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Stroke
JOURNAL OF THE AMERICAN HEART ASSOCIATION
American Stroke Association
A Division of American Heart Association

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Stroke published online Sep 28, 2006;
DOI: 10.1161/01.STR.0000245082.19294.ff
Stroke is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 0039-2499. Online ISSN: 1524-4628

Hyperoxic Exposure Leads to Nitrative Stress and Ensuing Microvascular Degeneration and Diminished Brain Mass and Function in the Immature Subject

Mirna Sirinyan, MSc; Florian Sennlaub, MD, PhD; Allison Dorfman, BSc; Przemyslaw Sapieha, PhD; Fernand Gobeil, Jr, PhD; Pierre Hardy, MD, PhD; Pierre Lachapelle, PhD; Sylvain Chemtob, MD, PhD

Background and Purpose—Neonates that survive very preterm birth have a high prevalence of cognitive impairment in later life. A common factor detected in premature infants is their postnatal exposure to high oxygen tension relative to that in utero. Hyperoxia is known to elicit injury to premature lung and retina. Because data on the exposure of the brain to hyperoxia are limited, we studied the effects of high oxygen on this tissue.

Methods—Rat pups were exposed from birth until day 6 to 21% or 80% O_2. Cerebral vascular density was quantified by lectin immunohistochemistry. Immunoblots for several proteins were performed on brain extracts. We assessed cerebral functional deficits by visual evoked potentials.

Results—Exposure of pups to hyperoxia leads to cerebral microvascular degeneration, diminished brain mass, and cerebral functional deficits. These effects are preceded by an upregulation of endothelial nitric oxide synthase (eNOS) in cerebral capillaries and a downregulation of Cu/Zn superoxide dismutase (SOD). The imbalance in nitric oxide (NO) production and antioxidant defenses favors the formation of nitrating agents in the microvessels revealed by increased nitrotyrosine (3-nt) immunoreactivity and decreased expression of NF-κB and the dependent vascular endothelial growth factor receptor 2. NOS inhibitors and eNOS deletion as well as an SOD mimetic (CuDIPS) restore vascular endothelial growth factor receptor-2 levels and nearly abolish the vasoobliteration. NOS inhibitors and SOD mimetic also prevent O_2-induced diminished brain mass and functional deficit.

Conclusions—Data identify NO and nitrating agents as major mediators of cerebral microvascular damage, ensuing impaired brain development and function in immature subjects exposed to hyperoxia. **(*Stroke*. 2006;37:000-000.)**

Key Words: antioxidant ■ brain ■ hyperoxia ■ nitric oxide ■ vasoobliteration

Newborns that survive very preterm birth have a high prevalence of cognitive impairment in later life.[1,2] Only part of such neurodevelopmental deficits can be attributed to major neurologic insults.[3] A factor of significant pathophysiological relevance shared by preterm infants is exposure to high oxygen tension relative to that in utero. It is well recognized that supraphysiological oxygen concentrations exert toxicity to the developing lungs and retina of the premature subject, in particular to the susceptible vasculature of these tissues.[4–6]

Oxygen-induced damage can largely be attributed to reactive oxygen species.[4,7] Premature subjects transition from an intrauterine environment with arterial oxygen tension levels markedly lower than those encountered ex utero and accordingly are particularly prone to insult from reactive oxygen species because their antioxidant defenses are not yet developed. Specifically, preterm babies have lower levels of the nonenzymatic antioxidants vitamins E, A, and C compared with term ones,[8] and the lungs and retina of immature subjects are deficient in superoxide dismutase,[9,10] because this enzyme along with catalase and glutathione peroxidase mature at term gestation.[11,12]

In the lung, exposure to high oxygen induces an increase in antioxidant enzymes[10] and a decrease in nitric oxide synthase (NOS) activity, possibly as a means of attenuating O_2 toxicity.[13] In contrast, in the retina, oxygen induces a microvascular degeneration, which is mediated to a large extent by an endothelial NOS (eNOS)-dependent increase in nitric oxide

Received April 3, 2006; accepted August 9, 2006.

From the Department of Pharmacology and Therapeutics (M.S., A.D., S.C.), McGill University, Montreal, Quebec, Canada); the Department of Pediatrics and Pharmacology (M.S., F.S., P.S., P.H., S.C.), Research Center of Hôpital Sainte-Justine, Université de Montréal, Quebec Canada; INSERM (F.S.), Unité 598, Institut Biomédical des Cordeliers, Paris, France; the Departments of Ophthalmology and Neurology–Neurosurgery (A.D., P.L.), McGill University, Montreal Children's Hospital Research Institute, Montreal, Quebec, Canada; and the Department of Pharmacology (F.G.), Université de Sherbrooke, Sherbrooke, Québec, Canada.

Correspondence to Sylvain Chemtob, MD, PhD, FRCPC, Departments of Pediatrics, Ophthalmology, and Pharmacology, Research Center, Hôpital Sainte-Justine, 3175 Côte Sainte-Catherine, Montreal, Quebec H3T 1C5, Canada. E-mail sylvain.chemtob@umontreal.ca

Stroke **is available at http://www.strokeaha.org** **DOI: 10.1161/01.STR.0000245082.19294.ff**

(NO).[7,14] Although NO can exhibit either cytoprotective or cytotoxic properties in retina (and other tissues),[15,16] NO-mediated retinal vasoobliteration[14] has recently been shown to be dependent on the redox potential of the tissue favoring oxidation and resulting in detrimental nitrative/nitrosative stress and microvascular degeneration.[7] At this point, whether the molecular responses of the brain to hyperoxia resemble those of the lung or conversely of the retina remains unknown.

In the brain, hyperoxia has been found to lead to neuronal cell death and a delay in brain growth in animal models.[17] In the present study, we proceeded to explore underlying mechanisms of hyperoxia-induced brain injury in the developing subject. We used an exposure to 80% O_2, equivalent to that used to develop a retinal vasculopathy as seen in retinopathy of prematurity.[7,14] We hereby show that hyperoxia diminishes cerebral antioxidant defenses and increases NO production resulting in increased nitration and in turn microvascular degeneration and diminished brain mass and function.

Materials and Methods

Sprague-Dawley rats (Charles River; Québec, Canada) were used according to a protocol of the Hôpital Sainte-Justine Animal Care Committee.

