Effects of Sustained Hyperoxia on Revascularization in Experimental Retinopathy of Prematurity

Xiaolin Gu,1,2 Sara Samuel,3 Mohamed El-shabrawey,4 Ruth B. Caldwell,1,2,3 Manuela Bartoli,1,2 Dennis M. Marcus,5 and Steven E. Brooks2,5,5

PURPOSE. To investigate the effects of prolonged hyperoxia on vascular recovery and glia survival after experimentally induced retinopathy of prematurity (ROP) in the mouse.

METHODS. The effects of hyperoxia on revascularization and vitreous neovascularization were compared between mice raised in 75% oxygen from postnatal day (P)7 to P12, followed by room air recovery and mice raised in 75% oxygen from P7 to P27. The status of astrocytes and Müller cells was evaluated by glial fibrillary acidic protein (GFAP) immunohistochemistry on retinal wholemounts and serial sections. A window of susceptibility to oxygen-induced vaso-obliteration was defined by comparing the extent of retinal vaso-obliteration resulting from 2 days of hyperoxia beginning on P7, P9, P11, P13, or P15.

RESULTS. Oxygen-induced vaso-obliteration of retinal capillaries was limited to the period between birth and P15. Paradoxically, revascularization was markedly accelerated and neovascularization markedly reduced in mice maintained in prolonged hyperoxia (P7–P27) compared with mice recovering in room air. The extended use of 75% oxygen during the recovery period was associated with preservation of astrocytes and Müller cells in the avascular retina.

CONCLUSIONS. The antiangiogenic effect of hyperoxia on retinal capillaries is strongly dependent on postnatal age. A protocol of continuous 75% supplemental oxygen accelerates recovery of inner retinal vasculature and prevents vitreous neovascularization, by a mechanism that may involve preservation of inner retinal glia. (Invest Ophthalmol Vis Sci. 2002;43:496–502)

Neovascularization of the vitreous is a major cause of visual morbidity. It occurs in a number of conditions characterized by compromise of the inner retinal circulation, including diabetic retinopathy, retinopathy of prematurity (ROP), and retinal vein occlusion. It is a sight-threatening aberration in the angiogenic repair process that occurs in response to retinal ischemia. The factors that control and compartmentalize the process of retinal revascularization are therefore of major clinical importance.

From the 1Department of Cellular Biology and Anatomy, the 2Vascular Biology Center, and the Departments of 3Ophthalmology and 4Pediatrics, Medical College of Georgia, Augusta, Georgia; and 5Faculty of Medicine, El-Mansoura University, El-Mansoura, Egypt.

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Corresponding author: Steven Brooks, Eye Consultants of Augusta, 503 Furys Ferry Road, Martinez, GA 30907; sbrooks@gabn.net.

Prior investigations have suggested that pathologic alterations in the inner limiting membrane and associated glia may be involved in promoting vitreous neovascularization.1–2 Increased expression of vascular endothelial growth factor (VEGF)3–5 and decreased expression of pigment epithelium-derived factor (PEDF) in the ischemic retina6 may also be involved, leading to overstimulation of angiogenesis.

Experimental work in animal models has demonstrated that supplemental oxygen can attenuate the severity of vitreous neovascularization in ischemic retinopathy,7–9 decrease the overexpression of VEGF,3,4 increase the expression of PEDF,6 and reduce degeneration of retinal astrocytes.8,9 However, a recently completed clinical trial in human infants with prethreshold ROP failed to demonstrate a statistically significant reduction in the rate of progression to threshold ROP in infants given supplemental oxygen.10 These apparently conflicting data led us to hypothesize that the timing of oxygen exposure may be a critical determinant of its effects on angiogenesis in the retina.

Using a well-established mouse model of ROP, we demonstrated a defined window of time in the early perinatal period during which breathing 75% oxygen profoundly inhibited angiogenesis and caused obliteration of retinal capillaries. Paradoxically however, in mice with continued exposure to 75% oxygen, accelerated recovery of previously damaged capillary beds and essentially complete inhibition of vitreous neovascularization were noted, compared with mice recovering in room air. The effects of hyperoxia on vascular recovery were associated with preservation of glia in the ischemic areas of the retina, suggesting their possible role in this process.

