Significant Reduction of the Panretinal Oxygenation Response after 28% Supplemental Oxygen Recovery in Experimental ROP

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PURPOSE. To test the hypothesis that after supplemental oxygen recovery (SOR) in the newborn rat model of retinopathy of prematurity (ROP) the preretal neovascular (NV) incidence and severity are decreased and the panretinal oxygenation ability is improved.

METHODS. Newborn rats were first raised in either room air (controls) or variable oxygen (50%/10%) for 14 days. The experimental rats were recovered during the next 6 days (until day 20) in either room air (21% O2) or supplemental oxygen (28%). All groups were then exposed to room air for an additional 6 days (until day 26). On day 20, magnetic resonance imaging (MRI) was used to determine the panretinal oxygenation response (ΔPO2, mm Hg) to a carbogen (95% O2/5% CO2) inhalation challenge. On days 20 and 26, the retinas from a different subset of control, room air–recovered, or SOR-recovered animals were analyzed using ADPase stained or fluorescein-labeled dextran infused retinal flatmounts.

RESULTS. On day 20, the panretinal ΔPO2 of the room air–recovered group (125 ± 5 mm Hg, mean ± SEM, n = 12) was significantly (P < 0.05) lower than that of the control group (179 ± 6 mm Hg, n = 11). The panretinal ΔPO2 value for the SOR group (87 ± 5 mm Hg, n = 7) was significantly (P < 0.05) lower than both the room air–recovered group and the control group. The NV incidence and severity were significantly reduced (P < 0.05) in the SOR animals compared with the room air–recovered animals. In contrast, on day 26 (after 6 days in room air), the NV incidence was statistically (P < 0.05) greater in the animals that had been exposed to SOR compared with room air–recovered animals.

CONCLUSIONS. After 28% SOR, the expected decrease in NV incidence and severity occurred but with an unexpected decrease in panretinal oxygenation ability. The present data strongly support an association between subnormal panretinal oxygenation ability and increased NV risk in the newborn rat ROP model. MRI appears to be a powerful new approach for quantitatively and noninvasively measuring retinal oxygenation and may be applicable to study other ischemic or ischemia-related retinopathies in addition to ROP, such as diabetic retinopathy, sickle cell retinopathy, macular degeneration, and glaucoma. (Invest Ophthalmol Vis Sci. 2000;41:1925–1931)

Experimental studies of retinopathy of prematurity (ROP) have demonstrated that supplemental oxygen recovery (SOR) reduces the risk of developing abnormal new preretal blood vessels (neovascularization, NV).1,2 These results helped to motivate the current National Eye Institute-sponsored clinical trial (STOP-ROP) to test the efficacy, safety, and costs of providing supplemental oxygen in moderately severe ROP (prethreshold ROP; http://www.nei.nih.gov). It is commonly thought that early changes in retinal oxygenation are strongly associated with the subsequent appearance of NV.3,4 Current techniques have not been available to measure retinal oxygenation in newborns. Thus, there is a gap in our understanding of the consequences of SOR on retinal oxygenation and its association with NV incidence and severity.

The majority of experimental efforts to study the effect of supplemental oxygen on the NV outcome involved the kitten ROP model.1,2 In this model, kittens are placed in a relatively high oxygen environment (>70%) for 4 to 5 days. This process produces extensive panretinal vessel obliteration. The animals were then allowed to recover in either room air (21% O2) or supplemental oxygen. In contrast, present-day premature infants are not exposed to constantly high oxygen levels. Instead, they frequently experience relatively smaller fluctuations above and below systemic normoxia.5 Recently, a newborn rat model of ROP has been developed with similarities to the clinical conditions.6–8 In this model, newborn rats are exposed to variable oxygen for the first 14 days and allowed to recover in room air for the next 6 days.6–8 This procedure produces NV in 100% of the eyes by day 20.6–8 The location of the NV at the border of the vascular and avascular retina and its morphology resembles that found in human ROP.6–8

Previously, we demonstrated a novel magnetic resonance imaging (MRI) method that noninvasively investigates retinal oxygenation in newborn rats. We found agreement between

