Role of Hypoxia during Normal Retinal Vessel Development and in Experimental Retinopathy of Prematurity

Wei Zbang,1 Yasuki Ito,1 Emily Berlin,1 Robin Roberts,1 and Bruce A. Berkowitz1,2

PURPOSE. To test the hypothesis that retinal hypoxia is present during vascular development in normal rat pups and in a newborn rat model of retinopathy of prematurity (ROP).

METHODS. Preretinal vitreous PO2 measurements were made during room air breathing using 19F magnetic resonance spectroscopy (MRS) and a perfluoro-15-crown-5-ether droplet in normal adult and newborn (postnatal day [P]1–P20) rats, and in newborn rats exposed first to 14 days of variable oxygen (before NV) and six additional days in room air after variable oxygen exposure (during NV). After each experiment, blood gas values were measured, and retinas were isolated. Retinas were adenosine diphosphatase (ADPase) stained, and flat-mounted to determine peripheral avascular extent and NV incidence and severity.

RESULTS. In the vascularized rat retina, no significant difference (P > 0.05) was found between the droplet-derived preretinal vitreous oxygen tension (24 ± 2 mm Hg, mean ± SEM, n = 18) and previously reported electrode-measured oxygen tension (22 ± 1 mm Hg). Only during normal retinal vessel growth (P1–P10) and before the appearance of NV was evidence for retinal hypoxia found at the border of the vascular and avascular retina. However, the mean PO2 (range, 24–28 mm Hg) over the vascular retina was not different (P > 0.05) between any of the newborn rat groups in this study.

CONCLUSIONS. 19F MRS of a perfluorocarbon droplet provides an accurate measure of preretinal vitreous oxygen tension in rats. These data support an important role of physiologic hypoxia in normal retinal circulatory development and raise the possibility that, in experimental ROP, retinal hypoxia is a necessary but not sufficient condition for the development of retinal NV. (Invest Ophthal Vis Sci. 2003;44:3119–3123) DOI: 10.1167/iovs.02-1122

The blinding complications of retinopathy of prematurity (ROP) are strongly associated with the development of retinal neovascularization (NV). Although the pathogenesis of the retinal NV is unknown, retinal hypoxia is commonly thought to cause (i.e., be a necessary and sufficient condition for) NV. Vascular endothelium growth factor (VEGF) is a potent hypoxia-inducible mitogen that was upregulated panretinally (i.e., over vascular and avascular retina) in a newborn rat ROP model and in a kitten oxygen-induced retinopathy model.1–4 Previously, we applied a functional magnetic resonance imaging (MRI) method to a newborn rat model of ROP before and during the appearance of NV and found that all retinal regions from superior ora serrata to inferior ora serrata had significantly lower changes in the preretinal vitreous oxygen tension during a carbogen inhalation challenge (i.e., a subnormal panretinal oxygenation response).3,4 Taken together, the above VEGF and functional MRI data suggest, but do not prove, that preretinal hypoxia is associated with the development of retinal NV in experimental ROP. However, a variety of factors (unrelated to hypoxia) could also be responsible for the panretinal upregulation of VEGF (e.g., hypoglycemia, and elevated free radical levels) and the appearance of a subnormal oxygenation response (e.g., vasospasm).

In this study, we tested the hypothesis that preretinal hypoxia is present in a newborn rat model of ROP. A novel 19F magnetic resonance spectroscopy (MRS) method was developed, validated, and applied to measure preretinal vitreous PO2 in the newborn rat. This method is based on the linear relationship between the spin-lattice relaxation rate (T1)−1 of the fluorine nuclide in metabolically inert perfluorocarbon liquids (PFC) and the steady state PO2 in the microenvironment around the droplet.5 PFCs are currently used as vitreous substitutes, among other applications, because they are nontoxic and have a density approximately twice that of water.6 PFC dissolves roughly one order of magnitude more oxygen than water under similar conditions.6 This makes the PFC (T1)−1, measured by 19F MRS, more sensitive to the oxygen tension than water.6 The key advantage of the 19F MRS approach is that it can, in principle, make preretinal vitreous PO2 measurements in eyes that are too small to accommodate an oxygen electrode (e.g., normal and ROP-affected eyes in rat pups).

