Reduced Severity of Oxygen-Induced Retinopathy in eNOS-Deficient Mice

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PURPOSE. Exposure of premature human infants to hyperoxia results in the obliteration of developing retina capillaries, leading to a vision-threatening retinopathy termed retinopathy of prematurity (ROP). The authors hypothesized that this process may be mediated in part by endothelial nitric oxide (NO)-derived oxidants such as peroxynitrite and tested this hypothesis in a mouse model of ROP.

METHODS. Normal mice, mice treated with the nitric oxide synthase (NOS) inhibitor Nω-nitro-L-arginine (L-NNA), and knockout mice carrying a homozygous targeted disruption of the gene for endothelial NOS (eNOS) were studied in an experimental model of ROP. Retinas were compared for extent of capillary obliteration in hyperoxia, vascular endothelial growth factor (VEGF) expression, nitrotyrosine formation, and vitreous neovascularization.

RESULTS. Oxygen-induced retinal vaso-obliteration was significantly reduced by L-NNA treatment (45% decrease from controls). The eNOS-deficient mice showed a similar reduction in vaso-obliteration (46% decrease from controls), and vitreous neovascularization was also substantially reduced (threefold decrease). Retinal nitrotyrosine formation, a measure of in situ peroxynitrite modification of proteins, was significantly elevated in normal mice during hyperoxia, in a spatial and temporal pattern consistent with a role in oxygen-induced vaso-obliteration. This was not seen in ENS-deficient mice. VEGF expression was similar in both groups of mice, although suppression in hyperoxia was slightly blunted in eNOS-deficient mice.

CONCLUSIONS. These data suggest a role for NO and peroxynitrite in the pathogenesis of ROP. Therapies aimed at modulation of eNOS activity may have therapeutic potential for preventing ROP. (Invest Ophthal Mol Vis Sci. 2001;42:222–228)

Retinopathy of prematurity (ROP) is a potentially blinding disorder of retinal vascular development affecting premature infants and is a leading cause of blindness in infancy.1 Experimental work in animals and clinical observations in human infants suggest that exposure of the developing retina to relative hyperoxia damages retinal capillaries and impairs vasculogenesis.2 The resulting vascular insufficiency ultimately produces inner retina hypoxia, leading to a poorly controlled process of retinal vascularization and the development of proliferative, vision-threatening retinopathy.

Sensitivity of retinal capillaries to oxygen appears to be limited to the developing retina. Maturation events that occur around the 36th postconceptional week in humans3 and the third postnatal week in mice4 render the vessels resistant to hyperoxia-induced obliteration. A role for oxygen-derived free radicals in mediating hyperoxia-induced vaso-obliteration is supported by studies showing that administration of exogenous antioxidants can attenuate retinopathy in certain animal models4–6 and possibly in humans as well.7 In addition, experimental oxygen-induced retinopathy is associated with the formation of lipid peroxides in the retina.8

Other investigators have proposed that premature downregulation of retinal vascular endothelial growth factor (VEGF) by hyperoxia,9–12 and markedly increased expression of pigment epithelium-derived factor (PEDF)13 are the key events leading to vaso-obliteration of newly formed capillaries. In both rats and mice, intraocular administration of VEGF before hyperoxia significantly reduces capillary obliteration, supporting the hypothesis that VEGF is a critical survival factor for developing capillaries in the retina.9,10