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the MRI retinal oxygenation measurement and that determined using an oxygen electrode in the normal rat retina under similar conditions. The MRI method measures the panretinal oxygenation response (ΔPO2, in mm Hg) produced during a carbogen (95% O2/5% CO2) inhalation challenge. Normally, carbogen breathing induces a relatively larger oxygenation response from the retinal circulation, compared with that produced during 100% oxygen breathing. It is thought that this larger oxygenation response is produced by minimizing the hyperoxia-induced vasoconstriction/autoregulation. However, in the vascular retina, if perfusion or perfusion reserve is low and/or retinal autoregulation dysfunctional, then a smaller than normal ΔPO2 will be produced during the carbogen challenge (see below). Previously, we found a subnormal panretinal oxygenation response to carbogen breathing before the development of NV in the newborn rat model of ROP. In the present study, we used the newborn ROP model to examine whether or not the panretinal oxygenation response is also subnormal during the appearance of NV in rats recovered from the variable oxygen procedure in either room air or 28% supplemental oxygen. The long-term objective of this research is to better understand the role of retinal oxygenation in the development of intraretinal and preretinal NV so that more effective diagnostic, treatment, and prevention strategies may be developed.

**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. Briefly, Sprague-Dawley mothers and litters (12–15 pups/litter) were housed in a modified pediatric incubator where the oxygen levels were varied every 24 hours between 50% and 10% oxygen for the first 14 days after birth. Note that the dams received were not always “proven breeders” and did not always produce enough milk for the pups; litters less than 12 pups were not used in this study. Rats were then allowed to recover in either room air (21% O2) or supplemental oxygen (28%) during the next 6 days (days 14–20). An additional subset of animals from each group was exposed to room air for the next 6 days. The different subsets of animals were examined either histologically on days 20 and 26 or by MRI on day 20 (during the appearance of NV). Animals studied by MRI were not studied histologically.

**MRI Examination**

The MRI procedure has been described in detail elsewhere. Briefly, on the day of the examination, urethane-anesthetized animals (0.083 ml of a 36% solution of urethane/20 g animal weight, intraperitoneal, freshly made daily; Aldrich, Milwaukee, WI) were gently positioned on a homemade MRI-compatible holder with their noses placed in a plastic nose cone and allowed to breathe spontaneously. Core temperature and pulse and hemoglobin oxygen saturation (data not shown) were continuously monitored and maintained. MRI data were acquired on a 4.7-T system using a two-turn surface coil (1.5-cm diameter) placed over the eye and a spin-echo imaging sequence (repetition time, TR, 1 second; echo time, TE, 22.7 msec; number of acquisitions NA 1; matrix size 128 × 256; slice thickness 1 mm; field of view 28 × 28 mm; sweep width 25,000 Hz; 2 min/image). The 2-minute image acquisition time provides a compromise condition between the conflicting demands of achieving high signal-to-noise and adequate resolution to observe oxygen filling of approximately half the vitreous in the newborn rat eye. A capillary tube (1.5-mm inner diameter) filled with distilled water was used as the external standard. Seven sequential 2-minute images were acquired as follows: six control images while the animal breathed room air and one image during carbogen breathing. The animals were switched over to carbogen at the same phase encode step near the end of the acquisition of the sixth image. This procedure was standardized for every animal. If some accident prevented this timing, the experiment was aborted and restarted again after a 10-minute “reset” time. Animals were returned to room air for 15 minutes (to allow recovery from the inhalation challenge) and removed from the magnet. While maintaining the core body temperature, blood from the abdominal aorta was collected immediately after a second 2-minute carbogen challenge and analyzed for PaO2, PaCO2, pH, and glucose concentration. After the blood collection, the animal was euthanized with an intracardiac potassium chloride injection.

Attempts to measure preretinal vitreous PO2 by subtracting images obtained during room air breathing and death (or hypoxemia) were not successful (data not shown). The major problem was that ocular perfusion pressure and eye shape changed from normal during death or hypoxemia. These changes significantly confounded quantitative pixel-by-pixel interpretation of the data. Furthermore, producing death or hypoxemia in humans is clearly difficult. Thus, we chose carbogen breathing because this method induces a maximum oxygenation response from the retinal circulation (compared with oxygen alone), makes detection of subtle differences between groups more robust and precise, and has clinical potential.

**Data Analysis**

To be included in the MRI part of this study, the animal must have demonstrated minimal movement (eye and/or head) during the MRI examination, nongasping respiratory pattern before the MRI examination (i.e., no repeated and visible difficulties in breathing enough to move the head), core temperatures in the range of 36.5°C to 38.5°C during the MRI examination, and PaO2 > 350 mm Hg and PaCO2 ranging from 45 to 65 mm Hg during the carbogen challenge. The number of animals examined by MRI on day 20 that satisfied the inclusion criterion for the room air control, room air-recovered, and SOR groups were 11, 12, and 7, respectively. Forty percent of the animals studied by MRI were not included in the final analysis because, primarily, they did not satisfy condition 4 above. This was because of poor maintenance of the animals’ core temperature during blood collection. Improvements in core temperature maintenance has brought the rejection rate to 5% to 10%.