MATERIALS AND METHODS

Animals and Materials

All experimental procedures were approved by the institutional Committee for the Use of Animals in Research and Education and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. C57Bl/6 mice were purchased from a commercial vendor (Harlan Sprague-Dawley, Indianapolis, IN). Biotinylated Griffonia simplicifolia lectin B4 and Texas red–conjugated avidin D were obtained from Vector Laboratories (Burlingame, CA). Mice were housed in cages in either room air or in a custom-built chamber in which the partial pressure of oxygen was maintained at 75% ± 2% and checked twice daily with an oxygen monitor.

Mouse Model of Proliferative Retinopathy and Protocols for Supplemental Oxygen

Oxygen-induced retinopathy was induced in newborn mice according to the protocol of Smith et al.11 On P7, newborn mice were placed along with their dams into 75% oxygen for up to 5 days (postnatal day [P]12), after which they were transferred back to cages in room air. Room temperature was maintained at 20°C, and illumination was provided by standard fluorescent lighting on a 12-hour light–dark cycle. Pups were nursed by their dams and given food (standard mouse chow) and water ad libitum.
In the mice receiving supplemental oxygen, the newborn mice and dams were kept in constant 75% oxygen from P7 to P27, with no periods of normoxia. Pups were removed at P17, P22, and P27 for analysis of retinal vascularization, vitreous neovascularization, and GFAP immunohistochemistry. One group of mice (n = 5) was returned to room air on P28 and their retinas analyzed for evidence of neovascularization on P32.

To determine the effect of postnatal age on the ability of 75% oxygen to induce vaso-obliteration, groups of mice (n = 5–6) were exposed to 2 days of constant 75% oxygen, beginning on P7, P9, P11, P13, or P15. At the end of the 2-day period of hyperoxia, the mice were killed by cervical dislocation, and their retinas analyzed for vaso-obliteration, as described later.

**Visualization of Retina Vasculature and Quantitation of Vaso-obliteration**

Vaso-obliteration and retinal vascular pattern were analyzed using retinal flatmounts labeled with biotinylated *G. simplicifolia* lectin B4 and Texas red–conjugated avidin D, as previously described. Retinae were viewed with fluorescence microscopy (Axiophot; Carl Zeiss, Chester, VA) and the images captured in digital format (Spot System; Diagnostic Instruments, Sterling Heights, MI). The central capillary dropout area was quantified from the digital images in masked fashion, using an imaging system (ILab Spectrum Scientific Image System; Signal Analytics, Vienna, VA).

**Quantitation of Vitreous Neovascularization**

Quantitation of vitreous neovascularization on P17 was performed using a modification of a technique described by Smith et al. Briefly, 10-μm-thick serial sections, each separated by at least 40 μm, were taken from around the region of the optic nerve. The hematoxylin and cosin-stained sections were examined in masked fashion for the presence of neovascular tufts projecting into the vitreous from the retina. The neovascular score was defined as the mean number of neovascular tufts per section found in eight sections (four on each side of the optic nerve) per eye.

**GFAP Immunohistochemistry**

Wholemount retinas were permeabilized with phosphate-buffered saline (PBS) containing 1% Triton X-100, and nonspecific antibody binding was blocked with 10% normal goat serum for 30 minutes. Retinae were incubated overnight at 4°C with rabbit polyclonal anti-GFAP antibody (Dako, Carpinteria, CA) diluted 1:100 in PBS containing 0.5% Triton X-100. After washing in PBS, retinas were incubated with Texas red–conjugated donkey anti-rabbit antibody at 1:500 (Molecular Probes, Eugene, OR) for 4 to 6 hours at 4°C. Retinae were washed with PBS, mounted on microscope slides in mounting medium (Vectashield; Vector Laboratories), and examined by fluorescence microscopy (Axiophot; Carl Zeiss).

