Acute Retinal Ischemia Inhibits Endothelium-Dependent Nitric Oxide–Mediated Dilation of Retinal Arterioles via Enhanced Superoxide Production

Travis W. Hein,[1,2] *Yi Ren,*[1,2] *Luke B. Potts,*[3] *Zhaoxu Yuan,*[1,2] *Enoch Kuo,*[1,2] *Robert H. Rosa, Jr,*[1,2] *and Lih Kuo*[1,2,3]

Purpose. Because retinal vascular disease is associated with ischemia and increased oxidative stress, the vasodilator function of retinal arterioles was examined after retinal ischemia induced by elevated intraocular pressure (IOP). The role of superoxide anions in the development of vascular dysfunction was assessed.

Methods. IOP was increased and maintained at 80 to 90 mm Hg for 30, 60, or 90 minutes by infusing saline into the anterior chamber of a porcine eye. The fellow eye with normal IOP (10–20 mm Hg) served as control. In some pigs, superoxide dismutase mimetic TEMPOL (1 mM) or vehicle (saline) was injected intravitreally before IOP elevation. After enucleation, retinal arterioles were isolated and pressurized without flow for functional analysis by recording diameter changes using videomicroscopic techniques. Dihydroethidium (DHE) was used to detect superoxide production in isolated retinal arterioles.

Results. Isolated retinal arterioles developed stable basal tone and the vasodilations to endothelium-dependent nitric oxide (NO)-mediated agonists bradykinin and L-lactate were significantly reduced only by 90 minutes of ischemia. However, vasodilation to endothelium-independent NO donor sodium nitroprusside was unaffected after all time periods of ischemia. DHE staining showed that 90 minutes of ischemia significantly increased superoxide levels in retinal arterioles. Intravitreal injection of membrane-permeable radical scavenger but not vehicle before ischemia prevented elevation of vascular superoxide and preserved bradykinin-induced dilation.

Conclusions. Endothelium-dependent NO-mediated dilation of retinal arterioles is impaired by 90 minutes of ischemia induced by elevated IOP. The inhibitory effect appears to be mediated by the alteration of NO signaling via vascular superoxide.

(*Invest Ophthalmol Vis Sci.* 2012;53:30–36) DOI:10.1167/iovs.11-8753

The neural retina tissue in patients exposed to acute periods of retinal ischemia suffers from blood flow deficit, which can lead to visual impairment and blindness.[1,2] Ocular diseases and complications such as acute angle-closure glaucoma, retinal vascular occlusion, and elevated intraocular pressure during vitreous surgery or after vitreoretinal surgery, are associated with retinal ischemia.[1–3] Currently, treatment modalities for restoring neural retina function are relatively limited. Experimental evidence demonstrating a reduction in retinal blood flow after the initial retinal ischemia suggests that dysfunction of arterioles, the major site for flow regulation, contributes to the persistent retinal damage.[4,5] However, studies are lacking to directly address whether vasomotor function of retinal arterioles is altered after acute retinal ischemia.

In the retinal circulation, the vascular endothelium is important in regulating local blood flow by the release of vasoactive factors such as the potent vasodilator nitric oxide (NO)[6] and vasoconstrictor endothelin-1.[7] It has been shown in pigs that endothelium-dependent arterial dilation and release of NO surrounding the retinal arterioles are reduced in vivo after acute retinal ischemia induced by asphyxia[4] and branch retinal vein occlusion,[8] respectively. However, in the latter study, NO levels were measured on the extraluminal side of arterioles, which hinders unequivocal identification of the source (vascular versus neuronal) of NO. Another phenomenon besides reduced NO bioavailability that has been observed during retinal ischemia is the enhanced production of oxygen-derived free radicals.[9] Elevated levels of superoxide anions could possibly react with vascular NO and reduce NO bioavailability.[10] However, the contribution of oxidative stress to vascular dysfunction in association with retinal ischemia is unclear. Therefore, the effect of retinal ischemia alone, independent of reperfusion, on the retinal arteriolar function and the possible underlying mechanisms contributing to the endothelial dysfunction related to NO bioavailability remain elusive.

In addition to the animal models described above, a frequently used model in a number of species for retinal ischemia is established using elevated intraocular pressure (IOP).[11–15] This model creates retinal ischemia by elevating and maintaining IOP above the retinal perfusion pressure, and correlates with the human diseases of acute angle-closure glaucoma, central retinal artery occlusion, and ophthalmic artery occlusion.[1,11,12] Therefore, in the present study, we used the elevated IOP model to test the hypothesis that acute retinal ischemia via elevation of IOP can lead to endothelial dysfunction and impair vasodilation by reducing NO bioavailability through enhanced levels of superoxide. To eliminate the confounding effects (i.e., neural/glial and hemodynamic factors) commonly encountered in in-vivo preparations, we used an

From the [1]Departments of Ophthalmology and [2]Surgery, Scott & White Eye Institute, and the [3]Department of Systems Biology and Translational Medicine, College of Medicine, Texas A&M Health Science Center, Temple, Texas.

Supported by Scott & White Research Foundation (TWH), Retina Research Foundation (LK), NIH NEI R01EY018420 (TWH), the Scott & White Research Foundation Ophthalmic Vascular Research Program (LK), the Kruse Family Endowment Fund (LK), and NIH NEI K08EY016143 (RHR).

Submitted for publication October 6, 2011; revised October 25, 2011; accepted October 27, 2011.

Disclosure: **T.W. Hein**, None; **Y. Ren**, None; **L.B. Potts**, None; **Z. Yuan**, None; **E. Kuo**, None; **R.H. Rosa, Jr**, None; **L. Kuo**, None

Corresponding author: Travis W. Hein, Departments of Ophthalmology and Surgery, College of Medicine, Texas A&M Health Science Center, 702 Southwest HK Dodgen Loop, Temple, TX 76504; thein@tamu.edu.