The MRI data were studied by first converting, on a pixel-by-pixel basis, signal intensity changes during carbogen breathing to oxygenation response values. All pixels along a 1-pixel-thick line (200 μm), drawn at the boundary of retina/choroid and vitreous from the superior ora at top, through the optic nerve, to the inferior ora at the bottom (identified by the clear
contrast differences between the preretinal vitreous, retina/choroid, and ciliary body/iris), were set to black. Next, a different 1-pixel-thick line was drawn in the preretinal vitreous space (immediately adjacent to the black pixels), and 54 pixels along this region of interest were extracted into a preretinal vitreous oxygenation response band. Each pixel (i.e., color band) is the median oxygenation response (volume averaged over a 1-mm section of preretinal vitreous in the nasotemporal direction) from across all retinas in that group at that distance from the optic nerve. Because these data were sampled from similar preretinal vitreous volumes, the potentially confounding effect of preretinal oxygen gradients on the retinal oxygenation measurement is minimized. Calculations suggest that oxygen diffusing from the hyaloidal circulation during a 2-minute carbogen challenge could confound interpretation of the regions within 0.5 mm from the optic nerve. Consequently, we did not analyze regions ≥0.5 mm from the optic nerve. To illustrate this, these regions were blanked out in the preretinal vitreous oxygenation response bands. No statistical evidence (P > 0.05) for an asymmetrical hemiretinal (i.e., superior to the optic nerve versus inferior to the optic nerve) oxygenation response was found in any group. Therefore, the superior and inferior hemiretinal values for each pixel equidistance from the optic nerve were averaged. The average values were used as the set of observations for each animal for further comparisons.

The hemiretinal averaged oxygenation responses for a superior-inferior pixel (excluding those within 5 mm of the optic nerve) for each group were not normally distributed and were compared using a Mann–Whitney rank sum (after log transformation) test, a Kolmogorov–Smirnov two-sample test, and a Kruskal–Wallis multiple comparison test. P < 0.05 was considered significant. To illustrate variations in the oxygenation response between animals within the same group, the averaged hemiretinal data are presented as a scattergram (Fig. 1). To illustrate the differences in the shape of the distribution of oxygenation responses between the groups, the data are also presented as a histogram (Fig. 1). To illustrate the spatial variations of the retinal oxygenation responses for each group, a median oxygenation response band for each group was constructed on a pixel-by-pixel basis from the individual oxygenation response bands for each animal in that group (Fig. 2). To illustrate the relationship between the MRI data (which represents a measure of retinal perfusion, see below) and retinal vessel patency, the composite median oxygenation response band for a group was superimposed on a representative fluorescein-labeled dextran-infused retinal flatmount for that group (Fig. 2).

**Histologic Analysis**

Separate experiments were performed, using animals not studied by MRI, to determine the peripheral retinal avascularity, incidence and severity of the NV, and vessel patency in the control, room air-, or supplemental oxygen-recovered groups on day 20 or, after further room air exposure, on day 26. The peripheral avascularity and incidence and severity of retinal NV were determined as previously described from the ADPase-stained flatmounts. To determine the extent of peripheral avascularity, the image of an ADPase-stained flatmount was captured by a CCD camera and analyzed using the program IMAGE (a freeware program available at http://rsb.info.nih.gov/nih-image/). As seen in Table 1, a smaller subset of flatmounts than that for NV incidence and severity was analyzed for peripheral retinal avascularity in the day 20 animals. We determined the NV incidence and severity soon after preparing the flatmount; however, the peripheral avascularity was measured at a much later time, and the ADPase stain faded to the point that not all the retinas could be analyzed. A larger subset of flatmounts was analyzed for peripheral retinal avascularity than for NV incidence and severity in the day 26 animals. These retinas were not used for NV incidence and severity determination because of damage to one or two retinal quadrants during flatmounting but were analyzed for peripheral avascularity in the remaining undamaged quadrants. To determine NV severity, three investigators independently scored each ADPase-stained retinal flatmount in terms of clock-hours of NV in a masked fashion. The median number of clock-hours retina of 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 0–12) occupied by abnormal vessel growth was determined. To compare the severity and avascularity, a two-sample Mann–Whitney rank sum test (two-sided) was used. To determine physiological patency of the retinal vessel, fluorescein-labeled dextran-infused retinal flatmounts were obtained from a subset of these animals using previously described methods.

