Effect of Brimonidine on Retinal and Choroidal Neovascularization in a Mouse Model of Retinopathy of Prematurity and Laser-Treated Rats

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PURPOSE. To determine whether chronic treatment with brimonidine (BRI) attenuates retinal vascular leakage and neovascularization in neonatal mice after exposure to high oxygen in a mouse model of retinopathy of prematurity (ROP), and choroidal neovascularization (CNV) in rats after laser treatment.

METHODS. Experimental CNV was induced by laser treatment in Brown Norway (BN) rats. BRI or vehicle (VEH) was administered by osmotic minipumps, and CNV formation was measured 11 days after laser treatment. Oxygen-induced retinopathy was generated in neonatal mice by exposure to 75% oxygen from postnatal day (P)7 to P12. BRI or VEH was administered by gavage, and vitreoretinal vascular endothelial growth factor (VEGF) concentrations and retinal vascular leakage, neovascularization, and vaso-obliteration were measured on P17. Experimental CNV was induced in rabbits by subretinal lipopolysaccharide/fibroblast growth factor-2 injection.

RESULTS. Systemic BRI treatment significantly attenuated laser-induced CNV formation in BN rats when initiated 3 days before or within 1 hour after laser treatment. BRI treatment initiated during exposure to high oxygen significantly attenuated vitreoretinal VEGF concentrations, retinal vascular leakage, and retinal neovascularization in P17 mice subjected to oxygen-induced retinopathy. Intravitreal treatment with BRI had no effect on CNV formation in a rabbit model of nonischemic angiogenesis.

CONCLUSIONS. BRI treatment significantly attenuated vitreoretinal VEGF concentrations, retinal vascular leakage, and retinal and choroidal neovascularization in animal models of ROP and CNV. BRI may inhibit underlying event(s) of ischemia responsible for upregulation of vitreoretinal VEGF and thus reduce vascular leakage and retinal-choroidal neovascularization. (*Invest Ophthalmol Vis Sci.* 2011;52:5424–5431) DOI:10.1167/iov.10-6262

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Ischemia has a well-established role in the pathogenesis of ocular diseases associated with retinal neovascularization, including retinopathy of prematurity (ROP) and proliferative diabetic retinopathy (PDR).¹ Retinal ischemia resulting from vaso-obliteration and cessation of normal growth of the vasculature during development in ROP² or from hyperglycemia-induced capillary dropout in PDR³ leads to the proliferation of abnormal microvasculature on the retinal surface. In ROP, the neovascularization usually regresses, but it can lead to irreversible vision loss if the vessels cause retinal traction and detachment, or if vascular leakage leads to scarring.⁴ Ischemia may also be involved in the choroidal neovascularization (CNV) that occurs in wet (exudative or neovascular) age-related macular degeneration (AMD).⁵ In wet AMD, fragile, leaky blood vessels from the choroid grow through Bruch's membrane into the retinal pigment epithelium (RPE) and proliferate in the sub-RPE and/or subretinal space. Vascular leakage, hemorrhage, and fluid accumulation associated with CNV can lead to rapid and severe vision loss in wet AMD.⁶

Vascular endothelial growth factor (VEGF), a vasopermeability⁷ and angiogenic⁸ factor that is upregulated by hypoxia,⁹ has a primary role in stimulating retinal neovascularization in ischemic retinopathies.¹ Elevated concentrations of VEGF have been demonstrated in the vitreous of patients with PDR.⁶ Further, treatment with anti-VEGF agents has been shown to decrease retinal neovascularization in patients with PDR¹⁰ as well as in an animal model of proliferative ischemic retinopathy.^{11–13} In a well-studied animal model of ROP, newborn mice exposed to 75% oxygen from postnatal day (P)7 to P12 and then returned to room air with normal oxygen content develop oxygen-induced retinopathy (OIR) characterized by hypoperfusion of the central retina during the period of exposure to high oxygen, followed by neovascularization at the junction between the vascular and avascular retina after the return of the animals to room air.⁴ The neovascularization presents as neovascular tufts extending into the vitreous and reaches a maximum at P17 to P21.⁴ Studies using the mouse OIR model have shown that retinal Müller cell expression of VEGF is increased within 12 hours after the return of P12 mice with oxygen-induced ischemia to normal air.¹⁴ Both systemic treatment beginning at P12 with kinase inhibitors that block VEGF receptor activation and intravitreal treatment at P12 with siRNA targeting VEGF have been shown to attenuate retinal neovascularization at P17 in this model.^{11,12} In previous studies, we have demonstrated that conditional knockout of VEGF in mouse Müller cells results in inhibition of retinal neovascularization and vascular leakage in OIR mice, as well as in streptozotocin-induced diabetic mice.^{15,16}

