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REDOX-DEPENDENT EFFECTS OF NITRIC OXIDE ON MICROVASCULAR INTEGRITY IN OXYGEN-INDUCED RETINOPATHY

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Abstract—Opposing effects have been ascribed to nitric oxide (NO) on retinal microvascular survival. We investigated whether changes in the redox state may contribute to explain apparent conflicting actions of NO in a model of oxygen-induced retinal vasoobliteration. Retinal microvascular obliteration was induced by exposing 7-day-old rat pups (P7) for 2 or 5 days to 80% O₂. The redox state of the retina was assessed by measuring reduced glutathione and oxidative and nitrosative products malondialdehyde and nitrotyrosine. The role of NO on vasoobliteration was evaluated by treating animals with nitric oxide synthase (NOS) inhibitors (*N*-nitro-L-arginine; L-NA) and by determining NOS isoform expression and activity; the contribution of nitrosative stress was also determined in animals treated with the degradation catalyst of peroxynitrite FeTPPS or with the superoxide dismutase mimetic CuDIPS. eNOS, but not nNOS or iNOS, expression and activity were increased throughout the exposure to hyperoxia. These changes were associated with an early (2 days hyperoxia) decrease in reduced glutathione and increases in malondialdehyde and nitrotyrosine. CuDIPS, FeTPPS, and L-NA treatments for these 2 days of hyperoxia nearly abolished the vasoobliteration. In contrast, during 5 days exposure to hyperoxia when the redox state rebalanced, L-NA treatment aggravated the vasoobliteration. Interestingly, VEGFR-2 expression was respectively increased by NOS inhibition after short-term (2 days) exposure to hyperoxia and decreased during the longer hyperoxia exposure. Data disclose that the dual effects of NO on newborn retinal microvascular integrity in response to hyperoxia *in vivo* depend on the redox state and seem mediated at least in part by VEGFR-2. © 2004 Elsevier Inc. All rights reserved.

Keywords—Nitric oxide synthase, Vasoobliteration, Retina, CuDIPS, VEGF, Free radicals

INTRODUCTION

Ischemic proliferative retinopathies such as retinopathy of prematurity (ROP) and of diabetes are characterized by a vasoobliterative phase leading to vascular rarefaction, which as a result of inner retinal hypoxia predisposes in turn to a vasoproliferative phase. The sensitivity of retinal microvascular endothelium to relatively high oxygen tension is particularly pronounced in the devel-

oping subject [1–4]. A notable mechanism implicated in oxygen-induced cytotoxicity relates to oxidative stress. Several lines of evidence point to a role for oxidant stress in the genesis of vasoobliteration in ROP: (1) impaired antioxidant defenses represented by a decreased ratio of reduced to oxidized glutathione is observed in subjects who develop ROP [3], (2) exposure to oxygen significantly reduces levels of Cu,Zn superoxide dismutase (SOD) activity in the retina [4] and more importantly triggers peroxidation [5], and (3) antioxidant supplementation attenuates the retinopathy in animals [4,6,7] including humans [8].

Interestingly, the free radical nitric oxide (NO) can exert either cytoprotective or cytotoxic effects on

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endothelium [9–17]. Accordingly, consistent with reports in other tissues [18–20], roles for NO generated from both endothelial (eNOS) and inducible (iNOS) isoforms of NO synthase (NOS) have been invoked in retinal vasoobliteration [21] and neovascularization [22,23]. Although it is generally assumed that higher concentrations of NO are associated with cytotoxicity [9,17,24], expression of the high-NO-generating iNOS has been found to correlate positively with vascular density in colorectal and breast carcinomas [16,25]. However, there is increasing evidence that the reaction product of superoxide ($O_2^{\bullet-}$) with NO, notably peroxynitrite, partakes in endothelial toxicity [9,17,24,26]. Upon hyperoxia exposure of newborn mice either eNOS-deficient or treated with the NOS inhibitor *N*-nitro-L-arginine (L-NA) from postnatal day (P)7 to P9, one observes diminished nitration products of peroxynitrite and retinal vasoobliteration [21]; of note, such short-term exposure to hyperoxia reduces superoxide dismutase (SOD) activity [4]. On the other hand, more prolonged exposure to hyperoxia surprisingly reverses the vasoobliteration, leading to accelerated revascularization [27], possibly due to a waning of the oxidative stress as glutathione generation augments [28]. Thus the deleterious or beneficial actions of NO on retinal microvasculature in response to hyperoxia might depend on the respective levels of other free radicals such as $O_2^{\bullet-}$ and the redox balance. We therefore hypothesized that NO in the presence of a redox potential shifted toward an oxidative environment, such as when $O_2^{\bullet-}$ concentrations are relatively high, elicits detrimental nitrosative stress to the retinal microvasculature *in vivo*, while comparable concentrations of NO when the redox balance is shifted toward a reduced environment favor endothelial cell survival.

Hence, we investigated the time-dependent changes and roles of NO and redox state in relation to retinal vasoobliteration in a rat pup model of oxygen-induced retinopathy. Our results indicate that upon exposure to hyperoxia an increase in NO along with a short-term oxidative shift in redox state is observed in the retina relatively depleted of microvasculature. Normalization of the redox potential by SOD mimetics, by enhancers of peroxynitrite degradation, or by NOS inhibition prevented the retinal vasoobliteration. However, upon longer duration of exposure to hyperoxia the redox state rebalanced and NOS inhibition aggravated the vasoobliteration. Respective sequential increases and decreases in the expression of vascular endothelial growth factor receptor (VEGFR)-2 were observed following NOS inhibition. Our findings reveal that the redox potential is an essential determinant of the effects of NO on retinal microvasculature *in vivo*, which seem mediated at least in part by VEGFR-2; observations

highlight that the use of NOS inhibitors in this condition must take into account the delicate balance between the dual actions of NO on retinal microvascular integrity.

