Vulnerability of the Retinal Microvasculature to Hypoxia: Role of Polyamine-Regulated K_{ATP} Channels

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PURPOSE. It is uncertain why retinal capillaries are particularly vulnerable to hypoxia. In this study, it was hypothesized that their specialized physiology, which includes being the predominant microvascular location of functional adenosine triphosphate-sensitive potassium (K_{ATP}) channels, boosts their susceptibility to hypoxia-induced cell death.

METHODS. Cell viability, ionic currents, intracellular calcium, and pericyte contractility in microvascular complexes freshly isolated from the rat retina were assessed using trypan blue dye exclusion, perforated-patch recordings, fura-2 fluorescence, and time-lapse videos. Chemical hypoxia was induced by antimycin, an oxidative phosphorylation inhibitor.

RESULTS. In freshly isolated retinal microvascular complexes, chemical hypoxia caused more cell death in capillaries than in arterioles. Indicative of the role of polyamine-dependent K_{ATP} channels, antimycin-induced capillary cell death was markedly decreased in microvessels treated with the polyamine synthesis inhibitor, difluoromethylornithine, or the K_{ATP} channel inhibitor, glibenclamide. These inhibitors also diminished the antimycin-induced hyperpolarization, as well as the antimycin-induced intracellular calcium increase, which was significantly dependent on extracellular calcium and was diminished by the inhibitor of calcium-induced calcium release (CICR), dantrolene. Consistent with the importance of the CICR-dependent increase in capillary cell calcium, dantrolene significantly decreased hypoxia-induced capillary cell death. We also found that activation of the polyamine/ K_{ATP} channel/ Ca^{2+} influx/CICR pathway not only boosted the vulnerability of retinal capillaries to hypoxia, but also caused the contraction of capillary pericytes, whose vasoconstrictive effect may exacerbate hypoxia.

CONCLUSIONS. The vulnerability of retinal capillaries to hypoxia is boosted by a mechanism involving the polyamine/ K_{ATP} channel/ Ca^{2+} influx/CICR pathway. Discovery of this pathway should provide new targets for pharmacological interventions to minimize hypoxia-induced damage in retinal capillaries. (*Invest Ophthalmol Vis Sci.* 2011;52:9345-9352) DOI:10.1167/iov.11-8176

This study addressed the question of why the capillaries of the retina are particularly prone to hypoxia-induced cell damage and death, which occurs during the course of a variety

of retinal vascular disorders. Here, we considered the idea that specialized physiological adaptations of the retinal capillaries boost their vulnerability to hypoxia.

Evidence is accumulating that within the circulatory system of the retina, there is functional specialization.¹⁻³ For example, most of the functional adenosine triphosphate-sensitive potassium (K_{ATP}) channels are located in the capillaries.² In contrast, the activity of voltage-dependent calcium channels (VDCCs) is minimal in this microvascular region, but is robust in the precapillary tertiary arterioles.³ An important operational result of this topographical distribution of ion channels is that the hyperpolarizing K_{ATP} current activated by vasoactive signals, such as adenosine, is generated almost exclusively in the capillaries² and must be transmitted proximally to microvascular sites where VDCCs are available to transduce the induced voltage change into a vasomotor response that alters blood flow.^{2,5} Although this functional specialization within the retinal microvasculature appears to be important for the effective regulation of local perfusion, we hypothesized that the abundance of K_{ATP} channels may boost the vulnerability of the capillaries to hypoxic damage.

How could an abundance of K_{ATP} channels boost capillary vulnerability to hypoxia? We posited that a hypoxia-induced drop in the ATP concentration activates the capillary K_{ATP} channels, whose function is inhibited by intracellular ATP. Due to the hypoxia-induced activation of K_{ATP} channels, increased K^+ efflux via these channels would cause hyperpolarization, which in turn, would increase the electrochemical gradient for the influx of calcium via nonspecific cation (NSC) channels, which are the predominant calcium-permeable ion channels expressed in retinal capillaries.⁴ Because increased intracellular calcium is known to exacerbate hypoxic damage in a variety of cell types,^{5,6} we proposed a working model in which the K_{ATP} channel-dependent increase in cell calcium boosts the vulnerability of retinal capillaries to hypoxia.

In addition to K_{ATP} channels, we hypothesized that endogenous polyamines play a role in establishing the vulnerability of retinal capillaries to hypoxia. These ornithine-derived molecules were of interest because we found previously² that the function of microvascular K_{ATP} channels, which are redox-sensitive,² is dependent on endogenous polyamines, whose catabolism generates H_2O_2 .⁷ Consistent with polyamines having a role in capillary cell death, these molecules are known to modulate death pathways in a variety of cell types,⁸ although its diversity of effects, which include enhancing and inhibiting cell death, remain confounding, and the mechanisms by which polyamines affect cell viability are incompletely understood. In this study, we tested the novel hypothesis that by regulating the function of K_{ATP} channels, endogenous polyamines may play a role in establishing the lethality of hypoxia in the capillaries of the retina.