Investigative Ophthalmology & Visual Science, January 2012, Vol. 53, No. 1

isolated vessel approach to assess whether retinal ischemia in vivo exerts a direct effect on retinal arterioles. The vasodilator function and levels of oxidative stress in these microvessels were assessed in vitro after 30, 60, or 90 minutes of ischemia.

METHODS

Animal Preparation and Elevated IOP Procedure

All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Scott & White Institutional Animal Care and Use Committee. Pigs (8–12 weeks old of either sex; 7–15 kg) purchased from Real Farms (San Antonio, TX) were sedated with tiletamine and zolazepam (Telazol; 4.4–6 mg/kg, intramuscularly; Fort Dodge Animal Health, Fort Dodge, IA), and anesthetized with isoflurane. Drops of topical 1% tropicamide and 1% cyclogyl were instilled into each eye twice during a 3- to 5-minute period to dilate the pupil and to allow visualization of the retina. The retinal vessels were observed before and after ischemia with a fundus camera (RetCam II; Clarity Medical Systems, Pleasanton, CA) to confirm vascular occlusion (Fig. 1). Then after administration of 0.5% proparacaine hydrochloride for topical anesthesia, a 100-μL Hamilton syringe with a 30-gauge needle and aid of an indirect ophthalmoscope was inserted into the vitreous cavity through the pars plana incision (located 4 mm away from limbus) of both normal control (sham without IOP elevation) and ischemia eyes. Balanced saline solution (Alcon Laboratories, Fort Worth, TX) was infused into the anterior chamber of the left eye to increase IOP to approximately 80 to 90 mm Hg for 30, 60, or 90 minutes. The right eye was left intact as a control. A tonometer (Tonopen; Medtronic Solan, Jacksonville, FL) was used to monitor the IOP. In some animals, 20 μL of vehicle (physiological salt solution; PSS) or superoxide dismutase mimetic TEMPOL (2 μmol) was injected intravitreally over the surfaces of retinal arterioles 15 minutes before elevating IOP. The amount chosen for administration of TEMPOL was based on pig volume of vitreous fluid (2 mL), which resulted in a concentration of 1 mM, and on evidence of reduced superoxide levels in retinal arterioles as assessed by dihyroethidium (DHE) staining in pilot studies. After retinal ischemia, heparin (1000 U/kg) was administered into the marginal ear vein to prevent clotting, and the eyes were enucleated and immediately placed in a moist chamber on ice.

Isolation and Cannulation of Microvessels

After enucleation, the anterior segments and vitreous bodies of the eyes were removed carefully under a dissection microscope.[16] The posterior segment was placed in a cooled dissection chamber (approximately 8°C) containing PSS with 1% albumin (USB, Cleveland, OH). Single retinal arterioles (0.6 to 1.0 mm in length) were carefully dissected out using a pair of microdissection forceps (Dumont; Fine Science Tools, Foster City, CA) with the aid of a stereomicroscope (model SZX12; Olympus, Melville, NY). After careful removal of any remaining neural/connective tissues, the arteriole was then transferred for cannulation to an acrylic resin vessel chamber containing PSS-albumin solution equilibrated with room air at ambient temperature. One end of the arteriole was cannulated using a glass micropipette filled with PSS-albumin solution, and the outside of the arteriole was securely tied to the pipette with 11 to 0 ophthalmic suture (Alcon Laboratories). The other end of the vessel was cannulated with a second micropipette and also secured with suture. After cannulation, the vessel and pipettes were transferred to the stage of an inverted microscope (model CKX41; Olympus) coupled to a video camera (Sony DXC-190; Labtek, Campbell, CA), video micrometer (Cardiovascular Research Institute, Texas A&M Health Science Center, College Station, TX), and a data acquisition system (PowerLab; ADInstruments, Colorado Springs, CO) for continuous measurement and recording of the internal diameter throughout the experiment. The cannulating pipettes were connected to independent pressure reservoirs. By adjusting the height of the reservoirs, the vessel was pressurized to 55 cm H_2O (40 mm Hg) intraluminal pressure without flow. This level of pressure was used based on pressure ranges that have been documented in retinal arterioles in vivo and in the isolated, perfused retinal microcirculation,[17] and was consistent with the estimated ocular perfusion pressure in humans as reported previously.[18] Arterioles with side branches and leaks were excluded from further study and all arterioles used developed basal tone.

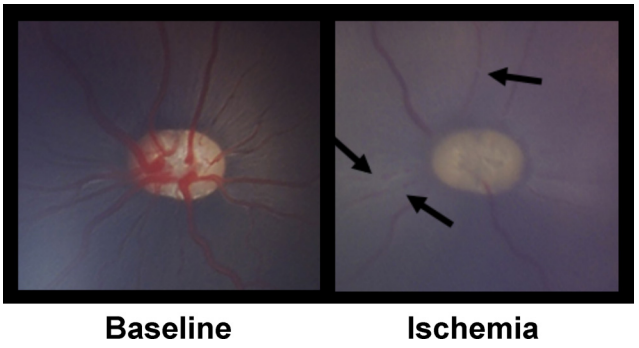

FIGURE 1. Verification of retinal ischemia in elevated IOP model. The porcine retinal circulation was observed before (baseline) and after ischemia with a fundus camera (RetCam II, Clarity Medical Systems) to confirm vascular occlusion. Under baseline conditions before IOP elevation, the perfusion of retinal vasculature was evident. Elevation of IOP to 80 mm Hg (ischemia) resulted in near obstruction of retinal arterioles with apparent box-carring of erythrocytes or segmentation of the blood column within the vessels (*arrows*), indicating lack of blood flow. Note also the pallor of the optic nerve head.

Experimental Protocols

Cannulated arterioles were bathed in PSS-albumin at 36°C to 37°C. After vessels developed a stable basal tone (approximately 60 minutes), vasodilator responses to endothelium-dependent NO-mediated agonists bradykinin (1 pM to 10 nM)[19,20] and neutralized L-lactate (10 mM),[21] and to endothelium-independent NO donor sodium nitroprusside (1 nM to 10 μM) were established. Vessels were exposed to each concentration of agonist for 4 to 5 minutes until a stable diameter was maintained. Drugs and chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated and were dissolved in PSS. Neutralized L-lactate was prepared by dissolving L-lactic acid in water followed by adjustment of the pH to 7.4 with NaOH (10 N).