MATERIALS AND METHODS

Animal preparation

Newborn Sprague–Dawley rats (Charles River, St-Constant, Québec, Canada) were used according to a protocol of Ste-Justine Hospital Research Center Animal Care Committee. Oxygen-induced retinopathy was induced in newborn rats according to an established protocol [7,29–32]. Rats were maintained along with their dams from postnatal day P7 to P12 in custom-built chambers at $80 \pm 0.1\%$ oxygen, controlled by a computer-assisted Oxycycler (BioSpherix Ltd., Redfield, NY). Control rats were kept in room air (21% O_2). We opted to expose rats to oxygen at this stage of development to elicit vasoobliteration unconfounded by arrest in normal neovascularization of the nerve fiber layer which occurs from P1 to P7. The continuous exposure to hyperoxia favors vasoobliteration [29,30,33], while alternate exposure to high and low concentrations of oxygen associated with a recovery period in normoxia following the period of hyperoxia facilitate development of neovascularization [1,34].

Pups were randomly selected to receive immediately before and during 80% O_2 exposure daily 50- μ L intraperitoneal injections of the following treatments ($n = 4$ per group): vehicle (50% dimethyl sulfoxide), nonselective NOS inhibitors L-NA (Sigma Chemicals, St. Louis, MO) ($10 \text{ mg} \cdot \text{kg}^{-1}$ twice), 7-nitroindazole Na (7-NINA; Tocris, Ellisville, MO) ($10 \text{ mg} \cdot \text{kg}^{-1}$ thrice), nNOS and eNOS inhibitor 1-(2-trifluoromethylphenyl)imidazole (TRIM; Cayman Chemical, Ann Arbor, MI) ($10 \text{ mg} \cdot \text{kg}^{-1}$), iNOS inhibitor *N*-(3-(aminomethyl)benzyl)acetamide dihydrochloride (1400W; Cayman Chemical) ($5 \text{ mg} \cdot \text{kg}^{-1}$ twice), SOD mimetic copper[II] [3,5-diisopropylsalicylate acid]₂ (CuDIPS; Calbiochem, Mississauga, Ontario, Canada) ($10 \text{ mg} \cdot \text{kg}^{-1}$), or degradation catalyst of peroxynitrite iron[III] porphyrin 5,10,15,20-tetrakis[4-sulfonatophenyl]prophyrinato iron chloride (FeTPPS, Calbiochem) (10 mg/kg) [35]. Control rats maintained at 21% O_2 received the same treatments. Rats were sacrificed by decapitation at P7, P8, P9, or P12 and retinas flat mounted and stained for endothelial cells as described below.

Vascular staining

Eyes were removed and fixed overnight at 4°C in 4% v/v formaline. The retinas were isolated and flat mounts were prepared for adenosine diphosphatase (ADPase) staining as described [29–31,36]. Briefly, retinas were washed in 50 mM Trizma-maleate buffer at 4°C and

incubated at 37°C in ADPase incubation medium (0.2 M Tris maleate, 3 mM lead nitrate, 6 mM MgCl₂, 1 mg/ml ADP); retinas were washed at room temperature with buffer and placed in a 1:10 dilution of ammonium sulfide for 1 min to allow reaction with ADPase. Retinas were mounted and photographed (MTI CCD-72, Dage, Michigan City, MI). Vascular density was calculated for the full retinal surfaces, using the imaging software Image-Pro Plus 4.5 (Media Cybernetics, Silver Spring, MD), and normalized relative to 21% O₂-exposed animals.

NADPH-diaphorase histochemistry

NADPH-diaphorase (NADPH-d), which reflects the activity of NOS isoforms, was performed on the retinal flat mounts of rat pups according to a previously described method [37–39]. Briefly, the eyes were fixed overnight in 4% v/v formaline. Retinas were isolated and incubated for 2 h at 37°C in a water bath in 0.1 M phosphate buffer (pH 7.4) containing 0.3% Triton X-100, 0.5 mM MgCl₂, 0.01 M sodium azide, 0.1% nitroblue tetrazolium, and 0.1% NADPH. Digital pictures of the flat mounts were taken with a Nikon DMX 1200 mounted on a Nikon SMZ800 stereomicroscope (Nikon, Japan) and analyzed with Image Pro Plus 4.5 software to localize NOS isoforms and evaluate relative activity according to staining intensity which was corrected for the background intensity.