VEGF is also an important mediator of CNV in wet AMD. VEGF has been localized with immunohistochemistry in surgically excised CNV tissue from patients with wet AMD,^{17,18} and intravitreal injections of anti-VEGF agents are used clinically in

first-line treatment of wet AMD.¹⁹ Both pegaptanib, an aptamer to VEGF, and ranibizumab, a recombinant humanized Fab fragment of a murine monoclonal anti-VEGF antibody, are approved for treatment of CNV in AMD. In animal models, laser photocoagulation of the choroid-RPE with disruption of Bruch's membrane reliably produces CNV.¹ Increases in VEGF mRNA expression by cells in the RPE and choroid have been demonstrated in a rat model of laser-induced experimental CNV,²⁰ and inhibition of VEGF receptor signaling with kinase inhibitors has been shown to almost completely eliminate CNV in a mouse model of laser-induced experimental CNV.²¹

The selective α_2 -adrenergic receptor agonist brimonidine (BRI) has been shown to preserve retinal function^{22–24} and promote retinal ganglion cell survival^{22,23,25–28} in animal models of retinal ischemia produced by transient ligation of ophthalmic vessels,^{24–26} transient pathologic elevation of intraocular pressure,^{22,23} laser-induced vascular coagulation,²⁷ or treatment with the vasoconstrictor endothelin-1.²⁸ Moreover, in a previous study from our laboratory that examined the effect of BRI treatment on diabetic retinopathy in rats with streptozotocin-induced diabetes, BRI treatment resulted in attenuation of both retinal VEGF expression and blood-retinal barrier breakdown in diabetic rats.²⁹ These results suggest that BRI may have beneficial effects in retinal disease associated with ischemia, increased expression of VEGF, and retinal or choroidal neovascularization, such as ROP and wet AMD. To test this hypothesis, we measured the effects of prolonged treatment with BRI or vehicle on CNV in the rat model of laser-induced experimental CNV and on vitreoretinal VEGF concentrations, retinal vascular leakage, and retinal neovascularization in the mouse OIR model.

METHODS

Animal Use Statement

All experiments with animals were designed and conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committees of the University of Oklahoma Health Sciences Center or the Allergan Institutional Animal Care and Use Committee.

Experimental CNV Model in Rats

Animals and Induction of CNV. Male Brown Norway (BN) rats weighing 250 to 300 g were obtained from Charles River Laboratories, Inc. (Wilmington, MA). The animals were maintained on a normal diet and were acclimated to the animal research facilities at Allergan for at least 1 week before experiments were initiated. After acclimation, the rats were weighed and then divided into treatment groups such that body weight was distributed similarly among the groups. The rats were anesthetized with a 1-mL/kg intramuscular injection of a 1:1 mixture of ketamine hydrochloride (65 mg/mL) and xylazine (11 mg/mL), and their pupils were dilated with a drop of 1% tropicamide and 10% phenylephrine HCl. Experimental CNV was induced by laser treatment, essentially as described previously.³⁰ Briefly, three to four laser spots surrounding the optic disc were applied with an argon laser (Novus 2000; Coherent Inc., Santa Clara, CA) to each eye between major retinal vessels. Each photocoagulation used a wavelength of 514 nm (green), a spot size of 100 μ m, a power of 110 mW, and an exposure time of 100 ms. A coverslip (18 mm) was used as a contact lens. Disruption of Bruch's membrane was confirmed by central bubble formation.³⁰ Both eyes were used in the analyses.

Drug Treatment and Evaluation of CNV. Systemic treatment with BRI or vehicle (VEH) was initiated 3 days before or at various times after laser treatment. BRI (1 mg/kg/d) or VEH (distilled water) was administered continuously with an osmotic minipump

(model 2ML2, 5 μ L/h; Alzet Osmotic Pumps, Cupertino, CA) inserted SC in the back of the animal as described previously.³¹ At 11 days after laser treatment, the animals were killed by CO₂ exposure, and CNV formation was assayed as described previously.³⁰ Briefly, the eyes were enucleated and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; 9 g/L NaCl, 0.232 g/L KH₂PO₄, and 0.703 g/L Na₂HPO₄ [pH 7.3]) for 1 hour. The anterior segment, crystalline lens, and retina were removed, and the remaining eye cups were washed with ICC buffer (0.5% BSA, 0% Tween 20, and 0.05% sodium azide in PBS) at 4°C and incubated for 4 hours at 4°C with a 1:100 dilution of a 1-mg/mL solution of isolectin IB₄ conjugated with Alexa Fluor 568. After incubation, the eye cups were washed with ICC buffer, radial cuts were made toward the optic nerve head, and the sclera-choroid/RPE complexes were flat mounted for fluorescence microscopy. The area of fluorescence was quantified with image analysis software (Metamorph; RPI, Natick, MA).