We report that in freshly isolated retinal microvascular complexes, the inhibitor of oxidative phosphorylation, antimycin A, causes substantially more cell death in the capillaries than in the precapillary arterioles. Experiments using the patch-clamp tech-

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nique, calcium-imaging, time-lapse photography, and the trypan blue viability assay provided evidence that the greater vulnerability of the capillaries to hypoxia-induced cell death is due to the activation of a pathway involving endogenous polyamines, hyperpolarizing K_{ATP} channels, calcium influx, and calcium-induced calcium release (CICR). Our experimental results indicate that activation of the polyamine/ K_{ATP} channel/ Ca^{2+} influx/CICR pathway is a previously unappreciated mechanism by which the vulnerability of retinal capillaries to hypoxia is boosted.

METHODS

Animal use conformed to the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the University of Michigan Committee on the Use and Care of Animals. This study used Long-Evans rats (Charles River, Cambridge, MA), which were maintained on a 12-hour alternating light/dark cycle and had unrestricted access to water and food.

Microvessel Isolation

A previously described tissue print technique² was used to isolate vast microvascular complexes from the retinas of rats, which were killed with a rising concentration of carbon dioxide. In brief, the procedure for microvessel isolation included the rapid removal of retinas, excision of adherent vitreous, and incubation for approximately 24 minutes at 30°C in Earle's balanced salt solution supplemented with 0.5 mM EDTA, 6 U papain (Worthington Biochemicals, Freehold, NJ), and 2 mM cysteine. Subsequently, retinas were placed in solution A, which consisted of 140 mM NaCl, 3 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM Na-HEPES, 15 mM mannitol, and 5 mM glucose at pH 7.4 with osmolality adjusted to 310 mOsm. After each retina was quadrisectioned, a retinal quadrant was positioned vitreal surface up onto the glass bottom of a chamber containing solution A and then very gently compressed by a 15 mm diameter glass coverslip (Warner Instrument Corp., Hamden, CT) onto which microvascular complexes adhered. This process was repeated several times with new coverslips. Typically, four or five coverslips containing microvascular complexes were obtained from a pair of retinas. Each studied microvascular complex included, from distal to proximal, a network of capillaries with abuminally located pericytes that appear as "bumps on a log" with a density of ≤ 4 per 100 μm , an approximate 400- μm long precapillary tertiary arteriole with ≥ 5 "dome-shaped" abluminal cell somas per 100 μm , and a secondary arteriole encircled by a layer of "doughnut-shaped" smooth muscle cells; these features have been characterized previously^{1,2,9} and are illustrated in Figure 1. Experiments were performed at room temperature (i.e., 22 °C to 23°C).

Model of Hypoxia

Chemical hypoxia was created by exposing isolated retinal microvascular complexes to the inhibitor of oxidative phosphorylation, antimycin A (5 μM).

Cell Viability Assay

Microvascular cells that failed to exclude trypan blue dye were classified as dead. As done previously,¹⁰⁻¹² the trypan blue assay was performed by exposing microvessel-containing coverslips to 0.04% trypan blue in solution A for 15 minutes. After washing in solution A, microvessels were examined at magnification $\times 100$ with an inverted microscope equipped with bright-field optics. Because differences in abluminal cell density made it straightforward to distinguish precapillary tertiary arterioles from the capillaries,¹ cell viability was tallied separately for these portions of the retinal microvasculature. For each microvascular region, the percentage of the surveyed cells that were trypan blue positive (i.e., dead), was calculated. Because trypan blue-containing cells typically were swollen, identification of these cells as being endothelial or abluminal was uncertain, and thus, subclassification of microvascular cells into these two types was not done.

Cell viability was initially quantified before the onset of antimycin exposure. In freshly isolated retinal microvascular complexes, cell viability was high (i.e., 96.7 ± 0.3 ; $n = 62$ microvascular complexes, and 94.9 ± 0.3 ; $n = 62$ in tertiary arterioles and capillaries, respectively). In some experiments, freshly isolated microvascular complexes were exposed to 0.5 μM glibenclamide or 1 μM dantrolene in solution A for 15 minutes before the addition of antimycin. In other experiments, microvascular complexes were incubated for 5 hours in solution A supplemented with the polyamine synthesis inhibitor, difluoromethylornithine (DFMO, 5 mM), before the addition of antimycin. At the time of the initial viability assay, the location on the coverslip of the assessed microvascular complex was carefully documented so that cell viability in the identical portion of the microvascular complex could be reassessed after a 20-hour exposure to 5 μM antimycin in solution A. After antimycin exposure, cell viability was again quantified using trypan blue.

Antimycin-induced cell death was calculated by subtracting the percentage of cell death that occurred during the 20-hour antimycin exposure from the percentage of cell death that occurred in during a 20-hour exposure to the vehicle used for the antimycin-containing solution (i.e., 0.1% ethanol in solution A); cell death in this control group was $2.1 \pm 0.8\%$ and $2.2 \pm 0.2\%$ in the tertiary arterioles and capillaries, respectively. To assess the effect on microvascular cell viability of the K_{ATP} activator, pinacidil, control experiments were performed using 0.05% dimethyl sulfoxide (DSMO), which was the

FIGURE 1. The vasculature of the rat retina. (A) Schematic drawing showing the portion of the rat retinal vasculature isolated by the tissue print procedure used in this study. Modified from Zhang et al.,⁹ with permission of the *Journal of Physiology*. (B) Differential interference contrast photomicrograph of a microvascular complex freshly isolated from the retina of an adult rat; modified from Matsushita and Puro,¹ with permission of the *Journal of Physiology*.

vehicle for pinacidil; in this control group, induced cell death was $2.1 \pm 0.8\%$ and $2.1 \pm 0.5\%$ in the tertiary arterioles and capillaries, respectively. Of note (see Fig. 4), the rates of cell death in 0.1% ethanol and 0.05% DMSO were not significantly different from those observed in microvascular complexes maintained for 20 hours in solution A without additives (i.e., $1.3 \pm 0.6\%$ and $2.8 \pm 0.7\%$ in tertiary arterioles and capillaries, respectively).