For GFAP labeling of cryosections, sections were brought to room temperature for 15 minutes and fixed in 4% paraformaldehyde for 6 minutes. After washing with PBS, sections were permeabilized with PBS containing 0.1% Triton X-100 and blocked with 1% bovine serum albumin for 30 minutes. Sections were incubated overnight at 4°C with rabbit polyclonal anti-GFAP antibody (Dako) diluted 1:100 in PBS containing 0.5% Triton X-100. After washing in PBS, sections were incubated with Texas red–conjugated donkey anti-rabbit antibody at 1:500 (Molecular Probes) for 4 to 6 hours at 4°C, washed with PBS, covered in mounting medium (Vectashield; Vector Laboratories) under a coverslip and examined by fluorescence microscopy (Axiophot; Carl Zeiss).

**Results**

**Defining the Window of Oxygen-Sensitive Angiogenesis in the Retina**

The data in Figure 1 illustrate that retinal capillaries were highly sensitive to 75% oxygen during the first 2 weeks of life, demonstrating widespread obliteration after 2 days of continuous exposure. They rapidly acquired tolerance to hyperoxia between P11 and P15 and were essentially insensitive thereafter. As previously reported, oxygen-induced vaso-obliteration spared the peripheral retina vasculature (Fig. 2A), a feature that contrasts sharply with human ROP.

**Effect of Hyperoxia on Retinal Revascularization**

Because of the vaso-oblitative effects of hyperoxia in the immature retina, we predicted that continuous exposure of pups to 75% oxygen would prevent or retard revascularization angiogenesis and result in a large area of persistent central retinal vaso-obliteration (Fig. 2A). On the contrary, revascularization of the central retina occurred at a much faster rate in mice maintained in continuous 75% oxygen than in mice returned to room air on P13 (Figs. 2B–G, and Fig. 3). Mice in prolonged hyperoxia demonstrated essentially complete revascularization of ischemic areas by P22 (Fig. 2F), compared with P27 in mice that recovered in room air (Fig. 2D). In addition, mice recovering in room air demonstrated abnormalities of the retinal vessels during the revascularization process. The central vessels were distorted and tortuous (Figs. 2B, 2C), similar to the features in PLUS disease in severe stages of human ROP.

**Effect of Hyperoxia on Vitreous Neovascularization**

As expected, extensive vitreous neovascularization was noted in mice exposed to 75% oxygen from P7 to P12 and returned to room air (Fig. 4). In contrast, mice kept in 75% oxygen from P7 to P27 showed essentially no neovascularization (Fig. 4, RA17), even when returned to room air from P28 to P32 (data not shown).

**Effect of Hyperoxia on Astrocytes and Müller Glia in the Retina**

In mice raised exclusively in room air, GFAP expression was seen in inner retinal astrocytes both centrally and peripherally (Figs. 5A, 5D and Figs. 6E, 6F), but not in Müller glia (Figs. 5A, 5D and Figs. 6E, 6F).
5D). In mice raised in 75% oxygen from P7 to P12 and then returned to room air, there was a profound loss of astrocytes from the central retina (Fig. 6A). In the peripheral retina, where capillaries remained intact, GFAP-positive astrocytes were preserved (Fig. 6B). In contrast, Müller cells demonstrated strong GFAP expression in the central retina where astrocytes and capillaries were absent (Figs. 5C, 5F), but showed no GFAP expression in the peripheral retina (Fig. 5G). In mice raised in 75% oxygen from P7 to P17, the distribution of glia and their GFAP expression were similar to those in mice raised in room air (Figs. 5B, 5E versus 5A, 5D; and Figs. 6C, 6D versus 6E, 6F), despite the presence of a large zone of central capillary obliteration. Thus, sustained hyperoxia effectively preserved astrocytes in the central retina in spite of extensive damage to the capillary networks and prevented the reactive expression of GFAP in the Müller glia.