Western blots

Expression of different NOS isoforms, VEGFR-1 and -2, and VEGF was evaluated by Western blot. Briefly, the retinas were isolated and snap frozen immediately after sacrifice. Each retina was resuspended in 75 µL of lysis buffer in H₂O (Tris-HCl 50 mM, pH 7.4, 1% Nonidet-P40, 0.25% Na-deoxycholate, NaCl 150 mM, EDTA 1 mM, complete protease inhibitor cocktail (Roche Diagnostics, Laval, Québec, Canada)). Tissues were snap frozen in liquid N₂ and thawed repeatedly (four times) to enhance tissue lysis. Tubes were kept on ice and vortexed every 10 min for 1 h followed by centrifugation at 21,000g for 10 min at 4°C. SDS-sample buffer (Tris-HCl 250 mM, pH 6.8, SDS 8%, glycerol 40%, β-mercaptoethanol 8%, bromophenol blue 0.04%, Boston BioProducts Inc., Ashland, MA) was added and samples were boiled for 5 min and then frozen at -20°C until used. Aliquots (50 µg of protein) were run on 7.5% SDS-PAGE. Proteins were transferred on polyvinylidene difluoride (PVDF) membrane, blocked for 4 h in TTBS 5% milk at room temperature, and probed with either nNOS (1:1000 dilution), iNOS (1:500 dilution), eNOS (1:1000 dilution) (BD Biosciences Pharmingen, San Diego, CA), VEGFR-2 (Flk-1/KDR) (1:250 dilution), or VEGFR-1 (flt-1) (1:1000 dilution) (Chemicon, Temecula, CA) overnight at 4°C. After washing with TTBS

(3 × 10 min) and incubating with a corresponding horseradish peroxidase-conjugated secondary antibody at room temperature for 90 min, membranes were washed (4 × 10 min) and incubated at room temperature in Western Blot Chemiluminescence Plus Reagent (Perkin-Elmer Life Sciences Inc., Boston, MA) for 3 min and then visualized with PhosphorImager (Fuji, Japan). Densitometry analyses were performed using Image Pro Plus 4.5 software.

Nitrite measurement

NO concentrations were assessed by measuring its major stable metabolite nitrite [39]. Retinas were immediately isolated and placed in a mixture of 500 µL Krebs buffer (mM: NaCl 120, KCl 4.5, CaCl₂ 2.5, MgSO₄ 1.0, NaHCO₃ 27, KH₂PO₄ 1.0, glucose 10) and 100 µL L-arginine (200 µM, Sigma Chemicals). Two retinas were pooled and placed in 37°C bath for 1 h. Preparation time prior to incubation was less than 1 min per eye. After the incubation, 100 µL buffer was passed through a rubber membrane with a 250-µL Hamilton microsyringe to avoid contact with surrounding air and injected into a chemiluminescence analyzer, Sievers 280i Nitric Oxide Analyzer (Sievers, Boulder, CO), to directly measure nitrite (NO₂⁻); KNO₂ was used as standard.

Reduced glutathione measurement

Pups prepared as described above were sacrificed at P7, P8, P9, or P12 to measure reduced glutathione (GSH) concentrations, a general marker of the redox state. Retinas were extracted and placed in 5% metaphosphoric acid (HPLC grade) in a volume (µL) five times the weight (mg). The samples were homogenized on ice with Omni 2000 (12,000 rpm, 2 × 10 s) and centrifuged for 1 min at 10,000g. The supernatant was frozen in liquid N₂ and stored at -80°C for GSH measurement; this whole procedure starting from tissue extraction lasted <4 min. On the day of the experiment, the supernatant was diluted 1:5 in HPLC-grade water and a standard curve for GSH (0.5 to 50 µM) was constructed in 1% metaphosphoric acid in HPLC-grade water. GSH was measured by capillary electrophoresis with the P/ACE MDQ Capillary Electrophoresis System (Beckman Coulter, Fullerton, CA) using eCAP capillaries (75 µm i.d. × 50 cm) [40]. Peaks were analyzed with computer software 32 Karat 5.0 (Beckman Coulter). Some samples were spiked with the standard to clearly identify GSH peaks.

Malondialdehyde and protein nitration assay

The product of peroxidation, malondialdehyde, was determined as previously described [41]. Briefly, less than 1 min after the animals were killed, each retina was isolated, ground 2 × 30 s with an Omni homogenizer in a buffer containing butyl-hydroxytoluene (BHT; 5 mM

Tris, 0.02% ASA, 0.5 mM EGTA), and centrifuged at 1000g for 10 min. To each sample was added 0.33% 2-thiobarbituric acid (TBA) in water mixed with glacial acetic acid and then the sample was heated at 95°C for 60 min. HPLC-grade *n*-butanol was added; samples were vortexed and spun down at 1000g for 10 min. The upper phase was read with spectrophotometer at 532 nm.

Tyrosine nitration was determined by slot-blot as described [42], as a marker of nitrosative stress. Briefly, retina was put in the buffer (~75 μ L) and sonicated 3×10 s on ice. Lysate was centrifuged at 12,000g at 4°C for 30 min. Tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl, pH 7.5) was added to final volume of 100 μ L. PVDF membrane was placed in Hybri-slot Manifold apparatus (BRL, MD), and 200 μ L TBS was added to each well; 100 μ L of TBS-diluted protein solution was allowed to passively diffuse for 3 h. The wells were then washed three times with 100 μ L TBS. Membrane was removed from apparatus, washed briefly with TTBS, and put in a blocking buffer for 2 h at room temperature. Membrane was incubated overnight at 4°C with antinitrotyrosine polyclonal antibodies (10 μ g/well, Upstate, Lake Placid, NY). Washes, secondary incubation, revelation, and densitometry techniques and analyses were the same as previously described above for Western blots. Membranes were stripped in buffer (100 μ M β -mercaptoethanol, 2% w/v SDS, 62.5 mM Tris-HCl, pH 6.7) at 60°C for 30 min with agitation, rinsed with TTBS for 30 min, and re probed with β -actin 1:40,000 (Novus Biologicals, Littleton, CO) monoclonal antibody to normalize for loading.