Oxygen-Induced Microvessel Degeneration

Effects of hyperoxia on neurovascular degeneration were studied by exposing pups from birth until postnatal (P) day 6 to 80% oxygen in chambers controlled by an Oxycycler (BioSpherix Ltd) consistent with an approximately 4-fold increase in Pa_{O_2} value normally detected in utero and as used to develop a model of retinopathy of prematurity,[7,14] exposure to ≤50% O_2 yields a less severe vasculopathy as reported for the retina.[18] Control animals were maintained in room air (21% O_2); Pa_{O_2} blood values taken from the left cardiac ventricle were 81±3 mm Hg in pups exposed to 21% O_2 and 332±26 mm Hg in animals exposed to 80% O_2 (n=3 per group). We exposed animals to O_2 at this stage of development because at this stage, their postnatal brain development corresponds to that of an infant born at approximately 24 weeks gestation.[19] Long-term effects of O_2 were studied by exposing rat pups to hyperoxia until P6 and returning them to 21% O_2 until P30; animals were otherwise exposed to identical lighting conditions (12-hour daily light cycles) and fed the same rodent chow. Rats were killed at either P6 or P30. Effects of hyperoxia exposure on cerebral microvascular density were also investigated in early postnatal $eNOS^{-/-}$ mice (provided by Dr P. D'Orléans-Juste, Université de Sherbrooke, Canada) by exposing them to O_2 for 2 days. $eNOS^{-/-}$ (KO) and $eNOS^{+/+}$ (WT) mice were bred on a C57BL/6 background. Isolated brains were weighed and fixed in 4% v/v formalin. Brain sections of 50 μm were permeabilized with methanol for 10 minutes (−20°C). These were incubated with TRITC-conjugated lectin *Griffonia simplicifolia* (endothelial cell marker; Sigma-Aldrich) (1:100) in phosphate-buffered saline overnight at room temperature. Sections were washed, visualized by epifluorescent microscopy, and vasculature quantified using Image-Pro Plus 4.5 software and normalized relative to 21% O_2-exposed rats;[7] given the specificity of the lectin and our focus on vasculature, background densitometry was not a concern. Quantification was averaged on 5 to 10 sections per animal and varied by <5%. This approach to measure vascular density has been abundantly used on another neural tissue, namely the retina.[5,7,14,16,20] The microvessel density in the cortical area of 80% O_2-exposed pups was compared with that in age-matched 21% O_2-raised pups, which was assigned a value of 100%. We measured vascular density as capillary length per surface area (mm/mm^2).

Effects of Antinitrating Agents in Hyperoxia-Exposed Rat Brains

Animals exposed to normoxia or hyperoxia for 6 days were injected intraperitoneally daily with vehicle or antinitrating drugs, namely *N*-nitro L-arginine methyl ester (L-NAME 20 mg/kg; Cayman Chemical), *N*-(3-[aminomethyl]benzyl) acetamidine dihydrochloride (1400W 10 mg/kg; Cayman Chemical), 1-(2-trifluoromethylphenyl) imidazole (Trim 10 mg/kg; Cayman Chemical), or Cu(II) (3,5-diisopropylsalicylate) (CuDIPs 10 mg/kg; Calbiochem). Rats were killed by decapitation at P1 or P6 and isolated brains were weighed and processed for vascular endothelial growth factor receptor-2 (VEGFR2) western blot at P1 or stained with lectin at P6 to quantify vessel density (see previously).

Visual Evoked Potential

Visual evoked potential (VEP) is a reliable and sensitive parameter to evaluate neurologic functional alterations. VEPs were recorded at P30 from control rats (n=5), rats formerly exposed to hyperoxia from P1 to P6 days (n=5), and rats exposed to hyperoxia and concomitantly treated with L-NAME, 1400W, Trim, or CuDIPs (n=5 for each drug-treated group; concentrations as described previously). Animals were anesthetized using a mixture of 85 mg/kg ketamine and 6 mg/kg xylazine and the pupils dilated with 1% cyclopentolate hydrochloride (Mydriacyl solution; Alcon Laboratories). Animals were placed in a recording chamber that included both flash stimulus as well as background light. A subdermal needle electrode (Grass model E2) was inserted under the scalp at the lambda suture and served as the active electrode, whereas reference (Grass model E6GH; Grass Instruments) and ground (Grass model E2) electrodes were placed in mouth and tail, respectively. VEPs were evoked to flashes of white light (0.9 log $cd/sec/m^{-2}$) presented against a background light of 30 cd/m^{-2}. Each response represents an average of 100 flashes (performed with Acknowledge data acquisition system; BIOPAC Systems Inc).

Western Blotting

Brains were isolated over a time course spanning from 6 hours to 6 days. Standard SDS-PAGE techniques were followed as previously described.[7] Primary antibodies were used according to the following conditions: eNOS (1:1000 dilution), nNOS (1:1000), iNOS (1:500) (BD Biosciences Pharmingen), Cu/Zn SOD (1:1000) (Calbiochem), nuclear factor kappa B (NF-κB) (1:500) (Zymed), or VEGFR2 (1:250) (Chemicon International). NF-κB detection was performed on nuclei isolated from rat brains at 4°C.[21] Equal protein loading was ensured by probing with 1:40 000 β-actin antibody (Novus Biologicals). Densitometry was measured in pixel intensity by Image-Pro Plus.

NADPH-Diaphorase Histochemistry

NADPH-diaphorase (NADPH-d), which reflects the activity of NOS isoforms, was performed on brain sections as previously reported.[7,16]

Immunohistochemical Analysis

Brains from O_2 and room air-exposed rats at P1 were fixed in 4% formalin and transferred to 30% sucrose overnight. Cryosections (10 μm) were fixed with methanol for 10 minutes (−20°C). Immunohistochemical analysis was performed as described[16,20,21] using TRITC-labeled lectin and antibodies against eNOS (polyclonal; Transduction Laboratories), 3-nitrotyrosine (3-nt, monoclonal; Transduction Laboratories), and VEGFR2 (polyclonal; Chemicon International). Alexa-conjugated secondary IgGs were then applied to slides (Molecular Probes), and nuclei were counterstained with DAPI (Molecular Probes). Sections were assessed using epifluorescent microscopy.