Electrophysiology

A microvessel-containing coverslip was positioned in a recording chamber (volume, 1 mL), which was initially perfused (approximately 1.5 mL per minute) with solution A. The reservoirs for the perfusates and the recording chamber were open to the air. Pipettes for perforated-patch recordings were filled with a solution consisting of 50 mM KCl, 65 mM K_2SO_4 , 6 mM $MgCl_2$, 10 mM K-HEPES, 60 $\mu g/mL$ amphotericin B, and 60 $\mu g/mL$ nystatin at pH 7.4 with the osmolarity adjusted to 280 mOsm. A recording pipette having a resistance of 5 to 10 M Ω was mounted in the holder of a patch-clamp amplifier (Axopatch 200B, MDS Analytical Technologies, Union City, CA). Positioning the tip of a recording pipette onto a pericyte located on the abluminal wall of a capillary was controlled with a piezoelectric-based micromanipulator (Exfo, Mississauga, Canada) while the pericyte-containing microvessel was viewed at magnification $\times 400$ with phase-contrast optics. With the application of suction to the back end of the pipette, a ≥ 10 G Ω seal formed. Recordings in which the access resistance became < 25 M Ω within 5 minutes after gigohm seal formation were used. As detailed previously,¹ approximately 95% of the current detected via a perforated-patch pipette sealed onto a pericyte was transmitted electronically via gap junction pathways from neighboring microvascular cells. Throughout each recording, the access resistance was monitored, and the recording was terminated if there was a significant change.

Currents were filtered with a four-pole Bessel filter, digitally sampled using an acquisition system (DigiData 1440A; MDS Analytical Technologies) and stored by a computer equipped with specialized software for data analysis and graphics display (pClamp version 10, MDS Analytical Technologies; and Origin version 8.1; OriginLab, Northampton, MA). For the generation of current-voltage (I-V) plots, currents were evoked at 10-second intervals by a negative to positive voltage ramp (52 mV s⁻¹), which was controlled by software (pCLAMP version 10, MDS Analytical Technologies); current was sampled at a rate of 2 kHz. Adjustment for the calculated liquid junction potential was made after data collection.

For the comparison of currents recorded in the absence and presence of antimycin, mean conductances calculated at 10 mV intervals from -100 mV to 0 mV were compared using Student's paired *t*-test. The membrane potential was defined as the voltage at which the recorded current was zero. For the data summarized (see Fig. 3B) the mean voltage before antimycin exposure was compared with the maximum hyperpolarization induced during an 8 ± 1 minute exposure to this inhibitor of oxidative phosphorylation. In some experiments, microvessels were exposed to 5 mM DFMO for approximately 20 hours before the onset of electrophysiological recordings. Because microvessels maintained in solution A for ≤ 5 hours or approximately 20 hours did not significantly affect the size to the hyperpolarization induced during exposure to antimycin, these data were pooled.

Calcium Imaging

Isolated retinal microvascular complexes were loaded with 5 μM fura-2AM (Molecular Probes, Eugene, OR) at 33°C for 90 minutes. After allowing the AM ester to be cleaved, a coverslip containing fura-loaded microvessels was positioned in a 200- μL recording chamber that was continuously perfused (approximately 1.5 mL per minute). Digital imaging was performed using a microscope (Nikon Eclipse E600 FN; Nikon, Tokyo, Japan), an optical sensor (Sensicam, Cooke Corp., Auburn Hills, MI), and a high intensity mercury lamp coupled to a

monochromator (Optoscan; Cairn Research Ltd., Faversham, UK); imaging equipment and data collection were controlled using software (MetaFluor, ver. 6.1; Molecular Devices, Sunnyvale, CA). Autofluorescence was not detected in the microvascular complexes. Endothelial cells were only minimally loaded with fura-2. Fluorescent intensities were measured every 10 seconds at 340 nm and 380 nm within regions of interest (ROIs), each of which encircled the soma of a single pericyte. As detailed previously for isolated retinal microvessels,³ there was minimal fura-derived fluorescence that was insensitive to free Ca^{2+} due to the sequestration of fura within cellular compartments or because of incomplete de-esterification of fura-AM; thus, because of the minimal amount of fluorescence detected from free Ca^{2+} -insensitive fura, Mn^{2+} quenching was not necessary. In each experiment, the background fluorescence of the optical system was measured by placing some ROIs in cell-free areas of the coverslip. After subtracting this background, the F_{340}/F_{380} ratio was calculated and converted to the intracellular calcium concentration by use of the equation of Grynkiewicz et al.,¹³ in which R_{min} and R_{max} were determined as detailed previously.¹⁴ For each monitored pericyte, the peak induced increase in calcium was determined during a 900-second exposure to antimycin.

