGTCCA
mouse *Tnfa*	GCCTCTTCTCATTCCTGCTTG	CTGATGAGAGGGAGGCCATT
mouse *Il6*	CTTCTTGGGACTGATGCTGGT	GGTCTGTTGGGAGTGGTATCC
mouse *Il1b*	CCCATTAGACAACTGCACTAC	GATTCTTTCCTTTGAGGCCC

human *ACTB*	GGGAAATCGTGCGTGACATT	AGTTTCGTGGATGCCACAGG
mouse *Actb*	CCAGGCATTGCTGACAGGAT	AGCCACCGATCCACACAGAG

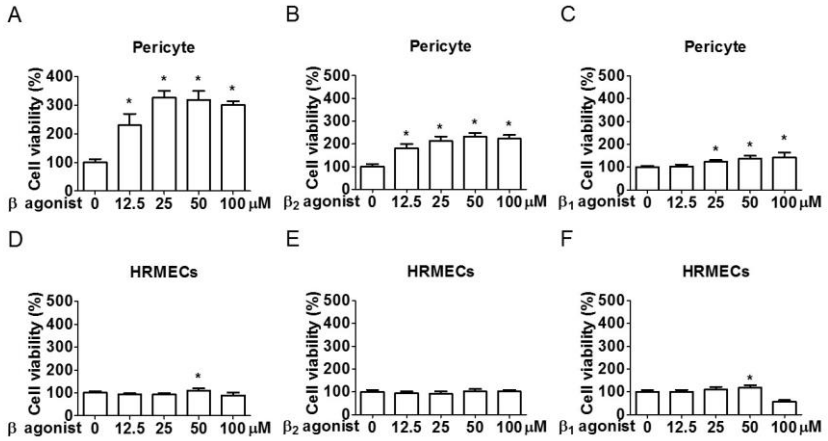

Figure 1. β-Agonists increase cell viability of pericytes.

The effects of β-agonists on the cell viability of pericytes and HRMECs were assessed by MTT assay. Pericytes and HRMECs were incubated for 48 h in complete medium with isoproterenol (β-agonist), salbutamol (β_2-agonist), or dobutamine (β_1-agonist) in the range of 12.5 μM to 100 μM. Isoproterenol, salbutamol, and dobutamine increased cell viability in pericytes (A-C) but not in HRMECs, except at a dose of 50 μM (D-F). The bar graph represents the mean ± SD of three independent experiments. * $P < 0.05$ by Student's *t*-test.

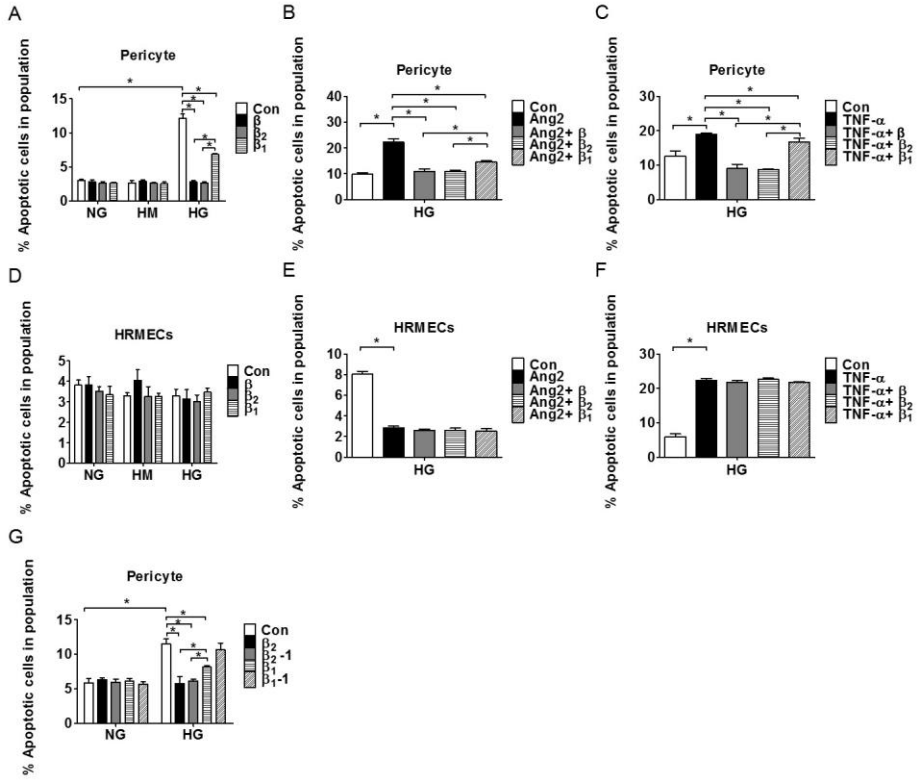

Figure 2-1. β_2-Agonists more effectively prevent pericyte apoptosis induced by high glucose, Ang2, and TNF-α than β_1-agonists.

The effects of β-agonists on the cell apoptosis of pericytes and HRMECs induced by high glucose, Ang2 (300 ng/mL), or TNF-α (100 ng/mL) were determined. Pericytes and HRMECs stained with annexin V fluorescein isothiocyanate and PI were analyzed by flow cytometry (A-G). Cell apoptosis was expressed as the percentage of apoptotic cells in the total cell population. The bar graph represents the mean ± SD of three independent experiments. The dosage of all types of β-agonists was 25 μM. β_1-1 agonist and β_2-1 agonist indicate xamoterol and formoterol, respectively. * $P < 0.05$ by Student's *t*-test.

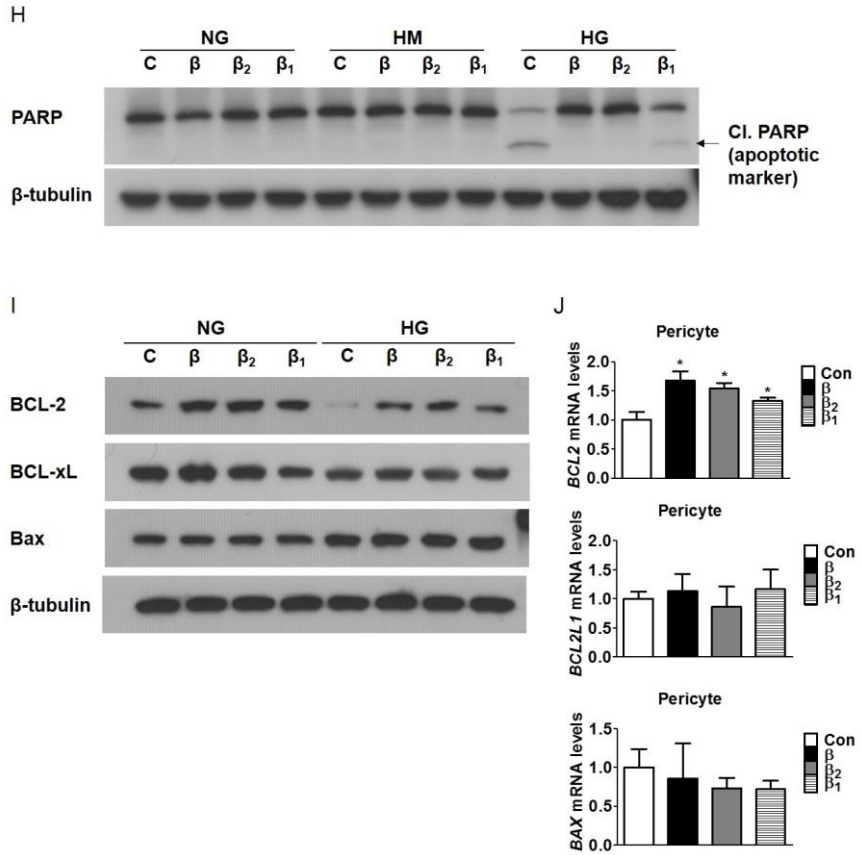

Figure 2-2. β_2-Agonists more effectively prevent pericyte apoptosis induced by high glucose, Ang2, and TNF-α than β_1-agonists.

(H) Western blot analysis of PARP cleavage was performed on lysates obtained from pericytes treated with isoproterenol, salbutamol, and dobutamine under normal glucose, high mannitol, and high glucose conditions for 48 h, and compared with the control. (I, J) Western blot and qRT-PCR quantification of BCL-2 (*BCL2*), BCL-xL (*BCL2L1*), and Bax (*BAX*) were performed on lysates obtained from pericytes treated with isoproterenol, salbutamol, and dobutamine

under normal glucose or high glucose conditions for 48 h, and compared with the control. The data represent three independent experiments. HG, high glucose (25 mM glucose); HM, high mannitol (5 mM glucose + 20 mM mannitol); NG, normal glucose (5 mM glucose). * $P < 0.05$ by Student's *t*-test.

