β2–Adrenergic Receptor Antagonism Attenuates CNV Through Inhibition of VEGF and IL-6 Expression

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METHODS. Mice were subjected to laser burns, inducing CNV, and were treated with an intravitreal β2-AR antagonist. After 3 and 5 days, total eye interleukin-6 (IL-6) and VEGF protein levels were measured, respectively. After 14 days, CNV was measured on choroidal–scleral flatmounts. The effects of β-AR signaling on VEGF and IL-6 expression were investigated in various mouse retinal and human RPE cells by using specific β-AR agonists and antagonists.

RESULTS. β2–Adrenergic receptor signaling increased Vegf mRNA expression by approximately 3- to 4-fold in mouse retinal microglia and pericytes in culture. β2–Adrenergic receptor signaling upregulated IL-6 mRNA expression between 10- and 60-fold in mouse retinal microglia, pericytes, RPE, and choroidal endothelial cells in culture. Intravitreal injection of β2-AR antagonist ICI 118,551 reduced CNV by 35% and decreased IL6 protein levels by approximately 50%. In primary human RPE cells, β2-AR activation also stimulated VEGF and IL-6 mRNA expression by 2- and 10-fold, respectively.

CONCLUSIONS. Anti-VEGF therapy for CNV is highly effective; however, some patients are resistant to therapy while others undergo repeated, frequent treatments. β2–Adrenergic receptor signaling is a potential therapeutic target because of its angiogenic and inflammatory properties.

Keywords: adrenergic antagonists, choroidal neovascularization, interleukin-6, VEGF

Exudative age-related macular degeneration (AMD) and diabetic retinopathy are leading causes of severe visual disability. Inhibition of vascular endothelial growth factor (VEGF) is the mainstay of treatment for both neovascular AMD1,2 and diabetic macular edema (DME).3,4 Despite these significant advances, several challenges remain in the treatment of both exudative AMD and DME. First, many patients require frequent and repeated intravitreal injections. Second, anti-VEGF treatment is possibly associated with systemic thromboembolic events5,6 and local adverse events including RPE tears7 and endophthalmitis.8 Third, some patients demonstrate resistance or tachyphylaxis toward anti-VEGF medications. Lastly, a small minority of patients do not respond to anti-VEGF therapy alone. These limitations have sparked investigations into new therapeutics targets and new modalities for inhibition of neovascularization.

Propranolol, a nonspecific β-adrenergic receptor (β-AR) antagonist, has become the gold standard for treatment of severe hemangioma of infancy.9 Tumor regressive properties from propranolol treatment stem from its ability to inhibit VEGF expression.10 Additionally, β2-AR signaling stimulates angiogenesis in chronic ischemia11 and propranolol inhibits endothelial tubulogenesis.12 These findings have resulted in research into the utility of β-AR antagonism in retinal neovascular diseases. In the oxygen-induced ischemic retinopathy (OIR) mouse, both propranolol treatment and specific β2-AR blockade cause reduced retinal neovascularization and VEGF expression.13,14 In retrospective clinical investigations, β-blocker treatment is correlated with reduced numbers of anti-VEGF injections in exudative AMD15 and fewer laser procedures in diabetic retinopathy.16 Recently, in patients with persistent retinal fluid despite maximal anti-VEGF therapy for exudative AMD, topical timolol-dorzolamide treatment in addition to anti-VEGF therapy improved retinal fluid.17 These studies led us to investigate the role of β-blockers in a laser-induced CNV model. In our prior study,18 we have found that intraperitoneal propranolol treatment reduces CNV area by 50%, and specific β2-AR blockade decreases VEGF expression in mouse choroidal endothelial and RPE cells in culture.

In the current follow-up study, we sought to determine if specific intravitreal β2-AR blockade inhibits CNV in mice. We next investigated the role of β-blocker treatment in the expression of VEGF and inflammatory cytokines in mouse retinal microglia, retinal pericytes, and choroidal endothelial and RPE cells in culture. And finally, we extended these results to human feral RPE cells in culture.
### MATERIALS AND METHODS

#### Reagents

Norepinephrine (NE) and propranolol were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Specific β-AR agonists and antagonists were purchased from Tocris (R&D Systems, Minneapolis, MN, USA). Table 1 summarizes the catalog numbers, concentrations, and vehicles used for each compound. The inhibition and activation constant concentrations (K) are also provided. The used concentrations in this study were chosen to maximize specific inhibition of each receptor.

