Specificity of Subnormal ΔPo_2 for Retinal Neovascularization in Experimental Retinopathy of Prematurity

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Purpose. To test the hypothesis that in an experimental model of retinopathy of prematurity (ROP) rat pups that are at risk for but do not have retinal neovascularization (NV) exhibit a normal oxygenation response to a hyperoxic inhalation challenge.

Methods. Newborn Sprague-Dawley rats were raised under two varied oxygen conditions (50/10 or 40/15, indicating percent of oxygen in the air on alternate days) for 14 days and then allowed to recover in room air. Functional magnetic resonance imaging was used to determine the retinal oxygenation response (increase in partial oxygen pressure in the vitreous over the room air value, or ΔPo_2, in mm Hg) to hyperoxic inhalation challenge. Adenosine diphosphatase (ADPase)–stained retinas were analyzed to determine the NV incidence and severity.

Results. On postnatal day (P)20, the 40/15 procedure produced significantly ($P < 0.05$) lower incidence of NV than the 50/10 protocol (8% vs. 99%, respectively). Retinal ΔPo_2 during carbogen breathing of the 40/15 animals that did not have evidence of NV was not different ($P > 0.05$) from that of normal age-matched animals; later time points were not examined. At P26 and P34, in 50/10 rats that no longer had NV, retinal ΔPo_2s during carbogen breathing were significantly ($P < 0.05$) lower than that of age-matched control pups. At P34 in control rats, but not in 50/10 rats, ΔPo_2 was 61% greater ($P < 0.05$) during carbogen breathing than during oxygen breathing.

Conclusions. The results from 40/15 experiments, together with the authors' previous data in 50/10 rats, which documented subnormal retinal ΔPo_2 before and during NV, provides additional support that subnormal retinal oxygenation to an inhalation challenge is an important event associated with the development of NV. In addition, 50/10 rats that no longer demonstrated NV had a persistent subnormal retinal ΔPo_2, suggesting a continuing risk of development of additional retinal complications after resolution of the NV in ROP. (*Invest Ophthalmol Vis Sci.* 2003;44:3551–3555) DOI:10.1167/iovs.03-0008

Improvements in neonatal care have resulted in the survival of very low birth weight infants who are at increased risk for retinopathy of prematurity (ROP).[1] The vision of these neonates is threatened with retinal vascular abnormalities, such as neovascularization (NV), during the preterm weeks as well as by potential later complications, such as myopia, strabismus, retinal distortion, retinal detachment, and reduced electroretinogram responses.[1–4] More work is needed to understand the nature of the vascular dysfunction associated with increased retinal risk of short- and long-term complications in ROP.

We have developed a novel functional magnetic resonance imaging (MRI) method for measuring the retinal oxygenation response to a hyperoxic inhalation challenge in the newborn and adult rat, rabbit, cat, and human.[5–11] In this technique, hyperoxic inhalation increases vitreous partial oxygen pressure over room air values (ΔPo_2). Because oxygen is paramagnetic, this ΔPo_2 produces an increase in the vitreous signal intensity on a T_1-weighted image.[11] Further, good agreement is found between the MRI-measured response and that determined by other investigators using an oxygen electrode in normal rat retina under similar conditions.[10] We, and others, have measured a roughly 50% improvement in retinal oxygenation in the rat during carbogen breathing in comparison with oxygen breathing.[10,12] Carbogen is a gas mixture of carbon dioxide (5%) and oxygen (95%) that has been used clinically, instead of 100% oxygen to minimize the vasoconstrictive effects of pure O_2 on retinal vessels. Comparing retinal ΔPo_2 during carbogen and 100% oxygen inhalation challenges can provide a measure of retinal autoregulatory capability.

We have used this functional MRI in a clinically relevant newborn rat ROP model to study whether subjects that were either going to have or already had NV had a subnormal retinal oxygenation response (i.e., the sensitivity of the ΔPo_2 measure to NV).[8] Newborn rats were exposed, from postnatal day (P)0 to P14, to an oxygen environment that alternated between 50% and 10% every 24 hours (50/10 rats). Between P14 and P20, the rats breathed room air and at P20 showed a high incidence (in 99% of the animals) and severity (six clock hours) of NV. A subnormal panretinal oxygenation response was found before (P14) and during (P20) the appearance of NV. In other experiments, we compared the retinal ΔPo_2 in 50/10 rats that recovered between P14 and P20 in either room air or 28% supplemental oxygen.[7] At P20, the experimental animals that recovered in supplemental oxygen had a decreased incidence and severity of NV, but an unexpected decrease in panretinal oxygenation. However, after six additional days in room air, the rats that had been exposed to supplemental oxygen demonstrated a significantly higher incidence of NV than did those that recovered in room air. Based on these data, we hypothesized that in experimental ROP, subnormal retinal ΔPo_2 is an important event associated with the development of NV and the persistence of complications.