In some experiments, freshly isolated microvascular complexes were exposed to 0.5 μM glibenclamide or 1 μM dantrolene in solution A for approximately 15 minutes before the calcium-imaging experiment; glibenclamide and dantrolene remained in the perfusates during the experiment. In other experiments, microvascular complexes were incubated for 18 to 20 hours in solution A supplemented with 5 mM DFMO before calcium imaging; the antimycin-induced increases in pericyte calcium in microvessels maintained for 1 to 5 hours and approximately 20 hours in solution A were not significantly different.

Time-Lapse Photography

Contractile responses of pericytes located in the capillary network of isolated retinal microvascular complexes were assessed with the aid of time-lapse photography, as detailed previously.¹⁴⁻¹⁷ In these experiments, a microvessel-containing coverslip was positioned in a perfusion chamber (volume, 200 μL) on the stage of an inverted microscope equipped with phase contrast optics (magnification $\times 325$) while images were recorded at 8-second intervals using a digital camera (Retiga 2000R) and commercially available software (QImaging, ver. 6.0; QImaging, Surrey, BC, Canada). Capillary pericytes were monitored before and during exposure to 5 μM antimycin. As in previous time-lapse studies of isolated retina microvascular complexes, determination of whether a pericyte contracted, relaxed, or remained unchanged during exposure to an experimental solution was made by careful visual inspection of the time-lapse movie.

In some experiments, solution A was supplemented with 0.5 μM glibenclamide or 1 μM dantrolene for 10 minutes before the instigation of time-lapse recording in which the blocker remained in the perfusate. Other experiments used a calcium-free solution consisting of solution A without $CaCl_2$ and with 3 mM EGTA. An additional series of time-lapse recordings were made using microvascular complexes that had been maintained for 20 hours in solution A supplemented with 5 mM DFMO; in control experiments, a 20-hour exposure to solution A without additives did not significantly affect the contractile response of pericytes to antimycin.

Chemicals

Unless otherwise noted, chemicals were from Sigma-Aldrich (St. Louis, MO).

Statistics

For the data are given as mean $\pm$ SE, probability was evaluated by the two-tailed Student's *t*-test. For the comparison of two groups, *P*-values of ≥ 0.05 indicated a lack of significant difference. For greater than two groups, the *P*-value for significance was adjusted using the Bonferroni correction. Data quantifying the contractile responses of ablu-

FIGURE 2. Antimycin-induced cell death in the capillaries and tertiary arterioles of isolated retinal microvascular complexes. (A) Cell death induced by a 20-hour exposure to 5 μ M antimycin. For each group, 14 experiments were conducted. * $P < 0.0001$. Microvessels were viewed with bright-field optics at magnification $\times 100$, and microvascular cells that failed to exclude trypan blue were classified as dead. Shown are the percentages of trypan blue positive cells in each microvascular region. (B) Antimycin-induced cell death in DFMO-treated microvessels. For each group, 17 experiments were performed. Induced cell death in the two groups was not significantly different. Of note, DFMO treatment significantly ($P < 0.0001$) lessened antimycin-induced cell death in the capillaries, but not in the tertiary arterioles.

minimal cells are given as the percentage of the total number of monitored abluminal cells that were observed to contract; statistical differences were evaluated using the Fisher exact test with the Bonferroni correction used to adjust the P -value for significance.

RESULTS

Initial experiments compared the vulnerability to chemical hypoxia of capillaries and precapillary arterioles located in microvascular complexes freshly isolated from the adult rat retina. Consistent with the capillaries being particularly vulnerable to hypoxia, a 20-hour exposure of isolated retinal microvessels to the inhibitor of oxidative phosphorylation, antimycin A (5 μ M), resulted in significantly ($P < 0.0001$) more cell death in the capillaries than in the tertiary arterioles (Fig. 2A).

What accounts for the greater vulnerability of capillaries to hypoxia-induced cell death? Based on the idea that certain specialized physiological features of the capillaries increase their vulnerability to various pathophysiological conditions, we hypothesized that endogenous polyamines, which play a prominent role in establishing capillary function,^{1–3} may render this portion of the retinal microvasculature particularly vulnerable to hypoxia. To assess this hypothesis, retinal microvessels were preincubated for 5 hours in a solution containing the inhibitor of polyamine synthesis, difluoromethylornithine (DFMO, 5 mM), and then subsequently exposed to 5 μ M antimycin for 20 hours in the continued presence of DFMO. As shown in Figure

2B, DFMO treatment significantly ($P < 0.0001$) decreased antimycin-induced capillary cell death and, as a result, effectively eliminated the relative vulnerability of the capillaries. These findings supported the hypothesis that endogenous polyamines play a key role in increasing the lethality of hypoxia in retinal capillaries.

How do endogenous polyamines increase the vulnerability of retinal capillaries to hypoxia? Because an important physiological action of polyamines, whose catabolism generates H_2O_2 ,⁷ is the functional regulation of oxidant-sensitive K_{ATP} channels in the capillaries,² we postulated that these ion channels may play a role in the polyamine-dependent increase in vulnerability to hypoxia. Consistent with K_{ATP} channels having such a role, perforated-patch recordings demonstrated that soon after the onset of antimycin exposure, there was the activation of a hyperpolarizing current that was sensitive to the K_{ATP} channel blocker, glibenclamide (Figs. 3A and 3B). In agreement with this hypoxia-induced activation of K_{ATP} channels being dependent on polyamines, the hyperpolarization induced during antimycin exposure was markedly ($P < 0.0001$) less in retinal microvessels treated with DFMO (Fig. 3B). Of note, although the antimycin-induced activation of the hyperpolarizing conductance was markedly attenuated by DFMO treatment, the resting membrane potential before antimycin exposure was somewhat more negative due to the increased outward current generated by inwardly rectifying potassium (K_{IR}) channels whose efflux of K^+ was no longer blocked by the endogenous polyamine, spermine.¹ Indicative