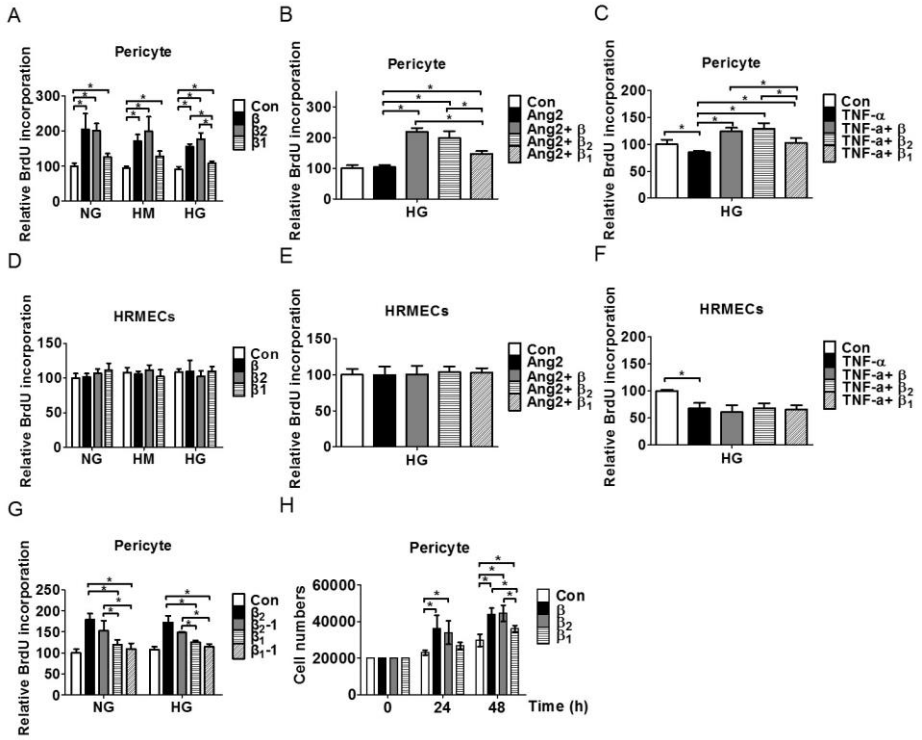

Figure 3. β_2-Agonists more effectively induce pericyte proliferation than β_1-agonists.

(A-G) Pericytes and HRMECs were treated with β-agonists for 48 h with or without Ang2 (300 ng/mL) or TNF-α (100 ng/mL) under high glucose conditions. Cell proliferation was measured by the BrdU incorporation assay. β_1-1 Agonist and β_2-1 agonist indicate xamoterol and formoterol, respectively. (H) Pericytes (2×10^4 cells/well) were seeded in 6-well culture plates. After 24 h and 48 h treatment with isoproterenol, salbutamol, and dobutamine under high glucose conditions, the number of pericytes was determined using a hemocytometer. The bar graph represents the mean ± SD of three independent

experiments. The dosage of all types of β-agonists was 25 μM. HG, high glucose (25 mM glucose); HM, high mannitol (5 mM glucose + 20 mM mannitol); NG, normal glucose (5 mM glucose). * $P < 0.05$ by Student's *t*-test.

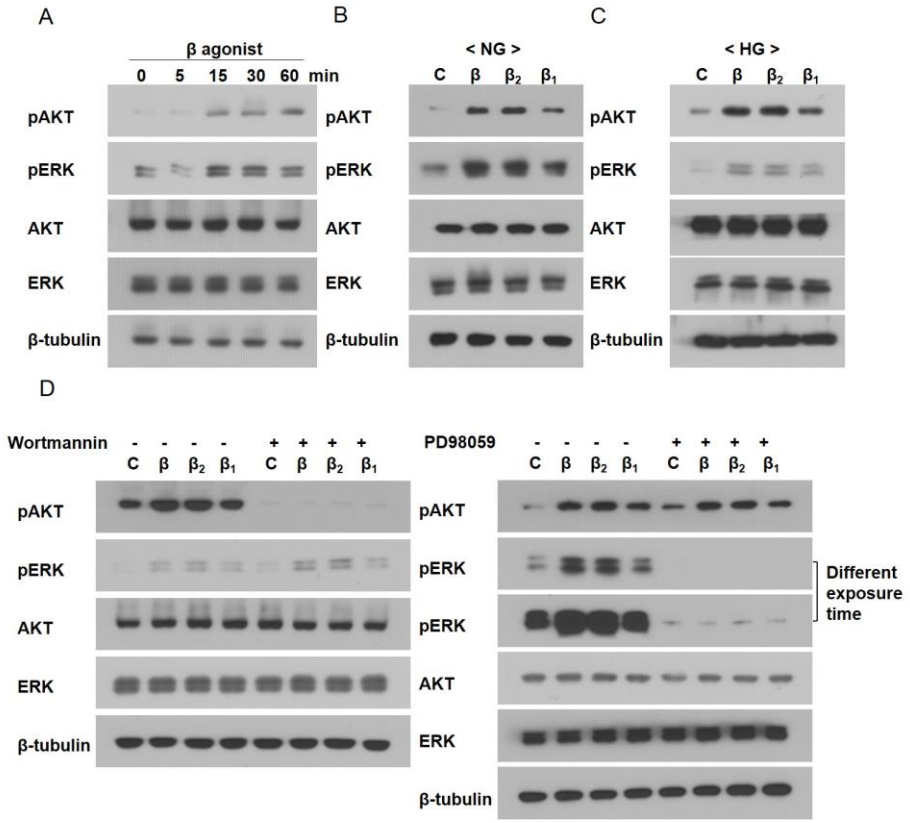

Figure 4-1. β-Agonists increase pericyte survival and proliferation via the PI3K/AKT and MAPK/ERK pathways.

(A) Western blot analysis of phospho-AKT (pAKT) and phospho-ERK (pERK) was performed on lysates obtained from pericytes treated with isoproterenol for 5, 15, 30, and 60 min. (B, C) Western blot analysis for pAKT and pERK was performed on lysates obtained from pericytes treated with isoproterenol, salbutamol, and dobutamine for 30 min in normal and high glucose conditions (HG; 25 mM). (D) Pericytes were preincubated with wortmannin (1 μM) or PD98059 (20 μM) for 1 h and treated with isoproterenol, salbutamol, and

dobutamine for 30 min in high glucose conditions. Determination of pAKT, AKT, pERK, and ERK was conducted by western blot with β-tubulin used as the loading control.

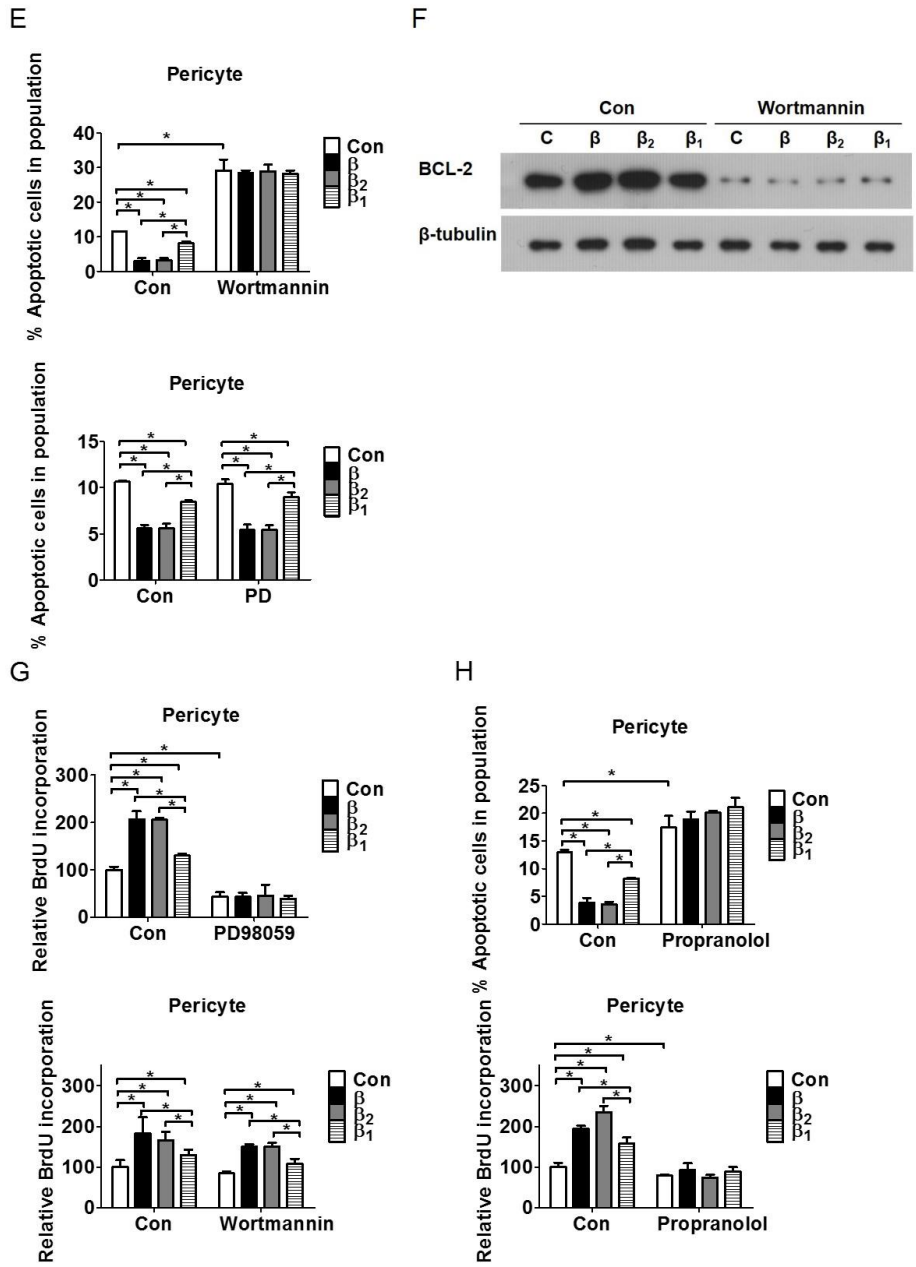

Figure 4-2. β-Agonists increase pericyte survival and proliferation via the PI3K/AKT and MAPK/ERK pathways.