There is increasing evidence, however, that nitric oxide (NO)-derived oxidants such as peroxynitrite may be important in situ mediators of oxidative injury. We therefore considered the possibility that they may have a pathogenic role in oxygen-induced retinopathy in developing retina. Evidence for peroxynitrite-mediated cellular injury has been reported in focal cerebral ischemia,14–17 myocardial ischemia-reperfusion,18 experimental models of retinal ischemia,19–21 and experimental autoimmune uveitis.22 Plasma 3-nitrotyrosine, a stable biochemical marker for peroxynitrite-mediated protein modification, has also been reported to be elevated in premature human infants who develop bronchopulmonary dysplasia23,24 and correlates with exposure to high concentrations of inspired oxygen. There are also considerable experimental data indicating that peroxynitrite-induced protein modifications can impair the function of cellular proteins25,26 and cause cytotoxicity.26–28 In vitro studies in our laboratory have shown that bovine aortic endothelial cells exposed to nonlethal concentrations of 3-morpholinosydnonimine (SIN-1), a donor of peroxynitrite, causes impairment of cell proliferation, migration, and VEGF-induced phosphorylation of the flk-1/KDR receptor.29

To study the putative role of endogenous NO in ROP, we used an established mouse model3 and compared the retinopathy observed in wild-type mice to that found in mice homozygous for a targeted disruption of the eNOS gene30 or mice treated with a potent pharmacologic inhibitor of NOS activity. Our findings indicate that eNOS deficiency, either through gene disruption or pharmacologic inhibition significantly protects the developing retina from oxygen-induced retinopathy.

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by a mechanism that appears to be independent of VEGF expression and pericyte distribution.

METHODS

Animals

These experiments were done using C57Bl/6 (eNOS +/+ ) and endothelial nitric oxide synthase (eNOS)-deficient (eNOS −/− ) mice. The eNOS −/− mice have been described in detail previously. All experiments were approved by the institutional Committee for Animal Use in Research and Education and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Oxygen-Induced Retinopathy

Oxygen-induced retinopathy was induced in newborn mice according to the protocol of Smith et al. On postnatal day 7 (P7) mice were placed along with their dam into a custom-built chamber in which the partial pressure of oxygen was maintained at 75%. Mice were maintained in 75% oxygen for up to 5 days (P12), after which they were transferred back to cages in room air (normoxia). Room temperature was maintained at 68°F, and rooms were illuminated with standard fluorescent lighting on a 12-hour light–dark cycle. Newborn mice were nursed by the dam and given food (standard mouse chow) and water ad libitum.

Pharmacologic Inhibition of NOS

ENOS +/+ mice (n = 7) were given intraperitoneal injections of L-NNA (500 mg/kg) on P7 and P8, immediately before and during exposure to 75% oxygen. Control mice (n = 6) were injected with phosphate-buffered saline (PBS) only on P7 and P8. Control and L-NNA-treated mice were maintained in the same oxygen chamber (75% oxygen) from P7 to P9. Analysis of retinal vaso-obliteration by oxygen on P9 was performed as described below.

Analysis of Vaso-Obliteration

Vaso-obliteration was assessed on P9 in mice exposed to 2 days of hyperoxia (P7–P9). Retinal vascular distribution was analyzed using retinal flat mounts (n = 6–7 retinas from different pups per group) labeled with biotinylated Griffonia simplicifolia lectin B4 (Vector Laboratories, Burlingame, CA) and Texas Red–conjugated Avidin D (Vector Laboratories) as described. Retinas were viewed with fluorescence microscopy (Axiophot; Zeiss, Chester, VA) and the images captured in digital format (Spot System; Diagnostic Instruments, Sterling Heights, MI). Central capillary dropout area was quantified from the digital images, in masked fashion, using the Metamorph Imaging System (Universal Imaging Corporation, West Chester, PA). Two-way analysis of variance (ANOVA) was used to compare the mean values between groups.

Analysis of Neovascularization

Extraretinal neovascularization was assessed on P17 in mice exposed to 5 days of hyperoxia (P7–P12) followed by 5 days in room air (P12–P17). Quantification of neovascularization was performed using a technique described by Smith et al. with minor modification. Briefly, hematoxylin and cosin-stained serial sections, separated by at least 40 μm, were examined in masked fashion for the presence of neovascular buds projecting into the vitreous from the retina. The neovascular score was defined as the mean number of neovascular tufts per section found in 16 sections (eight on each side of the optic nerve) per eye. The mean score from four eyes from four different pups was used for statistical analysis (two-way ANOVA) of group differences.