FIGURE 3. K_{ATP} channel function in retinal microvessels during chemical hypoxia. (A) Current-voltage relations were recorded initially in solution A, 8 ± 2 minutes after the onset of exposure to solution A supplemented with 5 μ M antimycin and 6 ± 3 minutes after the addition of 0.5 μ M glibenclamide to the antimycin-containing perfusate. I-V plots are the means of six experiments. Exposure to 5 μ M antimycin significantly ($P = 0.0182$) increased the ionic conductance, which was significantly ($P = 0.0268$) reversed by glibenclamide. *Inset*: Time course for the effect of antimycin on the membrane potential recorded via perforated-patch pipettes sealed onto capillary pericytes. Each data point is the mean of six recordings. (B) Effect of glibenclamide and DFMO on the hyperpolarization induced in retinal microvessels during chemical hypoxia. * $P \leq 0.0029$. The number of experiments for the antimycin, antimycin/glibenclamide, and antimycin/DFMO groups was six, six, and four, respectively. (C) Effect of glibenclamide on antimycin-induced cell death in retinal capillaries. * $P = 0.0004$. The number of experiments was 13 and six for the antimycin and antimycin/glibenclamide groups, respectively.

FIGURE 4. Capillary cell death induced during a 20-hour exposure to solution A without and with various additives. The following additives were used: 0.05% dimethyl sulfoxide (DMSO), which was the vehicle for pinacidil; 0.1% ethanol, which was the vehicle for antimycin; 5 μ M pinacidil, which is an activator of microvascular K_{ATP} channels²; 5 μ M antimycin, which is an inhibitor of oxidative phosphorylation; 5 mM DFMO, which is a polyamine synthesis inhibitor, and 100 μ M BaCl₂, which is a blocker of microvascular K_{IR} channels.¹ For each group, 10 $\pm$ 4 experiments were performed. Neither of the vehicles, DMSO or ethanol, significantly induced capillary cell death. Pinacidil did not significantly affect capillary cell death. Antimycin did significantly ($P < 0.0001$) increase cell death. In microvessels not treated with DFMO, barium did not significantly affect antimycin-induced cell death. DFMO significantly ($P < 0.0001$) decreased antimycin-induced cell death, and barium significantly increased ($P < 0.0001$) antimycin-induced capillary cell death in DFMO-treated microvessels.

that K_{ATP} channel activation increased the vulnerability of retinal capillaries to hypoxia, we observed that the inhibition of these channels by glibenclamide significantly ($P = 0.0004$) decreased antimycin-induced capillary cell death (Fig. 3C). Taken together, the results of this series of experiments supported the idea that the activation of polyamine-dependent K_{ATP} channels boosts the lethality of hypoxia in the capillaries of the retina.

Finding that the activation of K_{ATP} channels increases cell death in hypoxic capillaries, we asked whether activation of these channels affects the viability of capillary cells under normoxic conditions. Indicative that this is not the case, we found that exposure to the K_{ATP} channel activator, pinacidil, did not significantly affect the viability of capillary cells in microvascular complexes maintained in the absence of antimycin (Fig. 4). Thus, we concluded that the activation of K_{ATP} channels boosts cell death in hypoxic, but not in normoxic, capillaries.

How does the activation of polyamine-dependent K_{ATP} channels increase the lethality of hypoxia? To address this question, we considered the possibility that the activation of a hyperpolarizing K_{ATP} conductance in retinal capillaries would increase the influx of calcium, which at elevated concentrations can adversely affect the viability of metabolically compromised cells.^{5,6} Increased calcium influx during hyperpolarization seemed likely because nonspecific cation channels, rather than voltage-dependent calcium channels, are the predominant calcium-permeable ion channels in retinal capillaries.^{3,4} In agreement with this scenario, Figure 5A shows that soon after the onset of antimycin exposure, pericyte calcium increased significantly ($P < 0.0001$).

We wished to characterize the mechanism by which chemical hypoxia increased capillary cell calcium. To assess the role of polyamines and K_{ATP} channels, microvessels were treated

with DFMO or glibenclamide (Fig. 5B). In other experiments, the importance of calcium influx was determined using a perfusate lacking added calcium (Fig. 5B). We also assessed the role of calcium-induced calcium release (CICR) by using the CICR blocker, dantrolene (Fig. 5B). Consistent with a mechanism involving polyamines, K_{ATP} channels, calcium influx and CICR, each of the experimental conditions shown in Figure 5B resulted in a significant ($P \leq 0.0054$) decrease in the percentage of pericytes that had a relatively large antimycin-induced increase in intracellular calcium (i.e., an increase of ≥ 50 nM). Specifically, the percentage of pericytes in the control group with a maximum induced calcium increase of ≥ 50 nM was 19.1%, which was significantly more than the 0% ($P = 0.0309$), 0% ($P = 0.0309$), 0% ($P = 0.0075$), and 4.5% ($P = 0.0407$) detected in the DFMO, glibenclamide, low extracellular calcium, and dantrolene groups, respectively. Thus, the data in Figure 5B indicate that during hypoxia, relatively large increases in capillary cell calcium were mediated by a mechanism involving polyamines, K_{ATP} channels, calcium influx, and CICR. Supporting the importance of the polyamine/ K_{ATP} channel/ Ca^{2+} influx/CICR pathway in boosting the vulnerability of retinal capillary cells to hypoxia, antimycin exposure resulted in significantly ($P \leq 0.0014$) less capillary cell death in microvessels treated with DFMO (Fig. 2B), glibenclamide (Fig. 3C), or dantrolene (Fig. 5C).