Detection of Superoxide

Superoxide production in retinal arterioles was evaluated with the fluorescent dye DHE.[22] Retinal arterioles isolated from control and ischemia eyes without and with intravitreal injection of vehicle (saline) or TEMPOL were stained with DHE (4 μM) for 30 minutes. After being washed, arterioles were embedded in a formulation of water-soluble glycols and resins (Tissue-Tek O.C.T. Compound; Sakura Finetek, Torrance, CA) for cryostat sections. The embedded arterioles were cut into 10-μm thick sections and placed on glass slides. Images were taken with a fluorescence microscope (Axiovert 200; Carl Zeiss MicroImaging, Thornwood, NY). Fluorescence was detected with a 620/60 nm bandpass emission filter. Control and ischemia samples were placed on the same slide and processed under the same conditions. Settings for image acquisition were identical for control and experimental tissues. The resulting images were quantitatively analyzed for DHE fluorescence intensity using software developed by Wayne Rasband (ImageJ; National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html) with the following procedure. The average fluorescence intensity was determined for the DHE-treated control vessels from each experimental group and then all individual control and ischemia vessels from the same slide group were normalized to the corresponding average value.

Data Analysis

At the end of each functional experiment, the vessel was relaxed with 0.1 mM sodium nitroprusside in EDTA (1 mM) Ca^{2+}-free PSS to obtain its maximum diameter at 55 cm H_2O intraluminal pressure.[16] Diameter changes in response to vasodilator agonists were normalized to this maximum vasodilation and expressed as % maximum dilation. Data are reported as mean ± SEM and *n* value represents the number of animals studied. Student's *t*-test or ANOVA followed by Bonferroni multiple-range test was used to determine the significance of experimental interventions, as appropriate. A value of $P < 0.05$ was considered significant.

Results

Effect of Retinal Ischemia on NO-Mediated Vasodilation

In this study, all control ($n = 47$) and ischemia vessels ($n = 51$) developed a similar level of basal tone (constricted to 54 ± 2% and 58 ± 2%, respectively, of their maximum diameter; $P = 0.14$) at 36°C–37°C bath temperature with 55 cm H_2O intraluminal pressure. The average resting and maximum diameters were 54 ± 2 μm and 98 ± 2 μm for control vessels and 57 ± 3 μm and 97 ± 3 μm for ischemia vessels. Bradykinin dilated retinal arterioles isolated from control nonischemia eyes in a concentration-dependent manner after all three experimental time periods (Fig. 2). The vasodilator response to bradykinin was significantly reduced in eyes after exposure to 90 minutes, but not 30 or 60 minutes, of ischemia (Fig. 2). In addition, neutralized L-lactate, a putative endogenous vasodilator of retinal arterioles via endothelial production of NO,[21] caused dilation of retinal arterioles and this vasodilator response was inhibited after exposure to ischemia for 90 minutes (Fig. 3). The resting vascular tone was not altered by 90 minutes of ischemia (control: 52 ± 3% versus 90-minute ischemia: 58 ± 3%; $P = 0.12$). Furthermore, the dilation of retinal arterioles to the endothelium-independent NO donor sodium nitroprusside was not affected by 90 minutes of ischemia (Fig. 4).

Role of Superoxide in Retinal Arteriolar Dysfunction

To determine whether superoxide production is involved in the impairment of bradykinin-induced vasodilation, the membrane-permeable superoxide dismutase mimetic TEMPOL or vehicle (saline) was administered intravitreally to the ischemia and control eyes before 90 minutes of IOP elevation or normal pressure in these eyes. After treatment with TEMPOL, dilation of control and ischemia retinal arterioles to bradykinin was comparable (Fig. 5A). On the other hand, the reduction in dilation of ischemia vessels to bradykinin remained after intravitreal injection of vehicle (Fig. 5A). Neither TEMPOL nor vehicle altered the dilation of control or ischemia vessels to sodium nitroprusside (Fig. 5B). In addition, the basal tone of control and ischemia vessels was unaffected after TEMPOL treatment (control: 57 ± 3% versus ischemia: 61 ± 3%; $P = 0.33$).

Effect of Retinal Ischemia on Vascular Superoxide Production

In retinal arterioles isolated from nonischemia eyes (control), DHE fluorescence revealed sparse levels of superoxide in the vascular wall (Fig. 6). Exposure to 30 or 60 minutes of ischemia tended to slightly increase superoxide levels in retinal arterioles, but in a nonsignificant manner. By contrast, after 90 minutes of ischemia, retinal arterioles displayed a significant increase in superoxide levels in the vascular wall. Intravitreal injection of TEMPOL, but not vehicle, before elevation of IOP prevented the increase in ischemia-induced fluorescence signals for superoxide in the retinal arterioles (Fig. 6).

