Effect of chronic continuous or intermittent hypoxia and reoxygenation on cerebral capillary density and myelination

Amjad Kanaan, Reza Farahani, Robert M. Douglas, Joseph C. LaManna, and Gabriel G. Haddad. Effect of chronic continuous or intermittent hypoxia and reoxygenation on cerebral capillary density and myelination. *Am J Physiol Regul Integr Comp Physiol* 290: R1105–R1114, 2006. First published December 1, 2005; doi:10.1152/ajpregu.00535.2005.—Chronic hypoxia, whether continuous (CCH) or intermittent (CIH), occurs in many neonatal pathological conditions, such as bronchopulmonary dysplasia and obstructive sleep apnea. In this study, we explored the effect of CCH and CIH on cerebral capillary density and myelination. We subjected CD-1 mice starting at postnatal day 2 to either CCH 11% oxygen (O2), or CIH 11% O2 (4-min cycles), for periods of 2 and 4 wk followed by reoxygenation for 4 wk. Mice were deeply anesthetized and perfused. Brains were removed to fixative for 24 h, then paraffin-embedded. Coronal brain sections were taken for analysis. Immunocytochemistry for glucose transporter 1 was used to assess angiogenesis, and Luxol fast blue and fluoromyelin stains were used to assess myelination. Capillary density increased after 2-wk exposure to CIH and CCH. By 4 wk, capillary density increased in both CIH and CCH by 25% and 47%, respectively, in cortex and by 29% and 44%, respectively, in hippocampus (P < 0.05). There was a decrease in myelination in the corpus callosum of mice exposed to CCH (75% of control) and CCH (50% of control) (P < 0.05). Reoxygenation reversed the increased capillary density seen in CCH to normoxic values. However, dysmyelination that occurred in CCH-exposed mice did not show any improvement upon reoxygenation. We conclude that neonatal chronic hypoxia (1) induces brain angiogenesis, which is reversible with reoxygenation, and (2) irreversibly reduces the extent of myelination in the corpus callosum. This potential irreversible effect on myelination in early life can, therefore, have long-term and devastating effects.

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Kanaan, Amjad, Reza Farahani, Robert M. Douglas, Joseph C. LaManna, and Gabriel G. Haddad. Effect of chronic continuous or intermittent hypoxia and reoxygenation on cerebral capillary density and myelination. *Am J Physiol Regul Integr Comp Physiol* 290: R1105–R1114, 2006. First published December 1, 2005; doi:10.1152/ajpregu.00535.2005.—Chronic hypoxia, whether continuous (CCH) or intermittent (CIH), occurs in many neonatal pathological conditions, such as bronchopulmonary dysplasia and obstructive sleep apnea. In this study, we explored the effect of CCH and CIH on cerebral capillary density and myelination. We subjected CD-1 mice starting at postnatal day 2 to either CCH 11% oxygen (O2), or CIH 11% O2 (4-min cycles), for periods of 2 and 4 wk followed by reoxygenation for 4 wk. Mice were deeply anesthetized and perfused. Brains were removed to fixative for 24 h, then paraffin-embedded. Coronal brain sections were taken for analysis. Immunocytochemistry for glucose transporter 1 was used to assess angiogenesis, and Luxol fast blue and fluoromyelin stains were used to assess myelination. Capillary density increased after 2-wk exposure to CIH and CCH. By 4 wk, capillary density increased in both CIH and CCH by 25% and 47%, respectively, in cortex and by 29% and 44%, respectively, in hippocampus (P < 0.05). There was a decrease in myelination in the corpus callosum of mice exposed to CCH (75% of control) and CCH (50% of control) (P < 0.05). Reoxygenation reversed the increased capillary density seen in CCH to normoxic values. However, dysmyelination that occurred in CCH-exposed mice did not show any improvement upon reoxygenation. We conclude that neonatal chronic hypoxia (1) induces brain angiogenesis, which is reversible with reoxygenation, and (2) irreversibly reduces the extent of myelination in the corpus callosum. This potential irreversible effect on myelination in early life can, therefore, have long-term and devastating effects. Luxol fast blue; fluoromyelin; obstructive sleep apnea; angiogenesis; dysmyelination

CHRONIC CONTINUOUS HYPOXIA (CCH) and chronic intermittent hypoxia (CIH) occur in several disease states in early life, such as congenital heart disease, obstructive sleep apnea (OSA), bronchopulmonary dysplasia (BPD), and asthma. OSA affects up to 2% of children (1). Some of these diseases are rather frequent in occurrence and some, like BPD, have been increasing in incidence because of various factors, including the increase in survival of very low birth weight infants (60). Affected patients often have long-term pathological consequences (5), as well as cognitive and behavioral abnormalities or deficits (7, 8, 18, 45, 50, 64).