#### Animals

All research using mouse models of CNV was carried out in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the Institutional Animal Use and Care Committee of the University of Wisconsin School of Medicine and Public Health. Wild-type 6-week-old female C57BL/6j mice were housed on a 12-hour light–dark cycle and provided with food and water ad libitum. Laser-induced CNV experiments were performed as previously described.23 Briefly, 15 female mice were anesthetized and treated with three focal laser burns in each eye (30 retinas treated per group, 90 total burns per group). ICI 118,551 was dissolved in saline and delivered once approximately 30 minutes after laser treatment via intravitreal injection of 2 μL. For mRNA analysis, cells were preincubated with β-AR agonists for 2 hours in 24-well plates. Cells were then washed by DMG's laboratory in 70% Dulbecco's modified Eagle's medium containing 4.5 g/L D-glucose (catalog No. 11965; Invitrogen, Carlsbad, CA, USA). Synthesis of cDNA and quantitative PCR was performed identically as for various mouse cells (see Table 2 for primers).

#### Isolation and Culture of Human Fetal RPE Cells

Human fetal eyes were obtained from University of Washington Birth Defects Laboratory. Human fetal RPE cells were isolated as previously described.23 Human fetal RPE cells were cultured by DMG's laboratory in 70% Dulbecco’s modified Eagle’s medium containing 4.5 g/L D-glucose (catalog No. 11965; Invitrogen, Carlsbad, CA, USA), 30% F12 nutrient mixture containing L-glutamine (catalog No. 11765; Invitrogen), 1% antibiotic-antimycotic solution (catalog No. 1420; Invitrogen), and B27 (50X solution, catalog No. 17504; Invitrogen). Human fetal RPE cells were at passage 2 to 3. Cells were transferred to NS’s laboratory where they were cultured at 37°C with 5% CO2. Cells were not serum starved overnight, as they are cultured without serum. Human fetal RPE cells were treated with β-adrenergic agonists for 2 hours. RNA was extracted by using the Trizol RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). Synthesis of CDNA and quantitative PCR was performed identically as for various mouse cells (see Table 2 for primers).

#### Messenger RNA Analysis

For mRNA analysis, cells were preincubated with β-AR antagonists for 30 minutes before incubation with β-AR agonists for 2 hours in 24-well plates. Cells were then washed with 1X phosphate-buffered saline (PBS; Sigma), lysed in RLT plus (a guanidine-rich buffer; Qiagen), and frozen at −20°C.

#### Isolation and Culture of Choroidal and Retinal Endothelial Cells, Pericytes, Astrocytes, and RPE Cells

Choroidal endothelial, RPE cells, retinal endothelial cells, retinal astrocytes, and pericytes were isolated and cultured as previously described by us. All experiments were performed on cells between passage 5 and passage 15. All cells were maintained at 33°C with 5% CO2. All cells except for retinal pericytes were cultured on 1% gelatin-coated 60-mm dishes. Cells were not allowed to grow beyond 20 passages. Before experiments, cells were serum starved overnight in serum-free medium. Serum-free medium was identical to growth medium described previously except it lacked 10% FBS.

#### FIGURE 1. β2-Adrenergic receptor blockade attenuates CNV formation in mice. Mice were given a single intravitreal injection of saline (veh) or β2-AR antagonist (ICI-118,551, 0.03 μg per eye) on the same day as laser treatment. Choroidal neovascularization area was measured by ICAM-2 staining after 14 days (N = 27–29, **P < 0.01).
Messenger RNA was extracted by using RNaseasy Plus Mini Kit (Qiagen). The cDNA was synthesized by using Sprint RT Complete-Double PrePrimed (Clontech, Mountain View, CA, USA). Cytokine mRNAs were measured by quantitative PCR (Eppendorf, Hauppauge, NY, USA) and normalized to the housekeeping gene RpL13A by generating a DCt value. Primer sequences can be found in the following references or Table 2.18,24 Fold values were generated by normalizing to the vehicle control. Vehicle control samples were used to assay for baseline levels of β-AR.

Enzyme-Linked Immunosorbent Assay

Laser-induced CNV experiments were performed as described above. Four female mice per group were killed and eyes were harvested at days 3 or 5 post laser treatments. Eyes were combined from each animal to maximize protein yield. Whole eye tissue was homogenized and solubilized in ice-cold PBS buffer containing protease inhibitor (catalog No. 11836153001; Roche Biochemicals, Mannheim, Germany). The collected samples at day 3 post laser treatment were assayed for IL-6 protein by using mouse IL-6 ELISA kit (R&D Systems). Samples from day 5 post laser treatment were used for VEGF measurements with the mouse VEGF ELISA kit (R&D Systems).