In this study, we tested the specificity of the functional MRI measure (i.e., do subjects who are at risk for but do not have NV exhibit a normal oxygenation response?), by comparing retinal oxygenation responses in three groups of animals: control rats, rats with a history of varied oxygen exposure that were at risk for but did not exhibit NV, and rats with a history

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of varied oxygen exposure that no longer demonstrated retinal NV. Our hypothesis predicts normal retinal ΔPo_2 in both at-risk rat groups. The first at-risk group consisted of newborn rats exposed to a varied oxygen condition (40/15) that produces substantially reduced NV compared with the 50/10 condition described earlier.[13] The second at-risk group contained young rats that had been exposed to a 50/10 procedure until P14 and allowed to recover in room air for extended periods.[4] In these animals, NV had mostly regressed by P34.

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.

Animal Model

The newborn rat model of ROP has been described in detail elsewhere.[14] Briefly, Sprague-Dawley mothers and litters (12–15 pups per litter) were housed in a modified pediatric incubator where the oxygen levels were varied every 24 hours for the first 14 days after birth. Two conditions were examined in which the oxygen leveled alternated between 50% and 10% (50/10) or 40% and 15% (40/15). Rats were then allowed to recover in room air (21%) during either the next 6 days (until P20, 40/15 group), or 12 and 20 days (P26 and P34, 50/10 groups).

Functional MRI Examination

The functional MRI procedure has been described in detail elsewhere.[7,10] Briefly, on the day of the examination, urethane-anesthetized animals (0.083 mL of a 36% solution of urethane/20 g animal weight, intraperitoneally, freshly made daily; Aldrich, Milwaukee, WI) were gently positioned on an MRI-compatible homemade holder with the nose placed in a plastic nose cone that allowed the animal to breathe spontaneously. Core temperature was continuously monitored and maintained. MRI data were acquired on a 4.7-T system with a two-turn surface coil (1.5 cm diameter) placed over the eye and an adiabatic spin-echo imaging sequence (repetition time [TR] 1 second, echo time [TE] 22.7 ms, number of acquisitions [NA] 1, matrix size 128×256, slice thickness 1 mm, field of view 28×28 mm^2, sweep width 25,000 Hz, 2 minutes/image). A capillary tube (1.5 mm inner diameter) filled with distilled water was used as the external standard. Four sequential 2-minute images were acquired as follows: three control images while the animal breathed room air and one image during hyperoxia (either carbogen or 100% oxygen). In each animal, hyperoxia was started at the same phase-encoding step collected near the end of the third image. This procedure was followed exactly for every animal. Animals were returned to room air for 5 minutes to allow recovery from the inhalation challenge and were removed from the magnet. A second 2-minute inhalation challenge was performed outside the magnet, with care taken to not alter the spatial relationship between the animal's head and the nose cone. Blood from the abdominal aorta was collected immediately after a second 2-minute carbogen challenge and analyzed for Pao_2, $Paco_2$, and pH, as described previously.[8] Note that this second inhalation challenge (outside the magnet) is needed, because it is not feasible to routinely obtain an arterial blood sample from inside the magnet (>40 cm away from the magnet opening) from newborn rats. After the blood collection, the animal was killed with an intracardiac potassium chloride injection, the eyes were enucleated, and the retinas were flatmounted. The physiological data (i.e., blood gas level and core temperature) were normally distributed. Comparisons between groups were performed with an ANOVA.