(E) Apoptotic cell counts were assessed by FACS analysis 48 h after isoproterenol, salbutamol, and dobutamine treatment with or without wortmannin or PD98059 in high glucose conditions. (F) Pericytes were treated with isoproterenol, salbutamol, and dobutamine with or without wortmannin in high glucose conditions for 48 h. Determination of BCL-2 was conducted by western blot with β-tubulin used as the loading control. (G) Cell proliferation was assessed by the BrdU incorporation assay 48 h after isoproterenol, salbutamol, and dobutamine treatment with or without wortmannin or PD98059 in high glucose conditions. (H) Apoptotic cell counts and cell proliferation were assessed by FACS analysis and BrdU incorporation assay, respectively, after 48 h isoproterenol, salbutamol, and dobutamine treatment with or without propranolol (25 μM) in high glucose conditions. The dosage of all types of β-agonists was 25 μM. The bar graph represents the mean ± SD of three independent experiments. * $P < 0.05$ by Student's *t*-test.

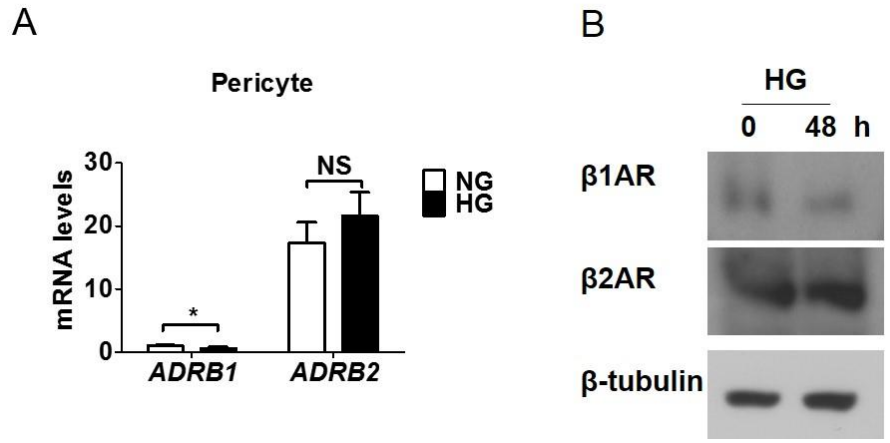

Figure 5. Expression of the β_2-adrenergic receptor is much higher than that of the β_1-adrenergic receptor in pericytes in high glucose conditions.

(A) The mRNA transcript level of β_1-AR (*ADRB1*) and β_2-AR (*ADRB2*) in pericytes exposed to normal and high glucose for 48 h was assessed by qRT-PCR. *ADRB1* and *ADRB2* mRNA levels were normalized to *ACTB* mRNA and reported as fold induction compared with *ADRB1* mRNA levels during normal glucose conditions. * $P < 0.05$ by Student's *t*-test. NS indicates not significant (* $P > 0.05$). (B) Western blot analysis of β_1-AR and β_2-AR protein expression was performed on lysates obtained from pericytes exposed to normal and high glucose for 48 h, and the protein β-tubulin was used as the loading control.

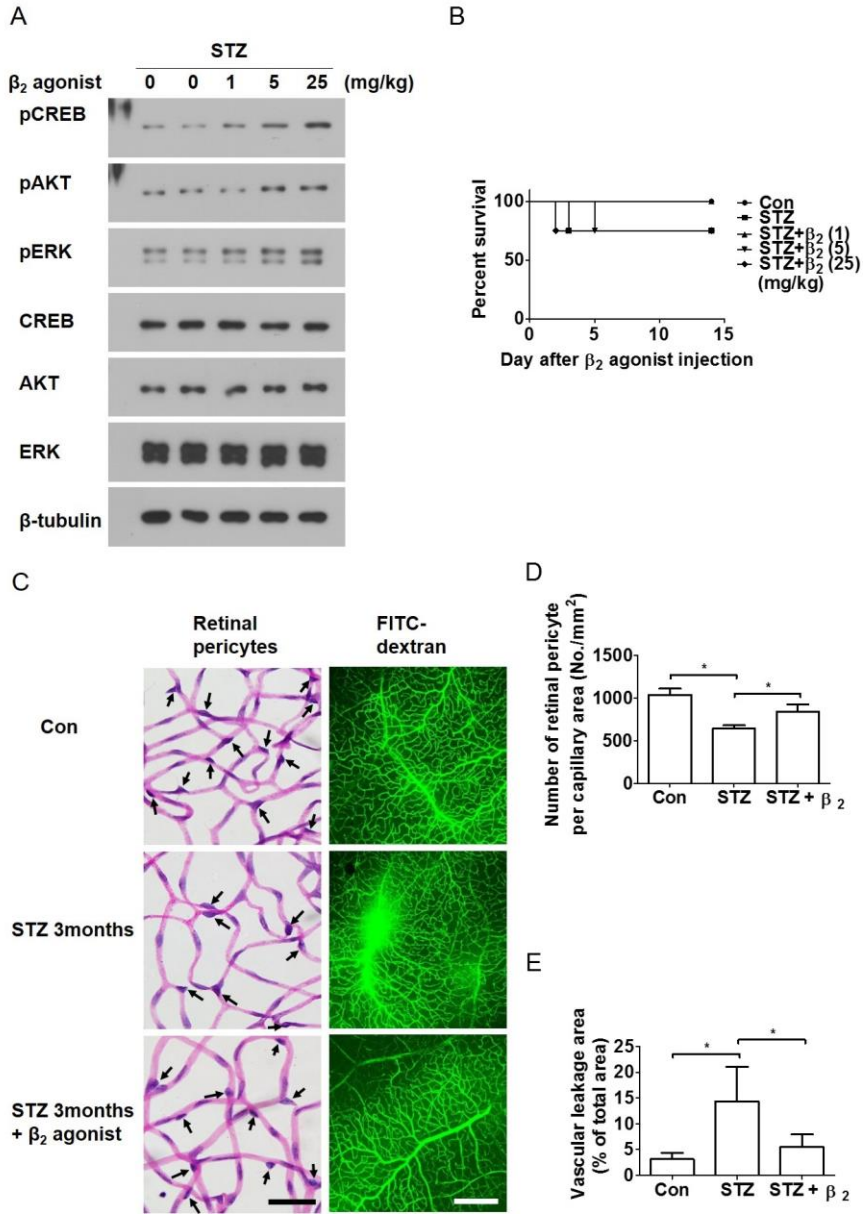

Figure 6-1. β_2-Agonists reduce vascular leakage by pericyte loss prevention in diabetic retinas.

(A, B) Following a 1-week streptozotocin induction, mice were administered

salbutamol daily by intraperitoneal injection for 15 days. (A) Western blot analysis of the phosphorylation of CREB, AKT, and ERK was performed on retinal lysates, and β-tubulin was used as the loading control. (B) Survival was monitored daily during the 15-day injection period. $n = 4$ mice per group. (C-E) Following a 1-week streptozotocin induction, mice were administered salbutamol (5 mg/kg), or PBS, daily by intraperitoneal injection for 11 weeks. Retinal vascular leakage and pericyte coverage were evaluated at 12 weeks after streptozotocin induction in mice. (C, D) The isolated retinas were digested with trypsin for pericyte evaluation. Pericytes were identified in periodic acid-Schiff- and hematoxylin-stained retinal digest preparations by morphologic criteria. Representative examples of retinal digest preparations are shown, and the arrows indicate representative pericytes. The number of pericytes was normalized to the area of capillaries (mm^2) in which they were counted. Original magnification ×400. Scale bar, 50 μm. * $P < 0.05$ by Student's *t*-test. (C, E) Retinal vascular leakage with FITC-dextran (70 kDa) was evaluated and the retinal vascular leakage area was quantified by the percentage of the vascular leakage area of the total image area. Original magnification ×100. Scale bar, 200 μm. * $P < 0.05$ by Student's *t*-test.