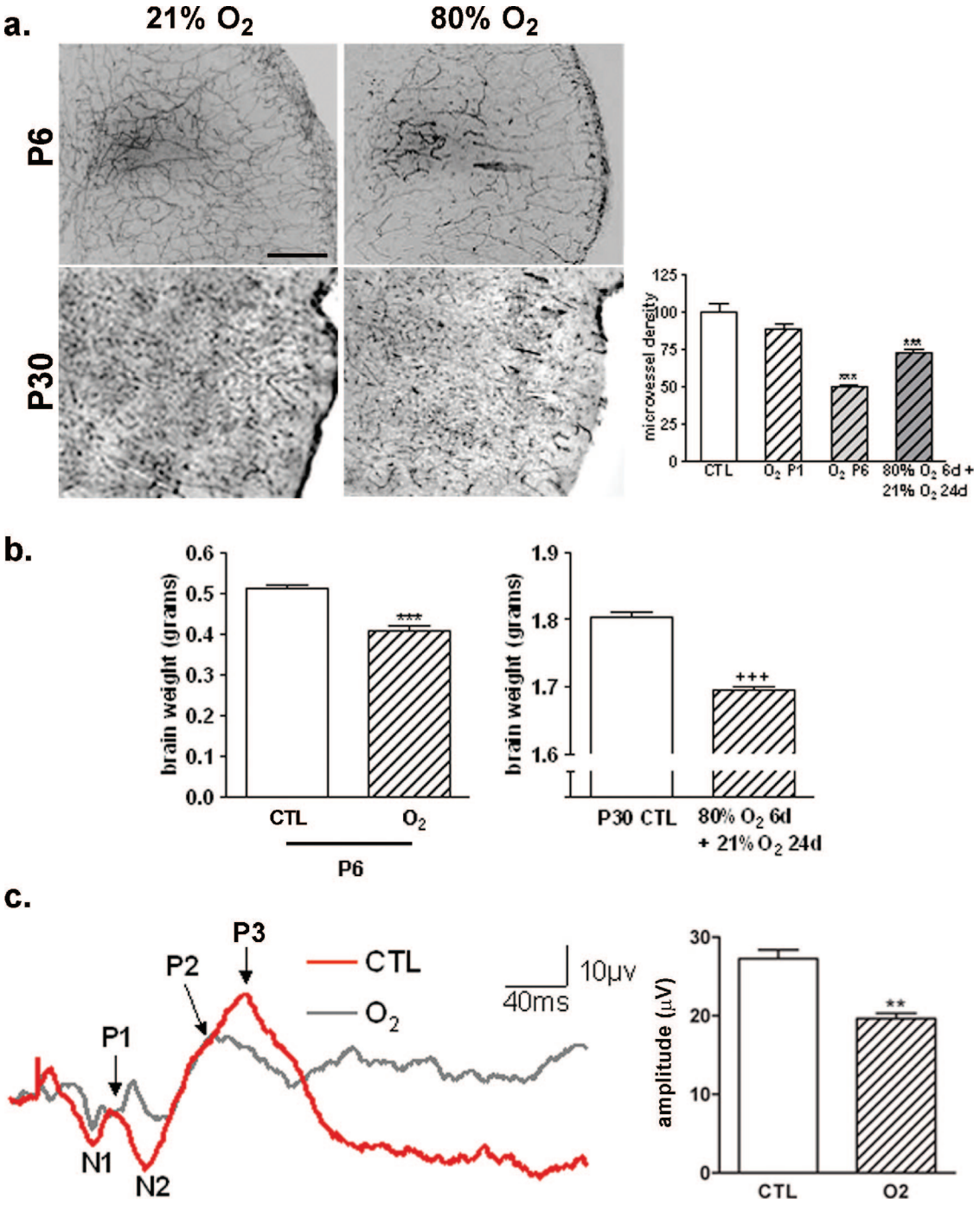

Figure 1. Effects of hyperoxia exposure on brain microvascular integrity and VEPs. (a) Lectin-stained brains from pups raised in normoxia (21% O_2) and those exposed to hyperoxia (80% O_2) until P6; also, lectin-stained brains for pups exposed to normoxia for 30 days versus those exposed to hyperoxia until P6 and returned to normoxia until P30. Scale bar=100 μm. Values in histogram are mean±SEM of vessel density in cortical region relative to that of 21% O_2-exposed rats, n=5 to 7 rats per group; ***$P<0.001$ compared with control (CTL). (b) Brain weights of CTL and O_2-exposed from birth to P6 after which exposure to normoxia was resumed until P30. Values are mean±SEM of 5 to 7 animals per group; ***$P<0.001$ compared with P6 CTL; +++$P<0.001$ compared with P30 CTL. (c) VEPs at P30 of normoxia-exposed rats (CTL) and of those exposed to 80% O_2 until P6 and then returned to normoxia until P30; records are VEPs of white light flashes. Values in histogram are mean±SEM of P_3 amplitude; 5 rats per group; **$P<0.05$ compared with CTL.

Immunoprecipitation of VEGFR2

Rat brains were isolated, homogenized in lysis buffer, and centrifuged at 8000 g for 10 minutes, and 3 mg of the resulting supernatant was reacted with anti-nt antibody (1:200) overnight at 4°C with the exception of controls. Protein A agarose beads were added to the cell lysate/antibody mixture, as well as the negative control, and rotated for 2 hours. Beads were washed with lysis buffer and samples were resolved by SDS-PAGE and probed for VEGFR2 as described previously.

Statistical Analysis

Data were analyzed by Student *t* test, one- or 2-way ANOVA, followed by post hoc Bonferroni test for comparison among means.

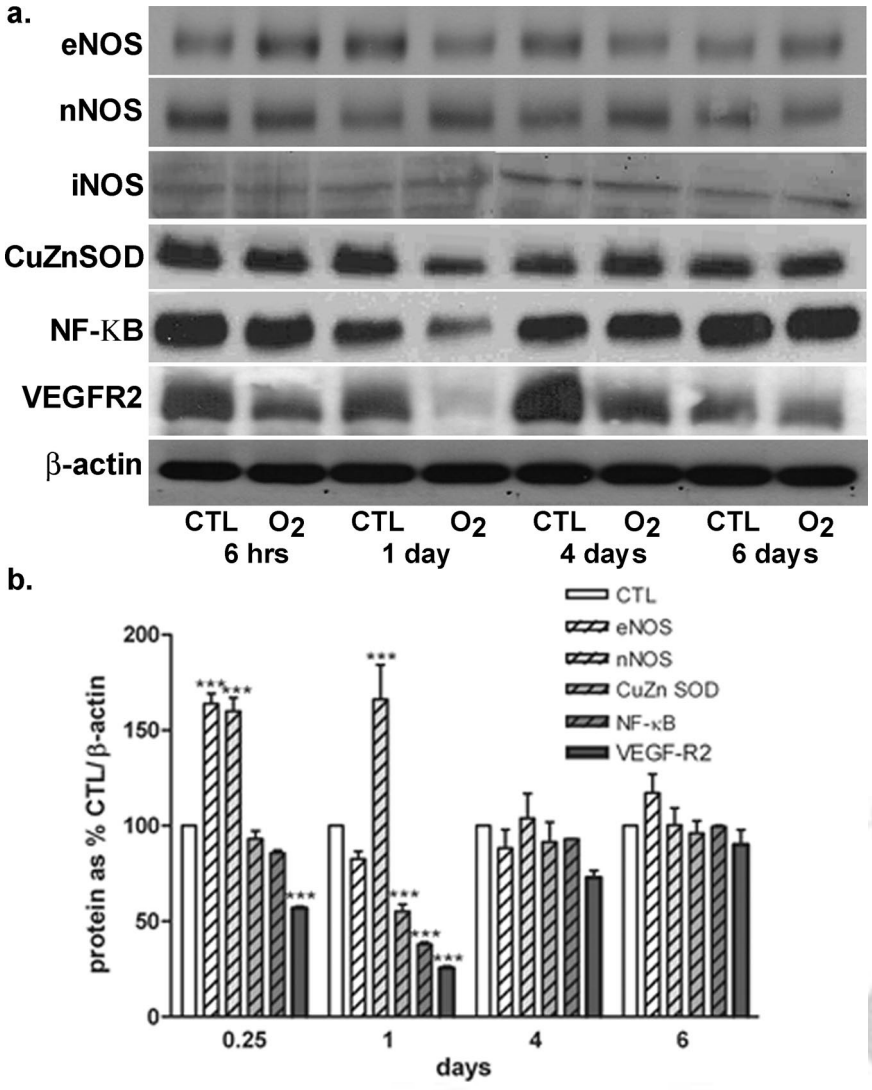

Figure 2. Expression of eNOS, nNOS, iNOS, Cu/Zn SOD, NF-κB, and VEGFR2 in rat brains after exposure to 80% O_2. a, Representative Western blots of eNOS, nNOS, iNOS, Cu/Zn SOD, NF-κB, VEGFR2, and β-actin (normalization standard) in brains (n=5 to 7) of pups exposed to 80% O_2 or room air from birth until P6. b, Values in histogram are mean±SEM of densitometry relative to that for β-actin; ***$P<0.001$ compared with corresponding time-dependent control (CTL) value.

Values are presented as mean±SEM. Statistically significance was set at $P<0.05$.