Effect of Hyperoxia on Histopathologic Changes in the Retina

Histologic analysis of retinas on P27 demonstrated that mice kept in 75% oxygen showed no major alterations compared with mice raised in room air (Fig. 7). Aside from the loss of capillaries in the central retina, the mice kept in hyperoxia showed excellent preservation of all layers of the retina. In contrast, mice returned from hyperoxia to room air on P13 showed scattered areas of retinal hemorrhage on P17 as well as neovascularization of the vitreous (data not shown), findings similar to those reported by Smith et al.11

DISCUSSION

It is well known that newly formed capillaries in the retina of premature infants and some species of newborn animals are sensitive to oxygen, a feature that predisposes them to ROP. It is not known, however, whether the oxygen sensitivity is a feature of angiogenesis in general or applies only to angiogenesis occurring during a specific period of early retinal development. If on the one hand it were a nonspecific feature of angiogenesis (e.g., by causing suppression of hypoxia-induc-
ible factors), it would be expected that recovery of injured retinal capillaries would be prevented or retarded in pups raised in continuous hyperoxia. If on the other hand the oxygen sensitivity is temporal, then continuous hyperoxia may actually facilitate vascular recovery by helping to relieve hypoxia during the recovery process. Resolving this question is important, not only for understanding and managing ROP but also for understanding how oxygen can be used to manage other ischemic retinopathies as well.

Previous studies have suggested that hyperoxia-induced suppression of VEGF causes endothelial cell apoptosis in nascent capillaries without a pericyte coating and that exogenous administration of VEGF can rescue retinal vessels from oxygen-mediated apoptosis. Although the molecular mechanisms are not well understood, the vaso-obliterative effects of hyperoxia are significantly reduced in mice without functional endothelial nitric oxide synthase (eNOS) or in mice given pharmacologic inhibitors of eNOS. However, our present data indicate that retinal capillary sensitivity to hyperoxia may be a unique feature of early retinal vascular development and not a feature of newly forming capillaries in general.

Our analysis in the mouse indicates that a relatively sharp transition occurs in the retina between P11 and P15, after which hyperoxia causes neither capillary vaso-obliteration nor suppression of angiogenesis. That this transition occurred under both normoxic and hyperoxic conditions suggests that the underlying mechanism is not closely regulated by oxygen tension, unless perhaps there are adaptive changes in key oxygen-sensitive control mechanisms.
Several clinical observations suggest that an analogous transition in oxygen sensitivity also occurs in the human retina. First, the proliferative phase of human ROP is more closely linked to postconceptional than to postnatal age.\textsuperscript{14,15} Data from two large clinical series demonstrate that the onset of neovascularization in ROP generally does not occur before 28 to 30 weeks after conception and peaks at 35 to 37 weeks.\textsuperscript{14,15} These observations suggest a programmed change in vascular physiology in which a change in oxygen sensitivity occurs during the period at 30 to 35 weeks after conception. Finally, established neovascularization in human ROP can persist or progress in spite of supplemental oxygen,\textsuperscript{10} implying that the antiangiogenic effects of oxygen in the human retina may also be temporal.

The mechanism by which capillaries acquire their tolerance to hyperoxia is not entirely clear. Data from Benjamin et al.\textsuperscript{13} indicate that capillary endothelia in the retina lose their sensitivity to oxygen when they acquire a pericyte coating and that VEGF and platelet-derived growth factor (PDGF)-B help mediate pericyte recruitment. Our observation of accelerated revascularization in continuous hyperoxia, a condition known to suppress VEGF expression, raises questions about the role that VEGF plays in the acquisition of oxygen tolerance. It also raises questions about the role that VEGF plays in driving the angio-

**FIGURE 6.** Distribution of inner retinal astrocytes as a function of oxygen-rearing conditions. GFAP-positive cells were visualized by immunohistochemistry in retina flatmounts. Representative areas from (A) central, avascular and (B) peripheral, vascular retina on P17 from a mouse raised in 75% oxygen from P7 to P12 and in room air from P13 to P17. (C) Central, avascular and (D) peripheral, vascular retina on P17 from a mouse raised in 75% oxygen from P7 to P17. (E) Central, vascular and (F) peripheral, vascular retina on P17 from a mouse raised in room air from P7 to P17. Scale square, 10 μm.
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Figure 7. Histology of retina on P27 as a function of oxygen conditions. Ten-micrometer-thick section of retina, stained with hematoxylin and eosin, from (A) an animal raised in room air and (B) an animal raised in 75% oxygen from P7 to P27. Sections demonstrate retina in the midperiphery. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium. Scale bar, 10 μm.