In addition to establishing a role for polyamine-dependent K_{ATP} channels, we considered the possibility that K_{IR} channels are involved in boosting the vulnerability of capillaries to hypoxia. K_{IR} channels were of interest because they are located in retinal capillaries¹ and their rectification is well known to be regulated by the polyamine, spermine,¹ which blocks K^+ efflux via these channels.^{18,19} To determine whether K_{IR} channel activity affected the vulnerability of capillaries to hypoxia, we exposed microvessels to antimycin in the presence of 100 μ M barium, which near-totally blocks the K_{IR} conductance in retinal microvessels.¹ As shown in Figure 4, barium did not significantly affect antimycin-induced capillary cell death in microvessels that had not been treated with DFMO. The lack of a role for K_{IR} channels was not unexpected because the outward conductance of these channels in the capillaries of the retina is normally minimal due to the strong rectification caused by spermine's blockade of K^+ efflux.¹ In contrast, our experiments performed with DFMO-treated microvessels, whose outward K_{IR} current are no longer blocked by endogenous spermine,² revealed that antimycin-induced capillary cell death was significantly ($P < 0.0001$) increased when barium was present (Fig. 4). Thus, in DFMO-treated capillaries, it appears that polyamine-gated K_{IR} channels have a protective role that lessens the vulnerability to hypoxia-induced cell death. However, when polyamine synthesis is not inhibited, the results summarized in Figures 2, 3, and 4 supported the conclusion that polyamine-dependent K_{ATP} channels play an important role in establishing the vulnerability of retinal capillaries to hypoxia.

Our observation that antimycin caused pericyte calcium to increase (Figs. 5A and 5B) raised the question of whether chemical hypoxia is associated with the contraction of these abluminal cells, whose contractile tone is calcium-sensitive.²⁰ This was of interest because pericyte contraction causes capillary lumens to narrow and thus could exacerbate hypoxia by diminishing the local perfusion oxygenated blood. As summarized in Figure 6, exposure to antimycin caused the contraction in 19% of the pericytes monitored by time-lapse photography ($n = 330$); with none of the monitored pericytes contracting before antimycin exposure, the effect of chemical hypoxia on pericyte contractility was highly significant ($P < 0.0001$). To assess the role of the polyamine/ K_{ATP} channel/ Ca^{2+} influx/CICR pathway in mediating the contraction of pericytes during antimycin exposure, we tested the effect of DFMO, glibencla-

(C) Effect of dantrolene (1 μ M) on antimycin-induced cell death in retinal capillaries. * $P = 0.0014$. The number of experiments was 13 and five for the antimycin and antimycin/dantrolene groups, respectively.

mid, low extracellular calcium, and dantrolene (Fig. 6). Under each of these experimental conditions, we found that the percentage of pericytes contracting during antimycin exposure was significantly ($P \leq 0.0054$; Fisher exact test) less than under control conditions. Based on these findings, we concluded that polyamines, K_{ATP} channels, calcium influx, and CICR play key roles in increasing the contractile tone of pericytes located on hypoxic retinal capillaries.

Taken together, the results of this study indicate that the polyamine/ K_{ATP} channels/ Ca^{2+} influx/CICR pathway boosts the vulnerability of retinal capillaries to hypoxia.

FIGURE 6. Effect of DFMO (5 mM), glibenclamide (0.5 μ M), calcium-free bath and dantrolene (1 μ M) on the percentage of pericytes that contract during exposure of retinal microvascular complexes to 5 μ M antimycin. * $P \leq 0.0017$ (Fisher exact test). For each group, 202 ± 49 pericytes were monitored by time-lapse photography.

DISCUSSION

The results of this study support the concept that the relatively high vulnerability of retinal capillaries to hypoxia is a consequence of their specialized physiology. Specifically, our studies have revealed that K_{ATP} channels, whose function is dependent on polyamine-driven oxidation,² not only serve a specialized operational role in the capillary network of the retinal vasculature,² but, as demonstrated here, the activation of these ion channels also contributes importantly to the pathologic effect of hypoxia.

A major conclusion of this study is that the lethality of hypoxia in retinal capillaries is markedly increased by a mechanism involving polyamines, K_{ATP} channels, calcium influx, and calcium-induced calcium release (Fig. 7). This is the first study to report that the activation K_{ATP} channels boosts the vulnerability of retinal capillaries to hypoxia. In addition, the essential role of endogenous polyamines in rendering capillary K_{ATP} channels capable of being activated during hypoxia is a previously unrecognized mechanism by which these ornithine-derived molecules can affect cell viability during pathophysiological conditions.