The central nervous system (CNS) is one of the most vulnerable organs to developmental injuries. In addition, since brain development continues after birth, any major insult during early life postnataally, such as prolonged hypoxia, may have drastic effects on the CNS’s development and function.

When the mammalian brain is exposed to hypoxia, several adaptive mechanisms are activated for compensation (29). Some of the systemic adaptations include an increase in ventilation, a drop in core temperature (41), and an increase in hematocrit. CNS adaptations also include transient increases in cerebral blood flow, increased glucose transport and glycolysis (22), decreased mitochondrial energy consumption (13), and vascular modifications, such as changes in cerebral capillary density (11, 23, 39).

In this study, we focused on two processes that continue to develop in early life in the CNS. One is vessel development and the other is myelin formation. Both processes can affect multiple functions in the CNS and are, themselves, potentially subject to perturbed maturation during oxygen deprivation.

Because hypoxia can take on various forms depending on the disease condition and because the consequence of hypoxia depends on the severity and duration of exposure (4, 44), we resorted to two paradigms: one is constant and the other is intermittent over prolonged periods. Furthermore, we concentrated on the cerebral cortex and hippocampus because these have been extensively studied by many investigators, including ourselves, and hence, comparisons among studies are rather straightforward. We hypothesized that exposure of newborn mice to chronic hypoxia (continuous or intermittent) will cause an increase in cerebral capillary density and may interfere with myelin formation (dysmyelination).

MATериалS and METHODS

Animal husbandry and surgery were conducted in conformity with the “Guiding Principles for Research Involving Animals and Human Beings” of the National Institutes of Health and was approved by the Albert Einstein College of Medicine Animal Institutional Committee.

Animal Housing and Hypoxia Exposure

Pregnant CD1 mice were obtained from Charles River Laboratories (Raleigh, NC). Postnatal day 2 (P2) CD-1 pups were housed with their dams in isobaric chambers. A combination of nitrogen (N2), oxygen (O2), and carbon dioxide (CO2) was injected into the chambers through a network of tubing to achieve a final concentration of 11% O2. Flow of gases into the chambers was controlled by the Oxycycler (model A44 × 0; BioSpherix, Redfield, NY), which, in turn, is controlled by ANA-Win2 software (ver. 2.4.17 developed by Watlow Anafaze, Watsonville, CA). The concentrations of O2 and CO2 were monitored by specific electrodes. A feedback system from these electrodes to the controller continuously adjusts the opening of a set of valves to maintain the required concentration of gases while the controller continuously compares the concentration of the gases with the set point.
of solenoid values that control the flow rate of each gas and, hence, dynamically maintains a desired final gas mixture. The level of ambient CO₂ in the chambers was kept at <0.01% via a flow-through system during experiments. Ambient temperature and humidity of gases were monitored and maintained at 22–24°C and 40–50%, respectively. For CCH, the O₂ level was maintained at 11 ± 0.1% constantly. For CIH, every cycle consisted of a 4-min period during which O₂ concentration was maintained at 11 ± 0.1% followed by a 4-min period at 21 ± 0.1%. The ramp time between the two levels took <1–2 min. This cycle was repeated throughout the hypoxia exposure experiments. Normal control mice were kept in the same room and were exposed to the same level of noise and light during the duration of each experiment. Mice were exposed to either CCH or CIH for 2 and 4 wk. Some of the mice, after 4 wk of exposure, were returned to normoxia for a period of 4 wk. Another group of mice was exposed, starting at P2, to 8 wk of chronic continuous hypoxia.