genesis associated with revascularization. Although VEGF expression is also known to be regulated in part by the concentration of glucose,16–20 additional studies specifically designed to evaluate VEGF expression are needed to resolve these questions. Other studies have suggested that type 2 astrocyte-derived fibroblast growth factor may act as a key survival factor for retinal capillaries.1 The increased survival of astrocytes that we observed in mice subjected to prolonged hyperoxia would be consistent with a role for an astrocyte-derived factor in promoting vascular recovery.

In addition to accelerated revascularization, we also observed a dramatic inhibition of vitreous neovascularization by exposure to continuous supplemental 75% oxygen. Although this may have been partly due to decreased expression of hypoxia-sensitive angiogenic growth factors (e.g., VEGF) or to increased expression of antiangiogenic factors (e.g., PEDF), it may also have been related to preservation of glia and an intact inner limiting membrane. That vitreous neovascularization was blocked, whereas retinal revascularization was simultaneously accelerated argues more strongly for a mechanism based on preserved compartmentalization and regulation rather than suppressed angiogenesis. These data corroborate several studies that have drawn attention to the possible role of glia cells in retinal vascular development and vitreous neovascularization.1,2,10,21–24

A role for glia degeneration in promoting vitreous neovascularization is also indirectly supported by data from other investigators who attempted to produce vitreous neovascularization by increasing the levels of VEGF, either by intravitreal injections25 or endogenous overexpression in the retina of genetically modified mice.26 Although the VEGF overexpression promoted the development of intraretinal microvascular abnormalities, the glia in the inner retina were not compromised, and vitreous neovascularization did not occur in those settings.25,26

Because our data on the rate of revascularization appear to contradict the findings of Chan-Ling et al.,8 it is important to mention several key differences between the studies that may help to reconcile the findings. First, it is not clear how the timing of supplemental oxygen in their studies relates to the intrinsic oxygen sensitivity of the retinal vessels in the kitten. This is an important point, because we have shown that the effect of hyperoxia on developing vessels can change dramatically as a function of age. Second, we used a constant level of supplemental oxygen throughout. Chan-Ling et al. used empirically derived regimens of supplemental oxygen in which the oxygen concentrations were frequently changed at 4-day intervals. The effect of these changes and their relationship to the period of oxygen sensitivity in the cat is difficult to determine and makes comparison with our results difficult. Finally, they showed a greater than 20-fold difference (23% vs. 1%) in revascularization between animals maintained in low levels (30% O2) of supplemental oxygen and those given high levels (50% O2), after 4 days, but only a 2-fold difference (83% vs. 42%) after 28 days.8 This finding may be interpreted as showing a relative acceleration in revascularization over time in animals exposed to high levels of supplemental oxygen. Thus, the findings of Chan-Ling et al. do not necessarily contradict our findings.

In conclusion, the effect of hyperoxia on vascular proliferation and remodeling is highly dependent on the developmental state of the retina and thus on the time frame during which it is administered. During the initial formation of retinal capillaries and vessels, hyperoxia causes profound disruption of vessel formation and leads to widespread vaso-obliteration. A critical and presumably permanent transition in oxygen sensitivity subsequently occurs (in normoxic or hyperoxic conditions), after which hyperoxia no longer causes obliteration of capillaries or suppression of angiogenesis. After this transition, the provision of continuous supplemental oxygen actually accelerates the process of retinal revascularization, while simultaneously preventing vitreous neovascularization. Although the mechanism for these effects is not clear, the increased survival of astrocytes and Müller glia in the avascular retinal tissue may be involved. We speculate that the survival of these cells supports revascularization and helps prevent neovascularization by maintaining appropriate tissue compartmentalization. Further studies are needed to explore the potential use of supplemental oxygen in ROP and other ischemic retinopathies.

References