Statistical analysis

Data were analyzed by one-way ANOVA followed by Tukey or Dunnett's tests for comparison among means. Statistical significance was set at $p < 0.05$. Values are presented as means \pm SE.

RESULTS

Expression of NOS isoforms, localization of NOS activity, and nitrite generation

Exposure to 80% O₂ from P7 to P9 or from P7 to P12 caused a significant vasoobliteration of the retinal vasculature compared to 21% O₂ controls, as expected; the vasoobliteration was slightly attenuated after the longer exposure to hyperoxia (Figs. 1b, 1f, and 1i). Expression of eNOS was augmented during exposure to hyperoxia relative to normoxia (Fig. 2a); nNOS was not augmented and iNOS was not detected under both conditions (data not shown). Similarly, intensity of NADPH-d staining on the vasculature (but not the parenchyma) increased comparably upon short- and longer-term exposure to hyperoxia (Fig. 2b), and there

was a corresponding increase in retinal NO₂⁻ concentrations which was inhibited by L-NA but not by the nNOS/iNOS-specific inhibitor TRIM (Fig. 2c), pointing to major role for eNOS as reported [21].

Glutathione, malondialdehyde, and nitrotyrosine concentrations

Because the redox state is largely dependent upon glutathione levels, which in turn influence nitration and peroxidation [43,44], we measured reduced glutathione concentrations, protein tyrosine nitration, and the advanced product of peroxidation malondialdehyde. Two days after exposure to hyperoxia (P7–P9) GSH concentrations were markedly decreased (by ~30%; Fig. 3a), and correspondingly malondialdehyde and nitrotyrosine levels increased (Fig. 3b). These changes (relative to normoxia) subsided as the hyperoxic period was extended for 5 days (Fig. 3).

Contribution of NO, peroxynitrite, and SOD on O₂-induced retinal microvascular degeneration

Microvascular degeneration occurs mostly within a couple of days of exposure to hyperoxia [45] and is mediated by NO [21] and by O₂^{•-} [4]. To decipher the interplay of NO and O₂^{•-} we exposed rat pups to short-term hyperoxia (P7–P9, when SOD is transiently down-regulated [4]) and to longer-term hyperoxia (P7–P12, when SOD levels renormalize around P8–P10 [4]), following treatments with NOS inhibitors L-NA or 7-NiNa, SOD mimetic CuDIPS, and peroxynitrite degradation catalyst FeTPPS. The oxygen-induced retinal vasoobliteration at P9 was markedly and comparably diminished by L-NA, 7-NiNa (data not shown), CuDIPS, and FeTPPS (Fig. 1); these treatments in normoxia-raised animals did not affect vascular density. In contrast, by P12 when the redox state had renormalized in hyperoxia-exposed rats (Fig. 3), L-NA treatment (P7–P12) resulted in augmented microvascular degeneration, while the earlier benefit of CuDIPS (and FeTPPS [not shown]) was sustained (Fig. 1); the selective n/iNOS inhibitor TRIM and the iNOS-specific inhibitor 1400W did not affect retinal vascular density (data not shown), consistent with lack of expression (iNOS) or its increase (nNOS) during hyperoxia.

VEGFR-1 and -2 expression and regulation by hyperoxia and NO

Because the growth and survival of the retinal microvasculature [45] is greatly affected by VEGFR-1 and -2 [46], we examined whether the effects of NOS inhibition on retinal vascular density in oxygen-exposed rat pups are associated with corresponding changes in these important receptors. The expression of VEGFR-1 and -2 was not affected by exposure to hyperoxia.