Results

Microvascular Degeneration, Diminished Mass, and Functional Deficit in the Brain of Hyperoxia-Exposed Rat Pups

Exposure to 80% O_2 from birth to P6 led to significant microvascular degeneration throughout the brain, more pronounced in the cortex, which began to be detected by 24 hours after exposure to hyperoxia (Figure 1a). The loss of vasculature was associated with a decrease in brain weight (Figure 1b). The decrease in microvascular density and brain weight persisted at P30 for pups exposed to hyperoxia for the first 6 postnatal days (Figure 1a, V). However, vessel density increased by P30, suggestive of reparative angiogenesis during the normoxic period (P7 to P30) (Figure 1a). Brain function at P30 (assessed by VEP, difficult to detect at earlier age) revealed decreased amplitude of the late component P_3 in the hyperoxia-exposed animals (Figure 1c), whereas early VEP components (N_1, P_1, N_2, P_2) were unaffected.

Expression of NOS, Cu/Zn SOD, NF-κB and VEGFR2 in Brains Exposed to Hyperoxia

NO from different NOS isoforms can exert cytotoxicity under certain circumstances.[7] We analyzed the expression of the 3 NOS isoforms. eNOS increased markedly by 6 hours on exposure to O_2 and decreased below control levels by 24 hours (Figure 2). nNOS exhibited an increase by 24 hours, which was not as pronounced as that seen after 6 hours for eNOS, but remained elevated until P4. By P6, eNOS and nNOS returned to control levels. iNOS expression remained unchanged (Figure 2).

The antioxidant enzyme Cu/Zn SOD catalyzes the conversion of $O_2^{\cdot-}$ anion into hydrogen peroxide. Cu/Zn SOD protein expression was downregulated at 6 hours and more markedly so 1 day after exposure to hyperoxia (Figure 2) and normalized subsequently. The acute reduction in SOD in the brain is consistent with that reported in the retina[22] and lungs.[10]

VEGFR2, which mediates vasoprotective effects of VEGF on neurovascular endothelium,[5] started to decrease during O_2 exposure by 6 hours and was heavily suppressed by 1 day

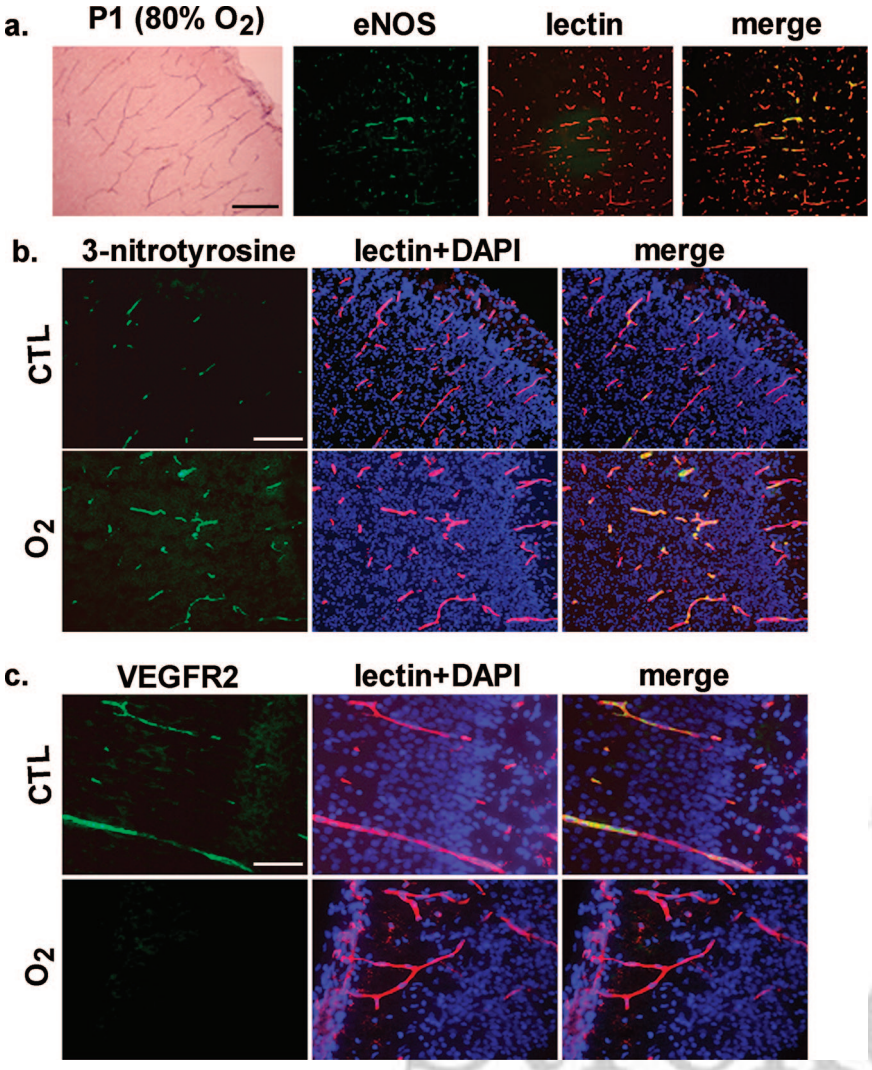

Figure 3. Expression of NADPH-d reactivity and immunofluorescence for eNOS, 3-nt, and VEGFR2 in brains after hyperoxic exposure. (a) NADPH-d staining in brain cortex 6 hours after O_2 exposure was localized along the microvasculature; eNOS (green) was mainly found on microvessels (red) as confirmed by colocalization (yellow). (b) 3-nt (green) in control (CTL) and hyperoxia-exposed pups at P1 in brain microvessels (red); merge depicts images of 3-nt and lectin staining. (c) VEGFR2 (green) at P1 reveals its localization in brain microvessels in CTL pups; merge reveals yellow staining of VEGFR2 and lectin. Hyperoxia resulted in virtual disappearance of VEGFR2 staining in microvessels (absence of yellow staining in middle panel). Blue staining (DAPI) identifies cell nuclei. Scale bar=10 μm. Images are representatives of 4 separate experiments.

(Figure 2). Immunohistology confirmed this marked reduction in VEGFR2 in microvasculature on O_2 exposure (Figure 3c); it should also be pointed out that VEGFR2 was largely localized to the endothelium (Figure 3c). By day 6, VEGFR2 expression returned to control levels. Interestingly, changes in levels of NF-κB, the transcription factor that regulates VEGFR2,[23] paralleled those of the receptor (Figure 2).

NADPH-Diaphorase Reactivity and Immunolocalization of eNOS and Nitrotyrosine

Strong NADPH-diaphorase reactivity was detected along the microvascular network of the brain cortex 6 hours after O_2 exposure (Figure 3a). This pattern matched eNOS immmunolocalization specifically to the endothelium (Figure 3a). Twenty-four hours after O_2 exposure, 3-nt levels (nitrative stress marker[24]) were markedly stronger in brain cortex microvasculature (Figure 3b).

Prevention of O_2-Induced Nitration and Microvascular Degeneration, Diminished Brain Mass, and VEGFR2 Expression

We determined the role of nitrative stress on brain microvascular degeneration and diminished brain mass by treating hyperoxia-exposed rat pups to NOS inhibitors or SOD mimetic. The NOS inhibitors (L-NAME inhibits all NOS isoforms, whereas Trim inhibits iNOS and nNOS but not eNOS) as well as SOD mimetic CuDIPs significantly attenuated O_2-induced 3-nt immunoreactivity, diminished microvascular degeneration, and preserved brain weight, whereas the iNOS-specific inhibitor 1400W was ineffective (Figure 4a through 4d). This presumed role of eNOS was corroborated in O_2-exposed $eNOS^{-/-}$ mice, which were protected against microvascular degeneration compared with $eNOS^{+/+}$ congeners (Figure 4d, bottom).

