Inhibition of Vitreoretinal VEGF Elevation and Blood–Retinal Barrier Breakdown in Streptozotocin-Induced Diabetic Rats by Brimonidine

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PURPOSE. To determine whether long-term brimonidine (BRI) treatment prevents the hyperglycemia-induced increase in vitreoretinal vascular endothelial growth factor (VEGF) expression and breakdown of the blood–retinal barrier (BRB) in streptozotocin (STZ)-induced diabetic rats.

METHODS. Brown Norway/Long-Evans rats were divided into three groups with similar distributions of blood glucose and body weight. Two groups received a single intravenous injection of STZ (65 mg/kg) and the remaining control group received vehicle. Drug treatment administered via miniosmotic pumps was initiated 1 or 6 weeks later. The STZ-induced diabetic rats were treated with BRI (1 mg/kg/d) or vehicle (VEH) and control nondiabetic rats were treated with VEH for 4 weeks. Vitreoretinal VEGF protein, vitreal glutamate, and BRB breakdown were then measured.

RESULTS. At 5 weeks after STZ treatment, STZ-treated diabetic rats demonstrated significantly elevated vitreoretinal VEGF expression, vitreal glutamate concentrations, and BRB leakage compared with nondiabetic control rats. Chronic BRI treatment had no effect on vitreal glutamate concentrations in diabetic animals but significantly decreased vitreoretinal VEGF expression and BRB breakdown to levels similar to those observed in control rats. BRI also significantly reduced BRB breakdown in aged diabetic rats at 10 weeks after STZ treatment.

CONCLUSIONS. BRI produced marked decreases in vitreoretinal VEGF and inhibition of BRB breakdown in diabetic rats. The mechanism for these effects may involve attenuation of retinal NMDA receptor activity by BRI. The results suggest that BRI would be useful for treatment of ocular diseases associated with BRB leakage, such as diabetic macular edema and retinopathy. (Invest Ophthalmol Vis Sci. 2010;51:1044–1051) DOI: 10.1167/iovs.08-3293

Diabetic retinopathy (DR) is a common complication of diabetes mellitus that is observed in 40% of diabetic patients 40 years of age and older in the United States,1 and it is a leading cause of visual impairment and blindness.2 DR classically has been considered to be a disease of the microvasculature. Vascular changes occurring in early (nonproliferative) DR include blood vessel dilation, capillary obstruction and degeneration, increased leukostasis, increased vascular permeability associated with breakdown of the blood–retinal barrier (BRB), loss of pericytes, and formation of microaneurysms. The advanced (proliferative) stage of DR is further characterized by neovascularization. However, DR is also associated with damage to nonvascular cells of the retina. Abnormalities in retinal electrophysiological activity can be measured before clinically detectable vascular lesions associated with DR,3 and degeneration of the inner nuclear layer of the retina and apoptotic degeneration of retinal ganglion cells (RGCs) in retina from diabetic patients have been described.4,5

Streptozotocin (STZ) destroys pancreatic islet β cells and is used to induce experimental diabetes in rodents. Adult rats treated with a single dose of STZ demonstrate hyperglycemia within 48 hours and are widely used as a model of insulin-dependent diabetes mellitus. STZ-induced diabetic rats demonstrate characteristics of the nonproliferative DR that occurs in humans, including blood vessel dilation,4 capillary degeneration,5 increased leukostasis,6 increased vascular permeability resulting from breakdown of the BRB barrier,7 loss of endothelial cells and pericytes from capillary beds,8 microaneurysm formation,9 changes in retinal electrophysiological activity,10 decreases in the thickness of the inner plexiform and inner nuclear layers of the retina,11 and decreases in RGC density that appear to be due to apoptotic cell death.11