METHODS

The animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the ARVO Statement for the use of Animals in Ophthalmic and Vision Research.

Normal Adult and Newborn Rats

In this study, 19F MRS was used to examine adult Sprague-Dawley rats (Hilltop Lab Animals, Chatsworth, CA) and postnatal day P1, P3, P6, P9, P14, and P20 rat pups.

Newborn Rat ROP Model

This model has been described in detail elsewhere.5,7 Briefly, Sprague-Dawley mothers and litters (12–15 pups/litter) were housed in modified pediatric incubators, where the oxygen levels were varied every 24 hours between 50% and 10% oxygen for the first 14 days after birth. Separate groups of animals were allowed to recover for the next 6 days in room air (until P20). This protocol produces retinal NV in 100% of the eyes.8 19F MRS was used to examine the ROP rats on day P14 (before the appearance of NV) and on day P20 (during the appearance of NV).
Perfluoro-15-Crown-5-Ether Droplet Injection Procedure

We chose the PFC perfluoro-15-crown-5-ether because of its high spectral signal-to-noise ratio (20 equivalent fluorines) and sensitivity to oxygen.6 PFC droplets were injected into the eye, either before the examination (typically 2 days before in metofane-anesthetized animals) or just before the animal was placed into the magnetic resonance imager in urethane-anesthetized animals. PFC droplet injections were performed with a 30-gauge blunt needle attached to a 5-μL syringe (Hamilton, Reno, NV) containing the PFC. To view the position of the needle in the vitreous, we examined the eye after dilation, using a contact lens and a surgical microscope with a coaxial light source. On the day of the Po2 measurement, we used MRI to determine the diameter and position of the droplet in the vitreous.

MRI Procedures

On the day of the experiment, rats were anesthetized with urethane (1.5 g/kg, intraperitoneally, 36% solution, freshly made daily) and gently positioned prone in the MRI cradle, with the left eye uppermost. Animals spontaneously breathed room air and their systemic physiologic parameters (rectal temperature, pulse, and hemoglobin oxygen saturation) were monitored and maintained during the entire experiment. After the urethane-anesthetized animal was placed in the magnet, transverse spin-echo (recovery time [TR] 1 second, echo time [TE] 22 ms, slice thickness 1 mm, number of acquisitions [NA] 1, 128 × 256 pixel2, field of view [FOV] 2.8 × 2.8 mm), and sagittal gradient recalled echo (TR 0.3 seconds, TE 7.2 ms, NA 1, 128 × 256 pixel2, slice thickness 1 mm, FOV 2.8 × 2.8 mm) images were collected. Because the PFC droplets do not contain protons, the droplet appears as a “black hole” on MRI. This allows its position and size in the vitreous to be readily measured. To position the droplet over the central vascularized retina, we took advantage of the fact that PFC liquids have a greater density than water and fall into the gravitationally dependent position. To position the droplet over the peripheral retina, we turned the cradle upside down.

After imaging, the surface coil was manually retuned (within 5 minutes) to the 19F frequency, and the static magnetic field homogeneity shimmmed on the 19F signal. Three saturation recovery T1 data sets were collected (10 r values, 5 minutes/T1 measurement). T1 was measured for each data set, using standard curve-fitting software on a computer work station (Avance; Bruker Instruments, Billerica, MA). The three T1’s were averaged to determine a mean Po2. The animal was removed from the magnet, and during room air breathing an arterial blood sample was collected for immediate blood gas measurement. The animal was then killed and the eyes removed for histologic analysis.