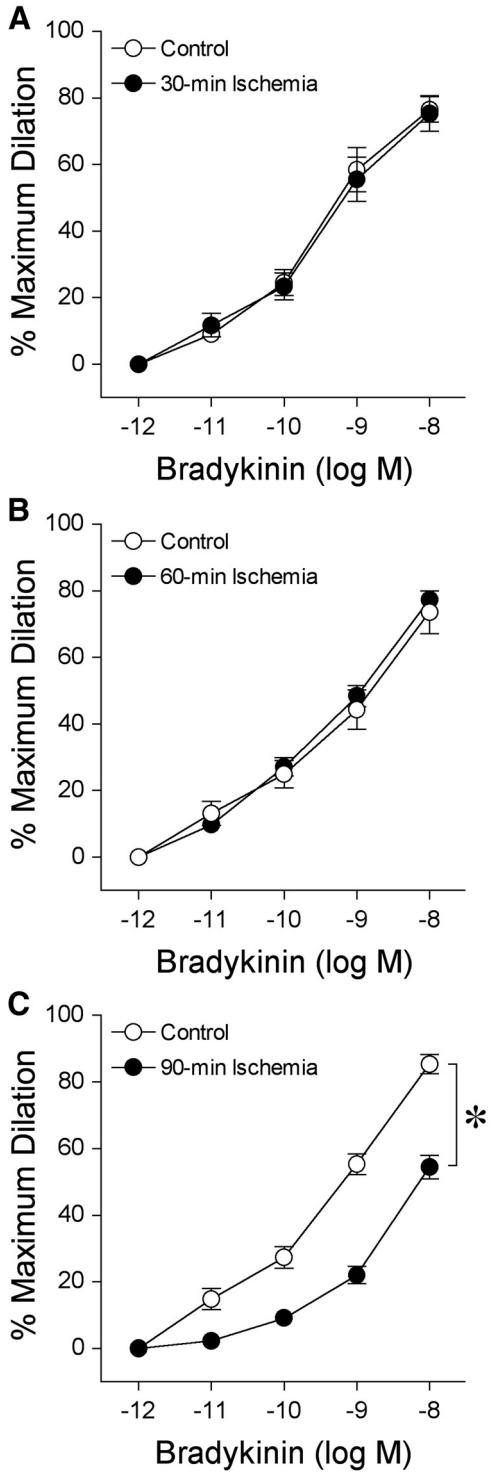

Figure 2. Effect of retinal ischemia on vasodilator response to bradykinin. Concentration-dependent dilation to bradykinin was assessed in porcine retinal arterioles isolated from normal eyes (control) or from eyes exposed to elevation of IOP (ischemia) for (**A**) 30 minutes ($n = 6$), (**B**) 60 minutes ($n = 7$), or (**C**) 90 minutes ($n = 12$). Bradykinin-induced dilation was significantly reduced in arterioles exposed to 90 minutes but not 30 minutes or 60 minutes of ischemia. *n*, number of animals (1–2 vessels from each eye per animal). *$P < 0.05$ versus control.

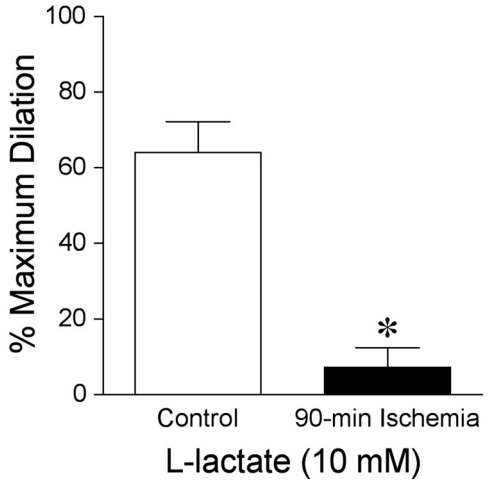

FIGURE 3. Effect of retinal ischemia on vasodilator response to L-lactate. L-lactate caused dilation of porcine retinal arterioles isolated from normal eyes (control), but this response was significantly reduced in arterioles exposed to 90 minutes of ischemia ($n = 5$). n, number of animals (1–2 vessels from each eye per animal). $^*P < 0.05$ versus control.

DISCUSSION

There is a paucity of information on the effect of acute ischemia on vasomotor function of the retinal microvasculature and the mechanism underlying development of the vascular pathophysiology is virtually unknown. Because retinal vascular disease is associated with ischemia and increased oxidative stress, it is imperative to investigate the effect of retinal ischemia on vasodilator function of retinal arterioles and to assess the possible role of oxygen-derived free radicals in vascular dysfunction. The salient findings of the present study demonstrate that exposure to elevated IOP for at least 90 minutes in the pig diminishes the ability of isolated retinal arterioles to dilate to bradykinin and to L-lactate, but not to sodium nitroprusside, after ischemia, and that pharmacological scavenging of superoxide anions preserves vasodilator function. Therefore, short-term retinal ischemia enhances oxidative stress, which subsequently compromises retinal endothelial function in terms of NO-mediated vasodilation. To the best of our knowledge, these results are the first evidence for a direct impact of elevated IOP-induced retinal ischemia on vasomotor function of retinal arterioles.

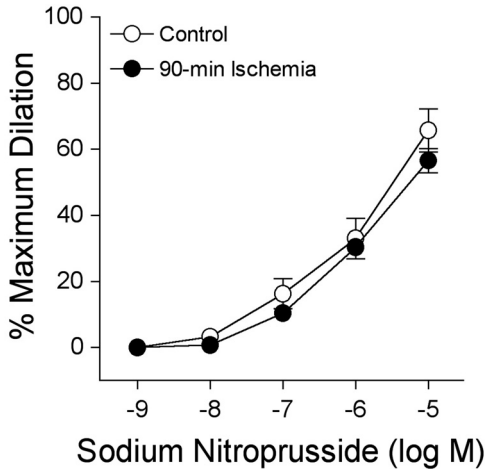

FIGURE 4. Effect of retinal ischemia on vasodilator response to sodium nitroprusside. Concentration-dependent dilation to sodium nitroprusside was assessed in porcine retinal arterioles isolated from normal eyes (control) or from eyes exposed to elevation of IOP (ischemia) for 90 minutes ($n = 7$). Sodium nitroprusside–induced dilation was comparable in control and ischemia vessels. n, number of animals (1–2 vessels from each eye per animal).