Surgery and Section Preparation

Mice were taken at each time point and were deeply anesthetized with isoflurane (Aerane, Baxter; Deerfield, IL), weighed, and transcardially perfused with 0.9% saline in 0.1 M phosphate buffer, pH 7.4 followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed, postfixed in the same fixative for 24 h at 4°C, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were exposed, starting at P2, to 8 wk of chronic continuous hypoxia. CIH for 2 and 4 wk. Some of the mice, after 4 wk of exposure, were returned to normoxia for a period of 4 wk. Another group of mice was exposed, starting at P2, to 8 wk of chronic continuous hypoxia.

Cerebral Capillary Density

Immunocytochemical staining for glucose transporter 1 (GLUT-1) was performed to assess brain microvascular density. Sections, 5 μm thick, were cut, deparaffinized, hydrated, and subjected to antigen retrieval at 90°C for 10 min using 0.01 M sodium citrate buffer. Subsequently, sections were incubated with a solution of 5% normal rabbit serum and 0.3% Triton X-100 in PBS; sections were then stained using a goat polyclonal anti-GLUT-1 antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) and a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA). Color detection was carried out with the use of avidin-biotin horseradish peroxidase solution, ABC kit, and the diaminobenzidine peroxidase substrate kit (Vector Laboratories). For all experimental groups, images from the parietal cortex and hippocampus, spanning about 150 μm in thickness, were taken with the use of a Leica light microscope (Leica Microsystems AG, Wetzlar, Germany) at ×20 objective. Image J software (National Institutes of Health image, http://rsb.info.nih.gov/ij/) was used to determine the number of GLUT-1-positive capillaries per field (area of this field with ×20 objective is 0.308476 mm²). Several images from both the cortex and hippocampus were taken from each brain, and the number of capillaries per field was averaged for each region separately. The obtained values from several animals were averaged, and results were expressed, for each region, as the mean number of capillaries per field ± SD. Quantification of capillary density was performed in the following experimental groups: 1) normoxia: 2, 4, and 8 wk; 2) CCH: 2, 4, and 8 wk; 3) CIH: 2 and 4 wk; and 4) CCH: for 4 wk followed by reoxygenation for 4 wk (n = 3–8 for each experimental group).

Myelin Staining

Staining with Luxol fast blue/cresyl violet. Sections were deparaffinized and hydrated with 95% alcohol in distilled water, then stained in Luxol fast blue (LFB) solution for 2 h at 60°C or overnight at 37°C. Slides were then washed in 95% alcohol three times and rinsed in distilled water. Differentiation was started by agitation for 10 s in a jar of 0.005% lithium carbonate. Sections were differentiated for 30–60 s in 70% alcohol. All slides were rinsed in distilled water, and staining was checked with a microscope. The above three steps were repeated until gray and white matter were clearly distinguishable. Nuclei were colorless, and myelin was turquoise on a very pale gray/blue background. When counterstaining with cresyl violet was desired, sections were stained in 0.1% cresyl violet in 1% acetic acid for 10 min and then washed in distilled water. They were then rinsed and differentiated in 70% alcohol until only nuclei and nissl substance were purple (~30 s). Sections were rinsed quickly in 100% alcohol and then mounted. Images of the corpus callosum were taken using the Leica AS LMD V4 laser microdissection microscope (Leica Microsystems AG) at ×4 and ×10 magnification.

Staining with fluoromyelin. Briefly, tissue sections were rehydrated in PBT (PBS + 0.2% Triton X-100) for at least 20 min and then bathed in FluoroMyelin Green fluorescent myelin stain solution (Molecular Probes, Invitrogen, Eugene, OR) diluted 1:300 in PBS for 20 min. Slides were washed three times for 10 min each with PBS and mounted with an aqueous antifade mounting medium such as ProLong or ProLong Gold antifade reagent. Images were visualized by Zeiss Axiosvert 200 M fluorescence microscope (Carl Zeiss Microlmaging, Thornwood, NY).

Images from both LFB and fluoromyelin stains were analyzed using MetaMorph 6.1 (Universal Imaging, Downingtown, PA). The average pixel intensity from the corpus callosum region was measured and subtracted from the background intensity. The extent of staining was used as a measure of the amount of myelin. Quantification of myelin stain was performed in the following experimental groups: normoxia: 4 and 8 wk; CCH: 4 wk; CIH: 4 wk; and CCH: 4 wk followed by reoxygenation for 4 wk (n = 4 for each experimental group). The results were expressed, for each group, as the mean pixel intensity in the corpus callosum ± SE.