VEGFR2 plays an important role in microvascular survival during hyperoxia[5] and is affected by hyperoxic-induced nitrative stress.[7] We determined whether this major factor is nitrated under hyperoxic conditions and studied its expression in O_2-exposed animals treated with L-NAME, Trim, and CuDIPs. VEGFR2 was specifically nitrated; this effect was blocked by L-NAME (Figure 5a). VEGFR2 nitration was associated with its decreased expression, which was also prevented by L-NAME, Trim, and CuDIPS (Figure 5b). These observations were corroborated in $eNOS^{-/-}$ mice which, contrary to their wild-type counterparts, did not exhibit decreased VEGFR2 expression (Figure 5c).

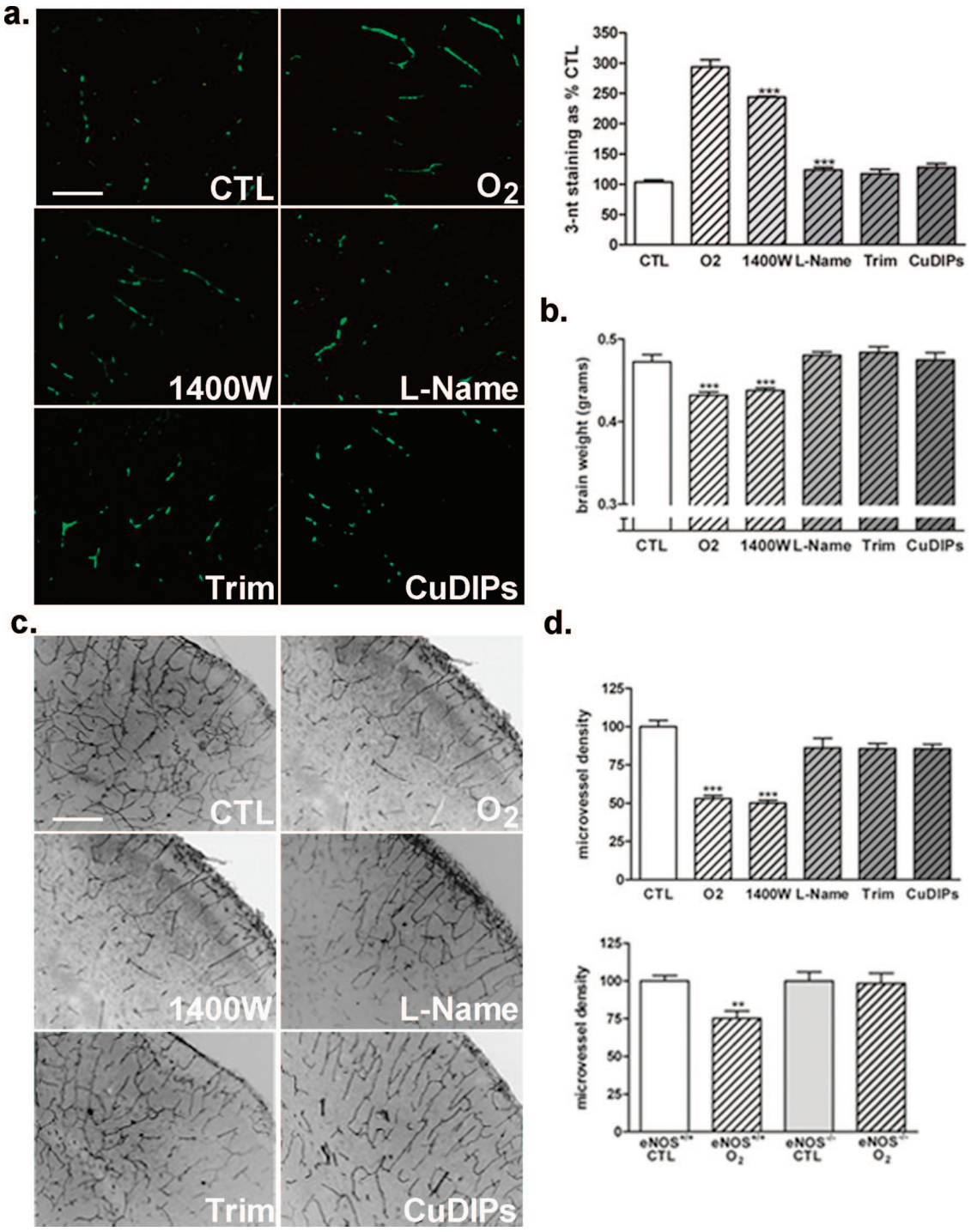

Figure 4. Effects of antioxidant treatment on O_2-induced brain vasoobliteration. (a) 3-nt immunoreactivity of hyperoxia-exposed pups treated with 20 mg/kg L-NAME, 10 mg/kg Trim, 10 mg/kg CuDIPs, and 10 mg/kg 1400W from birth to P6. (b) Brain weights of hyperoxia-exposed pups treated like in (a). (c) Representative photomicrographs of lectin-stained brain vascular of rat pups exposed to 80% O_2 from birth to P6 after treatment with vehicle, L-NAME, Trim, 1400W, or CuDIPS. Scale bar=20 μm. (d) Quantification of vessel density in cortical region of control (CTL) and O_2-exposed brains treated with drugs cited in (c) and in $eNOS^{-/-}$ and congener wild-type mice. Values in histograms are mean±SEM of 3-nt immunoreactivity (a), brain weight (b), and vessel density (d) relative to that in 21% O_2-exposed animals; n=8 brains per group. $^{**}P<0.05$ compared with CTL. Drug treatments in normoxia-exposed animals did not affect brain weight and vascular density when compared with untreated animals (not shown); the same applies to $eNOS^{-/-}$ normoxia-exposed mice compared with their wild-type congeners.

Inhibition of Nitrative Stress Prevents Altered Visual Evoked Potential

Finally, administration of L-NAME, Trim, and CuDIPs (but not 1400W) for the first 6 postnatal days during O_2 exposure maintained normal P_3 amplitudes (Table).

Discussion

The present study reveals that exposure of premature brains to hyperoxia leads to severe microvascular degeneration, diminished brain mass, and cerebral functional deficits. Hyperoxia is of significant pathophysiological relevance for