To be included in a study, an animal must have a single droplet of the correct diameter (0.7 mm) positioned in the preretal vitreous space. Not all the droplet injections were successful: up to 10% failed because the droplets leaked out of the eye immediately after injection, up to 10% of the droplets broke into multiple droplets and sampled different spatial locations, and up to 10% of the droplets were lodged too far anteriorly. Other acceptance criteria were (1) minimal movement (eye and head) during the examination, (2) a regular nongasping respiratory pattern before the examination, (3) rectal temperatures in the range of 35.0°C to 36.5°C, (4) PaO2 of more than 80 mm Hg, and (5) PaCO2 of 35 to 45 mm Hg. In addition, if the blood gas machine was not able to read a sample (e.g., due to a clot or excessive air in the capillary tube), the MR data were excluded. The number of animals that satisfied the inclusion criteria were as follows: adult (n = 3), P1 (n = 3), P2 (n = 1), P6 (n = 1), P9 (n = 1), P10 (n = 1), P14 (n = 6), and P20 (n = 11) and ROP before (n = 7) and during (n = 8) NV.

Calibration Conditions

Relaxation Rate/Po2. The spin-lattice relaxation rate of perfluoro-15-crown-5-ether in the vitreous is a function of oxygen tension and temperature.8 To measure the oxygen tension accurately, we have to estimate the retinal temperature and correct the relaxation rate versus oxygen concentration calibration curve. We derived the following calibration equation from triplicate perfluoro-15-crown-5-ether phantom studies at three different temperatures (32°C, 34°C, and 37°C) and three different oxygen levels (0%, 19.7%, and 100%). Using a linear analysis, we found that the droplet Po2 = 7.6(1/T1s)−Int(T)/slope(T), where 1/T1s is the relaxation rate, Int(T) is the calibration intercept as a function of temperature (T) [0.73215−(0.0094 × retinal temperature)]; slope(T) is the calibration slope as a function of T [0.02589− (0.0002 × retinal temperature)]; and 7.6 is a conversion factor between percentage oxygen level and pressure. At 37°C, our calibration parameters are similar to those previously published.8

Core/Retinal Temperature. We estimated the retinal temperature in the adult and newborn rats using two different methods. First, in separate groups of adult, newborn, and ROP rats, we positioned the tip of a thermocouple near the retina, using a surgical microscope. A separate temperature probe measured the core temperature. The room lights were then turned off, and retinal and core temperature were measured in triplicate. A linear relationship between retinal and core temperature was found. In the second approach, we determined whether the thermocouple temperature represented the temperature of the somewhat larger PFC droplet. Previously, we reported that the change in the 19F MRS chemical shifts of the PFC perfluorotributylamine (FTBA) are linearly related to temperature, but not to oxygen tension.7 To measure preretal vitreous temperature, a 0.5-μL FTBA droplet was placed on the retina (by using the procedure described earlier). Data were acquired in triplicate with a simple one-pulse sequence. 10 sets of 64 acquisitions/set, 1 K complex data points, and sweep width SW = 15,151 Hz. The rat’s core temperature was continuously monitored. In adult and newborn rats, there was also a linear relationship between core and retinal temperature. No statistical (P > 0.05) differences in the slope or intercept (ANCOVA analysis) were found when the thermocouple and 19F MRS FTBA methods were compared. However, there were differences between room air and ROP animals (P < 0.05). Linear analysis of the pooled thermocouple and FTBA data in room air or ROP animals resulted in the following equations: RA retinal temperature = 0.55 × rectal temperature + 13.5 (r = 0.99, P < 0.05); RO retinal temperature = 0.72 × rectal temperature + 6.9 (r = 0.9, P < 0.05). It was beyond the scope of this manuscript to determine the reason for these different temperature calibration curves in normal and experimental animals. Nonetheless, we estimated the preretal vitreous temperature from the core temperature, converted the core temperature to an estimated retinal temperature, with the appropriate equation, and then used this retinal temperature estimate to correct the relaxation rate/Po2 calibration curve.