The ΔPo_2 is detected as an increase in the signal intensity on a T_1-weighted image.[10] Previously, we validated that the MRI-measured ΔPo_2 was similar to that determined using an oxygen electrode in normal adult rat retina.[10] We measured the ΔPo_2 in the posterior vitreous (within roughly 200 μm from the retina, described later) as a measure of inner retinal oxygenation. It is important to note that steady state (room air) vitreous oxygen tension cannot be measured using this method, because many factors (e.g., vitreous temperature and protein content) affect the baseline preretinal vitreous water signal and its relaxation properties. In other words, simply obtaining an image of the eye during room air breathing alone is insufficient to measure retinal oxygenation. These factors are not likely to change on the short time scale between baseline and carbogen breathing. Thus, their contributions are expected to cancel and not contribute to the ΔPo_2 measurement.

Data Analysis

To be included in this study, the animal must have demonstrated (1) minimal eye movement during the MRI examination. Movement artifacts (typically seen in the phase encode direction) will confound interpretation of the vitreous signal intensity changes produced during the hyperoxic challenge; (2) a nongasping respiratory pattern before and after the MRI examination. If the animal is gasping (which occurred <1% of the time), the anesthetic had probably been improperly administrated (e.g., during administration, one of the vital organs or blood vessels was accidentally punctured). This could produce a change in systemic oxygenation unrelated to the retinal changes; (3) core temperatures in the range of 35.5°C to 36.5°C. Preliminary experiments (data not shown) found a strong association between core temperature and $Paco_2$ and Pao_2 levels. The effect of this correlation on the precision of the measurements was minimized by using a relatively tight range of temperatures; and (4) Pao_2 80 to 100 mm Hg and $Paco_2$ between 35 and 45 mm Hg during the oxygen challenge and Pao_2 more than 350 mm Hg and $Paco_2$ between 46 and 65 mm Hg during the carbogen challenge. Previously, we found that arterial oxygen levels above 350 mm Hg during a hyperoxic challenge are needed to produce a consistently large preretinal vitreous oxygenation response.[9] The range of acceptable arterial carbon dioxide levels is within the array of values expected during carbogen-breathing conditions.[10] In addition, tight control over the acceptable blood gas value range is needed to ensure adequate quality control of each sample. Occasionally, the blood gas machine was not able to read a sample (e.g., due to a clot or excessive air in the capillary tube). In this case, the MRI data were also excluded. These acceptance criteria are necessary for critical comparison of the retinal oxygenation response between groups while minimizing systemic differences. Because such tight criteria are used, only approximately 50% of the animals that started the study were used in the final analysis. Based on our previous experience in rats, a sample (n) of five or more is sufficient to draw statistical conclusions.

To determine retinal ΔPo_2, we calculated the signal intensity change that occurred in the vitreous during a hyperoxic challenge by performing a pixel-by-pixel subtraction of the average room air image from the hyperoxic image. Subtle shifting of the animals' position occasionally occurred during the experiment (e.g., settling on the gauze packing). Because the slice thickness (1 mm) was relatively large compared with the diameter of the eye (approximately 3 mm), partial volumes sampled in the MRI slice were similar if the eye subtly moved out of the imaging plane and the resultant retinal ΔPo_2 levels were not expected to be substantially affected. However, small movement can produce changes in the eye position within the plane of the image. In this case, because of the higher spatial resolution in plane, if all the images are not correctly aligned or coregistered, confounding subtraction artifacts can occur. To minimize potential subtraction artifacts due to possible in-plane movement, a warp affine image coregistration was performed on each animal using software written in house. A warp affine transformation is a mathematical procedure that allows correction for scaling and skewing distortions of the coordinate space.

After coregistration, the MRI data were transferred to a computer (Power Mac G4; Apple Computer, Cupertino, CA) and analyzed using the program NIH Image (http://rsb.info.nih.gov/nih-image/ developed by Wayne Rasband and provided in the public domain by the National

TABLE 1. Summary of Histology Results

	40/15	50/10		
	P20	P20	P26	P34
Peripheral avascularity (%)	< 1	11 ± 10	5.2 ± 1.7*	2.9 ± 0.05*
NV incidence (%)	8 (8/102)*	99 (153/155)	33 (3/9)*	16 (7/45)*
NV severity (clockhour), median (range)	1 (1–3)*	5 (1–12)	1 (1–2)*	1 (0.5–1.5)*

* $P < 0.05$, compared with 50/10 P20 group.