Due to the abundance of functional K_{ATP} channels in the capillaries and the paucity of these channels elsewhere in retinal vasculature,² the capillary network is specialized for the task of generating K_{ATP} channel-mediated voltage changes in response to vasoactive signals such as adenosine and dopamine.^{2,21} We have posited² that the initiation of K_{ATP} channel-dependent voltage responses at decentralized sites in the circulatory system of the retina is likely to enhance the spatial resolution of extracellular inputs and, as a consequence, to tighten the coupling of capillary perfusion to local metabolic demand. As shown previously, this functional specialization is

FIGURE 5. Assessment of the calcium component of the polyamine/ K_{ATP} channel/calcium pathway. (A) Time course for the increase in pericyte calcium during exposure to 5 μ M antimycin. Data points are the means of 63 monitored capillary pericytes. The steady state calcium concentration during antimycin exposure was significantly ($P < 0.0001$) greater than the concentration of pericyte calcium before antimycin exposure. (B) Distribution of the peak antimycin-induced increase in pericyte calcium in the absence (control) and presence of various inhibitors, as well as in a calcium-free bathing solution. The number of monitored pericytes was 63, 23, 23, 31, and 44 for the control (antimycin only), DFMO (5 mM), glibenclamide (0.5 μ M), calcium-free bath, and dantrolene (1 μ M) groups. Each bar shows the percentage of the total number of monitored pericytes whose maximum antimycin-induced increase in intracellular calcium was within the 10 nM range for that bar. Of note, for each experimental group, the percentage pericytes with an antimycin-induced increase of ≥ 50 nM was significantly ($P \leq 0.0407$; Fisher exact test) lower in the control group.

FIGURE 7. Model of how the polyamine/ K_{ATP} channel/ Ca^{2+} influx/CICR pathway boosts the lethality of hypoxia in retinal capillaries. In this model, a hypoxia-induced decrease in ATP results in the activation of capillary K_{ATP} channels, which are redox-sensitive channels whose function has been found to require polyamine-dependent oxidation.² Due to the opening of K_{ATP} channels, the membrane potential (V_m) of cells on hypoxic capillaries increases, and as a consequence, there is an increase in the electrical gradient for the influx of calcium via the nonspecific cation channels expressed in the retinal capillaries⁴; the paucity of functional voltage-dependent calcium channels in retinal capillaries³ limits their role. In hypoxic capillaries, the rise in capillary cell calcium caused by the hyperpolarization-induced increase in calcium influx is amplified by calcium-induced calcium release (CICR), and the resulting high level of intracellular calcium is proposed to boost the lethality of hypoxia. In addition, this model shows that activation of the polyamine/ K_{ATP} channel/ Ca^{2+} influx/CICR pathway causes abluminal pericytes on hypoxic capillaries to contract; the resulting narrowing of the capillary lumen and attenuation of local perfusion exacerbate the deficiency in oxygenation. Sites of action of the pharmacological inhibitors used in this study are shown.

dependent on the regulation of redox-sensitive K_{ATP} channels by endogenous polyamines,² whose catabolism generates H_2O_2 .⁷ However, despite the operational advantages provided by this functional specialization of the capillaries, this study revealed that the abundance of K_{ATP} channels in the capillaries also boosts the vulnerability of this portion of the retinal microvasculature to hypoxia.

The experimental observations presented in this study provide evidence supporting a model in which a hypoxia-induced decrease in intracellular ATP results in the activation of hyperpolarizing K_{ATP} channels and thereby, an increase in the voltage gradient for calcium influx via nonspecific cation channels and the subsequent triggering of a CICR-dependent boost in capillary cell calcium (Fig. 7). We propose that this increase in intracellular calcium is associated with an increased vulnerability to hypoxic cell damage, as has been found for other cell types.^{5,6} Our experiments also indicate that in addition to boosting the lethality of hypoxia, the hypoxia-induced activation of the polyamine/ K_{ATP} channel/ Ca^{2+} influx/CICR pathway causes the contraction of abluminal pericytes, whose constriction of the capillary lumen is likely to exacerbate capillary hypoxia by decreasing the perfusion of oxygenated blood.

In addition to the important role of K_{ATP} channels in establishing the vulnerability to hypoxia, our experimental findings suggest that under certain circumstances, the vulnerability of microvessels can also be modulated by K_{IR} channels, whose

rectification is regulated by the polyamine, spermine.^{1,18,19} However, at present the mechanism by which these channels affect hypoxia-induced cell death is not known. Furthermore, because capillary K_{IR} channels normally have strong inward rectification and thus, generate only a small outward current,¹ these channels do not appear to play a significant role in hypoxic capillaries and hence are not included in our current working model (Fig. 7).

Our conclusions concerning the role of the polyamine/ K_{ATP} channel/ Ca^{2+} influx/CICR pathway in boosting the vulnerability of retinal capillaries to hypoxia were based on experiments performed on microvascular complexes freshly isolated from the rat retina. An advantage of this experimental preparation is the ease with which capillaries and tertiary arterioles can be distinguished. This allowed us to quantitatively compare the vulnerability to hypoxia of these microvascular regions. Another experimental advantage of studying microvessels in isolation was the absence of potentially confounding effects caused by hypoxia-induced responses of nonvascular retinal cells. Also of importance, use of isolated microvessels made it relatively straightforward to use the patch-clamp technique to detect a hypoxia-induced K_{ATP} conductance, to perform calcium-imaging to detect a hypoxia-induced rise in pericyte calcium and to employ time-lapse photography to detect hypoxia-induced pericyte contractions. On the other hand, conclusions based on a study of microvessels isolated from the retina will require in vivo validation, although doing patch-clamping, calcium-imaging, and time-lapse photography in oculo appears to be technically unfeasible at present. Clearly, the possibility of species differences also warrants caution in extrapolating our findings with rodent retinal microvessels to the microvasculature of the human retina. In addition, to further clarify the effects of the polyamine/ K_{ATP} channel/ Ca^{2+} influx/CICR pathway on hypoxic capillary cells, it will be of interest to ascertain how this pathway affects hypoxia-induced apoptosis and/or necrosis in our antimycin model. Future studies are also required to characterize the response of the retinal microvasculature to hypoxic conditions that are less severe than the chemical hypoxia induced by antimycin. Overall, despite certain caveats and the need for additional analyses, the experimental approach used in this study has revealed a previously unappreciated mechanism that boosts the vulnerability of retinal capillaries to hypoxia.