Histologic Analysis

The percent peripheral avascularity and incidence and severity of retinal NV were determined as previously described from the adenosine diphosphatase (ADPase)-stained flatmounts.4,10 To determine the extent of peripheral avascularity, we captured the image of an ADPase-stained flatmount with a charge-coupled device (CCD) camera and analyzed on computer with NIH Image (available by ftp from zippy.nih.gov) or from http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). To determine NV severity, three investigators independently scored in a masked fashion each ADPase-stained retinal flatmount in terms of clock hours of NV. The median number of clock hours per retina recorded by the three investigators is reported. To determine the severity of NV, a clock face was mentally superimposed on the retinal surface and the number of clock hours (a score from 0 to 12) occupied by abnormal vessel growth was determined.
Statistical Analysis

The physiological (i.e., blood gas values, rectal temperatures, and blood glucose) data were normally distributed. Comparisons between groups were performed by ANOVA. To compare the NV severity and avascularity that were not normally distributed among the groups, a Kruskal-Wallis test was used. To compare NV incidences, a \( t \)-test (two-sided). Correlations were assessed using either linear regression or nonparametric approaches (Spearman \( R \), Kendall \( \tau \), and \( \gamma \)). If all three nonparametric methods found \( P < 0.05 \), the correlation was considered significant.

RESULTS

Normal Rat

Comparison of Preretinal Vitreous Droplet and Oxygen Electrode \( \text{PO}_2 \). In vascularized rat eyes (P14, P20, and adult\( ^{11} \)), no statistical difference (\( P > 0.05 \)) was found between the PFC droplet (24 \( \pm \) 2 mm Hg, \( n = 21 \); mean \( \pm \) SEM, P14, P20, and adult) and previously reported oxygen electrode data (adult: 22 \( \pm \) 1 mm Hg, \( n = 43 \); P20: 28 \( \pm \) 1 mm Hg, \( n = 32^{12-16} \)).

Preretinal Vitreous Droplet \( \text{PO}_2 \) Measurements during Normal Retinal Vessel Development. The droplet \( \text{PO}_2 \) in the preretinal vitreous from the P1 to P10 vascularized retinas was pooled, and the average was 27 \( \pm \) 2 mm Hg (\( n = 7 \); average ratio of droplet distance from the optic nerve head to extent of vascularized retina: 0.32 \( \pm \) 0.1; a distance ratio of 1 indicates that the droplet is exactly at the border of vascular and avascular retina). This pooled oxygen tension was not different (\( P > 0.05 \)) from the \( \text{PO}_2 \) measured over vascularized retina at P14 (before the appearance of NV) was 28 \( \pm \) 5 mm Hg (\( n = 7 \); average distance ratio: 0.47; range, 0.3–0.7) and at P20 (during the appearance of NV) was 26 \( \pm \) 4 mm Hg (\( n = 8 \); mean distance ratio 0.52; range, 0.3–0.7; Fig. 2). These oxygen tensions were not statistically different from the \( \text{PO}_2 \)s measured over vascularized retina in P1 to P10 control rats (27 \( \pm \) 2 mm Hg, see above) or in P14, P20, and adult vascularized rat retinas (24 \( \pm \) 2 mm Hg). However, because of the droplet and vitreous space sizes, it was not possible to obtain oxygen tension measurements at the border of vascular and avascular retina. The data scatter in the relatively small number of P14 ROP data points probably prevented linear regression analysis from achieving significance (\( r = -0.73, P = 0.06 \)). Nonetheless, in the P14 ROP animals, a significant correlation (\( r = -0.82, P = 0.02 \)) was found between the retinal \( \text{PO}_2 \) and ratio of droplet distance from the optic nerve head to extent of vascularized retina (Fig. 3).

At P20, in the experimental animals, a severe NV response was evident (Table 1). The \( \text{PO}_2 \), \( \text{PCO}_2 \), and temperature were similar between all the groups (Table 2). All the blood gas values fell within the expected range for room air breathing.