**RESULTS**

**Histology Analysis**

A summary of the peripheral avascularity incidence and severity and preretinal NV incidence and severity on days 20 and 26 is presented in Table 1. As expected, control animals did not have peripheral retinal avascularity or NV at either time point; these were statistically different (P < 0.05) from their respective parameters in the two experimental groups. Both experimental groups had a similar degree of peripheral avascular retina on day 20 (P > 0.05) but not on day 26 (P < 0.05). Statistical differences (P < 0.05) in peripheral avascularity on days 20 and 26 within the experimental groups were also found. The NV incidence and severity of the room air-recovered rats were significantly different (P < 0.05) from that of the animals recovered in supplemental oxygen on day 20. Furthermore, on day 26, the NV incidence of the room air-recovered animals was statistically different (P < 0.05) from that of the animals recovered in SOR; no differences in severity between those two groups was found (P > 0.05). Statistical differences (P < 0.05) in NV incidence and severity between the experimental groups on days 20 and 26 were also found. Representative fluorescein-labeled dextran-infused retinal flatmounts for the control, room air-recovered and supplemental oxygen-recovered animals on day 20 are presented in Figure 2. These data demonstrated physiological vessel patency panretinally in all groups.

**Systemic Physiology**

A summary of the blood parameters measured during a 2-minute carbogen challenge is present in Table 2. Although there were some differences between the groups, all the blood gas values fell within the expected range for carbogen breathing.
Figure 1 represents the group scattergrams, histograms, and descriptive statistics for the MRI retinal oxygenation response data for the age-matched control, room air–recovered, and supplemental oxygen–recovered groups on day 20. Figure 2 illustrates the preretinal oxygenation response bands for all three groups. Compared with the controls, the panretinal oxygenation response was approximately 30% lower (P = 0.0003, Kruskal–Wallis multiple comparison test) in the room air–recovered group and 50% lower in the supplemental oxygen–recovered group (P = 0.0003, Kruskal–Wallis multiple comparison test). Compared with the room air–recovered group, the panretinal ΔPo2 was approximately 30% lower in the supplemental oxygen–recovered group (P = 0.0003, Kruskal–Wallis multiple comparison test).

**DISCUSSION**

The underlying cause of the subnormal panretinal oxygenation response in the two experimental ROP groups in this study is not known. Observation of the superficial and deep retinal circulation under high magnification does not appear to be able to explain the MRI data because the retinas in this study were >90% vascularized with physiologically patent vessels (Fig. 2). We reasoned that the subnormal panretinal oxygenation may be due to dysfunction of the retinal vascular system but not to perturbations of retinal oxygen consumption, based on the following argument. The Po2 of the preretinal vitreous during room air breathing is a measure of the amount of oxygen supplied to the retina minus the amount consumed. During the carbogen challenge the amount of oxygen supplied...
to the retina increases approximately 400% (the retinal arterial oxygen levels change from 100 mm Hg to approximately 500 mm Hg during the challenge). This increase is likely much greater than the change in retinal oxygen consumption. Thus, $\Delta P_{O_2}$ is expected to reflect primarily the change in retinal oxygen supply and be sensitive to a variety of vascular physiological processes governing retinal oxygen supply during the carbogen challenge, such as retinal perfusion, perfusion reserve, and vessel autoregulation. Thus, we speculate that the variable oxygen exposure produced damage to some combination of retinal perfusion, perfusion reserve, or autoregulation. The SOR procedure used in this study appears to further damage the ability of retinal circulation to oxygenate. Experiments are ongoing in this laboratory to further examine these possibilities.

It is possible that the MRI retinal oxygenation response differences between the groups in this study were due to systemic physiological differences in the response to carbogen rather than to local retinal effects. To address this concern, various physiological parameters were measured and compared for each group. All the values measured fell within the expected range for carbogen breathing. Only the arterial blood oxygen tensions and glucose levels were significantly higher ($P < 0.05$) between the SOR group and each of the other 2 groups. Could the higher arterial oxygen tensions in the SOR group account for its relatively lower oxygenation response? This is considered unlikely because during carbogen breathing the hyperoxia occurs with hypercapnia. Because the arterial carbon dioxide levels are elevated to the same degree in all groups, and because hypercapnia-related vasodilation occurs even during hyperoxia, we do not expect that differences in arterial oxygenation of this magnitude between groups to account for the differences in retinal oxygenation response. Similarly, the higher blood glucose level seen in the
The present study supports the results of previous supplemental oxygen studies in the kitten. In addition, our finding of a similar NV severity at day 26 for animals exposed to either supplemental oxygen or room air (1 clock-hour) also appears to agree with the work of Chan–Ling et al. These authors report no differences in “vascular pathology” in kittens exposed to either room air or 50% oxygen for 8 days after stopping supplemental oxygen treatment. Unfortunately, there are no reports in the kitten literature on the effect of supplemental oxygen on NV incidence either immediately after supplemental oxygen or after some additional period in room air. Thus, our finding of a greater NV incidence 6 days after stopping supplemental oxygen, compared with the room air-recovered animals, is novel.