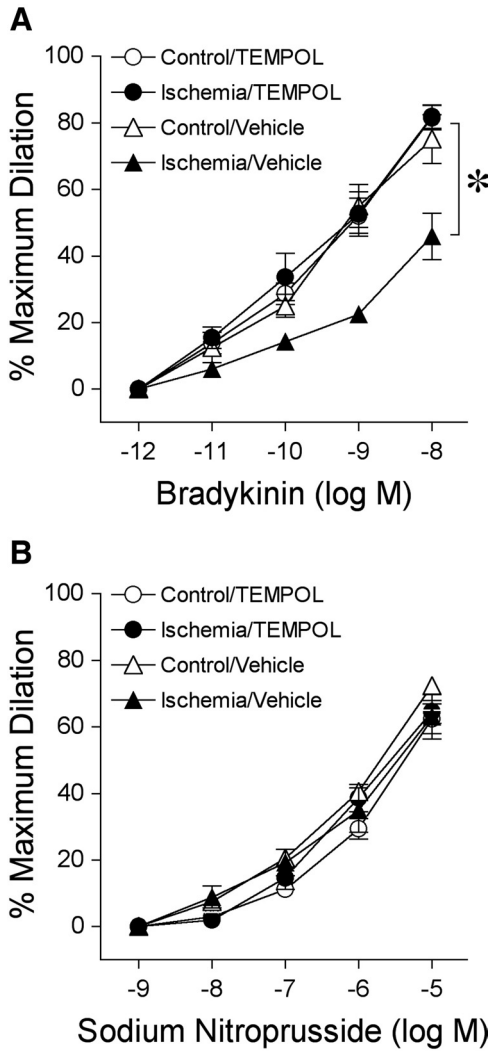

FIGURE 5. Role of superoxide in retinal ischemia-induced vasodilator dysfunction. Both eyes were administered intravitreal injection of superoxide dismutase mimetic TEMPOL (1 mM) or vehicle (saline) 15 minutes before 90 minutes of elevated IOP (ischemia) or normal IOP. (**A**) Pretreatment with TEMPOL ($n = 7$) but not vehicle ($n = 3$) prevented the reduction in dilation to bradykinin in the vessels from the ischemia eye. Superoxide scavenging did not alter the vasodilator response of vessels from normal eyes. (**B**) Dilation of control or ischemia vessels to sodium nitroprusside was unaffected by TEMPOL ($n = 6$) or vehicle ($n = 3$). n, number of animals (1–2 vessels from each eye per animal). $^*P < 0.05$ versus control/vehicle and TEMPOL-treated groups.

In the present study, we used an established in-vivo elevated IOP model[11–15] to characterize the retinal microvascular dysfunction associated with ischemia and then used an in-vitro approach to elucidate the putative cellular/molecular mechanisms. Because pig and human retinas exhibit similar characteristics in neuroanatomy, immunology, and vascular reactivity,[23–30] we used the pig as the experimental model. In regard to vascular function, we recently showed that dilations of isolated human retinal arterioles to pharmacological (bradykinin, adenosine, and endothelin-1) and physiological vasoac-

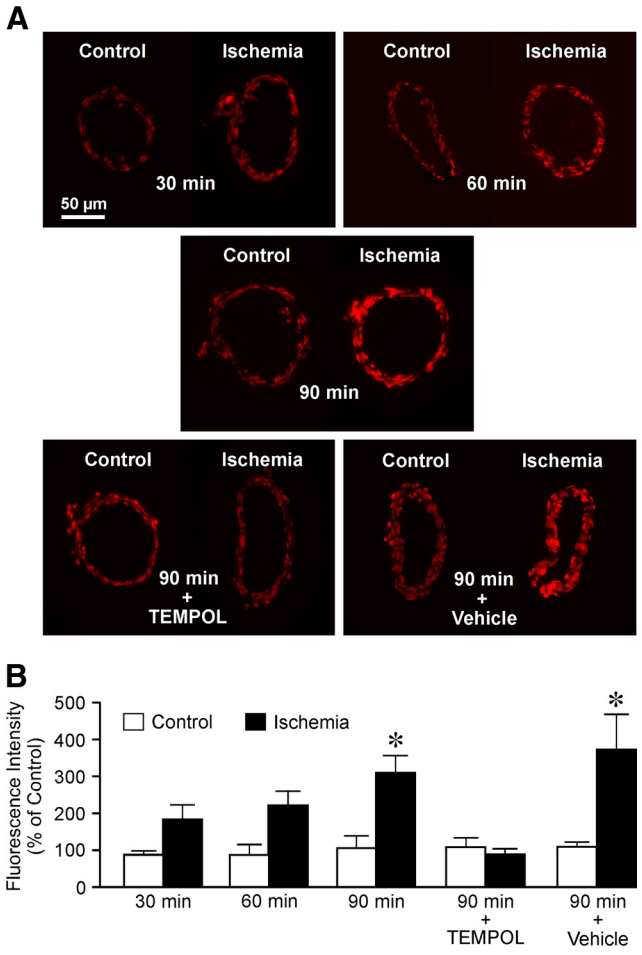

FIGURE 6. DHE fluorescence imaging of superoxide in retinal arterioles. (**A**) Retinal arterioles were isolated from eyes after 30, 60, or 90 minutes of exposure to normal IOP (control) or elevated IOP (ischemia). In another group, vessels were isolated from eyes administered either TEMPOL or vehicle (saline) before 90 minutes of normal IOP or elevated IOP. At identical settings for image acquisition, fluorescent signals in the vessel wall were markedly greater in the vessels exposed to 90 minutes of ischemia. Note the increased fluorescence reflecting superoxide levels in the endothelium and media (determined by the overlay of bright field image). Preischemia treatment with TEMPOL but not vehicle prevented the increase in superoxide signal in the vessels exposed to 90 minutes of ischemia. (**B**) Quantitative analysis of DHE fluorescence signals for control and ischemia vessel groups shown in (**A**). Data were obtained from 4 to 6 separate experiments per group. $^*P < 0.05$ versus control.

tive stimuli (flow-induced vasodilation and pressure-induced myogenic tone) were comparable to the responses in pig retinal arterioles.[30] Importantly, at a mechanistic level, human and porcine retinal arterioles both elicited NO-mediated dilation to bradykinin and to increases in flow, as well as Rho kinase-dependent myogenic tone and constriction to endothelin-1 in a comparable manner. Retinal ischemia was produced in the present study by elevating and maintaining IOP (80–90 mm Hg) above the retinal perfusion pressure. Several recent studies have also used this model at similar levels of elevated IOP in the pig to study the effect of ischemia on neural/glial cells and on gene/protein expression in neural retina and retinal vessels. The results from those studies demonstrated that short-term ischemia (60 minutes to 2 hours) followed by reperfusion was sufficient to promote Müller cell gliosis[31] and loss of ganglion cells,[32,33] and to increase expression of inflammatory proteins (mitogen-activated protein kinases and tumor necrosis factor alpha) in neural and vascular retina.[34,35] Our findings further support use of the porcine model and provide direct evidence for the effect of acute retinal ischemia alone on retinal arteriolar function.