Experimental CNV Model in Rabbits

Animals and Induction of CNV. Twenty-four adult Dutch Belted rabbits 6 to 7 months of age, weighing 2 to 2.5 kg, were used in the experiment. The animals were anesthetized by intramuscular injection of ketamine (50 mg/kg) and xylazine (5 mg/kg) before intraocular surgery to induce CNV. One eye of each animal was used as the study eye. Topical 1% tropicamide and 2.5% phenylephrine HCl were instilled in the study eye to dilate the pupil before intraocular surgery, fundus examination, and fluorescein angiography.

Experimental CNV was induced by subretinal injection of 50 μ L of a cocktail of angiogenic agents containing 100 ng of recombinant human FGF-2 (PeproTech, Inc., Rocky Hill, NJ) and 100 ng of lipopoly-saccharide (LPS; Sigma-Aldrich, St. Louis, MO), similar to a method described previously.³² The injection was made with a 30-gauge needle inserted through the retina, with injury of Bruch's membrane visualized by subretinal hemorrhage surrounding the needle tip. Six rabbits were injected subretinally with vehicle, but without injury to Bruch's membrane, to serve as a negative control. A Landers vitrectomy lens (Ocular Instruments, Inc., Bellevue, WA) was used to maintain clarity during the surgical procedure. Topical mydriatic ointment (1% atropine) and antibiotic ointment (bacitracin/neomycin/polymyxin) were applied after the procedure, to prevent complications such as inflammation-associated iris-lens adhesion.

Drug Treatment and Evaluation of CNV. BRI or VEH was delivered to the rabbit eyes by intravitreal injection at 1 hour, 3 days, 7 days, and 10 days after subretinal injection of FGF-2/LPS. At 14 days after the subretinal injection, the treated eyes were examined and photographed with a fundus camera to document changes in the vitreous, retina, choroid, and vasculature. CNV formation and vascular leakage were assessed by fluorescein angiography after intravenous administration of 0.2 mL of 5% fluorescein-dextran (MW 70,000; Sigma-Aldrich) and 0.25 mL of 10% fluorescein sodium (Akorn, Lake Forest, IL). The area of the CNV lesion was quantified by digital image analysis (ImageNet software; Topcon, Tustin, CA).

Experimental Oxygen-Induced Retinopathy Model in Mice

Animals and Treatment. OIR was induced in C57B6 mice using the protocol reported by Smith et al.⁴ Litters of newborn mice and their dams were placed in a 75% oxygen chamber from P7 to P12. The chamber contained enough food and water for 5 days and was opened only to allow drug administration to the animals. The mice were returned to room air with normal oxygen content on P12. BRI in water or VEH (water) was administered once daily by gavage, beginning on P10 or P12 and continuing through P16. Retinal neovascularization and vascular leakage were evaluated on P17 after 5 days of exposure of the animals to room air.

Retinal Angiography and Quantification. Retinal neovascularization and vaso-obliteration were evaluated by angiography in mice subjected to OIR, as described previously.^{4,15} P17 mice were

deeply anesthetized and then were perfused through the left ventricle with 1 mL of PBS containing 50 mg of high-molecular-weight (2000) fluorescein-dextran (Sigma-Aldrich). The eyes were enucleated and fixed in 4% paraformaldehyde for 24 hours. After removal of the lens, the retina was dissected and whole mounted with glycerol-gelatin. Quantification of vaso-oblivation and retinal neovascularization was performed as described previously.^{15,33} Images of retinal whole mounts taken at 4× magnification on an epifluorescence microscope (Olympus, Center Valley, PA) were imported into digital imaging software (Photoshop 7.0; Adobe Systems, San Jose, CA) and merged to produce an image of the entire retina. The freehand tool was used to outline areas of neovascular tuft formation as well as central avascular areas. The area of neovascularization and the avascular area (in pixels) were expressed as a percentage of the area of the whole retina (in pixels). To avoid bias, quantification of neovascularization and vaso-oblivation were performed by an observer masked to the animal treatment.