Statistical Analysis

For CNV, gene expression comparisons between cell lines, and ELISA, Student’s unpaired t-test was performed. For cell

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culture, each biological N was generated by an experiment on a unique passage day. Thus, Student’s paired t-test (two-tailed) was performed to compare two groups. For multiple comparisons, repeated-measures ANOVA was performed and posttests were done by using Bonferroni’s correction for multiple comparisons.

RESULTS

We have previously reported that daily intraperitoneal propranolol treatment reduces CNV area in the laser-induced mouse model. Using mouse RPE and choroidal endothelial cells (ChECs), we then demonstrated that both propranolol and specific β2-AR antagonism inhibit NE-induced VEGF expression in these cells. Our first aim of this study was to extend these results in vivo, showing that β2-blockade can inhibit CNV. For this study, we used a single intravitreal injection of the specific β2-AR antagonist ICI-118,551 at a dose of 0.03 μg per eye. This dose was chosen because we have previously demonstrated that a single intravitreal dose of propranolol at 0.3 μg per eye (0.03 mg/mL) could inhibit CNV in mice and is nontoxic in rabbits. In an initial pilot study, we performed a dose-escalation series by using 0.003 μg, 0.03 μg, and 0.3 μg ICI-118,551 per eye (not shown). We found that 0.03 μg per eye inhibited CNV formation. We then repeated

FIGURE 3. β2-Adrenergic receptor signaling upregulates VEGF expression in retinal microglia and pericytes. (A–B) Mouse retinal microglia and pericytes were preincubated with 1 μM propranolol for 30 minutes followed by incubation with vehicle (veh) or 10 μM NE for 2 hours (N = 4–5, *P < 0.05, ***P < 0.001 versus vehicle, #P < 0.05, ###P < 0.001 versus NE and vehicle). (C–D) Mouse retinal microglia and pericytes were preincubated with 1 μM β1 or 100 nM β2 and β3 antagonists for 30 minutes before 2-hour incubation with vehicle or 10 μM NE (N = 4–5, *P < 0.05, ***P < 0.001 versus vehicle, #P < 0.05, ###P < 0.001 versus NE). (E–F) Microglia and pericytes were incubated with 100 nM β1 and β2 or 1 μM β3 agonists for 2 hours (N = 4–5, *P < 0.05, ***P < 0.001 compared to vehicle).
this study and found that 0.03 μg ICI-118,551 inhibited CNV formation by 35% (Fig. 1).

Retinal endothelial cells,26 pericytes,27 microglia,28 Müller cells,29 and astrocytes29 are all sources of VEGF expression. In diabetic retinopathy, pericyte loss is the hallmark of early disease,30 Müller cells are key pathologic sources of VEGF expression,31 and microglia are important in the pathologic progression of diabetic eye disease.32,33 Therefore, we investigated the role of β-AR stimulation and VEGF expression in mouse retinal endothelial cells (RECs), retinal pericytes, retinal microglia, and retinal astrocytes (RASTs). The RASTs used in this study have characteristics of both astrocytes and Müller cells.21 We found that NE increased Vegf mRNA expression by 4.5- and 3.0-fold in retinal microglia and pericytes, respectively (Fig. 2A). Alternatively, NE had no effect on Vegf mRNA expression in RECs and RASTs (Fig. 2A). All four types of mouse retinal cells expressed all three β-AR types (Figs. 2B–D).

To determine which β-AR drives Vegf expression in retinal microglia and pericytes, we pretreated retinal microglia and pericytes with propranolol before NE stimulation. Propranolol completely blocked NE-stimulated Vegf expression in both cell types (Figs. 3A, 3B). Next, retinal microglia and pericytes were pretreated with specific β-AR antagonists before NE administration. The β1-AR antagonist had no effect on NE-driven Vegf expression (Figs. 3C, 3D). Alternatively, the β2- and β3-AR blockers reduced Vegf expression, compared to NE, although more completely in the presence of the β2-AR antagonist (Figs. 3C, 3D). To confirm this result, retinal microglia and pericytes were incubated with β-AR-specific agonists. Only the β2-AR agonist significantly increased Vegf expression, compared to vehicle, while the β3-AR agonist demonstrated only a trend in both cell types (Figs. 3E, 3F). In summary, the β2-AR predominantly regulated Vegf expression in retinal microglia and pericytes, with modest effects from the β3-AR.