Measurement of Retinal Nitrotyrosine

To analyze retinal nitrotyrosine, retinas were isolated, pooled, and frozen in liquid nitrogen (n = 14 retinas from 7 mice per group). Pooled retinas were homogenized in lysis buffer (20 mM Tris [pH 7.4], 2.5 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 40 mM NaF, 10 mM Na2P2O7, and 1 mM PMSF). Particular material was removed by centrifugation at 12,000g at 4°C for 30 minutes. Retinal proteins (25 μg) were immobilized onto a PVDF membrane (Bio-Rad, Hercules, CA) using the 40-well BioDot SF microfiltration unit (Bio-Rad). A dilution series of peroxynitrite-modified bovine serum albumin standard was also loaded in duplicate on each blot to generate a standard curve. The PVDF membranes were blocked with 5% dry milk and reacted with mouse monoclonal anti-nitrotyrosine antibody (Caym men, Ann Arbor, MI). The primary antibody was detected using a horseradish peroxidase–conjugated goat anti-mouse antibody and enhanced chemiluminescence (Amersham, Cleveland, OH). Intensity of immunoreactivity was measured using densitometry.

Quantitation of VEGF Protein Expression

Retinal proteins (100 μg, n = 20–28 pooled retinas from 10–14 mice per group) were electrophoresed on a 12% sodium dodecyl sulfate–polyacrylamide gel. Protein was transferred to nitrocellulose membranes and reacted with polyclonal anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive proteins were visualized using a horseradish peroxidase–conjugated goat anti-mouse antibody and enhanced chemiluminescence (Amersham). Membranes were stripped and reprobed with affinity purified anti-actin antibody (Sigma, St. Louis, MO) to verify equal protein loading. Signal intensity of the resulting bands was quantified by densitometry.

RESULTS

Hyperoxia-Induced Vaso-Obliteration

When normal mice are maintained in 75% oxygen from P7 to P9, extensive capillary obliteration occurs in the posterior retina (Fig. 1). This effect was significantly inhibited by treating the mice with the NOS inhibitor L-NNA or by targeted disruption of the eNOS gene (Fig. 1). Capillary obliteration in the L-NNA-treated mice was reduced by 43% compared with eNOS +/+ controls (P < 0.001; Fig. 2). Analysis in the eNOS −/− mice showed virtually identical results, with a 46% reduction in vaso-obliteration compared with eNOS +/+ mice (P < 0.001)(Fig. 2). Thus, all subsequent analyses were done with eNOS −/− mice. Additional control studies showed that when animals were maintained under normoxic conditions, the retina microvasculature of eNOS −/− mice was quantitatively similar to eNOS +/+ mice (Fig. 1). More detailed analyses of retina capillary density under room air conditions showed no differences between the eNOS −/− and eNOS +/+ mice on P9 or in adults (data not shown). As expected, the adult dams exposed to hyperoxia along with the pups showed no evidence of vaso-obliteration.

Neovascularization

Newborn mice raised in 75% oxygen for 5 days (P7–P12) and returned to room air develop extraretinal neovascularization by P17. In our studies the eNOS −/− mice developed significantly less extensive extraretinal neovascularization than the eNOS +/+ mice did. The mean number of extraretinal neovascular buds per cross section was approximately threefold higher in eNOS +/+ compared with eNOS −/− eyes (P < 0.05; Fig. 3). Neovascularization was most prominent at border zones between vascular and avascular tissue. Neovascularization did not occur in adult mice exposed to identical hyperoxic conditions or in room air controls.