Vascular endothelial growth factor (VEGF) is a hypoxia-induced angiogenic factor12 and a major vasopermeability factor13 that has emerged as a key mediator of BRB breakdown in DR and other retinal ischemic disease.14-16 Increases in VEGF protein expression have been documented in the retinas of STZ-induced diabetic rats,9,10,17 and vitreous concentrations of VEGF have been shown to be increased in patients with diabetic macular edema (DME)18 (Shippy SA, et al. IOVS 2008;49: ARVO EAbstract 169) or proliferative DR19 and to a lesser extent in diabetic patients without DR.18 VEGF is causally linked to the pathogenesis of diabetic retinopathy, playing an important role in leukocyte-mediated breakdown of the BRB and retinal neovascularization.14 Intravitreal injection of VEGF induces the retinal vascular changes that occur in experimental diabetes, including retinal leukostasis and concomitant BRB breakdown, whereas blockade of VEGF abolishes retinal leukostasis and vascular leakage in STZ-induced diabetic rats.14,15

Elevated glutamate concentrations in the vitreous humor have been demonstrated in patients with DR.20 Similarly, glutamate concentrations have been shown to be elevated in the retina of STZ-induced diabetic rats.21,22 The increase in vitreoretinal glutamate concentrations may occur secondary to oxidative stress-induced damage and dysfunction of glutamate transporters on retinal Müller cells,23 but other mechanisms are also possible, such as decreased metabolism of glutamate to glutamine24 and increased permeability of the BRB2 allowing...
plasma glutamate entry. An increase in the retinal expression of N-methyl-D-aspartate (NMDA)-type glutamate receptors has also been observed in experimental diabetes. Work from our laboratory has demonstrated that the elevation of VEGF expression and BRB breakdown in STZ-induced diabetic rats is blocked by the NMDA receptor channel blocker and uncompetitive antagonist memantine. Chronic memantine treatment also improves retinal function and reduces the RGC loss observed in diabetic rats. These results suggest that overactivation of NMDA receptors by elevated extracellular glutamate mediates the VEGF production, BRB breakdown, and RGC damage observed in DR.

The selective α2-adrenergic receptor agonist brimonidine has been shown to attenuate the increase in intracellular Ca produced by glutamate excitotoxicity in cultured RGCs, and a recent study demonstrated that α2-adrenergic receptor activation by brimonidine results in reduced NMDA receptor channel activity in RGCs in an ex vivo model. Further, brimonidine treatment has been demonstrated to prevent retinal damage and RGC loss in rats treated with NMDA. 

STZ-treated diabetic rats were treated with brimonidine (BRI; 0.01, 0.1, or 1 mg/kg daily) or VEH for 4 weeks. BRI or distilled water was administered continuously via an osmotic minipump (model 2ML2, 5 L/h; Alzet Osmotic Pumps, Cupertino, CA) inserted subcutaneously into the back of the animal, as described previously. Each minipump contained no fixative for 2 minutes at 66 mL/min to clear the dye. The animals were then divided into treatment groups such that body weight and blood glucose were measured at the end of the 4-week treatment period.

**Methods**

**Diabetic Animals and Drug Treatment**

Male Long-Evans (LE) rats and Brown Norway (BN) rats weighing 150 to 200 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 the experiments were initiated. All experiments were designed and conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and adhered to the guidelines of the Allergan Institutional Animal Care and Use Committee.

After acclimation, the rats were weighed and tail-snip baseline blood glucose was measured with a blood glucose monitoring system (One Touch Ultra Blood Glucose Monitoring; Lifescan, Milpitas, CA). The animals were then divided into treatment groups such that body weight and blood glucose were distributed similarly among groups. The animals were treated with a single intravenous injection of 65 mg/kg STZ or control vehicle (citrate buffer [pH 4.5]). After 3 days, tail-snip blood glucose was again determined to verify hyperglycemia (blood glucose concentration greater than 250 mg/dL) in the STZ-treated animals. The blood glucose concentration in the STZ-treated animals had typically increased from approximately 100 mg/dL to more than 400 mg/dL at this time. Drug treatment was initiated after 1 or 6 weeks when STZ-treated diabetic rats demonstrate significantly elevated VEGF expression and BRB breakdown. The nondiabetic control rats (CTL) were treated with a distilled water vehicle (VEH), and the STZ-treated diabetic rats were treated with brimonidine (BRI; 0.01, 0.1, or 1 mg/kg daily) or VEH for 4 weeks. BRI or distilled water was administered continuously via an osmotic minipump (model 2ML2, 5 L/h; Alzet Osmotic Pumps, Cupertino, CA) inserted subcutaneously in the back of the animal, as described previously. Each minipump was replaced with a fresh minipump after 2 weeks. After 4 weeks of continuous treatment, VEGF protein concentrations in the retina and vitreous fluid, glutamate concentrations in the vitreous fluid, and BRB breakdown were determined. Body weight and blood glucose concentration in each rat were also measured at the end of the 4-week treatment period.