Droplet \( \text{PO}_2 \) Measurements. The mean preretinal vitreous oxygen tensions over vascular retina at P14 (before the appearance of NV) was 28 \( \pm \) 5 mm Hg (\( n = 7 \); average distance ratio: 0.47; range, 0.3–0.7) and at P20 (during the appearance of NV) was 26 \( \pm \) 4 mm Hg (\( n = 8 \); mean distance ratio 0.52; range, 0.3–0.7; Fig. 2). These oxygen tensions were not statistically different from the \( \text{PO}_2 \)s measured over vascularized retina in P1 to P10 control rats (27 \( \pm \) 2 mm Hg, see above) or in P14, P20, and adult vascularized rat retinas (24 \( \pm \) 2 mm Hg). However, because of the droplet and vitreous space sizes, it was not possible to obtain oxygen tension measurements at the border of vascular and avascular retina. The data scatter in the relatively small number of P14 ROP data points probably prevented linear regression analysis from achieving significance (\( r = -0.73, P = 0.06 \)). Nonetheless, in the P14 ROP animals, a significant correlation (\( r = -0.82, P = 0.02 \)) was found between the retinal \( \text{PO}_2 \) and ratio of droplet distance from the optic nerve head to extent of vascularized retina (Fig. 3). No such correlation was found in P14 control rat pups (\( r = -0.77, P = 0.07 \); distance ratio, 0.39; range, 0.08–0.7), P20 control rats (\( r = -0.41, P = 0.21 \); distance ratio, 0.54; range, 0.08–0.8), and in P20 ROP newborns (\( r = 0.17, P = 0.69 \); distance ratio, 0.52; range, 0.3–0.7; data not shown).

DISCUSSION

In this study we developed and applied in rodents a novel \( ^{19} \text{F} \) MRS measurement of preretinal vitreous oxygen tension. Previously in rabbits, we reported reasonable agreement between

<table>
<thead>
<tr>
<th>Group</th>
<th>Avascuarity (%)</th>
<th>NV Incidence (%)</th>
<th>NV Severity (clock hour; range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROP P14</td>
<td>18.3 ( \pm ) 1.5</td>
<td>0 (30/30)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>ROP P20</td>
<td>8.8 ( \pm ) 1.8*</td>
<td>100 (11/11)*</td>
<td>4 (1-10)*</td>
</tr>
</tbody>
</table>

* Significantly different from ROP day-14 groups. \( P < 0.05 \).
the $^{19}$F MRS droplet measurement and that reported by oxygen electrodes.17 Here, we again find in adult and newborn rats agreement between the preretinal oxygen tension measured by the droplet approach and that obtained using an oxygen electrode. This agreement between the MRS and oxygen electrode data supports the procedures used in the present study to estimate the retinal temperature and convert the $T_1$ relaxation time to a $P_O2$. Previous oxygen electrode data indicate that preretinal vitreous oxygen measurements provide an estimate of the inner retinal oxygen tension.12

There are two major limitations of the present study. First, precise placement of a droplet over vascular or avascular retina was not always possible. This was not a concern in the study of normal retinal vessel development, because after birth the retinal vessels grow from the optic nerve head out to the periphery by P14. Thus, on different postnatal days, the droplet (which was positioned into approximately the same preretinal vitreous region) sampled different regions of the vasculature (e.g., the border of vascular and avascular retina). A second limitation is that the combination of low vitreous space volume and relatively large droplet size prevented the measurement of oxygen tension in the far peripheral (avascular) retina of the experimental ROP rat pups. Nonetheless, the present data underscore the use of $^{19}$F MRS as an accurate measure of preretinal vitreous oxygen tension in rodents’ eyes that are too small for oxygen electrode measurements.

The sensitivity of the droplet method to physiologically relevant retinal hypoxia was evaluated in P1 to P10 rat pups during normal retinal circulation development. It has been hypothesized that retinal vasularization is associated with retinal hypoxia (physiologic hypoxia) at the border of vascular and avascular retina.19 From P1 to P10, the retinal circulation grows from the central to the peripheral retina. Because of the relatively small vitreous space available for investigation, the droplet is typically positioned at approximately the same distance from the optic nerve in these newborn animals. Thus, a measure of oxygen tension at different spatial locations along the retinal circulation (i.e., the spatial oxygen gradient) can be obtained by examining rat pups at different postnatal ages. The present data support the physiologic hypoxia hypothesis, because retinal hypoxia was found during vessel development at the border of vascular and avascular retina, compared with the oxygen tension measured in the central retina. In addition, no evidence was found for a spatial oxygen gradient after normal retinal vessel development (i.e., at days P14 and P20). Together, these data provide for the first time direct evidence for an association between retinal hypoxia at the border of vascular and avascular retina and normal retinal circulation growth. In addition, the data underscore the detection sensitivity of the $^{19}$F MRS method to physiologically relevant retinal hypoxia.