Institutes of Health, Bethesda, MD) as previously described. Briefly, images obtained during room air breathing were averaged to improve the signal-to-noise ratio. All pixel signal intensities in the average room air image and the 2-minute carbogen image were then normalized to the external standard intensity. Signal intensity changes during carbogen breathing were calculated and converted to ΔPo_2, on a pixel-by-pixel basis, as previously described.[7] These data were analyzed as follows. First, the pixel values along a 1-pixel-thick line drawn at the boundary of the retina/choroid and vitreous were set to 255 (black). We estimate that the thickness of this line, based on the in-plane resolution (e.g., in the rat, a 28 × 28-mm^2 field of view was sampled by 128 × 256 data points) of 219 × 109 μm^2, is approximately 150 μm. The values in another 1-pixel-thick line drawn in the preretinal vitreous next to the black pixels were then extracted. This procedure minimized retinal-choroid pixel values from potentially contaminating those used in the final analysis ("pixel bleed") and ensured that similar preretinal vitreous space was sampled for each animal (minimizing the contribution from the very local preretinal oxygenation gradients next to the retinal surface[15]). The oxygenation responses from each pixel, excluding those within 5 mm of the optic nerve (to avoid potential contributions from residual hyaloid circulation), were obtained. To minimize potential confounding effects of age, the panretinal oxygenation responses were normalized to the mean ΔPo_2 of the respective age-matched control group. These normalized data were used for statistical comparisons.

The physiological parameters (i.e., blood gas levels and core temperatures) were normally distributed and are presented as the mean ± SEM. Comparisons between all groups were performed with an unpaired *t*-test. Comparison of retinal ΔPo_2 between control and experimental groups was performed with a generalized estimating equation approach. This method performs a general linear regression analysis that uses all the pixels in each subject and accounts for the within-subject correlation between adjacent pixels. In all cases, $P \leq 0.05$ was considered significant.

Histologic Analysis

Adenosine diphosphatase (ADPase)–stained flatmounts were analyzed to determine NV incidence and severity for each animal studied by functional MRI, as previously described.[16] Severity was determined only from retinas with some degree of NV. Three investigators independently scored each flatmount in clockhours of NV in a masked fashion. The median number of clockhours of NV per retina, determined from the conclusions of the three investigators, is reported. To determine 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. To compare the severity, a two-sample Mann-Whitney rank sum test (2-sided) was used. To compare the incidence, a χ^2 test was performed (2 × 2). $P < 0.05$ was considered significant.

RESULTS

Histologic Analysis

A summary of the percent of peripheral avascularity and preretinal NV incidence and severity at days P20 (40/15, 50/10), P26, and P34 (50/10) are presented in Table 1. As expected, at P20, the 50/10 group had significantly ($P < 0.05$) greater NV incidence and severity than the P20 40/15 group and P26 and P34 50/10 groups.

Systemic Physiology

A summary of core temperatures and blood parameters measured during the 2-minute carbogen and oxygen challenges is presented in Tables 2 and 3, respectively. All the blood gas data fell within the expected range for either 100% oxygen or carbogen breathing.[10]

Functional MRI

As shown in Figure 1, no significant ($P > 0.05$) difference in panretinal oxygenation response during carbogen breathing was found between P20 control and 40/15 animals that had no evidence of NV. Because there was no difference, panretinal ΔPo_2 was not measured at later time points in the 40/15 group. For comparison, note that, at P20 in a subset of 50/10 rat pups, retinal ΔPo_2 during carbogen breathing was subnormal ($P < 0.05$). At P26 and P34, the oxygenation response during carbogen inhalation of those animals in the 50/10 groups that did not have any evidence of NV were also significantly ($P < 0.05$) subnormal (Fig. 1). Only the P26 50/10 Pao_2 during carbogen breathing was significantly lower than in the age-matched

TABLE 2. Summary of Systemic Physiology during Carbogen Breathing

Group	PaO_2 (mm Hg)	$PaCO_2$ (mm Hg)	pH	Core Temperature (°C)
P20 control (n = 13)	456 ± 14	53 ± 2	7.34 ± 0.02	35.7 ± 0.1
P26 control (n = 5)	508 ± 4	54 ± 2	7.32 ± 0.01	36.2 ± 0.1
P34 control (n = 5)	515 ± 11	52 ± 2	7.34 ± 0.01	36.2 ± 0.2
P20 40/15 (n = 13)	487 ± 11	55 ± 1	7.33 ± 0.01	36.1 ± 0.1*
P20 50/10 (n = 7)	469 ± 12	54 ± 1	7.31 ± 0.08	36.0 ± 0.1
P26 50/10 (n = 6)	452 ± 16*	55 ± 1	7.33 ± 0.02	36.2 ± 0.1
P34 50/10 (n = 6)	526 ± 9	50 ± 1	7.36 ± 0.01	35.8 ± 0.2

* $P < 0.05$, compared with age-matched control.
Data are the mean ± SEM.