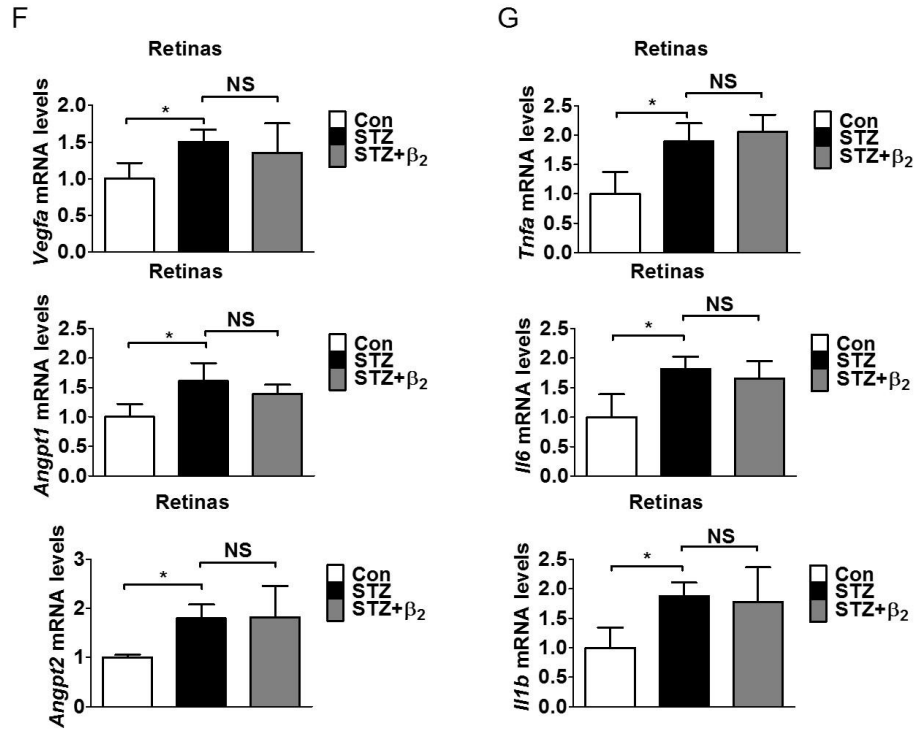

Figure 6-2. β_2-Agonists reduce vascular leakage by pericyte loss prevention in diabetic retinas.

(F, G) qRT-PCR for *Vegfa*, *Angpt1*, *Angpt2*, *Tnfa*, *Il6*, and *Il1b* were performed on lysates obtained from retinas in mice administered a daily intraperitoneal injection of salbutamol at 5 mg/kg for 11 weeks after 1 week of streptozotocin induction. *Vegfa*, *Angpt1*, *Angpt2*, *Tnfa*, *Il6*, and *Il1b* mRNA levels were normalized to *Actb* mRNA and reported as the fold induction compared with age-matched controls (Con). * $P < 0.05$ by Student's *t*-test. NS indicates not significant (* $P > 0.05$).

DISCUSSION

In this study, I demonstrated the effect of β-agonists on pericyte loss in DR. Although the nonselective β-agonist isoproterenol has been known to prevent retinal apoptosis and neurodegeneration in DR [16], the effect of β-agonists on retinal vascular leakage in DR has not yet been determined. Therefore, I determined whether β-agonists affected ECs to protect against vascular damage in DR. In contrast to a previous study [25], β-agonists did not affect EC viability, except at a 50 μM dose level (Figs 1D-F). Thus, I have focused on the effect of β-agonists on pericyte survival in DR.

Pericyte loss is one cause of vascular leakage in DR. Pericyte loss weakens the inner BRB and leads to capillary instability [22]. Although previous reports have shown that blocking sympathetic neurotransmission induced pericyte loss in mouse and rat retinas [28, 29], the effect of β-agonists on pericyte loss and vascular leakage in DR has not been fully elucidated. Based on my preliminary finding that the nonselective β-antagonist propranolol reduced pericyte survival and proliferation, I hypothesized that β-agonists may increase pericyte survival and proliferation, which may confer therapeutic benefits on the prevention of vascular leakage in DR.

In the present study, I demonstrated that β-agonists induced pericyte survival and proliferation under high glucose conditions (Figs 2A-C and 3A-C). Among the three types of β-AR agonists, β_2-agonists more effectively induced pericyte survival and proliferation than β_1-agonists in high glucose

conditions. To determine the basis of the different effects of β_1- and β_2-agonists on pericyte survival and proliferation, I found that the β_2-adrenoceptor was more highly expressed in pericytes than the β_1-adrenoceptor (Figs 5A, B). In addition, the expression ratio of β_2-AR to β_1-AR was increased in pericytes in high glucose conditions, because high glucose conditions induced the downregulation of β_1-AR expression but not β_2-AR expression (Figs 5A, B). Thus, the enhancement of β_2-agonists on pericyte survival and proliferation resulted from the increased expression of β_2-AR versus β_1-AR in pericytes in high glucose conditions. The β_2-agonist consistently demonstrated effective prevention pericyte loss in diabetic retinas of STZ mice (Figs 6C, D). I further determined the molecular mechanism by which β-agonists induced pericyte survival and proliferation in DR. I found that β-agonists increased BCL-2 expression by activation of the PI3K/AKT signaling pathway in pericytes, which in turn increased pericyte survival (Figs 4E, F). In addition, β-agonist-induced pericyte proliferation resulted from the activation of the MAPK/ERK signaling pathway (Fig. 4G). These results suggested that β-agonists induced pericyte survival and proliferation in DR via the PI3K/AKT and MAPK/ERK signaling pathways, respectively.

A variety of angiogenic factors and cytokines, including VEGF [33], Ang2 [22, 23, 34], TNF-α [23, 35, 36], and IL-6 [37], contribute to the development of pericyte loss or vascular leakage in diabetic retinas in DR. However, I found that β_2-agonists prevented pericyte loss in the diabetic retinas of STZ mice (Figs 6C, D) without perturbation of the expression of these angiogenic factors (Fig.

6F) and cytokines (Fig. 6G). Consistently, β_2-agonists prevented vascular leakage in the diabetic retinas of STZ mice (Figs 6C, E). These results suggested that β_2-agonists specifically stimulated β_2-AR in pericytes to activate the survival signaling pathways, but did not influence the levels of angiogenic factors and cytokines that are related to the development of pericyte loss in DR.

In this study, I proposed that β_2-agonists confer therapeutic benefits on the treatment of vascular leakage in DR. Moreover, I demonstrated the molecular mechanism by which β_2-agonists reduced pericyte loss and vascular leakage in DR. Although the systemic administration of β_2-agonists may cause side effects including pulmonary edema, myocardial ischemia, and cardiac arrhythmia, the use of local treatment modalities such as intravitreal injection or topical administration to eyes has shed new light on the potential therapeutic benefits of β_2-agonists for pericyte loss and vascular leakage in DR. However, I could not confirm that the β_2-agonists inhibit pericyte loss and vascular leakage in STZ mice through intravitreal injection or eye drop treatment due to experimental limitations. Pericyte loss and vascular leakage are observed more than 2 months after STZ induction in mice and rats [38, 39]. Unlike human eyes, however, since the mice eyes are very small, there is a significant limit to the number of intravitreal injection in mice. Therefore, I could not confirm that β_2-agonists through intravitreal injection prevent pericyte loss and vascular leakage because it is not possible to keep continuous intravitreal injections of β_2-agonists into mice more than 2 months after STZ administration. I also tried eye drop treatment because it was previously reported that eye drop treatment

of nonselective β-agonist isoproterenol could be used as treatment for DR [16]. In contrast to a previous study, however, β-agonists through eye drop treatment did not affect CREB phosphorylation of retinas, which indicated that β-agonists through eye drop treatment did not affect retinas (data not shown). Although β_2-agonists through local treatment modalities such as intravitreal injection or eye drop have not been shown to prevent pericyte loss or vascular leakage in STZ mice, since human eyes can be injected repeatedly by intravitreal injection, β_2-agonists through intravitreal injection may be possible to prevent vascular leakage without systemic side effects in DR.

In conclusion, I demonstrated that β-agonists prevented pericyte loss in diabetic mouse retinas, through the increase of pericyte survival and proliferation by activation of the AKT and ERK signaling pathways, respectively. Pericyte survival and proliferation was more effectively induced by β_2-agonists than β_1-agonists, which was attributed to the increased ratio of β_2-AR to β_1-AR expression in pericytes in high glucose conditions. Consequently, β_2-agonists prevented retinal vascular leakage in DR. In conclusion, I suggest β_2-agonists may be a potential therapeutic agent for the prevention of retinal vascular leakage in early DR.

CHAPTER 2

Effects of β-adrenergic receptor antagonists on pericyte survival in retinopathy of prematurity (ROP)

INTRODUCTION

ROP is an eye disorder that is the primary cause of blindness in pediatric patients [40]. ROP begins with delayed vascular growth, which results in retinal ischemia. In the retina, hypoxic conditions increase the stability of hypoxia-inducible factor-1 alpha (HIF-1α) and this subsequently leads to the upregulation of the expression of angiogenic factors [41]. Among the angiogenic factors, VEGF plays a major role in the formation of the vision-threatening condition ROP by inducing abnormal blood vessel formation and leading to breakdown of the blood-retinal barrier (BRB) [2]. Although laser ablation therapy is currently used for the treatment of ROP, alternative treatments are required owing to the invasiveness, cost, and only partial efficacy of the laser therapy.

