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% O2. 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 O2-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;37:2807-2815.)

Key Words: antioxidant ■ brain ■ hyperoxia ■ nitric oxide ■ vasoobliteration
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) acetamide 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 pups 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/m²) presented against a background light of 30 cd/m². 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. Primary antibodies were used according to the following conditions: eNOS (1:1000 dilution), nNOS (1:1000), and iNOS (1:500) (BD Biosciences Pharmingen), Cu/Zn SOD (1:1000) (Calbiochem), nuclear factor kappa B (NF-κB) (Zymed), or VEGFR2 (1:250) (Chemicon International). NF-κB detection was performed on nuclei isolated from rat brains at 4°C. 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.

Immunohistochemical Analysis

Brains from O₂ 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 using TRITC-labeled lectin and antibodies against eNOS (polyclonal; Transduction Laboratories), 3-nitrotyrosine (3-n, 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.
### 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 1.** Effects of hyperoxia exposure on brain microvascular integrity and VEPs. (a) Lectin-stained brains from pups raised in normoxia (21% O₂) and those exposed to hyperoxia (80% O₂) 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% O₂-exposed rats, n=5 to 7 rats per group; ***P<0.001 compared with control (CTL). (b) Brain weights of CTL and O₂-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% O₂ until P6 and then returned to normoxia until P30; records are VEPs of white light flashes. Values in histogram are mean±SEM of P3 amplitude; 5 rats per group; **P<0.05 compared with CTL.
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-\( \kappa \)B 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 \( O_2^- \) 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 2](http://stroke.ahajournals.org/)

**Figure 2.** Expression of eNOS, nNOS, iNOS, Cu/Zn SOD, NF-\( \kappa \)B, and VEGFR2 in rat brains after exposure to 80% \( O_2 \), a, Representative Western blots of eNOS, nNOS, iNOS, Cu/Zn SOD, NF-\( \kappa \)B, VEGFR2, and \( \beta \)-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 \( \beta \)-actin; ***\( P < 0.001 \) compared with corresponding time-dependent control (CTL) value.
Immunohistology confirmed this marked reduction in VEGFR2 in microvasculature on O2 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 O2 exposure (Figure 3a). This pattern matched eNOS immunolocalization specifically to the endothelium (Figure 3a). Twenty-four hours after O2 exposure, 3-nt levels (nitrative stress marker24) were markedly stronger in brain cortex microvasculature (Figure 3b).

Prevention of O2-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 O2-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 O2-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 hyperoxia5 and is affected by hyperoxic-induced nitrative stress.7 We determined whether this major factor is nitrated under hypoxic conditions and studied its expression in O2-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).
Finally, administration of L-NAME, Trim, and CuDIPs (but not 1400W) for the first 6 postnatal days during O₂ exposure maintained normal P₃ 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...
preterm infants that prematurely switch from an in utero environment of moderately low O2 tension to an extrauterine milieu of relatively high O2 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 cells27 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.
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 and more specifically with hyperoxia-induced increase in eNOS reactivity in brain and other tissues. (2) NO-elicited cytotoxicity is largely redox-dependent 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. (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 P3 wave arises from the visual cortex and enables to assess cortical function. 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. 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, but by acting as molecules that negatively regulate the expression of signaling events. For instance, tyrosine nitration has been described to downregulate plasma membrane receptors by enhancing susceptibility and targeting for proteasome degradation in endothelial cells. 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. Compelling evidence reveals more generalized diminished brain volume 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. However, these changes intimately depend on a functional vascular structure. The neural vasculature of the developing subject is particularly susceptible to oxidative stress. The neural vasculature of the developing subject is particularly susceptible to oxidative stress.

### Amplitude and Peak Time of VEP Components

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (composite)</th>
<th>Control (composite)</th>
<th>O2 (composite)</th>
<th>O2+1400W (composite)</th>
<th>O2+Trim (composite)</th>
<th>O2+CuDIPs (composite)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amp</td>
<td>8.91±4.83</td>
<td>7.27</td>
<td>7.27</td>
<td>7.27</td>
<td>10.90</td>
<td>16.36</td>
</tr>
<tr>
<td>PT</td>
<td>36.75±7.42</td>
<td>35.55</td>
<td>35.55</td>
<td>35.55</td>
<td>35.55</td>
<td>35.55</td>
</tr>
<tr>
<td>P1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amp</td>
<td>18.94±7.02</td>
<td>18.18</td>
<td>14.55</td>
<td>25.45</td>
<td>16.36</td>
<td>20.00</td>
</tr>
<tr>
<td>PT</td>
<td>46.63±3.35</td>
<td>46.67</td>
<td>52.22</td>
<td>46.67</td>
<td>46.67</td>
<td>46.67</td>
</tr>
<tr>
<td>P3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amp</td>
<td>32.45±5.30</td>
<td>27.27</td>
<td>19.64*</td>
<td>32.72</td>
<td>20.00*</td>
<td>27.27</td>
</tr>
<tr>
<td>PT</td>
<td>113.62±10.64</td>
<td>124.4</td>
<td>107.78</td>
<td>113.33</td>
<td>107.78</td>
<td>124.4</td>
</tr>
</tbody>
</table>

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).
Sirinyan et al  Hyperoxia Leads to Brain Microvascular Degeneration 2815

References


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

Mirna Sirinyan, Florian Sennlaub, Allison Dorfman, Przemyslaw Sapieha, Fernand Gobeil, Jr, Pierre Hardy, Pierre Lachapelle and Sylvain Chemtob

Stroke. 2006;37:2807-2815; originally published online September 28, 2006; doi: 10.1161/01.STR.0000245082.19294.ff

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/37/11/2807

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/