The exact mechanism underlying the reduction in NV incidence and severity on day 20 after SOR is not known. SOR is expected to elevate the retinal PO2, and this is thought to relieve the presumed retinal hypoxia that is hypothesized to play a key role in the development of NV in ROP. The reduction in NV incidence and severity in the SOR group on day 20, relative to the room air-recovered group, is consistent with the concept that elevated tissue oxygen levels relieve, to some extent, the presumed hypoxia. The relative increase in NV incidence in the SOR group on day 26, compared with room air-recovered animals may be due to the following. The retinal demand for oxygen is likely increasing due to continuing mat-

### Table 1. Summary of Histologic Analysis

<table>
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<tr>
<th></th>
<th>Day 20</th>
<th>Day 26</th>
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<tbody>
<tr>
<td></td>
<td>Room Air Control</td>
<td>Room Air-Recovered between Days 14 and 20</td>
</tr>
<tr>
<td>Avascular</td>
<td>0% (0/50)</td>
<td>79%* (77/98)</td>
</tr>
<tr>
<td>Avascularity,*</td>
<td>—</td>
<td>8.4 ± 0.8</td>
</tr>
<tr>
<td>NV incidence, %</td>
<td>0% (0/50)</td>
<td>100%* (112/112)</td>
</tr>
<tr>
<td>Severity† (clock-hour), median, range</td>
<td>—</td>
<td>6 (1–12)</td>
</tr>
</tbody>
</table>

Unless otherwise noted, data are presented as mean ± SEM.

* Determined only from retinas with avascular peripheral retina.
† Determined only from retinas with some degree of NV.
** P < 0.05, vs the age-matched control room air group.
*** P < 0.05, vs the age-matched room air-recovered group.
**** P < 0.05, vs the day 20 data for that group.

### Table 2. Summary of Blood Parameters (Mean ± SEM) Measured during a 2-Minute Carbogen Challenge

<table>
<thead>
<tr>
<th></th>
<th>Day 20</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Controls (n = 11)</td>
</tr>
<tr>
<td>Arterial Blood Parameters</td>
<td></td>
</tr>
<tr>
<td>PaO2, mm Hg</td>
<td>483 ± 8</td>
</tr>
<tr>
<td>PaCO2, mm Hg</td>
<td>56 ± 1</td>
</tr>
<tr>
<td>pH</td>
<td>7.27 ± 0.01</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>232 ± 6</td>
</tr>
</tbody>
</table>

* P < 0.05, vs age-matched control group.

** P < 0.05, vs room air-recovered group.

† Elevated due to the urethane anesthetic.
uration between days 20 and 26 in both the SOR and room air–recovered groups. However, because the oxygenation ability of the retinal circulation in the SOR group appears more impaired than in the room air–recovered group, a mismatch in oxygen supply and demand is likely to be relatively more severe in the SOR animals. This might lead to the relatively longer continuation of NV in a greater number of animals in the SOR group. The low oxygenation response observed in the present study is also consistent with the presence of hypoxia, but it cannot yet be unambiguously interpreted as a measure of hypoxia. Thus, the data in this work only indirectly provide evidence that the retina is hypoxic. Experiments in this laboratory are ongoing to directly measure retinal oxygen levels after SOR in experimental ROP.

In the present study, both room air-recovered and SOR groups had subnormal oxygenation responses during the appearance of NV. This result complements and extends our previous findings of a subnormal panretinal oxygenation response before the appearance of NV in this model. One weakness of the present study is that only 89% of the SOR animals developed NV on day 20. It is possible that some animals without NV were studied by MRI and may have skewed that group’s medians. However, retinas from animals without NV were studied by MRI and may have skewed that group’s medians. However, retinas from animals without NV are expected to have a relatively greater panretinal oxygenation response than retinas with NV. This would decrease, not increase, the differences in retinal oxygenation between groups. In addition, the relatively smaller panretinal oxygenation response in the SOR groups was associated with a relatively greater NV incidence on day 26, compared with the room air–recovered animals. Taken together, these data underscore our previous hypothesis that a subnormal panretinal oxygenation response is strongly associated with an increased risk of retinopathy in experimental ROP.

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References