Statistical analysis. Quantitative data were expressed as means ± SD or means ± SE. Statistical comparisons were performed by one-way ANOVA. In all cases, P < 0.05 was considered significant.

RESULTS

Systemic Physiological Changes in Response to Hypoxia

Hematocrit, total body weight and brain weight were measured at 4 wk. After 4 wk of exposure to hypoxia, hematocrit increased in CIH (48 ± 2%) by 14% and in CCH (55 ± 3%) by 33% (Fig. 1A) compared with control (42 ± 1.5%). Body weight was decreased in CIH and CCH at 2 wk and 4 wk. At 4 wk, the decrease in body weight was 13% in CIH (23 ± 3 g) and 38% in CCH (18 ± 4 g) (Fig. 1B) compared with their age-matched normoxic controls (27 ± 3 g) (P < 0.05). Brain weight at 4 wk was slightly lower in the experimental groups than control (Fig. 1C). However, when normalized to body weight (Fig. 1D), brain weight in control was not different from that in CIH, but was slightly lower than in CCH (P < 0.05). The body weight in the reoxygenated mice was no different from age-matched normoxic controls, as there was catch-up growth. Mice exposed to 8 wk of hypoxia had a high mortality rate of about 50%, and their weights were significantly lower, 34% less, than both normoxic control and reoxygenated mice (Fig. 1E).

Effect of Hypoxia and Reoxygenation Brain on Capillary Density

Cerebral capillaries were identified by GLUT-1 immunostaining, and the capillary density was quantified by counting the number of GLUT-1-positive capillaries per field at ×20 magnification (Fig. 2A). Hypoxia exposure for 2 wk resulted in an increase in capillary density in cerebral cortex by 18% and
32% in CIH and CCH, respectively (Fig. 2C), compared with control (P < 0.05). Similarly, in the hippocampus, the capillary density increased after 2 wk of exposure by 20% and 38% in CIH and CCH, respectively (Fig. 2D) (P < 0.05). After 4 wk of exposure, capillary density had increased further, in both CIH and CCH by 25% and 47%, respectively, in the cortex (Fig. 2E) and by 29% and 44%, respectively, in the hippocampus (Fig. 2F) compared with control (P < 0.05). Mice exposed to CCH for 4 wk and then returned to normoxia for a subsequent period of 4 wk (Fig. 3A) had no significant difference in their cerebral capillary density compared with age-matched normoxic controls (P < 0.05). Capillary density in mice exposed to 8 wk of CCH was significantly higher than 8-wk normoxic control mice (P < 0.05).

**Effect of Hypoxia and Reoxygenation on Myelination**

The extent of myelination was assessed in the corpus callosum by staining with both LFB and FluoroMyelin Green (Fig. 4, A and B). After 4 wk of exposure to hypoxia, the analysis of LFB-stained sections (Fig. 4A, a–c) showed a decrease in staining, and therefore, a decrease in myelination in the corpus callosum of CIH-exposed mice (75% of control) and CCH-exposed mice (50% of control) (P < 0.05) (Fig. 4C). Analysis of the fluoromyelin-stained sections (Fig. 4A, d–f) demonstrated a decrease in the fluorescence intensity and, therefore, a decrease in myelination in the corpus callosum in CIH (77% of control) and in CCH (60% of control) (P < 0.05) (Fig. 4D), confirming the results seen with LFB stain.

The decrease in myelination that occurred in CCH-exposed mice, compared with normoxic controls, did not show any reversal upon reoxygenation. Mice exposed to CCH for 8 wk and mice exposed to CCH for 4 wk followed by 4 wk of normoxia did not show a significant difference in myelin content from each other, but both were significantly less than control (normoxia 8 wk). The quantitative analysis of the images (Fig. 4B) revealed that the myelin content in the corpus callosum of the reoxygenated mice was 55% of control with LFB (Fig. 4E), and 75% of control with fluoromyelin (P < 0.05) (Fig. 4F).