In summary, our findings support the hypothesis that the lethal effect of hypoxia in the capillaries of the retina is markedly increased by a mechanism involving polyamines, K_{ATP} channels, calcium influx, and calcium-induced calcium release. Discovery of this pathway provides potential targets for new pharmacological interventions to limit cell death in hypoxic retinal capillaries.

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References

- Matsushita K, Puro DG. Topographical heterogeneity of K_{IR} currents in pericyte-containing microvessels of the rat retina: effect of diabetes. *J Physiol.* 2006;573:483–495.
- Ishizaki E, Fukumoto M, Puro DG. Functional K_{ATP} channels in the rat retinal microvasculature: topographical distribution, redox regulation, spermine modulation and diabetic alteration. *J Physiol.* 2009;587:2233–2253.
- Matsushita K, Fukumoto M, Kobayashi T, et al. Diabetes-induced inhibition of voltage-dependent calcium channels in the retinal microvasculature: role of spermine. *Invest Ophthalmol Vis Sci.* 2010;51:5979–5990.

4. Sakagami K, Wu DM, Puro DG. Physiology of rat retinal pericytes: modulation of ion channel activity by serum-derived molecules. *J Physiol*. 1999;521:637-650.
5. Seta KA, Yuan Y, Spicer Z, et al. The role of calcium in hypoxia-induced signal transduction and gene expression. *Cell Calcium*. 2004;36:331-340.
6. Yao H, Haddad GG. Calcium and pH homeostasis in neurons during hypoxia and ischemia. *Cell Calcium*. 2004;36:247-255.
7. Wang Y, Casero RA Jr. Mammalian polyamine catabolism: a therapeutic target, a pathological problem, or both? *J Biochem*. 2006;139:17-25.
8. Schipper RG, Penning LC, Verhofstad AA. Involvement of polyamines in apoptosis. Facts and controversies: effectors or protectors? *Semin Cancer Biol*. 2000;10:55-68.
9. Zhang T, Wu DM, Xu GZ, Puro DG. The electrotonic architecture of the retinal microvasculature: modulation by angiotensin II. *J Physiol*. 2011;589:2383-2399.
10. Kobayashi T, Puro DG. Loss of insulin-mediated vasoprotection: early effect of diabetes on pericyte-containing microvessels of the retina. *Invest Ophthalmol Vis Sci*. 2007;48:2350-2355.
11. Sugiyama T, Kawamura H, Yamanishi S, Kobayashi M, Katsumura K, Puro DG. Regulation of P2X₇-induced pore formation and cell death in pericyte-containing retinal microvessels. *Am J Physiol*. 2005;288:C568-C576.
12. Kodama T, Oku H, Kawamura H, Sakagami K, Puro DG. Platelet-derived growth factor-BB: a survival factor for the retinal microvasculature during periods of metabolic compromise. *Curr Eye Res*. 2001;23:93-97.
13. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem*. 1985;260:3440-3450.
14. Yamanishi S, Katsumura K, Kobayashi T, Puro DG. Extracellular lactate as a dynamic vasoactive signal in the rat retinal microvasculature. *Am J Physiol Heart Circ Physiol*. 2006;290:H925-H934.
15. Kawamura H, Kobayashi M, Li Q, et al. Effects of angiotensin II on the pericyte-containing microvasculature of the rat retina. *J Physiol*. 2004;561:671-683.
16. Kawamura H, Sugiyama T, Wu DM, et al. ATP: a vasoactive signal in the pericyte-containing microvasculature of the rat retina. *J Physiol*. 2003;551:787-799.
17. Wu DM, Kawamura H, Sakagami K, Kobayashi M, Puro DG. Cholinergic regulation of pericyte-containing retinal microvessels. *Am J Physiol*. 2003;284:H2083-H2090.
18. Lopatin AN, Makhina EN, Nichols CG. Potassium channel block by cytoplasmic polyamines as the mechanism of intrinsic rectification. *Nature*. 1994;372:366-369.
19. Ficker E, Taglialatela M, Wible BA, Henley CM, Brown AM. Spermine and spermidine as gating molecules for inward rectifier K⁺ channels. *Science*. 1994;266:1068-1072.
20. Puro DG. Physiology and pathobiology of the pericyte-containing retinal microvasculature: new developments. *Microcirculation*. 2007;14:1-10.
21. Wu DM, Kawamura H, Li Q, Puro DG. Dopamine activates ATP-sensitive K⁺ currents in rat retinal pericytes. *Vis Neurosci*. 2001;18:935-940.