Immunoblot Analysis. Vitreoretinal VEGF expression and albumin leakage were determined by immunoblot analysis. On P17, the animals were killed by CO₂ exposure, and retinal and vitreous tissue was isolated and homogenized by sonication at 4°C in lysis buffer (5 mM HEPES [pH 7.5], 50 mM NaCl, 0.5% Triton X-100, 0.25% sodium deoxycholate, 0.1% SDS, and 1 mM EDTA) containing 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM benzamidine, phosphatase inhibitor cocktails 1 and 2 (10 µL/mL; Sigma-Aldrich), and proteinase inhibitor cocktail set III (10 µL/mL; Calbiochem, San Diego, CA). The insoluble pellet was removed by centrifugation at 4°C, and the protein concentration of the supernatant was measured using a protein assay reagent kit (Bio-Rad Laboratories, Hercules, CA). Soluble protein (30 µg) was resolved by SDS-PAGE on 10% 1.0-mm 10-well minigels (NuPAGE Novex Bis-Tris; Invitrogen, Carlsbad, CA) and electrotransferred to a 0.2-µm-pore PVDF membrane (Invitrogen). The membrane was blotted with 1:1000 polyclonal goat anti-albumin antibody (Bethyl Laboratories, Montgomery, TX), 1:1000 monoclonal mouse anti-β-actin antibody (MA1-744; Affinity Bioreagents, CO), and 1:500 polyclonal rabbit anti-VEGF antibody (A2; Santa Cruz Biotechnologies). Peroxidase-linked anti-goat, -mouse, and -rabbit IgG antibodies (Amersham Biosciences, Buckinghamshire, UK) were used as secondary antibodies. Immunoreactive bands were detected by chemiluminescence (Super Signal West Dura Extended-Duration Substrate; Pierce, Rockford, IL). Images were captured (ChemiGenius Imaging Station; SynGene, Frederick, MD), and relative band density was determined (Genetools program; SynGene). The intensity of the β-actin signal was used as an endogenous control for loading. Data are expressed as the albumin:β-actin or VEGF:β-actin densitometric unit ratio.

Statistical Analysis

Descriptive statistics (mean ± SEM values shown in figures) were calculated on a spreadsheet (Excel; Microsoft Corp., Redmond, WA). Differences between treatment groups were evaluated with *t*-tests. Significance levels were set at *P* < 0.05, *P* < 0.01, and *P* < 0.001.

RESULTS

Effect of BRI on Laser-Induced CNV Formation in BN Rats

To determine the effect of BRI treatment on CNV formation in an animal model, we induced CNV by laser treatment of both eyes in BN rats. Systemic treatment of the animals with BRI (1 mg/kg/d) or VEH via osmotic minipumps was initiated 3 days before or 1 hour after laser treatment and was continued throughout the study. This dose of BRI was used because systemic treatment with 1/mg/kg/d BRI via osmotic minipumps was shown to have maximum effects on retinal VEGF expression and blood-retinal barrier breakdown in diabetic Long-Evans rats in a previously reported study.²⁹ At the end of the study, 11 days after laser treatment, the area of CNV was

quantified by analysis of fluorescence in flat-mount preparations of the sclera-choroid/RPE labeled with the endothelial and microglial cell marker isolectin IB₄ conjugated with Alexa Fluor 568. Continuous systemic treatment with 1 mg/kg/d BRI significantly reduced the area of the CNV lesion at 11 days after the induction of CNV, regardless of whether BRI treatment was initiated 3 days before or 1 hour after laser treatment (Fig. 1). When BRI treatment was initiated 3 days before laser treatment, the area of CNV was $11,919 \pm 1,128 \mu\text{m}^2$ in the BRI-treated animals compared with $19,185 \pm 1,522 \mu\text{m}^2$ in the vehicle-treated animals (*P* < 0.001), and when BRI treatment was initiated 1 hour after laser treatment, the area of CNV was $10,382 \pm 864 \mu\text{m}^2$ in the BRI-treated animals compared with $17,101 \pm 1,407 \mu\text{m}^2$ in the vehicle-treated animals (*P* < 0.001).

Time Dependence of the Effect of BRI on Laser-Induced CNV Formation in BN Rats

To determine the time dependence of the effect of BRI treatment on CNV formation in laser-treated BN rats, systemic treatment with BRI (1 mg/kg/d) or VEH via osmotic minipumps was initiated at 1 hour, 1 day, 3 days, or 5 days after laser treatment and was continued throughout the study. At the end of the study, 11 days after laser treatment, the area of CNV was quantified by analysis of isolectin IB₄ Alexa Fluor 568 fluorescence in flat-mount preparations, as described previously. Systemic treatment with 1 mg/kg/d BRI significantly reduced the area of the CNV lesion only when initiated within 1 hour after laser treatment (Fig. 2). There was no significant difference in the area of CNV between the BRI-treated animals and the vehicle-treated animals when systemic treatment was begun 1 day after laser treatment or at later times (Fig. 2).

Effect of BRI on Retinal Vascular Leakage in the Mouse OIR Model

To determine the effect of BRI treatment on retinal vascular leakage in mice subjected to OIR, newborn mice were placed in 75% oxygen from P7 to P12 and room air from P12 to P17. BRI (3 mg/kg) or VEH was administered by gavage once daily from P10 through P16. On P17, retinal vascular leakage was

FIGURE 1. Effect of BRI on laser-induced CNV formation in BN rats. Chronic treatment of BN rats with BRI (1 mg/kg/d) or vehicle using osmotic minipumps was initiated 3 days before (Pretreatment) or 1 hour after (Posttreatment) laser-induced CNV and was continued until 11 days after laser treatment. At the end of this period, the animals were killed, and CNV in the choroid-RPE was visualized by fluorescent labeling with isolectin IB₄ conjugated with Alexa Fluor 568. *Left:* representative images of isolectin IB₄ labeling in flat-mount preparations from rats treated with vehicle (A) or BRI (B). *Right:* quantitation of the area of fluorescence. Error bars, SEM. ****P* < 0.001 vs. VEH, *n* = 8 to 10 eyes (three to four laser spots per eye). Scale bar, 100 µm.