**Analysis of VEGF Protein and Glutamate**

Rats were killed by using CO2. The retinas and vitreous fluid were rapidly isolated and frozen in liquid nitrogen. For analysis of vitreal VEGF concentrations, vitreous fluid was pooled from both eyes of each rat, and the concentration of VEGF protein in the vitreous fluid was measured as described previously with an ELISA kit (R&D Systems Inc., Minneapolis, MN) that recognizes all the splice variants of VEGF. Vitreal VEGF concentrations were normalized to total protein determined by the Bradford method with a protein assay reagent kit (Bio-Rad Laboratories, Hercules, CA).

Glutamate concentrations in the vitreous fluid of each eye were measured by high-performance liquid chromatography tandem mass spectrometry (HPLC MS/MS) with a mass spectrometer (AB Sciex API 5000; Applied Biosystems [ABI], Foster City, CA), an autosampler (Shimadzu Scientific Instruments, Columbia, MS), and HPLC pumps (Shimadzu Scientific Instruments). HPLC was performed on a reversed-phase column (Luna C18, 30 × 2.0 mm, 5 μm particle size; Phenomenex, Torrance, CA) with a linear acetonitrile/10 mM ammonium formate gradient containing 0.5% formic acid at a flow rate of 50 μL/min. Mass spectrometric analysis was performed by multiple reaction monitoring (MRM) at m/z 148 → 84 for glutamate with a quantitation range of 0.1 to 600 ng.

**Western Blot Analysis**

After the appropriate treatment, the rats were killed with CO2. The retinas were dissected and homogenized by sonication at 4°C. Each retina was homogenized in 200 μL of 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 no 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM benzamidine, phosphatase inhibitor cocktails 1 and 2 (10 μL/mL; Sigma, St. Louis, MO), 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 with a protein assay reagent kit (Bio-Rad Laboratories). Soluble protein (80 μg) was resolved by SDS-PAGE on 4% to 12% 1.0-mm 10-well Bis-Tris minigels (NuPAGE Novex; Invitrogen, Carlsbad, CA) and electrotransferred to a 0.2-μm pore PVDF membrane (Invitrogen). The membrane was then cut into two pieces, and the upper and lower portions of the membrane were blotted with monoclonal anti-β-actin 1:10,000 (Catalog SC-7269; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-VEGF 1:200 (Catalog SC-7269; Santa Cruz Biotechnology) antibodies, respectively. Immunoreactive bands were visualized by enhanced chemiluminescence (PerkinElmer, Waltham, MA), and relative band density was determined on scanned autoradiographs with image-analysis software (FluorChem HD2; Alpha Innotech Corp., San Leandro, CA). The intensity of the β-actin signal, which was not different in diabetic and control rats (not shown), was used as an endogenous control for loading. Data are expressed as the VEGF:β-actin densitometric unit ratio.

**Measurement of BRB Breakdown**

BRB breakdown was measured using Evans blue dye, which irreversibly binds to plasma albumin. As described previously, briefly, Evans blue was dissolved in normal saline at 45 mg/mL. The animal was deeply anesthetized, and the right jugular vein and iliac artery were cannulated, and Evans blue solution was injected through the jugular vein at a dosage of 45 mg/kg. Blood (200 μL) was withdrawn from the iliac artery 2 minutes after Evans blue injection and then every 4 minutes for 120 minutes. The total volume of blood withdrawn was 1 mL for each animal. After the dye had circulated for 120 minutes, the chest cavity was opened and the animal was perfused through the left ventricle with a solution of 0.05 M citrate buffer (pH 3.5; 37°C) containing no fixative for 2 minutes at 66 mL/min to clear the dye. The
perfusion appeared clear at the end of this time. Immediately after perfusion, the eyes were enucleated and the retinas were carefully dissected under an operating microscope. Evans blue in the retinas and blood samples was quantitated as described previously. BRB breakdown was determined by the calculation: BRB breakdown = (retinal Evans blue in micrograms/retina dry weight in grams)/(time-averaged plasma Evans blue in micrograms/plasma volume in microliters × circulation time in hours) and was expressed as microliters plasma/gram retina dry weight per hour.