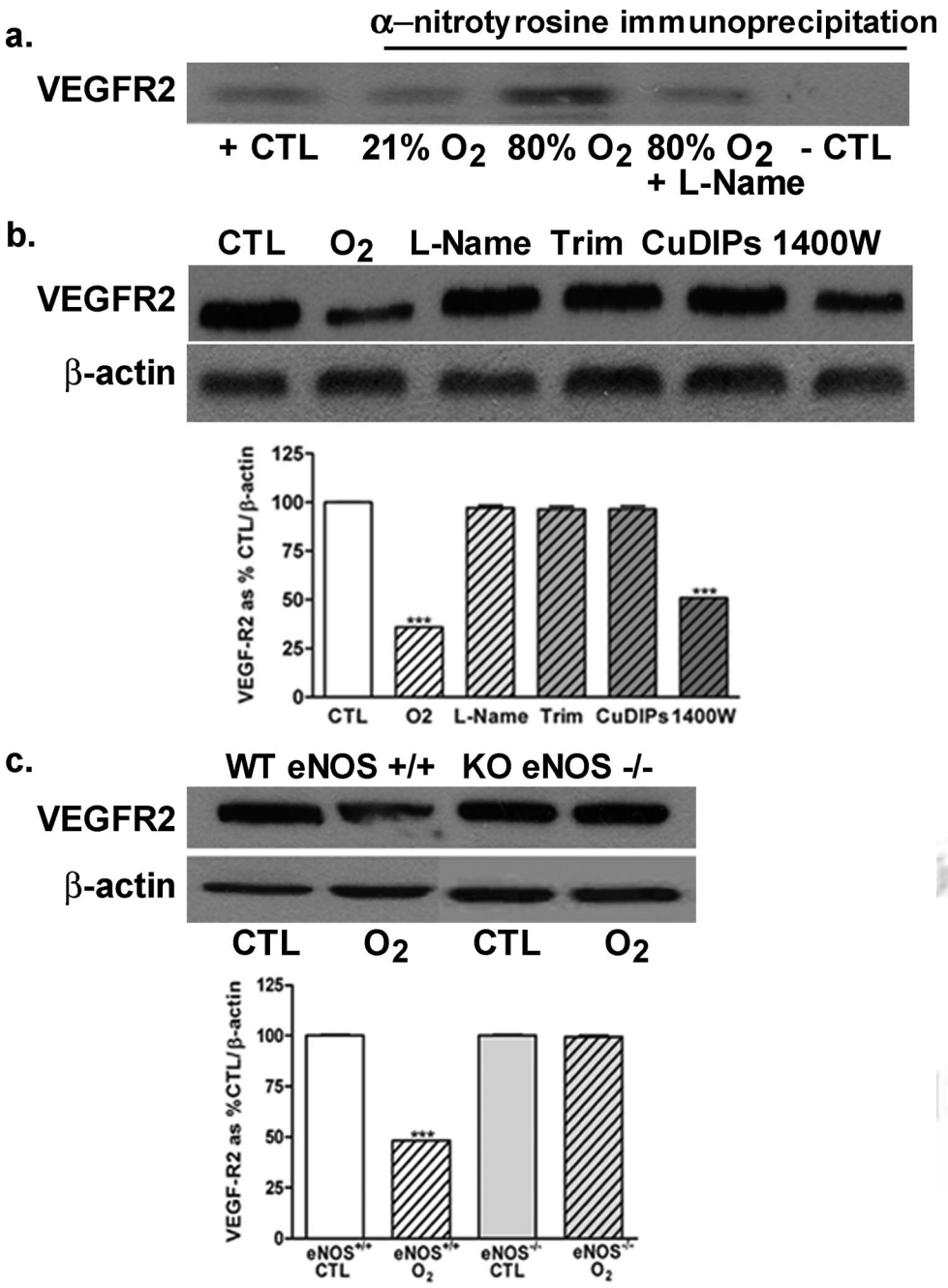

Figure 5. Effects of drug treatment or eNOS deletion on brain VEGFR2 nitration and expression after exposure to hyperoxia. (a) VEGFR2 expression in 3-nt immunoprecipitate in P6 animals treated or not with L-NAME (20 mg/kg intraperitoneal). Positive control (CTL) represents brain lysate, which did not undergo immunoprecipitation, whereas negative CTL was subjected to immunoprecipitation in the absence of 3-nt antibody. (b) VEGFR2 expression and corresponding densitometric analysis of rat pups injected intraperitoneally with L-NAME, Trim, CuDIPs, or 1400W while under hyperoxia as described in Figure 4 or of (c) CTL and O_2-exposed $eNOS^{+/+}$ or $eNOS^{-/-}$ mice. Blots were normalized to β-actin expression. Values in histograms are mean±SEM of 5 experiments. ***$P<0.001$ compared with CTL. Drug treatments in normoxia-exposed animals did not affect VEGFR2 expression when compared with untreated animals (not shown); the same applies to $eNOS^{-/-}$ normoxia-exposed mice compared with their wild-type congeners.

preterm infants that prematurely switch from an in utero environment of moderately low O_2 tension to an extrauterine milieu of relatively high O_2 concentration. Through its autoregulatory effects, hyperoxia leads to cerebral vasoconstriction in the developed subject, but this response is curtailed in the newborn.[25] However, hyperoxia leads to neuronal cell death and a delay in brain growth in animal models,[17,26] but the mechanisms underlying the neuropathology have not been investigated. Because NO is an important signaling molecule produced in various cell types in the brain, including cerebral endothelial cells[27] and exerts opposing effects on cell survival depending on the redox state,[7] we explored its role in brain injury after exposure to hyperoxia. Our data point to a prominent role for eNOS in hyperoxia-induced brain injury resulting in microvascular obliteration, brain cell death, diminished brain mass, and cerebral functional deficit.

Amplitude and Peak Time of VEP Components

Parameters	Control	Control (composite)	O_2 (composite)	O_2+L-NAME (composite)	O_2+1400W (composite)	O_2+Trim (composite)	O_2+CuDIPs (composite)
N1							
Amp	8.91±4.83	7.27	7.27	7.27	7.27	10.90	16.36
PT	36.75±7.42	35.55	35.55	35.55	35.55	35.55	35.55
P1							
Amp	18.94±7.02	18.18	14.55	25.45	16.36	20.00	21.82
PT	46.63±3.35	46.67	52.22	46.67	46.67	46.67	46.67
P3							
Amp	32.45±5.30	27.27	19.64*	32.72	20.00*	27.27	29.09
PT	113.62±10.64	124.4	107.78	113.33	107.78	124.4	124.4

Amplitude and peak time recordings of VEP parameters (N1, P1, P3) at P30 in normoxia control rats, hyperoxia-treated rats, and rats exposed to hyperoxia while receiving drug treatment. Amplitude (Amp) values in microvolts (μV); peak time (PT) values in milliseconds. Significantly different from control (*$P<0.05$).

Evidence for a significant involvement for eNOS during hyperoxia-induced brain injury includes: (1) an early increase (within 6 hours) in eNOS expression and NADPH-d reactivity, which reflects in situ NOS activity mostly confined to the microvasculature (Figures 2 and 3); findings are consistent with oxidative stress-inducing changes after ischemia[28] and more specifically with hyperoxia-induced increase in eNOS reactivity in brain and other tissues.[7,29] (2) NO-elicited cytotoxicity is largely redox-dependent[7] resulting in formation of peroxynitrite. Generation of the latter requires oxidation of NO by superoxide, which is favored by decreased levels of SOD. Indeed, an early decrease in Cu/Zn SOD expression in brain was observed in response to hyperoxia (Figure 2) as reported in other tissues.[10,22] (3) Accordingly, an early rise in indicators of nitrative stress (3-nt) mostly localized on endothelium and associated with cell death followed the corresponding early augmentation in eNOS (Figure 3 and supplemental Figure I, available online at http://stroke.ahajournals.org). (4) Pharmacological inhibition of eNOS and nNOS, but not of iNOS, or supplementation with SOD mimetic prevented hyperoxia-induced 3-nt reactivity, brain microvessel degeneration, as well as diminished brain mass and function (Figure 4, Table). The VEP P_3 wave arises from the visual cortex and enables to assess cortical function.[30] Our VEP data at P30 support the anatomic changes observed and modulated by effective treatments. (5) Finally, the specific role of eNOS in the early microvascular obliteration was confirmed in eNOS$^{-/-}$ mice (Figure 4) despite a "compensatory" increase in nNOS activity observed in these animals.[31] Nonetheless, the increase in nNOS between P1 and P4 (Figure 2), and the beneficial effect of Trim (Figures 3 and 5), does not exclude a contribution of nNOS in hyperoxia-induced injury.