FIGURE 2. Time dependence of effect of BRI on laser-induced CNV in BN rats. Chronic treatment of BN rats with BRI (1 mg/kg/d) or vehicle with osmotic minipumps was initiated 1 hour, 1 day, 3 days, or 5 days after laser photocoagulation and was continued until 11 days after lasering. At the end of this period, the animals were killed, CNV in choroid-RPE flat-mount preparations was visualized by fluorescent labeling with isolectin IB₄ conjugated with Alexa Fluor 568, and the area of fluorescence was quantified. Error bars, SEM. * $P < 0.05$ vs. VEH; $n = 8$ to 10 eyes (three to four laser spots per eye).

determined by immunoblot analysis of the concentration of albumin in homogenates of the retina/vitreous. The ratio of vitreoretinal albumin to β -actin was 1.32 ± 0.10 in the BRI-treated OIR mice compared with 2.09 ± 0.12 in the vehicle-treated OIR mice ($P < 0.01$, Fig. 3). The value for control P17 mice that had not been exposed to high oxygen was 1.23 ± 0.11 , suggesting that daily treatment with BRI from P10 to P16 reduced P17 retinal vascular leakage caused by previous exposure of the mice to high oxygen by approximately 90% (Fig. 3).

Effect of BRI on Retinal Vaso-obliteration and Neovascularization in the Mouse OIR Model

To determine the effect of BRI treatment on retinal neovascularization in mice subjected to OIR, newborn mice were placed in 75% oxygen from P7 to P12 and in room air from P12 to P17. BRI (3 mg/kg) or VEH was administered by gavage once daily from P10 through P16. On P17, the animals were perfused with high-molecular-weight fluorescein-dextran. Neovascularization was determined by angiography in whole-mount retinas (Fig. 4). Daily treatment with BRI from P10 to P16 significantly decreased retinal neovascularization at P17 in the mouse OIR model (Fig. 4C). The retinal area of neovascularization was $5.83\% (\pm 0.81\%)$ in the BRI-treated mice, compared with

FIGURE 3. Effect of BRI on retinal vascular leakage of albumin in mice subjected to OIR. Mice were placed in 75% oxygen from P7 to P12 and room air from P12 to P17. BRI (3 mg/kg) or VEH was administered by gavage once daily from P10 to P16. On P17, the animals were killed, retina and vitreous tissue was collected, and albumin and β -actin protein concentrations in vitreoretinal homogenates were determined by Western blot analysis. The same amount (30 μ g) of total vitreoretinal protein was loaded in each lane. (A) Representative immunoblots. (B) Summary of the densitometric quantitation. Error bars, SEM. ** $P < 0.01$ vs. VEH, $n = 4$ to 5.

FIGURE 4. Effect of BRI on retinal vaso-obliteration and neovascularization in mice subjected to OIR. Mice were placed in 75% oxygen from P7 to P12 and room air from P12 to P17. BRI (3 mg/kg) or VEH was administered by gavage once daily from P10 to P16. On P17, retinal vaso-obliteration and neovascularization were evaluated by angiography with high-molecular-weight fluorescein-dextran. Representative images of fluorescein-dextran in whole-mount retinas from BRI-treated (A) and VEH-treated (B) mice are shown. (B1) Conversion of image in (B) to show area of neovascularization (red). (C) Quantification of retinal neovascularization. The area of neovascularization is expressed as a percentage of the total area of the retina. Error bars, SEM. *** $P < 0.001$ vs. VEH, $n = 11$ to 17. (D). Quantification of vaso-obliteration. The central retinal avascular area is expressed as a percentage of the total area of the retina. Error bars, SEM. $P = 0.61$, $n = 10$ to 11.

$10.80\% (\pm 0.71\%)$ in the vehicle-treated mice ($P < 0.001$). The control P17 mice that had not been exposed to high oxygen demonstrated no retinal neovascularization (0%). Vaso-obliteration in the retinas was also evaluated to determine whether there is an effect of BRI treatment on the extent of ischemic injury in the OIR mice, which may explain BRI's effect on retinal neovascularization in this model. There was no significant difference in the area of avascular retina between the BRI-treated ($11.1\% \pm 0.55\%$) and vehicle-treated ($11.6\% \pm 0.71\%$) OIR mice ($P = 0.61$; Fig. 4D).