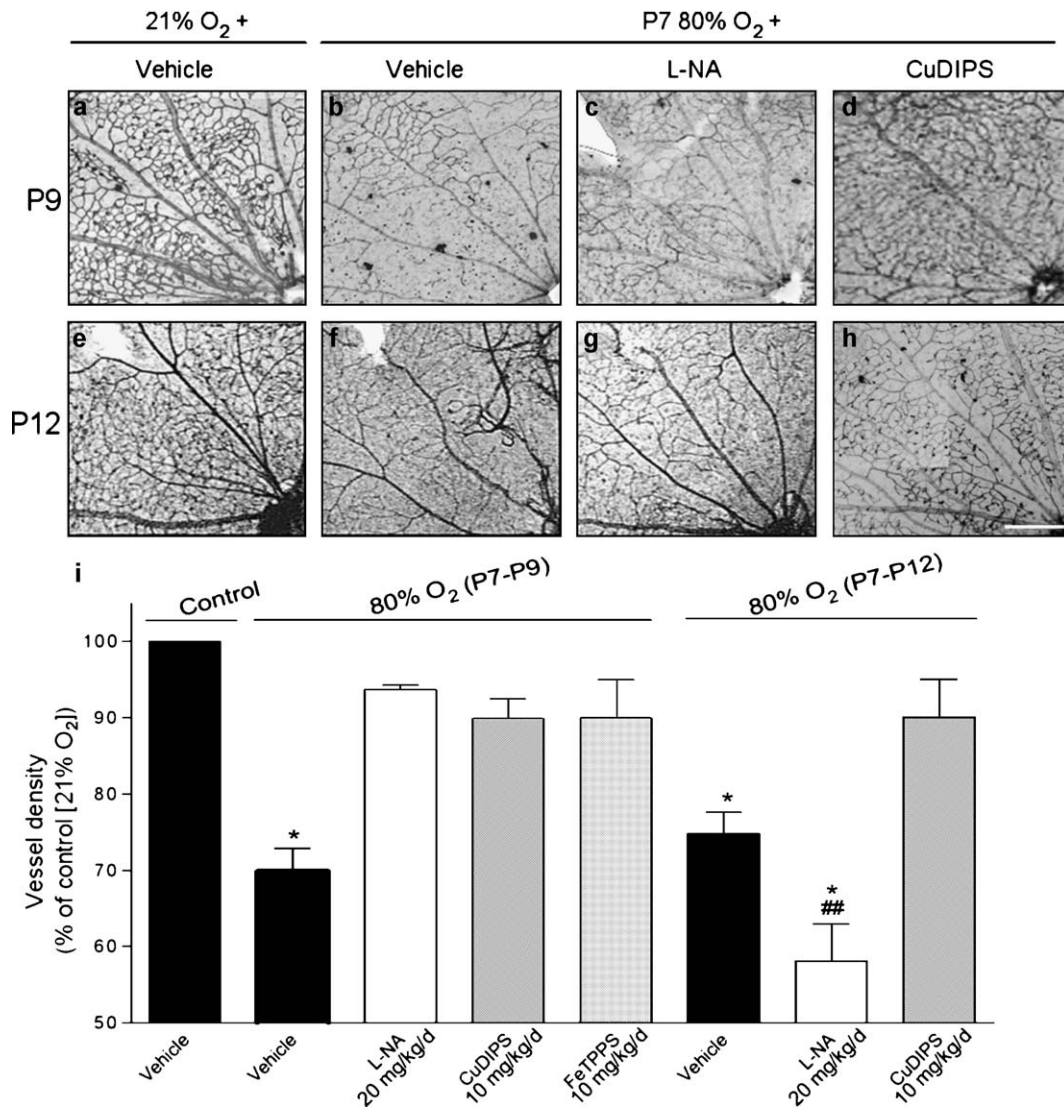


Fig. 1. Effects of NOS inhibition and antioxidants on oxygen-induced vasoobliteration. Rat pups exposed to oxygen (80% O₂) or room air from P7 to P12 were treated daily (ip) with L-NA (20 mg · kg⁻¹), CuDIPS (10 mg · kg⁻¹), FeTPPS (10 mg · kg⁻¹), or vehicle (DMSO). Rats exposed to 21% O₂ and treated with the same drugs did not exhibit changes in vascular density (not shown). Rats exposed to 80% O₂ and treated with the same drugs did not exhibit changes in vascular density (not shown). Scale bar, 250 μm. Values are mean ± SE of vessel density relative to that in vehicle-treated 21% O₂-exposed rats (controls); *n* = 3–5 retinas from different pups. **p* < 0.01 compared to all other values without asterisks; ##*p* < 0.05 compared to corresponding value of 80% O₂-exposed vehicle-treated rats P7–P12.

However, after 2 days of NOS inhibition in oxygen-exposed animals VEGFR-2 expression was significantly increased, and by 5 days of NOS inhibition the reverse was observed as VEGFR-2 expression decreased (Fig. 4); VEGFR-1 expression was unaffected. Noticeably, the changes in VEGFR-2 following NOS inhibition corresponded to those of vascular density (Fig. 1).

DISCUSSION

Exposure of immature retinal vasculature to high oxygen levels leads to microvascular degeneration in

various species, including humans [2,47]. This degeneration is mediated by NO [21] and occurs in the early phase of oxygen exposure [2,21]. During sustained oxygen exposure the microvasculature regenerates [27]. Assuming that eNOS activity is increased during more prolonged hyperoxia as suggested in some other tissues [48,49], one could presume an abated oxidative stress under this latter condition, since cytoprotective properties of NO [50] seem mostly observed when the redox balance is shifted toward a reduced environment, while the reverse is true for its cytotoxic properties [9,17,24,26]. We proceeded to investigate this inference on retinal