An interesting feature in this study is the downregulation of the prosurvival factor VEGFR2, which preceded cell death and vasoobliteration after exposure to hyperoxia (Figures 1 and 2, supplemental Figure I). There is increasing evidence that nitrating agents lead to extracellular death not only by directly inhibiting the respiratory chain,[32] but by acting as molecules that negatively regulate the expression of signaling events.[5,7,33] For instance, tyrosine nitration has been described to downregulate plasma membrane receptors by enhancing susceptibility and targeting for proteasome degradation in endothelial cells.[34] A similar paradigm appears to apply to VEGFR2, whereby its hyperoxia-induced downregulation was prevented by eNOS inhibitors and was undetected in eNOS$^{-/-}$ mice (Figure 5).

The poorer neurodevelopmental outcome observed in premature relative to term infants cannot for the most part be only attributed to specific major neurologic insults occurring during the perinatal period.[3] Compelling evidence reveals more generalized diminished brain volume[2] and point to more subtle structural changes that are likely involved such as alterations in neuritic extensions and in synaptogenesis, cerebellar injuries, and possibly cell migration.[4,35] However, these changes intimately depend on a functional vascular structure.[36] The neural vasculature of the developing subject is particularly susceptible to oxidative stress.[6,7,20] Our data suggest that early severe microvessel loss in response to hyperoxia is an important contributing factor to decreased brain function. The results uncover an important mechanism, specifically involving nitration-elicited vasoobliteration, which contributes to elucidate the impact of subtle cortical changes on neurocognitive functional outcome of former premature subjects.[1] Therapeutic strategies aimed at diminishing nitrative stress may have the potential of diminishing brain injury evoked by hyperoxic stress.

Acknowledgments

The authors thank Hendrika Fernandez for valuable technical assistance.

Sources of Funding

Supported by grants from the Canadian Institutes of Health Research, the Heart and Stroke Foundation of Canada, and the Fonds de la Recherche en Santé du Québec (FRSQ). M.S. holds a studentship from the Heart and Stroke Foundation of Canada; F.S. and S.C. are recipients respectively of fellowship and scientist awards from the Canadian Institutes of Health Research; S.C. also holds a Canada Research Chair (perinatology). P.H. is a recipient of a scholarship from the FRSQ, and F.G. is a recipient of a Junior 1 scholarship from the FRSQ and a researcher of the Canada Foundation for Innovation.

Disclosures

None.

References

1. Stewart AL, Rifkin L, Amess PN, Kirkbride V, Townsend JP, Miller DH, Lewis SW, Kingsley DP, Moseley IF, Foster O, Murray RM. Brain structure and neurocognitive and behavioural function in adolescents who were born very preterm. *Lancet*. 1999;353:1653–1657.
2. Abernethy LJ, Cooke RW, Foulder-Hughes L. Caudate and hippocampal volumes, intelligence, and motor impairment in 7-year-old children who were born preterm. *Pediatr Res*. 2004;55:884–893.
3. Hintz SR, Kendrick DE, Vohr BR, Poole WK, Higgins RD. National Institute of Child Health and Human Development Neonatal Research Network. Changes in neurodevelopmental outcomes at 18 to 22 months corrected age among infants of less than 25 weeks gestational age born in 1993–1999. *Pediatrics*. 2005;115:1645–1651.
4. Freeman BA, Crapo JD. Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. *J Biol Chem*. 1981;256: 10986–10992.
5. Alon T, Hemo I, Itin A, Pe'er J, Stone J, Keshet E. Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. *Nat Med*. 1995;1: 1024–1028.
6. Madan A, Penn JS. Animal models of oxygen-induced retinopathy. *Front Biosci*. 2003;8:d1030–d1043.
7. Beauchamp MH, Sennlaub F, Speranza G, Gobeil F Jr, Checchin D, Kermorvant-Duchemin E, Abran D, Hardy P, Lachapelle P, Varma DR, Chemtob S. Redox-dependent effects of nitric oxide on microvascular integrity in oxygen-induced retinopathy. *Free Radic Biol Med*. 2004;37: 1885–1894.
8. Baydas G, Karatas F, Gursu MF, Bozkurt HA, Ilhan N, Yasar A, Canatan H. Antioxidant vitamin levels in term and preterm infants and their relation to maternal vitamin status. *Arch Med Res*. 2002;33: 276–280.
9. Ogawa T, Ohira A, Amemiya T. Manganese and copper–zinc superoxide dismutases in the developing rat retina. *Acta Histochem*. 1997;99:1–12.
10. Morton RL, Das KC, Guo XL, Ikle DN, White CW. Effect of oxygen on lung superoxide dismutase activities in premature baboons with bronchopulmonary dysplasia. *Am J Physiol*. 1999;276:L64–L74.
11. Gerdin E, Tyden O, Eriksson UJ. The development of antioxidant enzymatic defense in the perinatal rat lung: activities of superoxide dismutase, glutathione peroxidase, and catalase. *Pediatr Res*. 1985;19: 687–691.
12. Fujii T, Ikeda Y, Yamashita H, Fujii J. Transient elevation of glutathione peroxidase 1 around the time of eyelid opening in the neonatal rat. *J Ocul Pharmacol Ther*. 2003;19:361–369.
13. Arkovitz MS, Szabo C, Garcia VF, Wong HR, Wispe JR. Differential effects of hyperoxia on the inducible and constitutive isoforms of nitric oxide synthase in the lung. *Shock*. 1997;7:345–350.
14. Brooks SE, Gu X, Samuel S, Marcus DM, Bartoli M, Huang PL, Caldwell RB. Reduced severity of oxygen-induced retinopathy in eNOS-deficient mice. *Invest Ophthalmol Vis Sci*. 2001;42:222–228.
15. Murohara T, Asahara T, Silver M, Bauters C, Masuda H, Kalka C, Kearney M, Chen D, Symes JF, Fishman MC, Huang PL, Isner JM. Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J Clin Invest*. 1998;101:2567–2578.
16. Sennlaub F, Courtois Y, Goureau O. Inducible nitric oxide synthase mediates the change from retinal to vitreal neovascularization in ischemic retinopathy. *J Clin Invest*. 2001;107:717–725.
17. Felderhoff-Mueser U, Bittigau P, Sifringer M, Jarosz B, Korobowicz E, Mahler L, Piening T, Moysich A, Grune T, Thor F, Heumann R, Buhrer C, Ikonomidou C. Oxygen causes cell death in the developing brain. *Neurobiol Dis*. 2004;17:273–282.
18. Penn JS, Henry MM, Wall PT, Tolman BL. The range of PaO2 variation determines the severity of oxygen-induced retinopathy in newborn rats. *Invest Ophthalmol Vis Sci*. 1995;36:2063–2070.
19. Dobbing J, Sands J, Gratrix CA. Cell size and cell number: a reconsideration of organ growth and catch-up potential. *Proc Nutr Soc*. 1979;38:99A.
20. Kermorvant-Duchemin E, Sennlaub F, Sirinyan M, Brault S, Andelfinger G, Kooli A, Germain S, Ong H, d'Orleans-Juste P, Gobeil F Jr, Zhu T, Boisvert C, Hardy P, Jain K, Falck JR, Balazy M, Chemtob S. Trans-arachidonic acids generated during nitrative stress induce a thrombospondin-1-dependent microvascular degeneration. *Nat Med*. 2005;11:1339–1345.
21. Gobeil F Jr, Dumont I, Marrache AM, Vazquez-Tello A, Bernier SG, Abran D, Hou X, Beauchamp MH, Quiniou C, Bouayad A, Choufani S, Bhattacharya M, Molotchnikoff S, Ribeiro-da-Silva A, Varma DR, Bkaily G, Chemtob S. Regulation of eNOS expression in brain endothelial cells of perinuclear EP_3 receptors. *Circ Res*. 2002;90:682–689.
22. Niesman MR, Johnson KA, Penn JS. Therapeutic effect of liposomal superoxide dismutase in an animal model of retinopathy of prematurity. *Neurochem Res*. 1997;22:597–605.
23. Illi B, Puri P, Morgante L, Capogrossi MC, Gaetano C. Nuclear factor-kappaB and cAMP response element binding protein mediate opposite transcriptional effects on the Flk-1/KDR gene promoter. *Circ Res*. 2000; 86:E110–E117.
24. Greenacre SA, Ischiropoulos H. Tyrosine nitration: localisation, quantification, consequences for protein function and signal transduction. *Free Radic Res*. 2001;34:541–581.
25. Hardy P, Peri KG, Lahaie I, Varma DR, Chemtob S. Increased nitric oxide synthesis and action preclude choroidal vasoconstriction to hyperoxia in newborn pigs. *Circ Res*. 1996;79:504–511.
26. Taglialatela G, Perez-Polo JR, Rassin DK. Induction of apoptosis in the CNS during development by the combination of hyperoxia and inhibition of glutathione synthesis. *Free Radic Biol Med*. 1998;25: 936–942.
27. Garthwaite J, Charles SL, Chess-Williams R. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature*. 1988;336:385–388.
28. Wei G, Dawson VL, Zweier JL. Role of neuronal and endothelial nitric oxide synthase in nitric oxide generation in the brain following cerebral ischemia. *Biochim Biophys Acta*. 1999;1455:23–34.
29. Atochin DN, Demchenko IT, Astern J, Boso AE, Piantadosi CA, Huang PL. Contributions of endothelial and neuronal nitric oxide synthases to cerebrovascular responses to hyperoxia. *J Cereb Blood Flow Metab*. 2003;23:1219–1226.
30. Ekert PG, Keenan NK, Whyte HE, Boulton J, Taylor MJ. Visual evoked potentials for prediction of neurodevelopmental outcome in preterm infants. *Biol Neonate*. 1997;71:148–155.
31. Al-Shabrawey M, El-Remessy A, Gu X, Brooks SS, Hamed MS, Huang P, Caldwell RB. Normal vascular development in mice deficient in endothelial NO synthase: possible role of neuronal NO synthase. *Mol Vis*. 2003;9:549–558.
32. Shiva S, Oh JY, Landar AL, Ulasova E, Venkatraman A, Bailey SM, Darley-Usmar VM. Nitroxia: the pathological consequence of dysfunction in the nitric oxide–cytochrome c oxidase signaling pathway. *Free Radic Biol Med*. 2005;38:297–306.
33. el-Remessy AB, Bartoli M, Platt DH, Fulton D, Caldwell RB. Oxidative stress inactivates VEGF survival signaling in retinal endothelial cells via PI 3-kinase tyrosine nitration. *J Cell Sci*. 2005;118:243–252.
34. Kotamraju S, Tampo Y, Keszler A, Chitambar CR, Joseph J, Haas AL, Kalyanaraman B. Nitric oxide inhibits H2O2-induced transferrin receptor-dependent apoptosis in endothelial cells: role of ubiquitin–proteasome pathway. *Proc Natl Acad Sci U S A*. 2003;100:10653–10658.
35. Miller SP, Ferriero DM, Leonard C, Piecuch R, Glidden DV, Partridge JC, Perez M, Mukherjee P, Vigneron DB, Barkovich AJ. Early brain injury in premature newborns detected with magnetic resonance imaging is associated with adverse early neurodevelopmental outcome. *J Pediatr*. 2005;147:609–616.
36. Vogel G. Developmental biology. The unexpected brains behind blood vessel growth. *Science*. 2005;307:665–667.