Dose and Time Dependence of the Effect of Brimonidine on Retinal Neovascularization in the Mouse OIR Model

To determine the dose and time dependence of the effect of BRI treatment on retinal neovascularization in mice subjected to OIR, newborn mice were placed in 75% oxygen from P7 to P12 and room air from P12 to P17. BRI (0.25, 0.5, 1, 2, or 3 mg/kg) or VEH was administered by gavage once daily from P10 to P16 or from P12 to P16. On P17, the animals were perfused with high-molecular-weight fluorescein-dextran and retinal neovascularization was determined by angiography in whole-mount retinas. The effect of BRI treatment on retinal neovascularization was dose dependent, and daily doses of 1, 2, and 3 mg/kg BRI given from P10 through P16 produced significant inhibition of retinal neovascularization at P17 in the mouse OIR model ($P < 0.001$; Fig. 5). The effect of BRI treatment on retinal neovascularization was also time dependent. Daily BRI treatment was effective in reducing retinal neovascularization only when treatment was begun at P10, during the period of exposure to high oxygen (Fig. 5). Daily treatment with 3 mg/kg BRI had no effect on neovascularization when given from P12 to P16, starting 2 to 3 hours after the animals were returned to room air (Fig. 5).

FIGURE 5. Time course and dose-response of BRI effect on retinal neovascularization in mice subjected to OIR. Mice were placed in 75% oxygen from P7 to P12 and room air from P12 to P17. Treated animals were given BRI (0.25, 0.5, 1, 2, or 3 mg/kg) or VEH by gavage once daily from P10 to P16 or from P12 to P16. On P17, retinal neovascularization was evaluated by angiography with high-molecular-weight fluorescein-dextran. The area of neovascularization is expressed as a percentage of the total area of the retina. Error bars, SEM. *** $P < 0.001$ vs. none, $n = 4$ to 17.

Effect of Brimonidine on Vitreoretinal VEGF Concentrations in the Mouse OIR Model

To determine the effect of BRI treatment on the concentration of VEGF in the retina and vitreous of mice subjected to OIR, newborn mice were placed in 75% oxygen from P7 to P12 and room air from P12 to P17. BRI (3 mg/kg) or VEH was administered by gavage once daily from P10 through P16. On P17, retina and vitreous tissue were collected, and the concentrations of VEGF in vitreoretinal homogenates were determined by Western blot analysis. The VEGF signal appeared as a dimer with an approximate molecular mass of 42 kDa. Daily treatment with BRI from P10 through P16 prevented the increase in vitreoretinal VEGF concentrations at P17 in the OIR mice (Fig. 6). The concentration of vitreoretinal VEGF, normalized to the concen-

FIGURE 6. Effect of BRI on vitreoretinal VEGF concentrations at P17 in mice subjected to OIR. Mice were placed in 75% oxygen from P7 to P12 and room air from P12 to P17 (OIR model) or remained in room air from P7 to P17 (control). BRI (3 mg/kg) or VEH was given by gavage once daily from P10 to P16. On P17, the animals were killed, retina and vitreous tissue was collected, and VEGF and β -actin protein concentrations in vitreoretinal homogenates were determined by Western blot analysis. The ratio of the VEGF concentration to the β -actin concentration was expressed as a percentage of the value in control animals treated with VEH. (A) Representative immunoblots. (B) Summary of the densitometric quantitation. Error bars, SEM. ** $P < 0.01$ vs. VEH/OIR, $n = 4$ to 5.

tration of β -actin and expressed as a percentage of the value in control animals treated with VEH that remained in room air ($100.0 \pm 2.3\%$), was $99.1\% (\pm 5.7\%)$ in the BRI-treated P17 OIR mice and $146.1\% (\pm 8.8\%)$ in the vehicle-treated P17 OIR mice ($P < 0.01$; Fig. 6). To determine the time course of the effect of BRI treatment on vitreoretinal VEGF, in an additional experiment we evaluated vitreoretinal VEGF concentrations in P14 OIR mice after daily treatment with BRI or vehicle from P10 through P13. The vitreoretinal VEGF concentration was significantly lower in the BRI-treated OIR mice than in the vehicle-treated OIR mice at P14 (Fig. 7).

Effect of BRI on CNV Formation Induced by Endotoxin and Growth Factor in Dutch Belted Rabbits

To determine the effect of BRI treatment on CNV formation in an animal model of nonischemic CNV, CNV was induced in one eye in Dutch Belted rabbits by a single dose of LPS and FGF-2 delivered subretinally through the retina with injury to Bruch's membrane. Eyes were treated with BRI (10 or 100 μ g) or VEH by intravitreal injection at 1 hour, 3 days, 7 days, and 10 days after the subretinal injection. CNV formation was evaluated by fluorescein angiography at 14 days after the induction of CNV. Repeated intravitreal treatment with 10 or 100 μ g BRI had no significant effect on the area of the CNV lesion at 14 days after the induction of CNV in this model system (Fig. 8). The area of CNV was $15.8 \pm 2.7 \text{ mm}^2$ in the animals treated with 10 μ g BRI, $16.7 \pm 4.6 \text{ mm}^2$ in the animals treated with 100 μ g BRI, and $14.8 \pm 2.5 \text{ mm}^2$ in the animals treated with vehicle.