β-Adrenoceptor antagonists (β-antagonists) are one of the most commonly used drugs in cardiovascular diseases, migraines, and anxiety disorders. The nonselective β-antagonist propranolol is known to be effective for the treatment of infantile hemangioma, a benign vascular tumor [42]. Propranolol specifically suppresses β-adrenergic stimulation by blocking β_1-AR and β_2-AR. Interestingly, propranolol has been reported to prevent pathologic retinal neovascularization and BRB breakdown by the suppression of increased VEGF in oxygen-induced ROP models (OIR) [17, 18]. However, it has also been reported that propranolol did not reduce VEGF expression, nor effectively protect against pathological neovascularization [43]. Although the mechanism

was not elucidated, propranolol aggravated pathological neovascularization at a slightly higher dose [43]. Despite the controversy, a clinical trial is in progress to assess propranolol treatment in ROP patients [44]. However, if propranolol is a risk factor for retinal damage, it may result in severe damage to the retinas of ROP patients because their retinas are unstable and incompletely developed. Therefore, the effect of propranolol on preclinical models of ROP, and the potential risk propranolol poses to retinas, needs to be confirmed.

Pericytes play an important role in protection of the eyes, helping to maintain the integrity of the inner BRB by surrounding ECs [21]. Pericyte loss not only causes macular edema by the induction of capillary instability and vascular leakage, but may cause microaneurysms and neovascularization by the proliferation of endothelial cells at the site of pericyte loss [15]. As pericytes also affect EC survival and apoptosis [45], it is important to maintain their survival during retinopathy. Based on previous reports that the loss of sympathetic neurotransmission induces pericyte loss in mouse and rat retinas [28, 29], and my findings that β-adrenergic receptor agonists increased pericyte survival in DR, I hypothesized that propranolol may induce pericyte loss, which in turn aggravates ROP.

In this study, I demonstrated that propranolol was inappropriate for ROP treatment because it results in the induction of pericyte loss. I found that propranolol substantially reduced pericyte survival and proliferation. In the experimental setting of a transwell co-culture model with ECs and pericytes, propranolol increased vascular permeability. Moreover, propranolol aggravated

the pathological neovascularization in OIR retinas. These results indicated that propranolol aggravated retinopathy by the induction of pericyte loss, and was not appropriate for ROP treatments.

MATERIALS AND METHODS

1. Cell cultures

Human umbilical vein endothelial cells (HUVECs, Lonza, Rockland, ME, USA), and human pericytes (PromoCell, Heidelberg, Germany) were maintained in EBM-2 media containing growth factors (Lonza, Rockland, ME, USA) and pericyte media containing growth factors (PromoCell), respectively. Cells were grown in an incubator with a humidified 5% CO_2 atmosphere, and an oxygen tension of either 140 mmHg (20% O_2 v/v, normoxic conditions) or 7 mmHg (1% O_2 v/v, hypoxic conditions).

2. Reagents and antibodies

Propranolol, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and FITC-dextran (70 kDa) were purchased from Sigma-Aldrich (St. Louis, MO, 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); BrdU cell proliferation ELISA kit (Roche, Indianapolis, IN, USA); Isolectin B_4 (Alexa Fluor 594 conjugated I21413, Invitrogen, Carlsbad, CA); anti-phospho-AKT, anti-phospho-ERK, anti-AKT, anti-ERK, 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).

3. Animals

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 (SNU-141008-2-2). C57BL/6J mice were purchased from the Central Laboratory Animal Inc. (Seoul, Korea). Eyes were collected under deep anesthesia and immediately frozen at -80 ℃ for quantitative RT-PCR, or were fixed in 4% paraformaldehyde for the estimation of retinal neovascularization.

4. Oxygen-induced retinopathy

To induce pathological retinal neovascularization, 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 daily from P12 to P16, and retinal vascularization was evaluated at P17.

5. Retinal neovascularization

Mice were euthanized by cervical dislocation, and the eyes were collected and fixed in 4% paraformaldehyde. The retinas were dissected and stained with Isolectin B_4 in 1 mM $CaCl_2$ in PBS (1:100 dilution). Then, the retinas were flat-mounted and viewed using a fluorescent microscope (Carl Zeiss, Jena, Germany).

6. Endothelial permeability assay

Permeability was measured by spectrophotometric assay of the flux of Evans blue (Sigma)-labeled bovine serum albumin (BSA; Sigma) across cell monolayers using a dual-chamber. HUVECs or pericytes were first seeded onto the abluminal side of the inverted transwell filter (Costar) at a density of 2×10^4 cells and allowed to adhere for 2 h. Then, the filter was flipped back and the HUVECs or pericytes were cultured for 1 day. HUVECs were then seeded onto the luminal side of the transwell filter at a density of 10^5 cells and co-cultured with HUVECs or pericytes in normoxic and hypoxic conditions, with or without propranolol, for an additional 48 h. Permeability was assayed using 0.67 mg/mL Evans blue dye diluted in growth medium containing BSA (40 mg/mL). Fresh growth medium was added to the lower chamber, and the medium in the upper chamber was replaced with Evans blue/BSA. After 10 min, the optical density of the lower chamber was spectrophotometrically measured at 650 nm (Tecan, Infinite M200PRO).

7. BrdU ELISA proliferation assay

To determine the proliferation of cells, the Cell Proliferation ELISA, BrdU assay (Roche) was used according to the manufacturer's protocol. Cells were cultured in 96-well plates and exposed to propranolol for 48 h. The cells were the labeled with 10 μM BrdU for 1 h and incubated with FixDenat solution for 30 min. The anti-BrdU peroxidase-conjugated antibody was added to the cells and incubated for 90 min at room temperature. After three washing steps, the bound peroxidase was detected by the subsequent substrate reaction. The measurements were performed on an ELISA plate reader at 450 nm. Three independent experiments were performed for each experimental condition.

8. Cell viability assay

To evaluate cell viability, the Sigma-Aldrich MTT labeling kit was used. In all experiments, 5×10^3 cells were seeded into 96-well plates. After 24 h, cells were treated with propranolol under normoxic conditions. The cells were then incubated with 100 μL of MTT (5 mg/mL) for 3 h. After solubilization of the blue formazan crystals with acidified isopropanol, the formazan levels were calculated following measurement of the absorbance intensity at 570 nm. Three independent experiments were performed for each experimental condition.

9. FACS analysis

In order to evaluate apoptosis, 5×10^5 cells were treated with propranolol (50 μM) 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.

10. 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.

11. Quantitative RT-PCR

RNAs were collected and isolated from cells using the RNeasy Plus Mini kit (Qiagen, Valencia, CA, USA). cDNAs were prepared from RNAs (1 μg) using 2.5 μM oligo-dT primers, 1 mM dNTPs, and MuLV reverse transcriptase. Quantitative real-time PCR (qPCR) assays were performed with SYBR Green PCR Master MIX (Applied Biosystems, Life Technologies, Gaithersburg, MD,

USA) using a 7900HT real-time PCR thermocycler (Applied Biosystems). The qPCR reaction conditions were 50 cycles of 95 °C for 5 s and 60 °C for 20 s. Quantitative real-time PCR was performed using the following primers: mouse *Vegfa* (forward: 5′-CCAAGATCCGCAGACGTGTA-3′; reverse: 5′-GCGTGGTGGTGACATGGTTA-3′), and mouse *Actb* (forward: 5′-CCAGGCATTGCTGACAGGAT-3′; reverse: 5′-AGCCACCGATCCACACAGAG-3′). The mean quantity was calculated from triplicate qPCR experiments for each sample and normalized to the control gene.

12. Western blot

Cells were harvested and lysed in radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail. Protein lysates were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were incubated with primary antibodies (1:1000) at 4 °C overnight and secondary antibodies (1:5000) at room temperature for 1 h. The membranes were incubated with enhanced chemiluminescence substrate (Pierce, IL, USA) and exposed to film.

13. Statistical analysis

Statistical analyses were performed using a standard two-tailed Student's *t*-test assuming unequal variances, and $P < 0.05$ was considered statistically

significant. Quantitative data and figures are reported as the mean ± SD.

RESULTS

Propranolol reduces pericyte viability

The MTT assay was used to determine the effect of propranolol on the cell viability of ECs and pericytes. Pericyte viability was dose-dependently reduced upon treatment with propranolol (Fig. 1A). The dose of propranolol required to reduce pericyte cell viability by 50% (ED50) was found to be approximately 50 μM (Fig. 1A). Pericyte viability was also reduced upon treatment with propranolol in a time-dependent manner (Fig. 1B). In contrast, propranolol exerted a smaller reduction on HUVEC viability than pericyte viability (Fig. 1C). At the ED50 for pericyte viability, propranolol only reduced HUVEC viability by 20% (Fig. 1C). Propranolol also reduced HUVEC viability in a time-dependent manner (Fig. 1D). These results indicated that pericytes are more sensitive to propranolol than HUVECs.