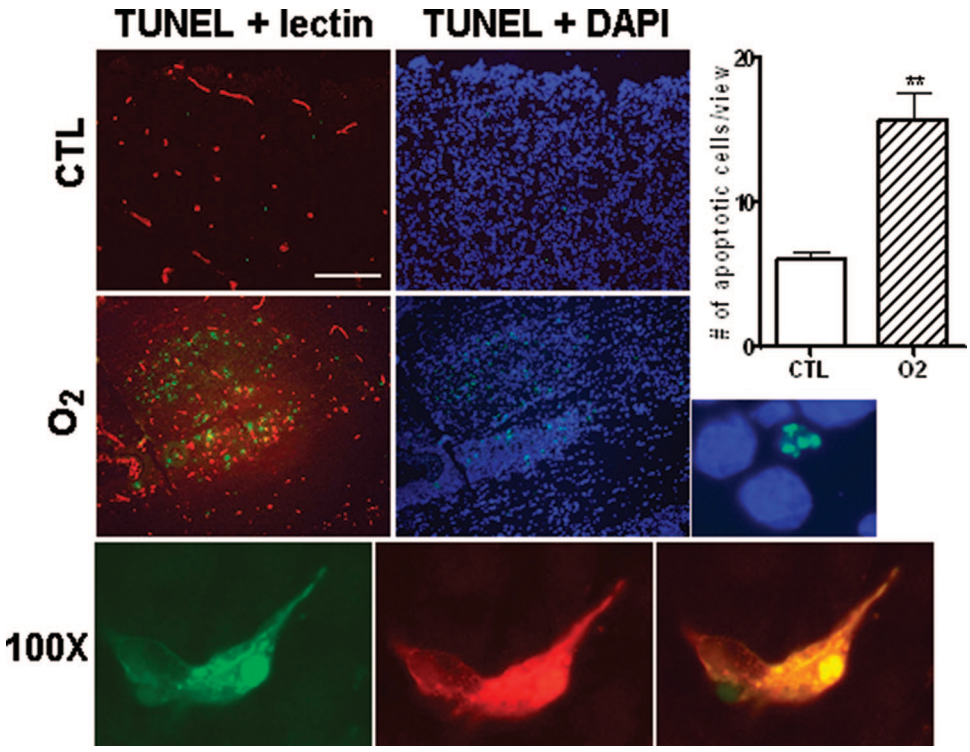

Figure I. Apoptosis in the cortex of 1-day-old brains detected by TUNEL staining. TUNEL-positivity (green) was scarce in the cortical region of CTL pups, whereas TUNEL-positive cells were abundant in O_2-exposed subjects. Blue staining (DAPI) identifies cell nuclei. TUNEL staining colocalized with lectin (red) in hyperoxia-exposed brains. 100× magnification (lower panel) shows a TUNEL-positive (green) endothelial cell (red), merge in yellow; TUNEL positivity was not only confined to vasculature. Images are representative of 3 independent experiments. Quantification (histogram) of the apoptotic cells was assessed by comparing the number of TUNEL-positive cells in the cortical region of CTL and O_2 pups in 10 cross-sections/group. Values are mean±SEM; $^{**}P<0.05$ compared to CTL values.