Statistical Analysis

Descriptive statistics (mean ± SEM in the figures) were calculated on a spreadsheet (Excel; Microsoft Corporation, Redmond, WA). Differences between treatment groups were evaluated by analysis of variance (JMP software; SAS Institute, Cary, NC). Pair-wise comparisons between groups were evaluated with the Tukey-Kramer HSD procedure to adjust for multiple comparisons. Significance levels were set at *P < 0.05 (**P < 0.01 / ***P < 0.001 / ****P < 0.001)*/ 

RESULTS

Effect of BRI on VEGF Protein Expression in Retina of STZ-Induced Diabetic Rats

The effect of prolonged BRI treatment on VEGF protein expression in the retinas of STZ-induced diabetic rats was determined by dividing the LE rats into three groups with similar distributions of blood glucose levels and body weights. Two groups were treated with a single intravenous dose of STZ to induce diabetes and the remaining control group was treated with vehicle. Seven days later, the animals began 4 weeks of drug treatment via osmotic minipump. STZ-treated animals were treated with 1 mg/kg/d BRI or VEH, and nondiabetic control animals were treated with VEH. Retinal VEGF protein expression was measured by Western blot analysis at the completion of treatment. A single VEGF band with a molecular mass between 14 and 19 kDa was detected in retinal samples from control and diabetic rats (Fig. 1A). In contrast, a previous study detected a single VEGF protein band with a molecular mass between 20 and 28 kDa in retinal samples from BN newborn rats. This difference in the molecular mass of the VEGF band observed may result from different VEGF protein isoforms in newborn and adult rats. Similar to previous observations, there was a significant, 10-fold increase in the expression of VEGF protein in the retinas of STZ-induced diabetic rats compared with nondiabetic control rats at 5 weeks after the induction of diabetes (Fig. 1). Treatment with BRI significantly reduced the expression of retinal VEGF protein in STZ-induced diabetic rats by ~60% (Fig. 1). The expression of retinal VEGF protein in BRI-treated STZ-induced diabetic rats was only fourfold higher than that observed in the nondiabetic control animals, and the difference was not statistically significant (*P > 0.05, Fig. 1).

Effect of BRI on VEGF Protein Concentrations in Vitreous Fluid of STZ-Induced Diabetic Rats

The effect of prolonged BRI treatment on the concentration of VEGF protein in the vitreous fluid of STZ-induced diabetic rats was determined by dividing LE rats divided into five groups with similar distributions of blood glucose concentrations and body weights. Four groups were treated with a single intravenous dose of STZ to induce diabetes, and the remaining control group was treated with vehicle. Seven days later, the animals began 4 weeks of drug treatment via osmotic minipump. The nondiabetic control animals were treated with VEH, and the STZ-treated animals were treated with BRI (0.01, 0.1, or 1 mg/kg daily) or VEH. VEGF protein concentrations in the vitreous fluid were quantitated at the completion of treatment. The concentration of VEGF protein (mean ± SEM) in the vitreous fluid of STZ-induced diabetic rats (182 ± 18 pg/mL) was significantly increased compared with the concentration in nondiabetic control rats (100 ± 10 pg/mL) at 5 weeks after the induction of diabetes (Fig. 2). Treatment with BRI at 0.01, 0.1, and 1 mg/kg/d dose-dependently inhibited the effect of STZ treatment on the VEGF concentration and reduced the concentration of VEGF protein in the vitreous fluid of STZ-induced diabetic rats to 135 ± 5, 112 ± 8, and 107 ± 12 pg/mL, respectively (Fig. 2). There was no significant difference between the concentration of VEGF protein in diabetic rats treated with 0.01, 0.1, or 1 mg/kg/d BRI and the concentration of VEGF protein in nondiabetic control rats.