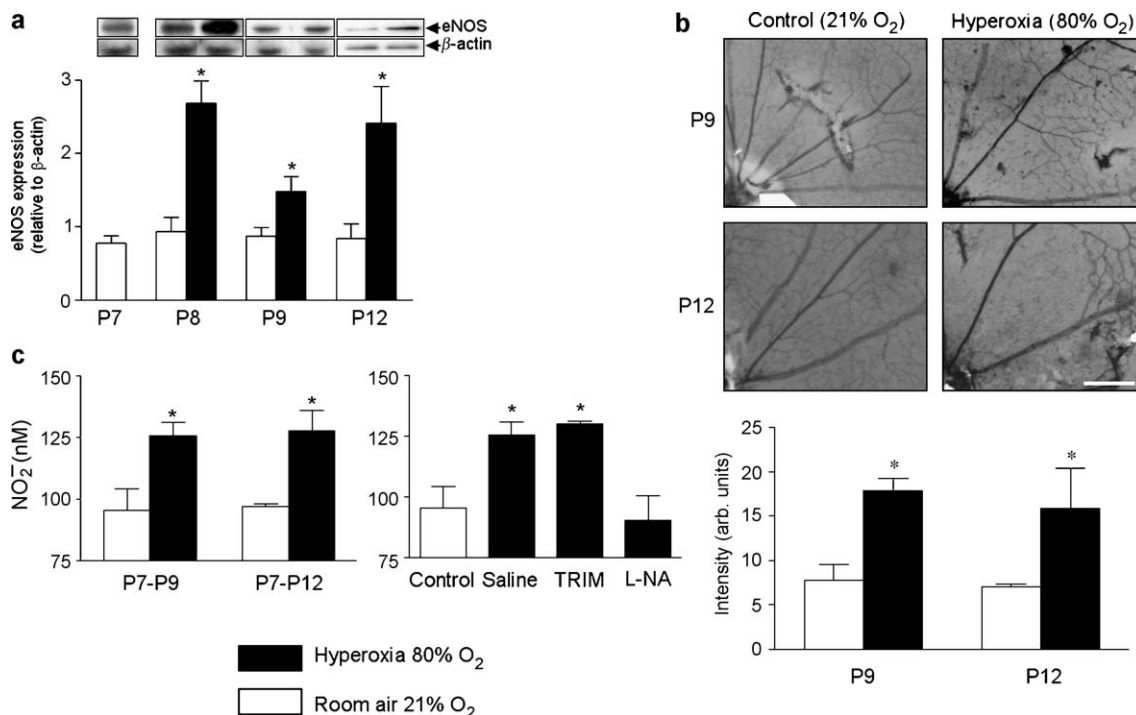


Fig. 2. Expression of eNOS, NADPH-d reactivity, and nitrite generation in retina exposed to hyperoxia. (a) Western blots and corresponding densitometric analysis of eNOS immunoreactivity in retinas of rat pups exposed to 80% O₂ or room air as in Fig. 1. Each experiment was repeated at least three times with samples from three separate experiments. Values are mean \pm SE of three experiments each repeated three times; **p* < 0.05 compared to same-day control (21% O₂). (b) NADPH-d reactivity in retinas of animals treated as in (a). Photomicrograph of retinas reveals NADPH-d-stained vasculature; histogram displays relative intensity of vascular staining by controlling for background intensity, which was unaltered by hyperoxia exposure. Scale bar, 125 μ m. Values are mean \pm SE of *n* = 3 or 4 retinas per treatment group; **p* < 0.05 compared to same-day control; arb. refers to arbitrary. (c) Nitrite generation in retinas of rat pups prepared as in (a). Range on abscissa (left panel) refers to period of exposure to hyperoxia. Animals exposed to hyperoxia from P7 to P9 received either vehicle (saline), L-NA, or TRIM. Values are mean \pm SE; *n* = 5 per treatment. **p* < 0.01 compared to values without asterisks.

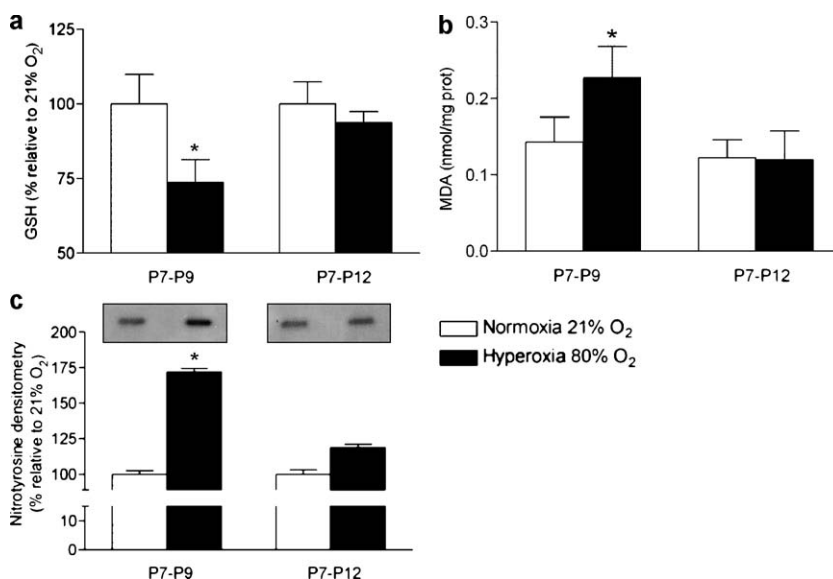


Fig. 3. Glutathione, malondialdehyde, and nitrotyrosine levels in retinas of rats exposed to hyperoxia. Rat pups were exposed to oxygen (80% O₂) or room air as in Fig. 1. Animals were killed at P9 or P12 to determine (a) reduced glutathione (GSH), (b) malondialdehyde (MDA) concentrations, and (c) nitrotyrosine immunoreactivity in retinas. Values are mean \pm SE each of four to nine different retinas; **p* < 0.05 compared to 21% O₂ controls.