Propranolol reduces pericyte survival and proliferation through the inhibition of the AKT and ERK signaling pathways

Next, I determined how propranolol affected apoptosis and proliferation in pericytes and HUVECs under normoxic and hypoxic conditions. To observe cell apoptosis and cell proliferation, annexin V/PI flow cytometric analysis and the BrdU cell proliferation assay were performed, respectively. Hypoxia alone was sufficient to induce pericyte apoptosis (Fig. 2A); however, propranolol synergistically induced pericyte apoptosis in both normoxic and hypoxic

conditions (Fig. 2A). Western blot analysis showed that propranolol increased cleaved PARP expression in pericytes, which is a marker of cell apoptosis (Fig. 2B). Although hypoxia alone did not affect pericyte proliferation, propranolol significantly reduced pericyte proliferation in both normoxic and hypoxic conditions (Fig. 2C). Propranolol also inhibited HUVEC proliferation in both normoxic and hypoxic conditions (Fig. 2F), but did not induce any changes in HUVEC apoptosis (Figs 2D, E).

To identify the mechanisms that mediate propranolol-induced changes in pericyte apoptosis and proliferation, I used a phospho-kinase array, which demonstrated that propranolol reduced AKT and ERK phosphorylation in pericytes (Fig. 3A). To confirm the effect of propranolol on the AKT and ERK signaling pathways, I used western blot analysis to examine the phosphorylation of AKT and ERK proteins in pericytes following treatment with propranolol. Propranolol reduced AKT and ERK phosphorylation in a time-dependent manner (Fig. 3B), and also reduced AKT and ERK phosphorylation in pericytes in both normoxic and hypoxic conditions (Fig. 3C). In contrast, propranolol did not affect AKT and ERK phosphorylation in HUVECs (Fig. 3D), but inhibited HUVEC proliferation by triggering G1 and G2 cell cycle arrest (Fig. 3E). These results indicated that propranolol preferentially increased pericyte apoptosis and suppressed pericyte proliferation through the inhibition of the AKT and ERK signaling pathways.

Propranolol increases vascular permeability and aggravates

neovascularization in oxygen-induced retinopathy

To determine the effect of propranolol on vascular permeability, I performed *in vitro* cell permeability assays (Figs 4A, B). First, I examined whether pericytes prevented endothelial cell permeability in an *in vitro* cell permeability assay. In a transwell cultured with cells in both the apical and basolateral sides, the extent of cell permeability with pericytes in the basolateral side was not significantly different from that with HUVECs in the basolateral side (Fig. 4A). However, the cell permeability with co-cultured with HUVECs and pericytes was lower than that with HUVECs in both sides of the transwell (Fig. 4A). These results indicated that pericytes have the capacity to reduce HUVEC permeability. Following the addition of propranolol to these experimental settings, the cell permeability of co-cultured HUVECs and pericytes was preferentially increased (Fig. 4B). Propranolol did not increase the cell permeability with cultured HUVECs in both sides of the transwell (Fig. 4B). These results indicated the importance of pericytes in controlling the EC barrier and that propranolol preferentially increased the vascular permeability by more sensitively affecting pericyte survival than EC.

Next, to examine the effect of propranolol on the neovascularization of OIR retinas in mice, I performed Isolectin B_4 immunofluorescence staining of the retinas of OIR mice (Fig. 5A). After the induction of OIR, mouse pups were intraperitoneally injected with propranolol from P12 to P16. Saline injected mice were used as the control. Compared to control retinas, OIR retinas showed increased levels of neovascularization and vaso-obliteration (Figs 5A-C). In

addition, OIR retinas with propranolol treatment significantly increased neovascularization and vaso-obliteration compared with OIR retinas without propranolol treatment (Figs 5A-C). Propranolol did not affect *Vegfa* mRNA in OIR mouse retinas (Fig. 5D). These results indicated that propranolol aggravated pathological neovascularization in retinas of OIR mice.

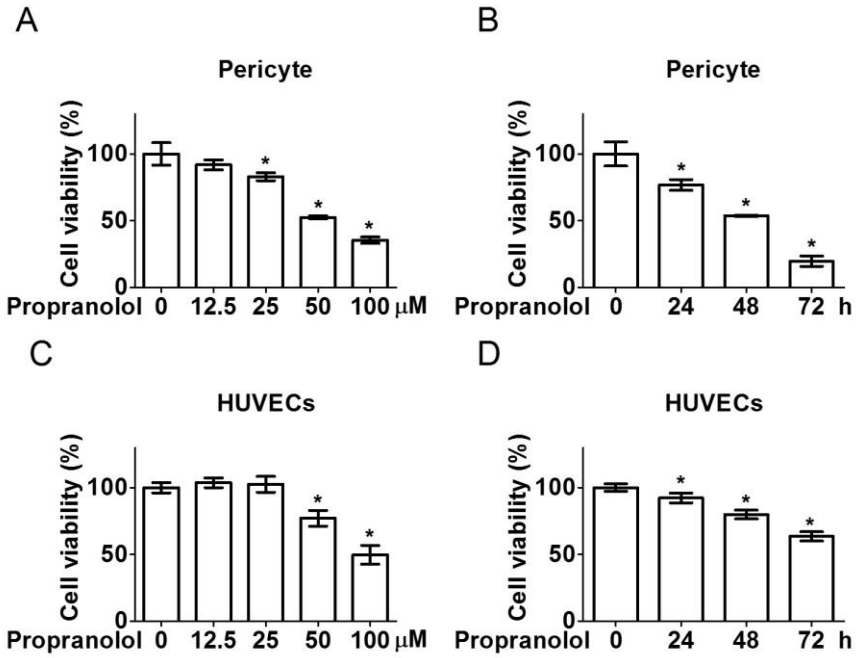

Figure 1. Propranolol reduces pericyte viability.

The effects of propranolol on the cell viability of pericytes and HUVECs were determined. Pericytes and HUVECs were incubated for 48 h with propranolol in the range of 12.5-100 μM (A, C) and compared with the control. Pericytes and HUVECs were incubated with 50 μM propranolol for 24, 48, and 72 h (B, D). Cell viability was assessed by MTT assay (A-D). The bar graphs represent the mean ± SD of three independent experiments. * $P < 0.05$ by Student's *t*-test.

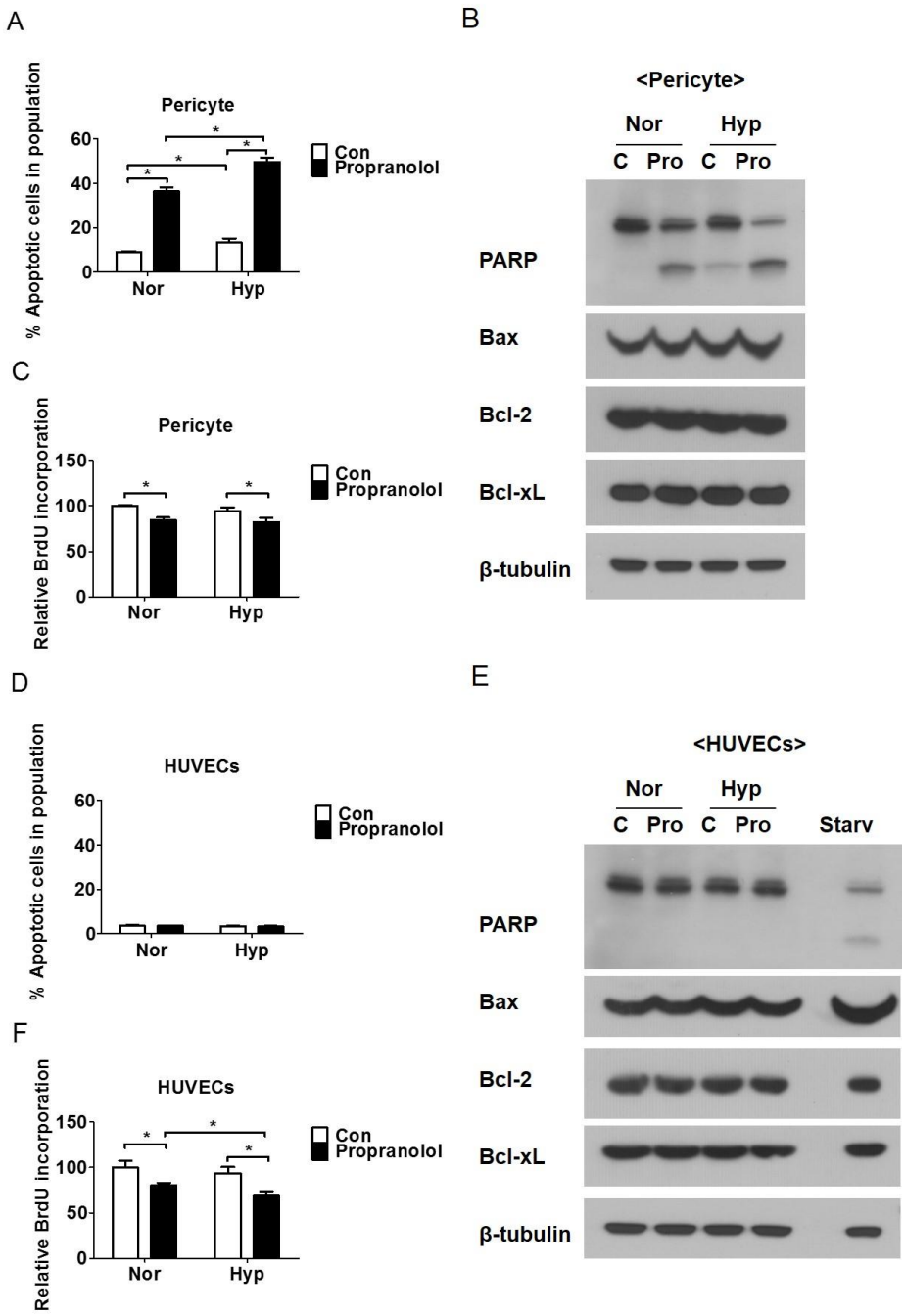
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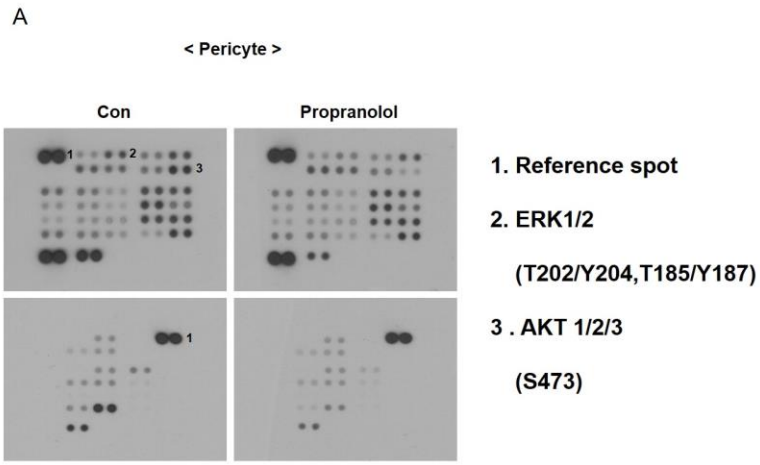
Figure 2. Propranolol reduces pericyte survival and proliferation.