Effect of BRI on VEGF Protein Concentrations in Vitreous Fluid of STZ-Induced BN and LE Diabetic Rats

To determine whether the effect of prolonged BRI treatment on VEGF protein concentrations in the vitreous fluid of STZ-induced diabetic rats is dependent on the strain of rats used as a model system, we distributed both BN and LE rats into three groups and treated them with CTL-VEH, STZ-VEH, or STZ-BRI (1 mg/kg/d), as described earlier. VEGF protein concentrations in the vitreous fluid were determined at the completion of treatment. Results in BN rats were similar to those in LE rats
The concentration of VEGF protein (mean ± SEM) in the vitreous fluid of STZ-induced diabetic BN rats (259 ± 7 pg/mL) was significantly increased compared with the concentration in nondiabetic control BN rats (157 ± 20 pg/mL) at 5 weeks after the induction of diabetes, and long-term treatment with BRI significantly inhibited the effect of STZ treatment on VEGF concentrations and reduced the concentration of VEGF protein in the vitreous fluid of STZ-induced diabetic BN rats to 140 ± 3 pg/mL, a concentration comparable to that observed in nondiabetic VEH-treated BN rats (Fig. 3). In comparison, the concentration of VEGF protein in the vitreous fluid was 164 ± 18 pg/mL in nondiabetic control LE rats, 228 ± 17 pg/mL in diabetic LE rats, and 153 ± 6 pg/mL in BRI-treated diabetic LE rats (Fig. 3). These results indicate that the attenuation of VEGF protein concentrations in the vitreous fluid of STZ-induced diabetic rats by BRI is not dependent on the use of LE rats as the model system.

**Effect of BRI on BRB Breakdown in STZ-Induced Diabetic Rats**

The effect of long-term BRI treatment on BRB leakage in STZ-induced diabetic rats was determined by distributing BN rats into three groups and treating them with CTL-VEH, STZ-VEH, or STZ-BRI (1 mg/kg/d), as described earlier. BRB leakage was measured at the completion of treatment by the accumulation of albumin-bound Evans blue dye in retinal tissue after perfusion to clear the albumin-bound dye from the vasculature. This measure suggests BRB leakage but is not a quantitative measure of permeability alone, as changes in vascular parameters such as surface area of the vessels, capillary pressure, and oncotic pressure, as well as clearance of the dye, can affect the results. Leakage of the BRB (mean ± SEM) was significantly increased from 9.1 ± 1.0 µL plasma/gram retina × hour−1 in nondiabetic control rats to 22.9 ± 2.7 µL plasma/gram retina × hour−1 in STZ-treated diabetic rats at 5 weeks after the induction of diabetes (Fig. 4). Treatment with BRI significantly attenuated the effect of STZ treatment on BRB breakdown and reduced vascular leakage in STZ-induced diabetic rats to 12.2 ± 2.9 µL plasma × gram retina dry weight−1.

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932966/)

**Figure 2.** Dose–response effect of BRI on VEGF protein concentrations in the vitreous fluid of diabetic LE rats. The rats were treated with a single intravenous dose of STZ or vehicle control. Seven days later, the STZ-treated animals were treated with one of three dosages of BRI (STZ-BRI) or VEH (STZ-VEH) and the control animals were treated with VEH (CTL-VEH) for 4 weeks via osmotic minipump. At the end of treatment, the animals were killed, vitreous fluid was collected, and vitreal VEGF protein concentrations were determined by ELISA. Error bars, SEM. **P < 0.01 vs. CTL-VEH, ***P < 0.001 vs. STZ-VEH, n = 4–5.