**DISCUSSION**

In this study, we have made a number of salient observations. Although CIH and CCH studies have been presented in
Fig. 2. Changes in cortical and hippocampal capillary density during prolonged exposure to hypoxia both continuous and intermittent (2 and 4 wk). A and B: glucose transporter-1 (GLUT-1)-stained sections of cortex and hippocampus in normoxia, CIH, and CCH at 2 and 4 wk of exposure. C–F: Capillary density analysis of GLUT-1-stained sections and graphical representation showing a significant increase in CIH and to a greater extent in CCH compared with age-matched normoxic controls both at 2 wk ($n = 4$) and 4 wk ($n = 8$). Values are expressed as means ± SD. *$P < 0.05$ compared with controls (normoxia). **$P < 0.05$ compared with CCH.
past publications, this is the first study, to our knowledge, that
investigated vascular density and myelination in these two
conditions. We have demonstrated in this work that CCH and
CIH cause a significant increase in capillary density in both
cortex and hippocampus after 2 wk and 4 wk of exposure. Of
major interest is that, upon reoxygenation, there is reversal of this
increased capillary density in CCH, the condition we studied.
Unlike the reversal in capillary density with reoxygenation, the
dysmyelination that occurred with CCH and CIH never improved
when the CCH-exposed group was reoxygenated.

Capillary Density in Hypoxia

As we have described in this work, CCH induces a marked
increase in capillary density in the neonatal mouse brain, and
this increase seems to be reversible upon reoxygenation. This
neonatal response to CCH is similar to that observed in the
adult rat brain, in which chronic hypoxia increases capillary
density (23), and this increase is reversed by reoxygenation
(21). We saw the same type of response in both the cortex and
hippocampus. Although these two regions are known to have
different energy requirements and blood supply (27), there was
no major difference in the relative increase in capillary density
between cortex and hippocampus.

Theoretically, the decrease in brain size seen with hypoxia
may contribute to the increase in capillary density. We reported
a 5–10% decrease in absolute brain weight with hypoxia and,
when normalized to body weight, there was no difference, but
rather a slight increase in brain weight in hypoxia compared
with control. We do not believe that the minimal changes in
brain size with hypoxia contribute significantly to the almost
50% increase in capillary density measured. One caution that
should be noted in this argument is the assumption that the
decrease in brain size is homogeneous.

Another potential limitation to the method of quantifying
capillary density is related to counting capillaries using
GLUT-1, which is known to increase in expression with
hypoxia (10, 21), leading to a more intense stain in the hypoxia
group. Although the intensity is important in detecting and
counting capillaries, even faintly stained capillaries can be
seen. We do not believe that we overestimated the increase in
capillary density during hypoxia because GLUT-1 expression
increases.

Because the brain is very sensitive to hypoxic stress, adap-
tive mechanisms, including those at the gene level, are acti-
vated very quickly, even after brief exposures (29). Some of
these mechanisms include increased erythropoiesis, cerebral

Fig. 3. Changes in cortical and hippocampal capillary density during prolonged exposure to continuous hypoxia for 4 wk, followed by reoxygenation (ReOx)
for 4 wk. A: GLUT-1-stained sections of cortex and hippocampus in 1) normoxia and 2) CCH at 8 wk. B and C: capillary density analysis of GLUT-1-stained sections and graphical representation showing an increase in CCH 8 wk compared with either 8-wk normoxia or 4-wk CCH followed by reoxygenation for 4 wk (n = 4). Values are expressed as means ± SD. *P < 0.05 when compared with controls (normoxia). **P < 0.05 compared with CCH.
blood flow (26), glucose transport (22), glycolysis, decreased mitochondrial energy consumption, and angiogenesis. Clearly, these mechanisms attempt to minimize the mismatch between supply and demand in states when ATP production is limited. The observation that reoxygenation (after a hypoxic period) induced regression of capillary density supports the belief that there is a continuous matching of capillary density and tissue oxygen level (24) and that the angiogenesis/regression seen with hypoxia/reoxygenation may only be an augmentation of an ongoing physiological process (49).