Accumulating evidence suggests that retinal ischemia due to incompetent flow regulation and/or abnormal vasomotor function initiates or participates in the pathogenesis of several retinal diseases, including retinal vascular occlusion, acute angle-closure glaucoma, and diabetic retinopathy.[1,36] Although experimental studies have shown that a brief episode of retinal hypoxic-ischemia in pigs can impair endothelium-mediated dilation to acetylcholine in the retinal circulation and reduce retinal blood flow,[4,5] the direct effect of ischemia on retinal arterioles and the underlying mechanisms responsible for this diminished vasodilator response are not completely understood. The lack of this basic information hampers our understanding of the development/progression of vascular abnormalities associated with retinal ischemia. Therefore, in the present study we used an isolated vessel approach[16,20,21,30,37] to directly characterize the vasomotor response of small retinal arterioles, the major regulators of retinal blood flow, after retinal ischemia under a defined environment. We first established the temporal effect of retinal ischemia on vasodilator function and found that 30 and 60 minutes of elevated IOP were insufficient to hinder dilation of retinal arterioles to endothelium-dependent NO-mediated agonist bradykinin. On the other hand, exposure to ischemia for 90 minutes led to significant reduction of vasodilation to bradykinin. Because endogenous tissue levels of bradykinin are increased during acute ischemia,[38,39] the release of this peptide might be important to blood flow regulation during inadequate supply of nutrients. Our current finding on the impairment of bradykinin-induced vasodilation may have clinical implication in the development of retinal vascular disease. Because vasodilation to endothelium-independent NO donor sodium nitroprusside was unaffected after all time periods of ischemia, it appears that acute retinal ischemia selectively impaired endothelial function. Interestingly, Hayreh and colleagues[40–42] demonstrated narrowing of retinal vessels and irreversible damage of the neural retina (visual evoked response) after occlusion of the central retinal artery for at least 105 minutes in monkeys. The slight differences in retinal tolerance (105 minutes vs 90 minutes in the present study) may be related to species (monkey versus pig), ischemia model (direct arterial occlusion versus elevated IOP) or approach (in vivo versus in vitro assessment of retinal circulation). Nonetheless, these studies together support comparable time-dependent susceptibility of the retinal circulation to acute retinal ischemia. Although we did not examine the influence of reperfusion on vascular function, the temporal findings in our study are consistent with an earlier report that 90 minutes of retinal ischemia in the rat, but not 30 or 60 minutes, followed by reperfusion was required to cause sustained retinal edema.[43] Ischemia alone was not evaluated in this earlier study, but the 90 minutes of ischemia seems sufficient to elicit both functional and structural changes in the retinal microcirculation.

In addition to the reduction of bradykinin-induced NO-mediated dilation, acute retinal ischemia also reduced the dilation of retinal arterioles in response to the endogenous metabolic factor L-lactate. A responsive change in retinal arteriolar diameter to vasoactive metabolites is essential in terms of regulating retinal blood flow to match metabolic demands of the retinal tissue.[44] Lactate is a major product of retinal tissue metabolism that has been proposed to be an important signal for modulating retinal microvascular tone. The putative regulatory role of lactate is based on the observations in animal and human studies showing that intravitreal[45] and intravenous[46,47] administration of L-lactate, as well as exercise-induced hyper-

lactatemia,[48] evokes dilation of retinal arteries and increases retinal blood flow, respectively, in vivo. Similar to our earlier study, we used neutralized L-lactate to prevent the potential influence of pH on vascular tone. Our previous study showed that neutralized L-lactate caused endothelium-dependent NO-mediated dilation of porcine retinal arterioles.[21] The current findings on impaired vasodilation to bradykinin and L-lactate support the view that pathophysiological and physiological vasodilator agonists are susceptible to detrimental actions of acute 90-minute retinal ischemia in the context of impairing endothelium-dependent NO-mediated vasodilation.

A potential mechanism contributing to the ischemia-induced vascular dysfunction could be increased oxidative stress within the neuronal and/or vascular cells. It has been shown that reactive oxygen species can be increased in the retinal tissue during acute retinal ischemia[9] and elevated IOP.[49,50] Furthermore, an earlier study surmised that elevated IOP reduces NO-mediated increase in blood flow via enhanced production of reactive oxygen species leading to reduction of electroretinogram, based on the evidence that antioxidants in the absence but not presence of NO synthase blockade (nitro-L-arginine) preserved neuronal function.[49] However, it remains unclear whether ischemia elicits generation of reactive oxygen species within the retinal vasculature and subsequently affects vasomotor function. Reactive oxygen species such as superoxide anions can alter NO-mediated reactivity directly by inactivating NO[51] and/or inhibiting NO synthase activity.[52] Therefore, we investigated whether superoxide plays a role in vascular dysfunction. Our data support the idea of NO deficiency because the intravitreal administration of the membrane permeable superoxide dismutase mimetic TEMPOL before 90 minutes of elevated IOP preserved the postischemia dilation of isolated retinal arterioles to bradykinin. The salutary effect of TEMPOL seems to be specific because it did not affect resting basal tone or vasodilation to SNP. Further support for superoxide production was revealed with DHE staining showing that 90 minutes of retinal ischemia is capable of generating TEMPOL-sensitive superoxide in the vascular wall of retinal arterioles. These findings in the retinal vasculature extend previous evidence in vivo showing that free radical scavengers prevented ischemia (45 minutes)-induced increase in oxygen-derived free radical production in the rabbit retina and prevented neuronal dysfunction (loss of neuronal b wave during electroretinogram measurement).[50] Although the possible contribution of specific vascular oxidant enzymes to the impaired NO-mediated dilation remains unclear, our current data support the idea that increased oxidative stress within the vascular wall during acute retinal ischemia contributes to endothelium-dependent vasodilator dysfunction.