Previously, we measured in a newborn rat ROP model a subnormal panretinal oxygenation response to a hyperoxic inhalation challenge at P14 and P20 and speculated that a subnormal response was a surrogate marker of retinal hypoxia.3,4 The data in the current work do not support this hypothesis. No evidence was found for panvascular retinal hypoxia before (P14) or during (P20) the appearance of NV in experimental ROP (Fig. 2). It is possible that intraretinal hypoxia is present but not detectable in the preretinal vitreous space. We had previously argued that the retinal oxygenation response to an inhalation challenge is a measure of the ability of the retinal circulation to supply oxygen during the provocation but does not reflect changes in oxygen consumption during the challenge.4 In this case, it is not clear how intraretinal hypoxia, but not preretinal vitreous hypoxia, would be associated with a subnormal panretinal response measured in the preretinal vitreous space. It was not possible to simultaneously measure the preretinal vitreous oxygen tension and oxygenation response because of the droplet-displaced vitreous that is needed for the provocation experiment. Based on these considerations, the present data do not support the idea that the previously reported subnormal panretinal oxygenation response is a marker of retinal hypoxia.

Two (not mutually exclusive) hypotheses are commonly put forth to explain why hypoxia may cause the growth of retinal NV: (1) that hypoxia at the border of vascular and avascular retina occurs at an inappropriate time compared with the needs of the retina and (2) that there is a critical (i.e., lower) level of hypoxia needed for the development of NV. However, these hypotheses do not seem to explain the available experimental data. For example, in the ROP rats, hypoxia would be expected at the border of vascular and avascular retina in all 12 clock hour areas and/or VEGF diffusion in the small vitreous volume (approximately 11 $\mu$L), so that all 12 clock hours of retina would be exposed to VEGF. However, only approximately six clock hours of NV were found in the

<table>
<thead>
<tr>
<th>Group</th>
<th>$P_{O2}$ (mm Hg)</th>
<th>$P_{CO2}$ (mm Hg)</th>
<th>pH</th>
<th>Core Temperature ($^\circ$C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1-P10 ($n = 7$)</td>
<td>85 ± 3</td>
<td>40 ± 2</td>
<td>7.48 ± 0.02</td>
<td>35.2 ± 0.1*</td>
</tr>
<tr>
<td>P14 ($n = 6$)</td>
<td>112 ± 7*</td>
<td>41 ± 0.4</td>
<td>7.32 ± 0.02</td>
<td>36.6 ± 0.3</td>
</tr>
<tr>
<td>P20 ($n = 11$)</td>
<td>97 ± 3</td>
<td>41 ± 1</td>
<td>7.44 ± 0.01</td>
<td>35.8 ± 0.1</td>
</tr>
<tr>
<td>ROP P14 ($n = 7$)</td>
<td>91 ± 4</td>
<td>41 ± 2</td>
<td>7.41 ± 0.02</td>
<td>35.7 ± 0.2</td>
</tr>
<tr>
<td>ROP P20 ($n = 8$)</td>
<td>95 ± 2</td>
<td>36 ± 1</td>
<td>7.46 ± 0.01</td>
<td>35.6 ± 0.1</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SEM.

*Significantly different from P20 group, $P < 0.05$. 