In the adult rat, the increase in capillary density is dependent on the duration of hypoxia. This response begins after 1 wk of hypoxia and peaks between 2 and 3 wk (21, 30). If vascular density is dependent on hypoxia duration, then it would be reasonable to suspect that CIH would have a smaller effect on density than CCH. Our data indeed demonstrated that this is the case. However, we believe that this response is more complicated than just the duration because 1) the relative increase in capillary density at 2 wk was not drastically different from that at 4 wk in CCH and 2) CIH includes a phase of reoxygenation, which might result in intermittent hyperoxic insults.

Although the phenomenon of increased capillary density with chronic hypoxia has been well established, the mechanisms underlying this increased density have just started to be elucidated. Hypoxia induces an increase in the expression of
HIF-1 which, in turn, upregulates the expression of vascular endothelial growth factor (VEGF), which induces angiogenesis in both newborn (25, 38, 46) and older brain (9, 12, 56). This hypoxia-induced angiogenesis is highly dependent on the interplay between VEGF and other non-HIF-regulated proangiogenic factors, of which angiopoietin is considered to have a critical role. Angiopoietin 1 (Ang-1) is essential in the interaction between the capillary endothelial cells and the surrounding supporting cells and confers stability (48) to the existing vessels through its binding to the Tie 2 receptor, a receptor tyrosine kinase, specifically found on endothelial cells. Ang-1 is constitutively expressed in the adult, unlike its endogenous antagonist (35) angiopoietin-2 (Ang-2), which is only expressed in areas of vascular remodeling and highly vascularized tumors. Ang-2 disrupts cell-cell and cell-matrix interactions of endothelial cells, thereby, facilitating angiogenesis when VEGF levels are high (47, 49). However, when VEGF levels are low, Ang-2 induces endothelial cells to undergo apoptosis and regression of vessels (49). This may be the mechanism by which the capillary endothelial cell death and regression.

Hypoxia-induced angiogenesis is not a phenomena restricted to the brain. Exposure to hypoxia causes an increase in capillary density in the myocardium (40, 61, 66). Hypoxia-induced angiogenesis is well known as a crucial step in growth and progression of tumors (17), and it plays a role in the pathophysiology of several disease entities like rheumatoid arthritis (62) and diabetic retinopathy (36).

Myelination in Hypoxia

As stated earlier, we particularly focused on myelination because this is a fundamental step in the maturation of the CNS and has not been studied in any major way as a function of oxygenation in early life. The myelination process is carried out by oligodendrocytes and is mostly postnatal. In mice, myelination begins postnatally, and the peak age of myelin accumulation is approximately P20 (P18–P25) (58). This is such a critical period that, in general, injury to myelin that occurs in the first few weeks of life may not be reversible. Many factors have been shown to interfere with proper myelination, including nutrition, ethanol, drugs (high-dose anticonvulsants) (51), and oxidative stress. In this work, we have observed that hypoxic exposure in newborn mice, whether in models of CIH or CCH, results in a significant decrease in the extent of myelination in the corpus callosum compared with age-matched controls. The only other study that has shown similar results reported that the number of myelinated axons and myelin lamellae in the infant rat corpus callosum was decreased by exposure to prolonged hypobaric hypoxia (31). In this study, we not only focused on CCH but also on CIH. Although CIH seems to have a more modest effect on myelination than CCH, we do not know the full effect of CIH on myelin function and axonal development. In fact, in similar
paradigms in the rat, CIH had a more severe effect on neuronal cell survival than CCH (19, 43).

Unlike capillary density and body weight, which reversed trends upon return to normoxia, the dysmyelination in the CCH-exposed group was irreversible with reoxygenation. In fact, the dysmyelination was even worse, albeit not significantly, with reoxygenation. Even after a few hours of exposure to continuous hypoxia, irreversible injury has been reported to occur in oligodendrocytes or axons (28, 32). Because of the possibility of irreversible injury, hypoxic insults in early life may result in long-term neurodevelopmental sequelae (64). As shown in many studies, there is a high correlation between exposure to mild and moderate neonatal hypoxia, both chronic and intermittent, and adverse cognitive and behavioral outcomes in children (18, 63).