(A-F) The effects of propranolol on the survival and proliferation of pericytes and HUVECs under normoxic and hypoxic (1% O_2) conditions were determined. Pericytes and HUVECs were incubated for 48 h with or without 50 μM propranolol in normoxic and hypoxic conditions. (A, D) Pericytes and HUVECs were stained with annexin V FITC and PI and analyzed by flow cytometry. Cell apoptosis was expressed as the percentage of apoptotic cells in the total cell population. The bar graphs represent the mean ± SD of three independent experiments. (B, E) Western blot analysis of cleaved PARP, Bax, Bcl-2, and Bcl-xL was performed on lysates of pericytes and HUVECs obtained from the same experimental procedures. Serum starvation for 48 h was used as a positive control for the apoptosis of HUVECs (E). β-Tubulin was used the loading control. (C, F) Cell proliferation was measured by a BrdU incorporation assay. The bar graphs represent the mean ± SD of three independent experiments. * $P < 0.05$ by Student's *t*-test.

A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Propranolol
1
2
3
1
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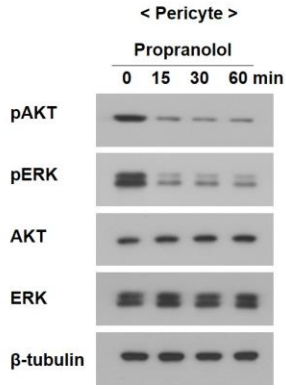
3 . AKT 1/2/3
(S473)

B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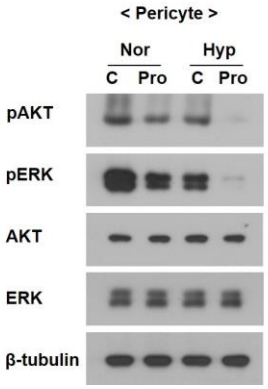
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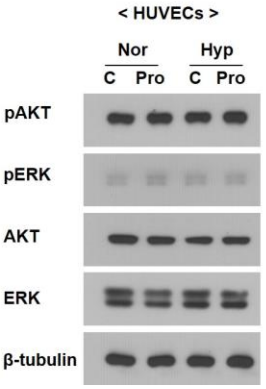
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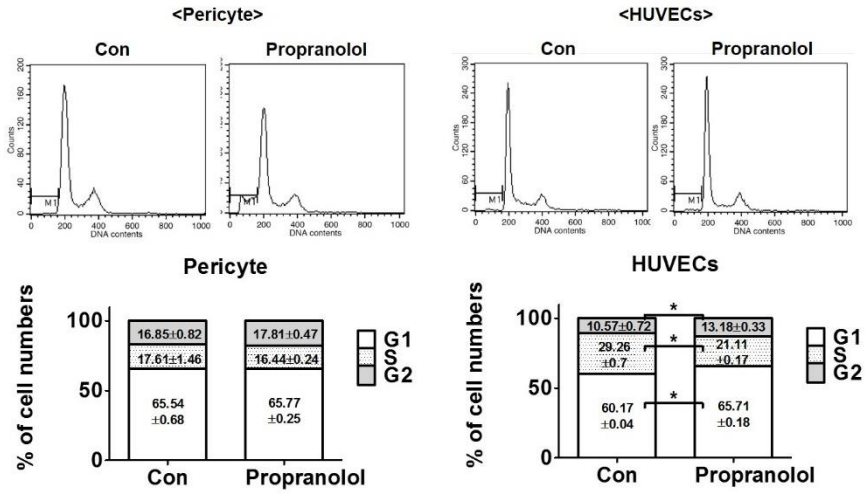
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M1
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0
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17.61±1.46
65.54
±0.68
17.81±0.47
16.44±0.24
65.77
±0.25
10.57±0.72
29.26
±0.7
60.17
±0.04
13.18±0.33
21.11
±0.17
65.71
±0.18
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G1
S
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Figure 3. Propranolol reduces pericyte survival and proliferation by inhibition of the AKT and ERK signaling pathways.

(A) Expression of protein phosphorylation was investigated in propranolol-induced pericytes for 30 min compared with control (Con) pericytes using a phospho-kinase antibody array kit. (B) Western blot analysis for phospho-AKT (pAKT), phospho-ERK (pERK), AKT, and ERK was performed on lysates obtained from pericytes treated with 50 μM propranolol for 15, 30, and 60 min. (C, D) Western blot analysis of pAKT, pERK, AKT, and ERK was performed on lysates obtained from pericytes and HUVECs treated with propranolol for 30 min in normoxic and hypoxic (1% O_2) conditions. The protein β-tubulin was used as a loading control. (E) The cell cycle was analyzed by flow cytometry. Pericytes and HUVECs were incubated with propranolol for 48 h in normoxic conditions and cellular DNA was labeled with propidium iodide and analyzed. The graph represents the mean ± SD of three independent experiments. * $P < 0.05$ by Student's *t*-test.

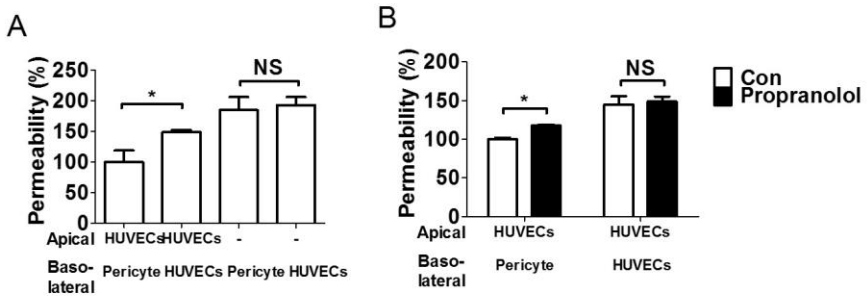

Figure 4. Propranolol increases vascular permeability.

Permeability across functional EC layers was determined by measurement of the absorbance at 650 nm with a spectrophotometer. (A) The permeability of HUVECs co-cultured with pericytes, HUVECs co-cultured with HUVECs, HUVEC monolayers, and pericyte monolayers was estimated. Permeability was expressed as a percentage relative to the HUVECs co-cultured with pericytes. (B) The permeability of HUVECs was estimated after co-culture with pericytes or HUVECs with or without 50 μM propranolol for 48 h. The permeability was expressed as a percentage relative to the co-culture of HUVECs with pericytes without propranolol treatment. The bar graph represents the mean ± SD of three independent experiments. * $P < 0.05$ by Student's *t*-test. NS indicates not significant (* $P > 0.05$).

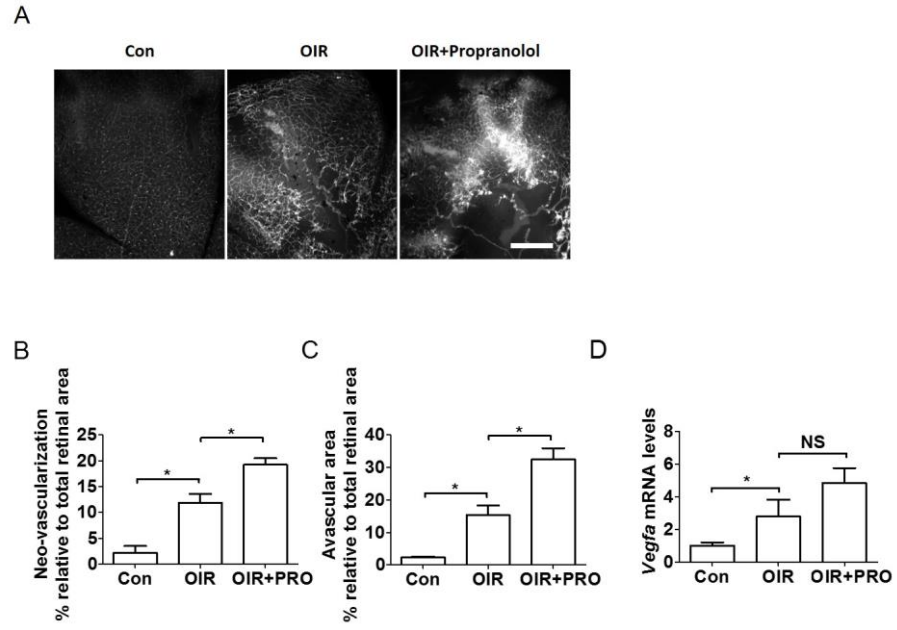

Figure 5. Propranolol aggravates neovascularization in oxygen-induced retinopathy.