DISCUSSION

This study demonstrated that treatment with BRI significantly decreases retinal neovascularization in neonatal mice subjected to OIR, an animal model of ROP, and significantly decreases CNV in rats with laser-induced rupture of Bruch's membrane. The effect of BRI treatment on retinal and choroidal neovascularization was time dependent and was seen only when treat-

FIGURE 7. Effect of BRI on vitreoretinal VEGF concentrations at P14 in mice subjected to OIR. Mice were placed in 75% oxygen from P7 to P12 and room air from P12 to P14. BRI (3 mg/kg) or VEH was given by gavage once daily from P10 to P13. On P14, the animals were killed, retina and vitreous tissue was collected, and VEGF and β -actin protein concentrations in vitreoretinal homogenates were determined by Western blot analysis. The ratio of the VEGF concentration to the β -actin concentration was expressed as a percentage of the value in OIR animals treated with VEH. (A) Representative immunoblots. (B) Summary of the densitometric quantitation. Error bars, SEM. * $P < 0.01$ vs. VEH/OIR, $n = 3$ to 5.

FIGURE 8. Effect of BRI on CNV formation induced by FGF-2 and LPS in Dutch belted rabbits. A solution containing 100 ng of FGF-2 and 100 ng of LPS was injected subretinally with injury of Bruch's membrane to induce CNV in 1 eye of each rabbit. BRI (10 or 100 µg) or VEH was delivered to experimental eyes by intravitreal injection at 1 hour, 3 days, 7 days, and 10 days after the induction of CNV. CNV formation was monitored with fluorescein angiography and a fundus camera at 2 weeks after the induction of CNV, and the area of CNV was quantified. Error bars, SEM; $n = 6$.

ment was initiated in the presence of ischemia, under circumstances in which VEGF has a primary role in stimulating neovascularization. These findings suggest that BRI may be useful for treatment of disease associated with retinal and choroidal neovascularization in humans.

Ischemia has a primary role in the pathogenesis of retinal neovascularization in ROP, PDR, and retinal vein occlusions. VEGF induced by hypoxia stimulates vascular endothelial cell proliferation and new vessel formation in these ischemic retinopathies.²¹ In the mouse model of ROP, retinal ischemia is induced by hyperoxia from P7 to P12. Oxygen-induced vasoobliteration is rapid, and the central zone of vasoobliteration reaches a peak by P9.³⁴ When the mice are returned to room air at P12, the central avascular retina becomes hypoxic, resulting in the upregulation of retinal VEGF expression, followed by retinal neovascularization.^{4,14} In the present study, daily oral treatment with BRI significantly decreased vascular leakage and the elevation of vitreoretinal VEGF concentrations in mice subjected to OIR. BRI treatment dose dependently inhibited retinal neovascularization in this model only when treatment was begun at P10 under ischemic conditions, before the return of the animals to normal air and to the subsequent induction of VEGF. A critical period for the BRI effect may be the first several hours after returning the mice to room air on P12, since BRI treatment starting 2 to 3 hours after the return to room air is ineffective. The timing of the BRI effect is consistent with an action on VEGF induction by hypoxia rather than protection from oxygen-induced injury. Daily BRI treatment starting on P10 resulted in reduced vitreoretinal levels of VEGF at P14, before the observed effect of BRI treatment on retinal neovascularization at P17, as well as at P17. As BRI treatment was begun at P10, after the critical period of vasoobliteration in the OIR model,³⁴ we did not anticipate a significant difference in vasoobliteration between the BRI- or vehicle-treated OIR mice, and none was observed.

The role of ischemia and hypoxia in the development of CNV is less clear. Hypoxia is unlikely to have a direct role in the CNV associated with conditions such as ocular histoplasmosis, pathologic myopia, or choroidal rupture.²¹ Alterations in choroidal blood flow have been demonstrated in patients with nonexudative AMD, however, suggesting that ischemia is involved in the etiology of CNV that develops as nonexudative AMD progresses to wet AMD.⁵ Inflammation, oxidative damage, and alterations in the extracellular matrix in the RPE may also contribute to the development of CNV in wet AMD.^{1,35,36}