Nitrotyrosine Formation

Nitrotyrosine is a stable end product of the reaction between peroxynitrite and free or protein-bound tyrosine residues.
On P9, low levels of retinal nitrotyrosine were found in eNOS+/+ and eNOS−/− mice raised in normoxia (Fig. 4). In contrast, eNOS+/+ mice reared in hyperoxia from P7 to P9 showed significant formation of retinal nitrotyrosine (P < 0.05), whereas the level in eNOS−/− mice was not significantly greater than that found in room air (Fig. 4). Immunolocalization studies revealed that nitrotyrosine expression occurred primarily in the inner retina (Fig. 5), consistent with a putative role in vaso-obliteration. Localization to specific cell types was not possible. Adult eNOS+/+ mice, which do not develop retinopathy, demonstrated low levels of retinal nitrotyrosine in room air and hyperoxia.

**VEGF Expression**

ENOS−/− and eNOS+/+ mice demonstrated low levels of retinal VEGF protein in normoxia at P7 (Fig. 6). Exposure to hyperoxia from P7 to P9 produced a decline in VEGF protein in the retinas of both strains of mice on P9. However, the relative decline in eNOS−/− was only 30% (P > 0.05), compared with 77% (P < 0.05) in the eNOS+/+ mice. After 5 days of hyperoxia (P7-P12), both the eNOS+/+ and the eNOS−/− mice demonstrated a marked increase in VEGF on return to room air (P13), consistent with upregulation induced by tissue hypoxia.

**DISCUSSION**

The development of oxygen tolerance is a hallmark of retinal vascular maturation. The clinical importance of this process is most strikingly revealed by the sight-threatening retinopathy that affects premature infants exposed to a relatively hyperoxic environment. The molecular mediators of vaso-obliteration, as well as the mechanisms that ultimately confer oxygen toler-
Vasoreactivity, are thus of considerable scientific and clinical importance. Because this pathologic process may also represent an exaggeration of the normal developmental process of microvascular remodeling in immature vascular beds, its study in ROP may have broader applicability in developmental vascular biology.

Recent studies have suggested that oxygen-induced alterations in retinal VEGF and PEDF expression are central to the vaso-obliterative process and that the acquisition of a pericyte covering is the key development event that confers oxygen-resistance to retina capillaries. Although direct oxidative injury to capillaries also plays a part in the pathogenesis of ROP, its exact role and the identity of the mediators remain poorly defined.

Our studies in a mouse model reveal that NO may be an important factor in oxygen-induced retinopathy in the newborn period. Mice lacking functional eNOS were partially protected from hyperoxia-induced injury and subsequent proliferative retinopathy. In addition, the systemic administration of an NOS inhibitor (L-NNA) to wild-type mice led to a similar level of vaso-protection compared with the eNOS knock-out, strongly...
supporting a putative role for NO in the vaso-obliterative proc-

ess.

Further study is needed to establish a definitive mechanism for the protective effect of NOS inhibition or deletion. However, the reduction in nitrotyrosine levels (a marker of per-

oxynitrite-mediated protein modification) in eNOS−/− mice compared with eNOS+/+ controls suggests a reduction in NO-mediated oxidative stress. A contributory role for peroxyni-

trite in the pathogenesis of vaso-obliteration is further sup-

ported by our findings that (1) increased formation of immu-

noreactive nitrotyrosine is observed early in the course of vaso-obliteration, (2) immunolocalization of nitrotyrosine is most intense in the inner retina, in the proximity of retina 

vessels, and (3) eNOS+/+ adult mice exposed to hyperoxia do not develop retinopathy and do not show increased nitro-

tyrosine.

The observation that nitrotyrosine formation did not increase significantly in the retinas of eNOS−/− mice, despite the fact that nNOS is normally expressed (alSha-

brawey M, Caldwell R, unpublished data, June 1999) suggests that nNOS does not produce quantitatively significant levels of peroxynitrite in the setting of ROP. Although this does not exclude nNOS from involvement in ROP, the fact that a nonspecific inhibitor of NOS activity produced quantitatively similar levels of vaso-protection compared with eNOS gene disruption also suggests that eNOS may be the primary NOS isoform involved in vaso-obliteration. However, the fact that NOS inhibition and eNOS knock-out provided only partial protection against retinopathy indicates that oxygen-sensitivi-

ty of developing retina capillaries is only partly mediated by NO-dependent mechanisms.