(A) Representative images of retinal flat mounts from P17 mice with oxygen-induced retinopathy treated with vehicle or propranolol (2 mg/kg/day) daily from P12 to P16. Retinal flat mounts were stained with Alexa Fluor 594 conjugated Isolectin B4. Original magnification ×50. Scale bar, 400μm. (B, C) The extent of the neovascularization area (neovascular tuft) and avascular area (vaso-obliteration) were quantitatively evaluated as a percentage of the total retinal area. * $P < 0.05$ by Student's *t*-test. (D) qRT-PCR for *Vegfa* expression was performed on lysates obtained from P17 mice with oxygen-induced retinopathy treated with propranolol (IP, 2 mg/kg/day) or vehicle control daily

from P12 to P16. *Vegfa* mRNA levels were normalized to *Actb* mRNA and reported as the fold induction compared with the control. The bar graph represents the mean ± SD. * $P < 0.05$ by Student's *t*-test. NS indicates not significant (* $P > 0.05$).

DISCUSSION

In this study, I examined the suitability of propranolol in the treatment of ROP disease. Previous studies have shown that propranolol reduced neovascularization and vascular leakage in the OIR mouse model by downregulation of VEGF expression [17]. In addition, propranolol inhibited EC proliferation and reduced the increased VEGF levels in ECs [46, 47]. In the present study, although propranolol reduced HUVEC proliferation (Fig. 2F), propranolol aggravated neovascularization in the retinas of OIR mice (Figs 5A-C). In contrast to previous reports, propranolol did not reduce VEGF expression in the retinas of OIR mice (Fig. 5D). This is consistent with a previous study, which showed that a high dose of propranolol worsened both the regrowth of normal vessels and pathological neovascularization in OIR [43], and that propranolol did not reduce VEGF levels in the retinas of diabetic rats and OIR mice [2, 43]. Based on the findings that propranolol inhibited EC proliferation (Fig. 2F), but aggravated neovascularization in the retinas of OIR mice (Figs 5A-C) even at similar conditions of VEGF levels in the retinas of OIR mice (Fig. 5D), I hypothesized that propranolol may affect pericytes, which regulate EC proliferation and neovascularization.

The vascular layers of retinas consist mainly of ECs, pericytes, astrocytes, and microglia [48]. Pericytes are important in the prevention of vascular leakage by surrounding ECs [21]. In addition, neovascularization occurs at the site of pericyte loss through proliferating ECs [15]. Thus, pericytes contribute

to the regulation of neovascularization in pathological retinal conditions. Elevated vascular leakage and neovascularization observed in ROP patients [49, 50] emphasized the role of pericytes in the development of ROP. In ROP cases in which patients experience pericyte loss, vascular leakage and neovascularization may be exacerbated. Therefore, I hypothesized that propranolol may induce pericyte loss in retinas and exacerbate the symptoms of ROP.

Propranolol is a nonselective β_1- and β_2-antagonist. β-Adrenergic receptors are expressed in pericytes as well as in ECs [51-54]. However, the effects of propranolol on pericytes are largely unknown. In this study, I found that propranolol more significantly affected the cell viability of pericytes than ECs (Figs 1A-D). At a dose able to induce pericyte apoptosis (Figs 2A, B), propranolol did not induce EC apoptosis (Figs 2D, E). In accordance with a previous study [54], I also found that propranolol inhibited pericyte proliferation (Fig. 2C). Moreover, propranolol significantly reduced AKT and ERK phosphorylation in pericytes (Figs 3A-C). Given that AKT and ERK signaling pathways are involved in pericyte survival and proliferation [55], I proposed that propranolol inhibited pericyte survival and proliferation via the AKT and ERK signaling pathways.

Infantile hemangioma (IH) is the most common vascular tumor in children, and occurs in 5-10% of all infants [56] and up to 30% of premature infants [57]. The treatment options for IH are corticosteroids, surgical excision, vincristine, interferon, or cyclophosphamide [58, 59]. However, these treatments have

limitations and severe side effects [58]. Since 2008, propranolol has been a feature of IH treatment because it was reported that propranolol effectively reduced hemangioma growth with few side effects [42]. Some IH patients who were treated with propranolol experienced adverse side effects such as apneas, bradycardias, hypotension, and hypoglycemia [60], and removal of these side effects would be beneficial to safe treatments. Previously, it was known that IH was associated with the occurrence and severity of ROP in preterm infants [61]. Thus, IH patients would be more likely to have a high occurrence of ROP in preterm neonates. However, based on the present study, propranolol offers a high possibility to aggravate retinopathy in ROP patients. Thus, I suggested that propranolol treatment for IH may aggravate retinopathy, because IH is more likely to accompany ROP.

In conclusion, I demonstrated that propranolol treatment aggravated the vascular leakage and neovascularization by pericyte loss in OIR. This is the first demonstration of the toxic effects of propranolol in retinas, and indicated that propranolol was not suitable for the treatment of ROP.

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국문 초록

망막병증은 망막에 지속적이거나 심각한 손상이 생겨 발생하는 질병이다. 망막병증의 대표적인 질병으로는 당뇨병 망막병증과 미숙아 망막병증이 있다. 당뇨병 망막병증과 미숙아 망막병증의 주된 원인은 비정상적인 새로운 혈관생성과 혈액누수이다. 망막에서의 혈관내피성장인자 (VEGF)의 증가 또는 혈관주위세포 (pericyte)의 결손이 당뇨병 망막병증과 미숙아 망막병증에서 발생하는 혈액누수와 혈관신생을 유발시키는 원인으로 알려져 있다. 따라서 혈관주위세포의 결손을 막는 것이 망막병증의 치료제로 사용 될 수 있다. 또한 교감신경전달 결손이 마우스와 랫 망막의 혈관주위세포의 결손을 유발시킬 수 있다는 보고와 같이 베타-아드레날린 수용체 신호전달이 혈관주위세포에 중요한 역할을 줄 수 있다는 관련 보고들이 나오고 있다. 이 연구에서는 망막 질환들에서 베타-아드레날린 수용체 작용제와 길항제가 혈관주위세포 결손에 미치는 영향에 대해 알아보았다. 베타-아드레날린 수용체 작용제는 당뇨병 망막에서 증가된다고 알려진 고혈당, Ang2, TNF-α에 의해 유도되는 혈관주위세포의 세포사멸을 막아주었다. 또한 베타-작용제는 고혈당에서 혈관주위세포의 증식을 유도하였다. 반대로 베타-아드레날린 수용체 길항제는 저산소 환경에서 오히려 혈관주위세포의 세포사멸을 유도하

였고 증식을 억제시켰다. 베타-작용제는 혈관내피세포의 생존과 증식에는 영향을 주지 않았으며, 베타-길항제는 오직 혈관내피세포의 증식에만 약간의 감소를 유도하였다. 베타-작용제와 길항제 모두는 PI3K/AKT 와 MAPK/ERK pathway 를 통해 혈관주위세포의 생존과 증식에 관여하였다. 재미있게도, 베타 2-작용제가 베타 1-작용제보다 효과적으로 혈관주위세포의 생존과 증식에 관여하였데 이는 혈관주위세포에 존재하는 베타 2-수용체가 베타 1-수용체보다 고혈당에서 많이 발현되어 있기 때문이었다. 또한 베타 2-작용제는 당뇨 마우스 모델에서 효과적으로 혈관주위세포의 결손과 혈액누수를 막았다. 반면 베타-길항제는 OIR 모델에서 혈관주위세포의 결손을 유도시킴으로서 오히려 혈액누수와 혈관신생을 악화시켰다. 이것으로 베타 2-작용제는 혈관주위세포의 생존과 증식을 통해 당뇨시 망막에서의 혈관주위세포 결손을 막아주기 때문에 당뇨병 망막병증 치료제로 이용가능함을 알 수 있었다. 반면 베타-길항제는 혈관주위세포 결손을 유도시키기 때문에 미숙아 망막병증 치료제로 적절하지 않으며, 오히려 미숙아 망막병증 환자들이 베타-길항제 사용시 주의가 필요하다.

주요어 : 당뇨병 망막병증, 미숙아 망막병증, 베타-아드레날린 수용체 작용제, 베타-아드레날린 수용체 길항제, 혈관주위세포 결손, 혈액누수, 혈관신생
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