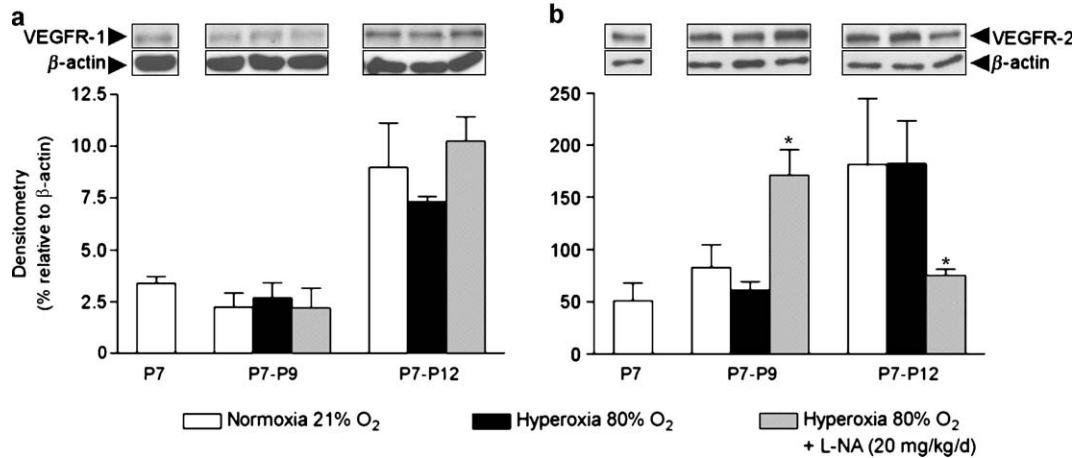


Fig. 4. Modulation of vascular endothelial growth factor receptor-1 and -2 expression in retinas by hyperoxia and NOS inhibition. Rat pups were prepared as described in Fig. 1. VEGFR-1, VEGFR-2, and β -actin expression were determined by Western blot analysis; VEGFR-1 and -2 densitometric analyses controlled for those of β -actin are presented in histograms. Values are mean \pm SE, each of four experiments. * $p < 0.05$ compared to corresponding same-day values without asterisks.

microvasculature of the developing subject. Our findings reveal comparable increases in retinal NO levels in the early and later response to hyperoxia largely contributed by eNOS, which in turn exert opposing effects on retinal microvascular integrity depending upon the redox state of the tissue and seem partly mediated by VEGFR-2. These observations highlight the complex dual role of NO during the progressive genesis of retinovascular pathology and the delicate balance disrupted by interfering simply with actions of this major mediator.

Nitrosative stress has been shown to be important in NO-mediated endothelial cell injury [21,26]. NO provides the biological precursor for these nitrating agents. NO can form nitrating agents in a number of ways, including reaction with superoxide to form peroxynitrite and through enzymatic oxidation of nitrite to form NO₂ [17,26]. The redox state of the tissue is crucial in the generation of nitrating agents [51]. In the retina, oxygen-induced microvascular degeneration is dependent not only on NO [21] but also on O₂^{•-} [4]. The endogenous Cu,Zn SOD is transiently down-regulated in oxygen-exposed animals [4] and normalizes during longer-term exposure of rat pups (P8 to P10) [4]. This decrease in activity of an important free radical scavenger could lead to high O₂^{•-} levels and a high redox state in the retina during short-term exposure to oxygen, favoring the generation of toxic nitrating agents and the consumption of reduced glutathione [52]. Thus, to detect changes in the redox state we measured GSH levels [44]. One notes an early drop in GSH with a rise in peroxidation and nitration products (Fig. 3), which is associated with a marked retinal vasoobliteration prevented by the SOD mimetic CuDIPS, the peroxynitrite degradation catalyst FeTPPS, and the NOS inhibitor L-NA (Fig. 1). However, in an apparent paradox, as exposure to hyperoxia is prolonged

(for 5 days), microvascular degeneration was not exacerbated (Fig. 1) despite sustained increased concentrations of NO (Fig. 2c); in fact, by further prolonging exposure to hyperoxia, normal revascularization was enhanced [27]. Interestingly, as the period of exposure to hyperoxia is extended, the redox state rebalanced (Fig. 3) as reported in other tissues [28]; consequently, as would be expected, CuDIPS and FeTPPS did not exert additional effects on retinal microvascular integrity by extending the treatment to P12 (Fig. 1). Correspondingly, protein tyrosine nitration increased in short- but not in longer-term O₂-exposed animals (Fig. 3), predictably due to diminished generation and rapid ongoing disposal [53] of these nitrated products. Along with these redox-dependent changes NO is likely to exert prosurvival/proliferative effects [50]. Of relevance, the cytoprotective effects of NO may signal activation of the prosurvival/pro-proliferative receptor VEGFR-2 [11–14,18,19,54,55] and modulate its expression [14,56,57] (see below). Furthermore, we observed a significant increase of protein tyrosine nitration in short- but not in long-term-exposed animals, suggesting that nitrating agents are generated only in short-term hyperoxia (Fig. 2). Accordingly, (partial) removal of NO during longer-term exposure to hyperoxia may curtail microvascular survival and ensuing density as observed in the present study with L-NA (Fig. 1), especially since expression of VEGF per se also decreases, while this is not the case in normoxia-raised animals. Altogether, our findings underline the importance of the redox state, rather than simply the NO concentration as proposed [15,24], in governing opposing actions of NO on retinal vasculature in vivo; in this process observations also highlight the differences in NO actions during short- and longer-term exposure to hyperoxia in the developing retina.

Using a similar model exposed to hyperoxia at the same time of retinovascular development, our findings on short-term effects of L-NA on vascular density are consistent with those reported [21]. However, a preretinal neovascular role for NO arising largely from eNOS based on mice with disrupted eNOS genes has been documented in oxygen-induced retinopathy by the same authors and others [21,22]. Preretinal neovascularization occurs during the posthyperoxic ischemic phase. These latter observations may at first seem incongruent with the present observations based on the redox-dependent effects of NO, since oxidant stress is also detected during ischemia [58] and microvascular cytotoxicity would be predicted rather than survival/proliferation. However, exposure to the diminished eNOS activity in animals with disrupted eNOS gene took place throughout the hyperoxic and the posthyperoxic ischemic phases [21,22]; thus, one cannot distinguish the specific time of action of NO, which is further complicated by compensatory increases in the activities of nNOS especially [59].