In conclusion, we have demonstrated for the first time that retinal ischemia elicited by 90-minute elevation of IOP impairs endothelium-dependent NO-mediated dilation of isolated porcine retinal arterioles. The mechanism underlying the inhibitory effect of retinal ischemia involves enhanced levels of superoxide in arterioles. This acute retinal ischemia model in the pig will be a useful tool to investigate the direct impact of ischemia on vascular reactivity in the retinal microcirculation. The results obtained from the present studies provide further knowledge toward our understanding of the pathogenesis of retinal vascular disease associated with acute retinal ischemia.

Acknowledgments

The authors thank Wenjuan Xu and Hongfang Liu for their expert technical assistance.

References

1. Osborne N, Casson R, Wood J, et al. Retinal ischemia: mechanisms of damage and potential therapeutic strategies. *Prog Ret Eye Res.* 2004;23:91–147.
2. Hayreh SS. Acute retinal arterial occlusive disorders. *Prog Ret Eye Res.* 2011;30:359–394.
3. Bek T. Inner retinal ischaemia: current understanding and needs for further investigations. *Acta Ophthalmol.* 2009;87:362–367.
4. Gidday JM, Zhu Y. Endothelium-dependent changes in retinal blood flow following ischemia. *Curr Eye Res.* 1998;17:798–807.
5. Lin J, Roth S. Ischemic preconditioning attenuates hypoperfusion after retinal ischemia in rats. *Invest Ophthalmol Vis Sci.* 1999;40:2925–2931.
6. Goldstein IM, Ostwald P, Roth S. Nitric oxide: a review of its role in retinal function and disease. *Vis Res.* 1996;36:2979–2994.
7. Polak K, Luksch A, Frank B, et al. Regulation of human retinal blood flow by endothelin-1. *Exp Eye Res.* 2003;76:633–640.
8. Donati G, Pournaras C, Pizzolato G, Tsacopoulos M. Decreased nitric oxide production accounts for secondary arteriolar constriction after retinal branch occlusion. *Invest Ophthalmol Vis Sci.* 1997;38:1450–1457.
9. Bonne C, Muller A, Villain M. Free radicals in retina ischemia. *Gen Pharmacol.* 1998;30:275–280.
10. Gryglewski RJ, Palmer RMJ, Moncada S. Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature.* 1986;320:454–456.
11. Smith G, Baird C. Survival time of retinal cells when deprived of their blood supply by increased intraocular pressure. *Am J Ophthalmol.* 1952;35:133–136.
12. Büchi E, Suivaizdis I, Fu J. Pressure-induced retinal ischemia in rats: an experimental model for quantitative study. *Ophthalmologica.* 1991;203:138–147.
13. Dollery C, Henkind P, Kohner E, Paterson J. Effect of raised intraocular pressure on the retinal and choroidal circulation. *Invest Ophthalmol Vis Sci.* 1968;7:191–198.
14. Parrish R, Gass J, Anderson D. Outer retina ischemic infarction–a newly recognized complication of cataract extraction and closed vitrectomy. Part 2. An animal model. *Ophthalmology.* 1982;89:1472–1477.
15. Cao W, Drumheller A, Zaharia M, et al. Effects of experimentally induced ischemia on dopamine metabolism in rabbit retina. *Invest Ophthalmol Vis Sci.* 1993;34:3140–3146.
16. Hein TW, Yuan Z, Rosa RH Jr, Kuo L. Requisite roles of A_{2A} receptors, nitric oxide, and K_{ATP} channels in retinal arteriolar dilation in response to adenosine. *Invest Ophthalmol Vis Sci.* 2005;46:2113–2119.
17. Kulkarni P, Joshua IG, Roberts AM, Barnes G. A novel method to assess reactivities of retinal microcirculation. *Microvasc Res.* 1994;48:39–49.
18. Nagaoka T, Takahashi A, Sato E, et al. Effect of systemic administration of simvastatin on retinal circulation. *Arch Ophthalmol.* 2006;124:665–670.
19. Jeppesen P, Aalkjaer C, Bek T. Bradykinin relaxation in small porcine retinal arterioles. *Invest Ophthalmol Vis Sci.* 2002;43:1891–1896.
20. Nagaoka T, Hein TW, Yoshida A, Kuo L. Simvastatin elicits dilation of isolated porcine retinal arterioles: role of nitric oxide and mevalonate-Rho kinase pathways. *Invest Ophthalmol Vis Sci.* 2007;48:825–832.
21. Hein TW, Xu W, Kuo L. Dilation of retinal arterioles in response to lactate: role of nitric oxide, guanylyl cyclase, and ATP-sensitive potassium channels. *Invest Ophthalmol Vis Sci.* 2006;47:693–699.
22. Zhang C, Hein TW, Wang W, Kuo L. Divergent roles of angiotensin II AT_1 and AT_2 receptors in modulating coronary microvascular function. *Circ Res.* 2003;92:322–329.
23. Bloodworth JJ, Gutgesell HJ, Engerman R. Retinal vasculature of the pig. Light and electron microscope studies. *Exp Eye Res.* 1965;4:174–178.
24. Rootman J. Vascular system of the optic nerve head and retina in the pig. *Br J Ophthalmol.* 1971;55:808–819.
25. Beauchemin ML. The fine structure of the pig's retina. *Albrecht Von Graefes Arch Klin Exp Ophthalmol.* 1974;190:27–45.