The sensitivity of the droplet method to physiologically relevant retinal hypoxia was evaluated in P1 to P10 rat pups during normal retinal circulation development. It has been hypothesized that retinal vasularization is associated with retinal hypoxia (physiologic hypoxia) at the border of vascular and avascular retina.19 From P1 to P10, the retinal circulation grows from the central to the peripheral retina. Because of the relatively small vitreous space available for investigation, the droplet is typically positioned at approximately the same distance from the optic nerve in these newborn animals. Thus, a measure of oxygen tension at different spatial locations along the retinal circulation (i.e., the spatial oxygen gradient) can be obtained by examining rat pups at different postnatal ages. The present data support the physiologic hypoxia hypothesis, because retinal hypoxia was found during vessel development at the border of vascular and avascular retina, compared with the oxygen tension measured in the central retina. In addition, no evidence was found for a spatial oxygen gradient after normal retinal vessel development (i.e., at days P14 and P20). Together, these data provide for the first time direct evidence for an association between retinal hypoxia at the border of vascular and avascular retina and normal retinal circulation growth. In addition, the data underscore the detection sensitivity of the $^{19}$F MRS method to physiologically relevant retinal hypoxia.

Previously, we measured in a newborn rat ROP model a subnormal panretinal oxygenation response to a hyperoxic inhalation challenge at P14 and P20 and speculated that a subnormal response was a surrogate marker of retinal hypoxia.3,4 The data in the current work do not support this hypothesis. No evidence was found for panvascular retinal hypoxia before (P14) or during (P20) the appearance of NV in experimental ROP (Fig. 2). It is possible that intraretinal hypoxia is present but not detectable in the preretinal vitreous space. We had previously argued that the retinal oxygenation response to an inhalation challenge is a measure of the ability of the retinal circulation to supply oxygen during the provocation but does not reflect changes in oxygen consumption during the challenge.4 In this case, it is not clear how intraretinal hypoxia, but not preretinal vitreous hypoxia, would be associated with a subnormal panretinal response measured in the preretinal vitreous space. It was not possible to simultaneously measure the preretinal vitreous oxygen tension and oxygenation response because of the droplet-displaced vitreous that is needed for the provocation experiment. Based on these considerations, the present data do not support the idea that the previously reported subnormal panretinal oxygenation response is a marker of retinal hypoxia.

Two (not mutually exclusive) hypotheses are commonly put forth to explain why hypoxia may cause the growth of retinal NV: (1) that hypoxia at the border of vascular and avascular retina occurs at an inappropriate time compared with the needs of the retina and (2) that there is a critical (i.e., lower) level of hypoxia needed for the development of NV. However, these hypotheses do not seem to explain the available experimental data. For example, in the ROP rats, hypoxia would be expected at the border of vascular and avascular retina in all 12 clock hour areas and/or VEGF diffusion in the small vitreous volume (approximately 11 $\mu$L), so that all 12 clock hours of retina would be exposed to VEGF. However, only approximately six clock hours of NV were found in the

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933710/)
ROP group. Similarly, in a branch retinal vein occlusion model in the adult pig in which all ischemic foci were hypoxic (measured with an oxygen electrode) before the appearance of NV, only approximately 45% of these ischemic retinas showed development of NV. It seems unlikely that the presence of hypoxia would be inappropriate only for some retinal locations or VEGF in the vitreous would lead to NV for some but not other retinal regions. It is not possible, with the present data, to test these alternative hypotheses definitively, because problems with positioning the droplet (described earlier) prevented a measure of the magnitude of the hypoxia at the border region in the P14 ROP animals (i.e., it had to be inferred from the data in Fig. 3). We speculate that worse-than-normal hypoxia at the border of vascular and avascular retina in experimental ROP animals does not cause the phenotype change from normal to abnormal vessel development. Instead, the presence of hypoxia at the border of vascular and avascular retina in P1 to P10 control animals and in P14 ROP pups raises the possibility that hypoxia is not a causative factor in the development of retinal NV. The possibility that retinal hypoxia is a necessary but not sufficient condition for the development of NV could also explain the results of Pournaras. Experiments are on going to explore this hypothesis more fully.

Acknowledgments

The authors thank John Penn and Rob Linsenmeier for their help and encouragement with this project and Rod Braun and Tim Kern for suggestions that greatly improved the manuscript.

References