![Figure 3](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932966/)

**Figure 3.** Effect of BRI on VEGF protein concentrations in the vitreous fluid of diabetic BN and LE rats. The rats were treated with a single intravenous dose of STZ or vehicle control. Seven days later, the STZ-treated animals were further treated with 1 mg/kg/d BRI (STZ-BRI) or VEH (STZ-VEH) and the control animals were treated with VEH (CTL-VEH) for 4 weeks via osmotic minipump. At the end of treatment, the animals were killed, vitreous fluid was collected, and vitreal VEGF protein concentrations were determined by ELISA. Error bars, SEM. *P < 0.05 and **P < 0.01 vs. CTL-VEH, n = 4–5 (BN rats) and n = 3–5 (LE rats).
Effect of BRI on BRB breakdown in the retinas of diabetic BN rats. The rats were treated with a single intravenous dose of STZ or vehicle control. Seven days later, the STZ-treated animals were further treated with 1 mg/kg/d BRI (STZ-BRI) or VEH (STZ-VEH), and the control animals were treated with VEH (CTL-VEH) for 4 weeks via osmotic minipump. At the end of treatment, BRB breakdown in the retinas of the animals was determined by the Evans blue technique. Error bars, SEM. **P < 0.01 vs. CTL-VEH, *P < 0.05 vs. STZ-VEH, n = 5.

Effect of BRI on BRB Breakdown in STZ-Induced Aged Diabetic Rats

To determine whether the effect of long-term BRI treatment on BRB breakdown in the retinas of STZ-induced diabetic rats is dependent on the duration of diabetes, we divided LE rats into five groups and treated them with CTL-VEH, STZ-VEH, or STZ-BRI (0.01, 0.1, or 1 mg/kg daily) as described earlier, except that treatment with VEH or BRI was begun 6 weeks after STZ treatment. BRB breakdown was determined at the completion of treatment at 10 weeks after the induction of diabetes. Leakage of the BRB (mean ± SEM) was significantly increased from 9.2 ± 1.1 μL plasma × gram retina dry weight$^{-1}$ × hour$^{-1}$ in nondiabetic aged control rats to 38.5 ± 8.1 μL plasma × gram retina dry weight$^{-1}$ × hour$^{-1}$ in STZ-treated aged diabetic rats at 10 weeks after the induction of diabetes (Fig. 5). Treatment with BRI at 0.01, 0.1, and 1 mg/kg/d significantly attenuated the effect of STZ treatment on BRB breakdown and dose-dependently reduced BRB leakage in STZ-induced aged diabetic rats to 19.8 ± 4.8, 11.9 ± 2.1, and 11.2 ± 2.5 μL plasma × gram retina dry weight$^{-1}$ × hour$^{-1}$, respectively (Fig. 5). There was no significant difference between BRB leakage in aged diabetic rats treated with 0.01, 0.1, or 1 mg/kg/d BRI and BRB leakage in nondiabetic aged control rats. These results suggest that BRI reduces BRB breakdown in diabetic rats even after an extended period of hyperglycemia.

Effect of BRI on the Glutamate Concentration in Vitreous Fluid of STZ-Induced Diabetic Rats

To address the possibility that vitreal glutamate is elevated in STZ-induced diabetic rats and BRI affects this increase in glutamate, we distributed LE rats into three groups treated with CTL-VEH, STZ-VEH, or STZ-BRI, as described earlier. The concentration of vitreal glutamate in the vitreous fluid was determined at the completion of treatment at 5 weeks after the induction of diabetes. The concentration of vitreal glutamate (mean ± SEM) in STZ-induced diabetic rats (1764 ± 457 μM) was significantly increased compared with the concentration in nondiabetic control rats (603 ± 130 μM) after 5 weeks of diabetes (Fig. 6).

In this study prolonged treatment with BRI significantly attenuated the elevated expression of VEGF in the retina of diabetic rats and the increased concentration of VEGF in the vitreous, without affecting the vitreal glutamate concentration. Long-term BRI treatment also dose dependently attenuated the increase in retinal vascular leakage and BRB breakdown in diabetic rats. The effects of BRI were not dependent on the particular strain of rats used as the model system, as similar results with respect to VEGF concentrations in vitreous fluid were observed in BN as well as LE diabetic rats. In most of the experiments described herein, treatment with BRI was begun after 1 week of diabetes, but results were similar when the duration of diabetes was extended and treatment was begun after 6 weeks of diabetes. These results suggest that BRI could be useful for treatment of DR in humans, who typically develop the clinical manifestations of DR after several years of diabetes.