On the basis of our findings, we speculate that the acquisi-

tion of oxygen tolerance by developing retina vessels may be due in part to expression of more effective peroxynitrite scav-

gen systems or to a decline in eNOS activity. This speculation is supported by the work of previous investigators, who provided data suggesting that antioxidant expression is re-

duced in developing tissues including the retina. Glutathione in particular, may be especially important in peroxynitrite scavenging and antioxidant activity in premature infants, yet little is known about its expression in the developing retina. We are also not aware of any quantitative data regarding the in vivo activity of eNOS in relation to stage of retina development and are currently conducting studies to address these issues.

A second mechanism for improved oxygen tolerance in the l-NNA-treated and eNOS-deficient mice may involve the effect of NO on VEGF expression. The role of VEGF as an endothelial cell survival factor is well established, and VEGF withdrawal in high oxygen has been shown to correlate closely with the apoptosis and regression of newly formed capillaries. Moreover, previous investigations have shown that NO donors inhibit VEGF expression in the arterial wall in response to balloon angioplasty and in rat lungs during acute and chronic hypoxia. It is thought that NO and VEGF are reciprocally regulated such that stimulation of VEGF’s receptor flk-1/KDR activates eNOS, leading to NO formation. NO then inhibits VEGF production in adjacent cells by a paracrine feedback mecha-
nism involving inhibition of AP-1 binding to the VEGF promoter. Given this relationship, the lack of eNOS activity in the knockout and l-NNA-treated mice may help explain the observed diminution in VEGF suppression in oxygen, as well as the larger rise in VEGF in hypoxia, in the eNOS−/− compared with eNOS+/+ mice. These differences in VEGF expression may have contributed to the improved oxygen tolerance of the retinal capillaries in the eNOS−/− and l-NNA-treated mice.

**FIGURE 6.** Western blot comparing VEGF protein expression in the reti-

nas of (A) eNOS+/+ and (B) eNOS−/− mice raised either in room air until P7 (P7, Normoxia), room air until P7 and then 75% oxygen from P7 to P9 (P9, 2 d 75% O2), or room air until P7, 75% oxygen from P7 to P12, and room air from P12 to P13 (P13, 24 hour hypoxia). Graphs show mean densitometry value of VEGF signal from corresponding Western blot. Error bars, SE.
In terms of clinical applicability of eNOS inhibition to human ROP, it is important to note that we did not observe major alterations in retinal structure or vasculature in the eNOS−/− mice. Vorwerk et al.46 have provided quantitative data showing that there is no statistically significant difference between eNOS−/− and eNOS+/+ mice in terms of the cell numbers in the retinal ganglion cell, and inner or outer nuclear layers. In addition we have demonstrated that retina capillary density in eNOS−/− and eNOS+/+ mice is not significantly different (al-Shabrawey M, Caldwell R, unpublished data, June 1999). These findings are especially important if drug- or gene-based therapies to inhibit eNOS are to be used to treat premature infants with ROP.

In conclusion, targeted disruption of the eNOS gene as well as pharmacologic inhibition of NO/vasodilator activity leads to improved oxygen tolerance of immature retina capillaries in mice without significantly interfering with normal retinal vascular development or causing significant structural abnormalities of the retina. The improved oxygen tolerance appears to be due in part to a reduction in peroxynitrite-mediated cytotoxicity and results in reduced neovascularization. Further studies are necessary to better define the molecular mechanisms of retinal vasculo-oblitration in hyperoxia, including the role of pericytes as well as to better understand the role of NO in normal retina development and function.

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