Since the laser-induced CNV model is a highly inflammatory process, we investigated the cytokine profile in retinal microglial cells incubated with NE. We found that after 2 hours of NE treatment, only Interleukin-6 (IL-6) mRNA was increased by 7-fold in microglial cells (Fig. 4A). To determine the β-AR responsible for NE-driven Il-6 expression, we pretreated retinal microglial cells with propranolol before NE stimulation. Propranolol significantly decreased NE-stimulated Il-6 expression (Fig. 4B). Next, we pretreated retinal microglial cells with β-AR–specific antagonists. The β2-AR blocker significantly reduced NE-stimulated Il-6 expression (Fig. 4C).

In cancer and endothelial cells, β-AR activation increases VEGF and IL-6 expression.34–36 Therefore, we hypothesized that the β2-AR also regulates IL-6 expression in ChECs, RPE cells, and pericytes. We tested this hypothesis by repeating the above experiments with propranolol, specific β-AR antagonists, and specific β-AR agonists. In the presence of propranolol, NE-stimulated Il-6 expression was completely blocked in ChECs, RPE cells, and pericytes (Figs. 5A, 5D, 5G). After
pretreatment with specific β-AR blockers, only the β2-AR antagonist significantly decreased IL-6 expression, compared to NE treatment, in ChECs, RPE cells, and pericytes (Figs. 5B, 5E, 5H). And finally, only the β2-AR agonist significantly increased IL-6 expression in ChECs, RPE cells, and pericytes were incubated with β-AR agonists identically to Figures 5C and 5F (N = 3–4). For all experiments *P < 0.05, **P < 0.01, ***P < 0.001 compared to vehicle, #P < 0.05, ##P < 0.01, ###P < 0.001 compared to NE.

As previously discussed, retrospective15,16 and prospective17 clinical trials have demonstrated the efficacy for β-blocker therapy in neovascular AMD and DME. To support these findings in vitro, we tested if β2-AR agonists could increase VEGF and IL-6 expression in primary human fetal RPE cells. We found that both NE and specific β2-AR agonists increased VEGF mRNA expression 2-fold (Fig. 7A). Similarly, NE and β2-AR activation elevated IL-6 expression 10-fold and 9-fold, after 5 days. We chose these time points from previously published results in the laser-induced CNV model, demonstrating these time points are the maximum levels of IL-637 and VEGF expression38 respectively. We found that VEGF protein was unchanged (Fig. 6A), while IL-6 expression was reduced by 38% (Fig. 6B).

To confirm these findings in vivo, we repeated the laser-induced CNV experiments in mice, comparing saline vehicle to intravitreal β2-AR antagonist ICI-118,551 at 0.03 μg per eye as described in Figure 1. We harvested eyes for total protein to measure IL-6 protein levels after 3 days and VEGF expression after 5 days.
Pranolol treatment in OIR mice reduces VEGF expression and pathologic retinal neovascularization. In follow-up studies, specific β2-AR inhibition similarly has decreased VEGF expression and neovascularization. Lastly, genetic deletion of the β1- and β2-AR mitigates retinal neovascularization in OIR mice. These studies demonstrate that β2-AR antagonism reduces neovascular retinal disease by both pharmacologic and genetic methods, supporting our results.

In retinal microglial cells and pericytes, we found that the β3-AR had an intermediate effect on VEGF and IL-6 expression. The β3-AR has previously been shown to affect VEGF expression and retinal neovascularization. In cultured retinal and choroidal endothelial cells, β3-AR agonism stimulates migration and proliferation. In cultured retinal explants, hypoxia increases β3-AR expression, and β3-AR signaling regulates VEGF expression. In wild-type and β1/β2-AR knockout OIR mice, β3-AR agonism increases VEGF expression and pathologic retinal neovascularization. These results suggest an additional role for the β3-AR in VEGF expression and neovascular retinal disease, in agreement with our results.