VEGF has an important role in DR because it causes vascular leakage, which can result in DME at any stage of the disease, as well as neovascularization in advanced DR. Most moderate
vision loss in diabetic patients can be attributed to DME.54 The elevation of VEGF expression in more advanced stages of DR is stimulated by hypoxia resulting from ischemia55 and is most pronounced in patients with active neovascularization.36 The stimulus for the early elevation of VEGF expression in the retina of diabetic animals, however, has not been completely determined.5,7 The destruction of single capillaries may not lead to significant hypoxia,7,57 and other factors such as high glucose concentrations, cytokines, and activation of advanced glycation end products, PKCβ, and angiotensin II have been shown to increase VEGF expression58 and could have a role in the diabetic retina. Hyperglycemia is associated with oxidative stress and free radical production,17 and these rather than ischemia may be the stimulation for the increased expression of VEGF in early DR, because expression of VEGF is elevated before significant capillary occlusion is observed in STZ-induced diabetic rats.59

Induction of diabetes with STZ is associated with hyperglycemia and significant weight loss.15 In our experiments, no supplemental insulin was administered to prevent weight loss, and no animals were excluded from analysis. Long-term daily treatment with BRI had no effect on the hyperglycemia and weight loss in diabetic animals, suggesting that the effects of BRI on retinal VEGF expression and BRB breakdown are most likely mediated by a local mechanism rather than by a metabolic, systemic mechanism. Consistent with this suggestion, we found that the vitreoretinal concentrations of BRI as measured by LC-MS/MS after 4 weeks of treatment in our paradigm are more than sufficient for biological activity. Mean vitreal and vitreous fluid of diabetic LE rats. The rats were treated with a single intravenous injection of streptozotocin (STZ-BRI) or VEH. Mean body weight in kg (SEM) and mean blood glucose in mg/dL (SEM) were measured in STZ-induced diabetic animals after 4 weeks via osmotic minipump. At the end of treatment, the animals were killed, vitreous fluid was collected, and the vitreal glutamate concentration of each eye was determined by HPLC MS/MS. Error bars, SEM; *P < 0.05 vs. CTL-VEH, n = 11. **P < 0.01 vs. CTL-VEH, n = 11. Figure 6. Effect of BRI on glutamate concentrations in the vitreous fluid of diabetic LE rats. The rats were treated with a single intravenous dose of STZ or vehicle control. Seven days later, the STZ-treated animals were further treated with 1 mg/kg/d BRI (STZ-BRI) or VEH (STZ-VEH), and the control animals were treated with VEH (CTL-VEH) for 4 weeks via osmotic minipump. At the end of treatment, the animals were killed, vitreous fluid was collected, and the vitreal glutamate concentration of each eye was determined by HPLC MS/MS. Error bars, SEM; *P < 0.05 vs. CTL-VEH, n = 22.

not find any significant effect of BRI on basal BRB leakage in control nondiabetic rats (data not shown). Our previous study showed that the NMDA receptor competitive antagonist memantine attenuates vitreoretinal VEGF concentrations and BRB breakdown in STZ-induced diabetic rats, suggesting that a hyperglycemia-induced increase in extracellular glutamate and subsequent overactivation of NMDA receptors is involved in the increase in VEGF expression and BRB leakage observed in DR.10 Increases in extracellular glutamate and overactivation of NMDA receptors have also been implicated in the pathologic course of ischemic retinal injury. In a rat model of retinal ischemia, pretreatment with BRI prevents the elevation of the glutamate concentration in the vitreous fluid and preserves retinal function,26 and pretreatment with memantine reduces RGC loss.43 These observations led us to investigate the possibility that the mechanism of action of BRI in the diabetic retina involves a decrease in the concentration of extracellular glutamate. However, prolonged treatment with BRI did not affect the elevation of the glutamate concentration in the vitreous fluid of STZ-induced diabetic rats, suggesting that the mechanism of action of BRI in decreasing VEGF protein expression and BRB breakdown does not involve the extracellular glutamate concentration. Studies of retinal VEGF mRNA expression in STZ-induced diabetic rats have produced conflicting results, with VEGF mRNA concentrations reported to be increased,37,41 decreased,42,43 or unchanged44 in diabetic rats compared with levels in control rats. These results suggest that regulation of VEGF protein expression in STZ-induced diabetic rats is primarily posttranslational.41 The effect of BRI on VEGF protein expression also appears to be posttranslational, as in this study, mRNA for VEGF was not significantly upregulated in STZ-induced diabetic rats, and long-term BRI treatment had no effect on the expression of VEGF mRNA in STZ-induced diabetic rats (data not shown).