TABLE 3. Summary of Systemic Physiology during 100% Oxygen Breathing

Group	PaO_2 (mm Hg)	$PaCO_2$ (mm Hg)	pH	Core Temperature (°C)
P34 Control ($n = 8$)	549 ± 20	38 ± 1	7.44 ± 0.01	36.4 ± 0.2
P34 50/10 ($n = 9$)	537 ± 8	39 ± 2	7.42 ± 0.01	35.8 ± 0.1*

* $P < 0.05$, compared with P34 control
Data are the mean ± SEM.

control. However, no significant ($P > 0.05$) differences were found in retinal ΔPo_2 during a carbogen challenge between 50/10 rats at P20, P26, and P34. In addition, at P34 in control rats, as expected, ΔPo_2 was 61% greater ($P < 0.05$) during carbogen breathing than during 100% oxygen breathing (Fig. 2). However, in P34 50/10 rats, there was no significant difference ($P > 0.05$) between carbogen and 100% oxygen-inhalation challenges (Fig. 2).

DISCUSSION

In this study, we tested the specificity of a subnormal retinal ΔPo_2 during a carbogen inhalation challenge for risk of NV in two cases in which retinal NV either did not develop or had resolved. In our previous studies, we had found that a subnormal retinal ΔPo_2 during carbogen inhalation was a sensitive marker of NV, because a subnormal retinal ΔPo_2 was found before (P14) and during (P20) the appearance of retinal NV in newborn rats that had been exposed to a 50/10 varied oxygen environment.[7,8] Our hypothesis predicted that the retinal ΔPo_2 during a carbogen challenge in animals without retinal NV would be normal. In the first case, a mild varied oxygen regimen (40/15) was examined because it had been reported that this procedure produced minimal incidence and severity of NV.[13] We confirmed a minimal risk of NV in animals exposed to a 40/15 condition (Table 1) and found a normal ΔPo_2 in retinas that did not have NV (Fig. 1). The question of whether retinas with NV in the 40/15 group had a subnormal panretinal ΔPo_2 was not examined in this study, because so few of the 40/15 animals exhibited development of retinal NV (incidence 8%), and the functional MRI examination was performed before we knew which eyes had NV. Nonetheless, these considerations strongly support our hypothesis that a subnormal retinal ΔPo_2 during carbogen breathing is an important event associated with the development of retinal NV.

We also examined the specificity of retinal ΔPo_2 after the regression of NV in animals with a history of varied oxygen exposure (50/10). In this study, a high incidence of NV (99%) at P20 associated with the 50/10 conditions was followed by a progressive reduction in NV after P20. We expected a normal retinal ΔPo_2 during carbogen breathing in those animals that no longer had retinal NV. Somewhat surprisingly, in the 50/10 group, retinas that had had NV but were no longer at risk of NV (P26 and P34) demonstrated a subnormal retinal oxygenation response to a carbogen inhalation challenge (Fig. 1). It seems unlikely that retinal NV would appear later than P34 in 50/10 rats.[4] To interpret the functional MRI data properly in relation to NV risk, it appears to be important to establish whether the retina has already demonstrated NV. Work is ongoing to determine whether, in experimental ROP, subnormal retinal ΔPo_2 after NV regression is a marker for future retinal complications.[5,17]

The underlying cause of the subnormal panretinal oxygenation response to carbogen breathing in the 50/10 group is not known. Systemic differences between the experimental and control groups do not appear to be responsible, because no significant differences in arterial blood gases during a carbogen