To further decipher mechanisms by which NO exerts its divergent effects during short- and longer-term hyperoxia, we studied the expression of VEGFR-1 (flt1) and VEGFR-2 (flk1/KDR), which are major receptors that are modulated by oxidant stress [60] in models of ROP [61]. Inhibition of NOS during the early period of exposure to hyperoxia associated with a nitrosative stress resulted in increased VEGFR-2 expression and preservation of retinal microvascular density (Figs. 1 and 4), whereas NOS inhibition during the longer duration of exposure to hyperoxia when the redox state was rebalanced led to decreased VEGFR-2 expression and aggravated vasoobliteration.

The absence of effects of hyperoxia or NOS inhibition on VEGFR-1 receptor may result from differential regulation of VEGFR-1 and -2. The expression of VEGFR-2 is regulated by NF κ B, [56] unlike that of VEGF and VEGFR-1 which is mainly regulated by the hypoxia-inducible factor [62]. Accordingly, the most marked changes in expression of these receptors in oxygen-induced retinopathy are observed during the posthyperoxic ischemic period when immunoreactivity is found to increase albeit mainly is confined to preretinal neovascular buds [61,63]. However, during hyperoxia coincidental increases in NO and reactive oxygen species may interfere with NF κ B activation [57] and in turn in VEGFR-2 expression [56]. Of interest, VEGFR-2 expression was slightly reduced by short-term hyperoxia; although VEGFR-2 expression appeared unchanged during longer exposure to hyperoxia (Fig. 4), by correcting for an approximate 30% loss of microvasculature (Fig. 1), expression of this receptor would be increased correspondingly. Accordingly, NOS inhibition

in the presence of a nitrosative stress would be expected to secure NF κ B activation but in its absence would activate it [64] and result in corresponding changes in VEGFR-2 expression [56] as we have observed (Fig. 4), consistent with reports in other tissues on redox-dependent effects of NOS inhibition on VEGFR-2 expression [14,65,66]. Finally, although some have attributed a cytoprotective role mostly for VEGFR-1 [46] which is not modulated by NOS inhibition in the present study, one cannot exclude a cell survival role for VEGFR-2 [67].

Taken together, our studies highlight that the redox state is a very important modulator of the complex opposing actions of NO on microvascular endothelium integrity in particular in oxygen-induced retinopathy and that these effects seem likely in part dependent upon expression of VEGFR-2. A diagram depicting the sequential contribution of NO, its synthases, antiox-

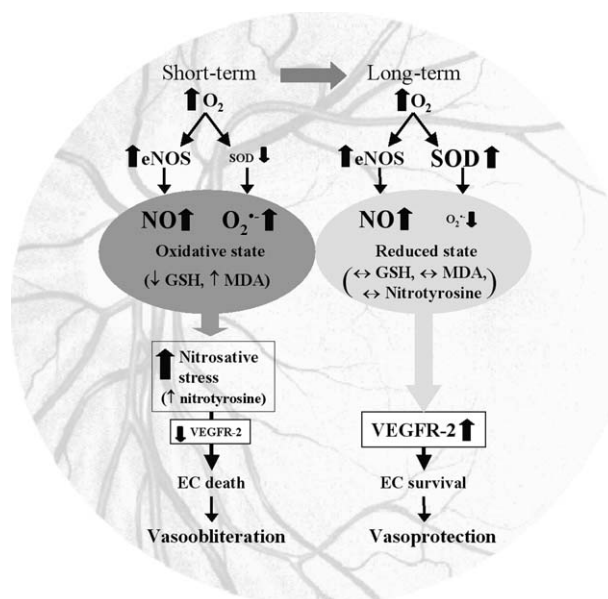


Fig. 5. Effects of NO on retinal microvascular integrity during short- and longer-term exposure to hyperoxia. Short-term exposure to hyperoxia increases eNOS expression and decreases SOD activity in the retina [4]. NO in the presence of an oxidative environment (associated with decreased GSH and increased MDA levels) leads successively to a nitrosative stress (resulting in increased nitrotyrosine formation), a down-regulation of VEGFR-2, and a microvascular degeneration. Upon longer exposure to hyperoxia, eNOS expression remains up-regulated but SOD activity renormalizes [4]; this reestablishes a reduced redox state (resuming normal GSH levels and decreased MDA and nitrotyrosine concentrations), which leads consecutively to increased VEGFR-2 expression (relative to short-term hyperoxia and to diminished microvascular density) and to endothelial cell survival. The symbols and abbreviations refer to the following: \uparrow increase; \downarrow decrease; \leftrightarrow same as room air control; eNOS, endothelial nitric oxide synthase (activity); SOD, superoxide dismutase (activity); NO, nitric oxide; $O_2^{\bullet-}$, superoxide anion; MDA, malondialdehyde; GSH, reduced glutathione; VEGFR-2, VEGF receptor 2; EC, endothelial cell.

idants, and oxidants, and VEGFR-2 during short- and longer-term hyperoxic stress is presented in Fig. 5. To our knowledge this is the first time that such a mechanism is uncovered in the same tissue in vivo, which provides an explanation for the apparent contradictions with regard to the role of NO on retinal (and possibly other tissue) endothelial cell survival. In addition, the present findings may also apply to ischemia during which an oxidative stress is observed [58]; in this case NOS activity resulting in a nitrosative stress [68] inhibited revascularization of the ischemic retina [23]. In sum the use of NOS inhibition in conditions such as oxygen-induced retinopathy model of ROP must be weighed against the delicate balance between the dual actions of NO on retinal microvascular integrity; we propose that antioxidants might be a safer alternative.

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