Recent studies have demonstrated α2-adrenergic receptor-mediated modulation of NMDA receptor channel function in prefrontal cortex pyramidal neurons and retinal ganglion cells.26,45 Activation of α2-adrenergic receptors has been shown to cause a decrease in NMDA receptor currents in these neurons. The effects of α2-adrenergic agonists are blocked by treatments that elevate cAMP concentrations26 and are mimicked by a protein kinase A inhibitor,45 suggesting that decreases in cAMP concentrations and protein kinase A activity mediate the modulation of NMDA receptor channel activity. These observations suggest a possible mechanism for the effects of BRI in the diabetic retina, in that BRI may decrease vitreoretinal VEGF protein concentrations and BRB breakdown in STZ-induced diabetic rats by modulating the function of NMDA receptors and decreasing NMDA channel activity, thus preventing the increase in intracellular Ca2+ activation of protein kinase C, increase in VEGF production, and BRB breakdown that would otherwise result from hyperglycemia/oxidative stress-induced overactivation of retinal NMDA receptors. In this scenario, the retinal cells involved could be Müller cells, RGCs, or both, since both cell types have been shown to express NMDA receptors.24,46,47 α2-adrenergic receptors48 (Lai R, et al. IOVS 2008;49:ARVO E-Abstract 4931; Lonngren U, et al. IOVS 2008;49:E-Abstract 6112), and VEGF.49,50 Other potential mechanisms for the effects of BRI on VEGF expression and BRB breakdown are possible, however, and further studies are needed to explore the possible mechanisms for the effects of BRI in diabetic retina.

BRI treatment (1 mg/kg daily for 4 weeks) had no significant effect on either the basal concentration of VEGF in the retina and vitreous fluid of nondiabetic control rats or BRB leakage in nondiabetic control rats (data not shown). It is likely that upregulation of NMDA receptors observed in diabetes52 and/or elevation of extracellular glutamate with subsequent activation...
of NMDA receptors is needed for BRI to affect vitreoretinal VEGF expression and vascular permeability. As it is likely that a basal level of VEGF activity is needed for normal processes such as growth of blood vessels in wound healing and to provide collateral circulation around obstructed blood vessels, treatment with BRI may be devoid of potential side effects associated with anti-VEGF treatments (bevacizumab, ranibizumab) that are expected to decrease basal VEGF activity. As side effects of steroid treatment include cataract development and increases in intraocular pressure,51,52 BRI may also be safer than steroids for the treatment of retinal disease associated with elevated vitreoretinal VEGF expression and BRB leakage.

BRI eye drops reduce intraocular pressure and are used clinically in the treatment of glaucoma and ocular hypertension.53 It has been proposed that BRI that reaches the retina after topical administration may also be neuroprotective and act directly on RGCs to promote their survival and prevent glaucomatous optic nerve damage.54 Although many studies have shown that BRI treatment promotes the survival and helps maintain the function of RGCs in models of optic nerve injury,55,56 to our knowledge, there has been only a single previous report of the effects of BRI in the diabetic retina. In a preliminary report by Mondal et al.,55 clinically significant DME developed in 0% (0/25) of patients who were treated with a placebo and 20% (5/25) of patients who were treated with a placebo (artificial tears). Patients treated with BRI on average had no new microaneurysms, suggesting a possible mechanism involving effects on the vasculature. In the present study, we have shown that BRI prevents the rise in VEGF expression and the breakdown of the BRB that lead to loss of retinal function in DR through a novel mechanism, distinct from the mechanism of anti-VEGF medications or steroids. These results suggest that BRI could be useful for the treatment of ocular diseases that result from oxidative stress and/or ischemia and involve VEGF-mediated vascular leakage or neovascularization, such as non-proliferative and proliferative DR, DME, retinopathy of prematurity, ME secondary to retinal vein occlusion, and wet age-related macular degeneration.

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