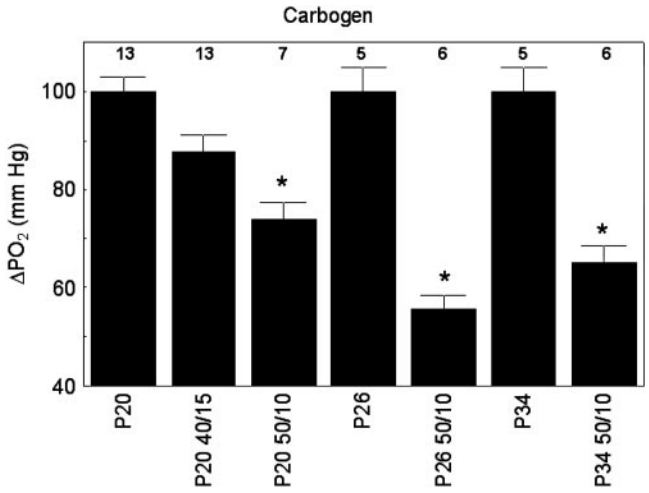

FIGURE 1. Panretinal ΔPo_2 (mean ± SEM) during a carbogen-inhalation challenge in control (P20, P26, and P34), 40/15 (P20), and 50/10 (P20, P26, and P34) groups. *$P < 0.05$, compared with age-matched control rats. The number of animals used to generate these data are shown *above* each bar. Data are normalized to the mean ΔPo_2 of the respective age-matched control group.

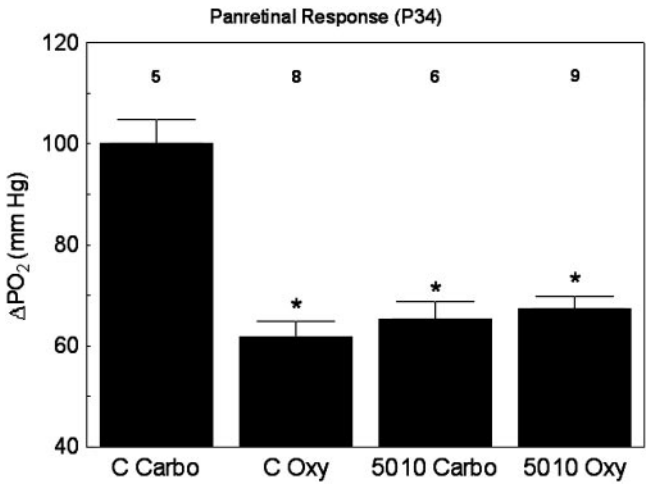

FIGURE 2. Comparison of panretinal ΔPo_2 (mean ± SEM) during carbogen and 100% oxygen inhalation challenges at P34 in control rats and 50/10 rats. *$P < 0.05$, compared with control rats breathing carbogen. To aid the comparison, the P34 control and 50/10 data presented in Figure 1 are reproduced in this figure. Control rats demonstrated a 61% greater ($P < 0.05$) retinal ΔPo_2 during a carbogen-inhalation challenge than during 100% oxygen breathing. However, in the P34 50/10 rats, there was no significant difference ($P > 0.05$) between carbogen and 100% oxygen-inhalation challenges. The number of animals used to generate these data are shown *above* each bar. The panretinal oxygenation responses are normalized to the mean ΔPo_2 of the P34 control group.

challenge were found (Table 2). The retinal oxygenation response primarily reflects changes in oxygen supply during the inhalation challenge, because the Pa_{O_2} increases approximately 400% (from room air levels of 100 mm Hg to approximately 500 mm Hg during the challenge), and this is much greater than the change in retinal oxygen consumption. ΔP_{O_2} is expected to be sensitive to a variety of vascular physiologic processes governing retinal oxygen supply during the carbogen challenge, such as vessel autoregulation. As expected, in P34 control rats, a 61% greater ($P < 0.05$) panretinal ΔP_{O_2} was measured during carbogen breathing compared with that during 100% oxygen inhalation (Fig. 2). In contrast, in the P34 50/10 animals, no significant difference ($P > 0.05$) in retinal ΔP_{O_2} during carbogen and 100% oxygen was found (Fig. 2). In addition, we did not find any difference in panretinal ΔP_{O_2} during 100% oxygen breathing in control and 50/10 rats at P34. The similar ΔP_{O_2} found during the two inhalation challenges supports the notion that at least one of the defects in the 50/10 animals after regression of the NV is an inability to respond adequately to a carbogen challenge (i.e., a functional vasospasm). Studies are ongoing to determine whether treatments that correct the autoregulatory defect, also normalize retinal ΔP_{O_2} and prevent the development of NV.

Acknowledgments

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