Despite the above findings, there are additional studies that disagree with our results. In a replicative study by a different group, propranolol has no effect on VEGF expression or retinal neovascularization in OIR mice. Other studies suggest protective roles for β-AR signaling in the retina. Genetic deletion of the β1-AR results in acellular capillaries and pericyte ghosts, hallmark signs of early nonproliferative diabetic retinopathy. Similarly, β2-AR knockout mice demonstrate reduced inner retinal thickness, increased inner retinal cell apoptosis, and reduced electroretinogram amplitudes. It is of note in this study that β2-AR knockout mice on a mixed B6/129 background were compared to wild-type B6 mice. Additionally, treatment with a combined β1- and β2-AR agonist in β2-AR knockout mice on a mixed background increases retinal thickness and restores electroretinogram changes. And finally, treatment with the same β1/β2-AR agonist in diabetic rats reduces acellular capillaries and pericyte ghosts, increases retinal thickness, and normalizes the electroretinogram. The major difference between these studies and our results are the mixed background mouse strains and the chronic nature of the treatments. Comparison between mixed B6/129 mice and B6 mice could certainly introduce strain-dependent effects, which are independent of the β2-AR. Additionally, chronic loss of β-AR signaling through genetic deletion or long-term β-AR agonism via pharmacologic treatment is a different experimental system from acute β-AR blockade through a single intravitreal injection.
We found that intravitreal β2-AR antagonism did not reduce VEGF protein levels in the laser-induced CNV mouse. This study was likely limited by the use of total eye protein rather than retina or choroidal protein only. Additionally, all of our in vitro effect sizes were much larger for IL-6 than VEGF, consistent with this finding. Propranolol and β2-AR antagonism have been previously shown to reduce VEGF protein levels in the OIR mouse. Studies using either retina and choroidal protein only or with immunohistochemical methods to identify the in vivo cell types responsible will be subject of future studies in our laboratory.

The laser-induced CNV mouse model is a highly inflammatory model. We therefore investigated many cytokines and their regulation by β-AR signaling in microglial cells. We found that only IL-6 mRNA expression was significantly upregulated by NE treatment. Furthermore, β2-AR signaling regulated IL-6 mRNA expression in mouse retinal pericytes, RPE, and ChEcs. These results were confirmed in the laser-induced CNV model in vivo and in human fetal RPE cells in vitro. The relevance of our findings is supported by multiple previous studies in humans and mice. In patients with exudative AMD, aqueous humor IL-6 levels correlate with CNV size and macular thickness. In patients with diabetes, aqueous IL-6 levels are higher in patients with diabetic retinopathy, DME, and proliferative disease. In the laser-induced CNV model, IL-6 inhibition reduces CNV size and VEGF expression. Additionally, these data agree with prior studies in macrophage, cancer, and endothelial cells. Many patients with neovascular AMD are resistant to anti-VEGF therapy. The pleiotropic effect of β2-AR blockade to inhibit both VEGF and IL-6 expression may explain why topical dorzolamide-timolol is effective in patients with persistent macular edema secondary to neovascular AMD. Additionally, β2-AR blockade may be highly advantageous in inflammatory CNV formation in diseases such as punctate inner choroidopathy and multifocal choroiditis with panuveitis.

We were surprised to find that β2-AR signaling influences VEGF and IL-6 expression in retinal pericytes, RPE cells, ChEcs, and retinal microglial cells. All of these cell types have been previously identified as important sources of VEGF in CNV or DME. Interestingly, this effect was not ubiquitous with no β-AR regulation of VEGF in RECs and RASTs. Future studies will investigate if this result is confirmed in vivo and the mechanistic differences between these cell types. This study had several limitations and considerations before clinical translation. First, the laser-induced CNV model is a highly inflammatory model that simulates diseases such as ocular histoplasmosis, punctate inner choroidopathy, multifocal choroiditis with panuveitis, or postchroroidal rupture CNV more than it models neovascular AMD. However, these other types of CNV remain clinically relevant. Second, primary mouse retinal cell lines lose typical characteristics with passages. However, our results were replicated in primary human fetal RPE cells (passage 2-3), demonstrating that passage-related effects are unlikely. Third, a single intravitreal injection of a small molecule inhibitor such as IC 118,551 has pharmacokinetic limitations, which are completely unknown and need further investigation. Therefore, the ideal drug delivery technique would be a sustained release implant to reduce systemic effects while delivering sustained local therapy.

In summary, β2-AR antagonism reduced CNV area and decreased expression of VEGF and IL-6 in retinal pericytes, RPE cells, and ChEcs. Furthermore, β2-AR agonism stimulated VEGF and IL-6 expression in human RPE cells. These studies suggest that β2-blockade could be a future antiangiogenic and anti-inflammatory therapy for CNV and potentially for retinal neovascularization as well.

References