26. Simoens P, De Schaepdrijver L, Lauwers H. Morphologic and clinical study of the retinal circulation in the miniature pig. A: Morphology of the retinal microvasculature. *Exp Eye Res.* 1992; 54:965–973.
27. De Schaepdrijver L, Simoens P, Pollet L, Lauwers H, De Laey JJ. Morphologic and clinical study of the retinal circulation in the miniature pig. B: Fluorescein angiography of the retina. *Exp Eye Res.* 1992;54:975–985.
28. Chandler MJ, Smith PJ, Samuelson DA, MacKay EO. Photoreceptor density of the domestic pig retina. *Vet Ophthalmol.* 1999;2:179–184.
29. Yang P, Chen L, Zwart R, Kijlstra A. Immune cells in the porcine retina: distribution, characterization and morphological features. *Invest Ophthalmol Vis Sci.* 2002;43:1488–1492.
30. Hein TW, Rosa RH Jr, Yuan Z, Roberts E, Kuo L. Divergent roles of nitric oxide and Rho kinase in vasomotor regulation of human retinal arterioles. *Invest Ophthalmol Vis Sci.* 2010;51:1583–1590.
31. Wurm A, Iandiev I, Uhlmann S, et al. Effects of ischemia-reperfusion on physiological properties of Muller glial cells in the porcine retina. *Invest Ophthalmol Vis Sci.* 2011;52:3360–3367.
32. Kyhn MV, Warfvinge K, Scherfig E, et al. Acute retinal ischemia caused by controlled low ocular perfusion pressure in a porcine model. Electrophysiological and histological characterisation. *Exp Eye Res.* 2009;88:1100–1106.
33. Kyhn MV, Klassen H, Johansson UE, et al. Delayed administration of glial cell line-derived neurotrophic factor (GDNF) protects retinal ganglion cells in a pig model of acute retinal ischemia. *Exp Eye Res.* 2009;89:1012–1020.
34. Gesslein B, Hakansson G, Carpio R, et al. Mitogen-activated protein kinases in the porcine retinal arteries and neuroretina following retinal ischemia-reperfusion. *Mol Vis.* 2010;16:392–407.
35. Gesslein B, Hakansson G, Gustafsson L, Ekstrom P, Malmsjo M. Tumor necrosis factor and its receptors in the neuroretina and retinal vasculature after ischemia-reperfusion injury in the pig retina. *Mol Vis.* 2010;16:2317–2327.
36. Margalit E, Sadda S. Retinal and optic nerve diseases. *Artif Organs.* 2003;27:963–974.
37. Rosa Jr RH, Hein TW, Yuan Z, et al. Brimonidine evokes heterogeneous vasomotor response of retinal arterioles: diminished nitric oxide-mediated vasodilation when size goes small. *Am J Physiol Heart Circ Physiol.* 2006;291:H231–H238.
38. Kamiya T, Katayama Y, Kashiwagi F, Terashi A. The role of bradykinin in mediating ischemic brain edema in rats. *Stroke.* 1993;24: 571–575.
39. Pan HL, Chen SR, Scicli GM, Carretero OA. Cardiac interstitial bradykinin release during ischemia is enhanced by ischemic preconditioning. *Am J Physiol Heart Circ Physiol.* 2000;279:H116–H121.
40. Hayreh SS, Weingeist TA. Experimental occlusion of the central artery of the retina. IV: Retinal tolerance time to acute ischaemia. *Br J Ophthalmol.* 1980;64:818–825.
41. Hayreh SS, Weingeist TA. Experimental occlusion of the central artery of the retina. I. Ophthalmoscopic and fluorescein fundus angiographic studies. *Br J Ophthalmol.* 1980;64:896–912.
42. Hayreh SS, Kolder HE, Weingeist TA. Central retinal artery occlusion and retinal tolerance time. *Ophthalmology.* 1980;87:75–78.
43. Szabo ME, Droy-Lefaix MT, Doly M, Carre C, Braquet P. Ischemia and reperfusion-induced histologic changes in the rat retina. Demonstration of a free radical-mediated mechanism. *Invest Ophthalmol Vis Sci.* 1991;32:1471–1478.
44. Delaey C, Van de Voorde J. Regulatory mechanisms in the retinal and choroidal circulation. *Ophthalmic Res.* 2000;32:249–256.
45. Brazitikos PD, Pournaras CJ, Munoz JL, Tsacopoulos M. Microinjection of L-lactate in the preretinal vitreous induces segmental vasodilation in the inner retina of miniature pigs. *Invest Ophthalmol Vis Sci.* 1993;34:1744–1752.
46. Ido Y, Chang K, Williamson JR. NADH augments blood flow in physiologically activated retina and visual cortex. *Proc Nat Acad Sci U S A.* 2004;101:653–658.
47. Garhofer G, Zawinka C, Resch H, et al. Effect of intravenous administration of sodium-lactate on retinal blood flow in healthy subjects. *Invest Ophthalmol Vis Sci.* 2003;44:3972–3976.
48. Garhofer G, Kopf A, Polska E, et al. Influence of exercise induced hyperlactatemia on retinal blood flow during normo- and hyperglycemia. *Curr Eye Res.* 2004;28:351–358.
49. Veriac S, Tissie G, Bonne C. Oxygen free radicals adversely affect the regulation of vascular tone by nitric oxide in the rabbit retina under high intraocular pressure. *Exp Eye Res.* 1993;56:85–88.
50. Muller A, Pietri S, Villain M, et al. Free radicals in rabbit retina under ocular hyperpressure and functional consequences. *Exp Eye Res.* 1997;64:637–643.
51. Li J, Shah A. Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology. *Am J Physiol Regul Integr Comp Physiol.* 2004;287:R1014–R1030.
52. Du XL, Edelstein D, Dimmeler S, et al. Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site. *J Clin Invest.* 2001;108:1341–1348.