Although an important role for VEGF in CNV formation in wet AMD has been established, the stimulus for the increased expression of VEGF in wet AMD has not been clearly defined. Along with hypoxia,⁹ oxidative stress³⁷ and cytokines including interleukin-6 and transforming growth factor- β ³⁸ have been shown to induce expression of VEGF in cell culture and animal models. Studies have demonstrated elevated levels of protein and lipid oxidative modifications in Bruch's membrane and RPE tissue from AMD patients,³⁵ and inflammatory cells are present in CNV tissue from patients with wet AMD.⁶

In the rat model of laser-induced CNV formation, laser burns surrounding the optic disc are used to disrupt Bruch's membrane. Photocoagulation of the choriocapillaris can be expected to lead rapidly to local ischemia in choroid-RPE. Further, RPE cell damage/death and mobilization of inflammatory cells occur within 1 day of laser treatment, before new vessel formation.³⁰ In the present study, chronic systemic treatment with BRI decreased CNV formation in the rat laser-induced CNV model only when treatment was initiated before or within 1 hour after lasering. Brimonidine treatment initiated 1 day or later after laser treatment had no effect on CNV formation in this model. These results suggest that BRI acts early in the pathway of events that lead to CNV formation after laser treatment. It is likely that BRI's effect on CNV formation was secondary to an effect on VEGF, because VEGF has been shown to be an important mediator of CNV formation in the rodent laser-induced CNV model,²¹ and BRI treatment attenuated the elevation in vitreoretinal VEGF concentration in mice in the OIR model in the present study and was shown to attenuate the increase in retinal VEGF expression in the diabetic rat retina in a previous study.²⁹ The mechanism of the effect of BRI treatment on VEGF concentrations has not been determined.

Although chronic systemic BRI treatment inhibits CNV in the rat laser-induced CNV model, and we have observed similar inhibition of laser-induced CNV by intravitreal injection of BRI in rats (results not shown), in the rabbit model of experimental CNV produced by subretinal injection of FGF-2 and LPS with injury to Bruch's membrane, four intravitreal injections of BRI given at 1 hour after the subretinal injection and within the next 10 days had no effect on CNV formation at 2 weeks after the subretinal injection. In this rabbit model, primary CNV develops in the area of the injury to Bruch's membrane within 2 weeks after the subretinal injection and is believed to result directly from the activity of the exogenous angiogenic factors, with no role of ischemia in CNV formation.³² Therefore, the lack of effect of BRI on primary CNV formation in this model suggests that BRI may attenuate neovascularization only under conditions such as ischemia in which VEGF has a primary role in stimulating neovascularization.

The beneficial effects of BRI treatment on retinal and choroidal neovascularization in animal models of ROP and CMV are likely to be mediated by inhibition of a pathway activated by ischemia that leads to upregulation of VEGF expression (Fig. 9). Evidence from the laser-induced rat CNV model suggests that this pathway involves activation of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway leading to induction of transcription factor hypoxia-inducible factor (HIF)-1, which activates transcription of VEGF.³⁹ Activation of the extracellular signal-regulated kinase (ERK) signaling pathway is also needed for ischemia-induced upregulation of VEGF.³⁹ The effects of BRI treatment on neovascularization were time dependent and were seen when treatment was initiated before or during the ischemic insult. Similarly, in previous studies in which BRI was shown to have beneficial effects on retinal ganglion cell survival in animal models of transient retinal ischemia, BRI treatment had to be administered before or within a brief period after the ischemic episode to protect

FIGURE 9. Schematic of a potential mechanism for the effect of BRI on retinal and choroidal neovascularization in animal models of ROP and CMV. Ischemia produced by hyperoxia or laser photocoagulation leads to increased expression of VEGF, which stimulates blood-retinal barrier breakdown and retinal and choroidal neovascularization. Brimonidine may inhibit the underlying event(s) of ischemia responsible for the upregulation of VEGF and thus attenuate VEGF expression, vascular leakage, and retinal and choroidal neovascularization.

against retinal ganglion cell loss.^{23,25} The mechanism for the neuroprotective effects of BRI after transient ischemia is likely multifactorial and may involve reduction in extracellular glutamate concentrations,²³ increased expression of neurotrophic factors,⁴⁰ and activation of intrinsic cell survival signaling pathways.⁴⁰ The neuroprotective effects of BRI might also involve modulation of *N*-methyl-D-aspartate (NMDA) receptor function.⁴¹ Whether any of these BRI mechanisms can also affect the upregulation of VEGF requires further investigation.

Previous studies have shown that BRI treatment promotes the survival and helps maintain the function of retinal ganglion cells in animal models of ischemic and mechanical optic nerve injury^{22–28} and prevents the elevation in VEGF expression and vascular leakage in rats with streptozotocin-induced diabetes.²⁹ The results of the present study provide further evidence that BRI inhibits pathways triggered by ischemia that lead to VEGF expression and neovascularization, as well as pathways triggered by ischemia that lead to neuronal death.

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