The importance of nutrition in myelin development during early life is well established (6, 34, 52–54). It can be argued that, in this model, disruption in myelin formation could be attributed, in part, to poor nutrition. Another similar debate has already taken place in the literature related to hypoxia and body and organ size. A similar question was asked as to whether the reduction in body size is attributed to hypoxia per se or nutritional issues during hypoxia. Studies done in our laboratory have shown that culling the hypoxia-exposed litter from 8 to 4 pups or rotating mothers in the exposure chamber does not contribute to the reduction in body weight seen with hypoxia (unpublished observations). This strongly suggests that maternal responses (42) showed that neonatal growth retardation during moderate (15% O$_2$) or severe (10% O$_2$) hypoxic exposure can be almost entirely attributed to hypoxia per se or nutritional factors during hypoxia. Studies done in our laboratory have shown that culling the hypoxia-exposed litter from 8 to 4 pups or rotating mothers in the exposure chamber does not alleviate the reduction in body weight seen with hypoxia (unpublished observations). This strongly suggests that maternal nutritional effects do not play a major role and that it is hypoxic stress that induces this retarded growth. Evidence in the literature exists to support this contention. Mortola et al. (42) showed that neuronal growth retardation during moderate (15% O$_2$) or severe (10% O$_2$) hypoxic exposure can be almost entirely attributed to hypoxia and is not mediated by the maternal responses (42). A number of other studies have been done at high altitudes on children in the Andes, Ethiopian highlands, and Asian Himalayas, and it was clear that even when controlled for nutrition and other factors, there was a delay in linear growth of well-nourished children and that these patterns are established very early in life (20). In spite of such data on body growth and hypoxia, we cannot totally rule out the possibility that nutritional factors could have affected myelination, although we favor the idea that hypoxia, rather than inadequate nutrition, affected these mice.

Many alterations in protein and gene expression occur in the neonatal brain subjected to hypoxia in both neuronal axons and oligodendrocytes (33, 55, 57, 59, 65). The effect of hypoxia highly depends on the duration of exposure, as well as the developmental age. Oligodendrocytes and their precursors are the CNS cells most sensitive to hypoxic damage during early stages of development and become more resistant to hypoxia as they mature (2). It is believed that hypoxic injury to these cells contributes to the pathogenesis of myelination disturbances (57). Alternatively, axonal damage by hypoxia may present as potential mechanisms of injury (59). Whether the hypoxia-induced white matter injury is an oligopathy or an axonopathy (15), several mediators have been implicated in this process, and these include: glutamate, cytokines, and reactive oxygen and nitrogen species (3). A better understanding of the hypoxia-induced injury cascade, in both gray and white matter, which leads to dysmyelination, will help develop therapeutic strategies for a timely intervention in affected neonates.

Previous studies, including studies done by our laboratory, have investigated the potential basis for growth and development under hypoxic conditions. For example, microarray studies from Curritin et al. (14) on mice subjected to sublethal postnatal hypoxia showed an upregulation of VEGF and its receptor Flk-1 and downregulation platelet-derived growth factor-α and myelin-associated glycoprotein (MAG), as well as changes in many other genes involved with glial maturation, vasculogenesis (14). Marti et al. (37) reported an upregulation of erythropoietin (EPO) gene expression in the mammalian brain with hypoxia (37). Also, a hypoxia-induced downregulation of MAG and Nogo-A genes was reported by Weiss et al. (65) in rodent brain. Bernaudin et al.’s microarray study (10) of hypoxia/reoxygenation in neonatal rat brain showed an upregulation of HIF-1α, VEGF, EPO, GLUT-1, adenomendullin, propyl 4-hydroxylase alpha, metallothionein-1, MAP kinase phosphatase-1, RNA-binding protein CUG-BP E3-like factor (Celf), 12-lipoxygenase, and tissue-type plasminogen activator genes. We have published and have currently in press microarray data demonstrating changes in growth factor expression with hypoxia in kidney and in heart (16). Currently, we are using microarray studies to elucidate these changes in brains exposed to chronic continuous and intermittent hypoxia.

In summary, we have shown that exposure to hypoxia in the newborn stimulates an increase in cerebral capillary density and reduces myelination in the corpus callosum. We also showed that with subsequent reoxygenation, only the increase in capillary density was reversible but not the dysmyelination. Because of the possibility of residual pathology, we believe that neonatal chronic exposure to low oxygenation, whether constant or intermittent, such as in bronchopulmonary dysplasia or obstructive sleep apnea should be alleviated to prevent any long-term deficits later on in life.

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