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Decreased VEGF expression and microvascular density, but increased HIF-1 and 2 α accumulation and EPO expression in chronic moderate hyperoxia in the mouse brain

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Abstract

Normal brain function is dependent on continuous and controlled oxygen delivery. Chronic moderate hypoxia leads to angiogenesis, suggesting a modulatory role for oxygen in determining capillary density. The objective of this study was to determine physiologic and brain angiogenic adaptational changes during chronic moderate normobaric *hyperoxia* in mice. Four-month old C56BL/6J mice were kept in a normobaric chamber at 50% O₂ for up to 3 weeks. Normoxic littermates were kept in the same room outside the chamber. Freshly collected or fixed brain specimens were analyzed by RT-PCR, Western blot analysis and immunohistochemistry. Results show accumulation of hypoxia inducible factors 1 and 2 α (HIF-1 and 2 α), and increased expression of erythropoietin (EPO), cyclooxygenase-2 (COX-2) and angiopoietin-2 (Ang-2). Conversely, vascular endothelial growth factor (VEGF), and VEGF receptor-2 (KDR/Flk-1), Peroxisome proliferator-activated receptor gamma coactivator 1- α (PGC-1 α) and prolylhydroxylase-2 (PHD-2) expressions were decreased. VEGF mRNA level was diminished but there was no change in HIF-1 α mRNA and von Hippel Lindau E3 ubiquitin ligase (VHL) protein expression. Microvascular density was significantly diminished by the end of the 3rd week of hyperoxia. Overall, our results are: 1) increased expression of the potent neuroprotective molecule, EPO; 2) diminished expression of the potent angiogenic factor, VEGF; and 3) decreased microvascular density. We can, therefore, conclude that brain microvascular density can be controlled by HIF-independent mechanisms, and that brain capillary density is a continuously adjusted variable with tissue oxygen availability as one of the controlling modulators.

Keywords

COX-2; Ang-2; PGC-1 α ; PHD-2; brain angioplasticity

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1. Introduction

The brain has a high metabolic rate; its oxygen demand exceeds that of all other organs except the heart (Diringer, 2008). Although oxygen is essential for animal survival, it may become toxic at an elevated partial pressure (Allen et al., 2009; Archibald, 2003).

The cells of the brain, especially neurons, are vulnerable to the deleterious effects of excessive reactive oxygen species (ROS) produced during hypoxic and hyperoxic oxidative stress (Bitterman, 2004; Lee et al., 2005). Hence, the partial pressure of oxygen in the brain parenchyma is tightly controlled, and normal brain function is delicately sensitive to continuous and controlled oxygen delivery (Dore-Duffy and LaManna, 2007).

The brain maintains its optimal continuous supply of oxygen and nutrients by systemic and brain physiologic and angiogenic adaptational changes (Boero et al., 1999). Angiogenesis is a complex process which requires the coordinated production and interaction of multiple vascular regulating factors among which HIF, VEGF, COX-2 and Ang-2 are the critical ones (Carmeliet, 2003; Pichiule and LaManna, 2002). The oxygen-sensing HIF/PHD/VHL dependent pathway plays a central role in cellular adaptation to oxygen fluctuations (Jaakkola et al., 2001; Miro-Murillo et al., 2011; Mole and Ratcliffe, 2008). Under normoxic conditions PHD hydroxylates prolyl residues in the HIF- α subunits that are then recognized by the VHL-E3 ubiquitin ligase complex that marks them for degradation by the proteasome (Ivan et al., 2001; Mole and Ratcliffe, 2008). Various factors including hypoxia, growth factors, ROS, ketosis, and increase in extracellular pH (acidosis) are known to induce accumulation of HIF- α (Lopez-Lazaro, 2006; Lu et al., 2002; Lum et al., 2007; Mekhail et al., 2004; Puchowicz et al., 2008). When HIF- α accumulates in the cytoplasm it translocates into the nucleus and binds with the HIF- β subunit to form active HIF, which promotes expression of genes that contain hypoxia-response element (HRE) and that are involved in cellular response to the adverse milieu (Ivan et al., 2001; Jaakkola et al., 2001).

The VEGF and EPO genes are among those that are inducible by HIF (Berra et al., 2003; Semenza, 2007). VEGF-A is a potent endothelial cell mitogen and a key regulator of physiological and pathological angiogenesis (Ferrara et al., 2003). It acts through two distinct tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1), and is essential for endothelial cell differentiation as well as the sprouting of new capillaries from preexisting vessels (Ferrara et al., 2003; Hosford and Olson, 2003). Flk-1 appears to be the dominant signaling receptor mediating angiogenesis, whereas the much weaker Flt-1 appears to be involved in VEGF autoregulation (Brekken et al., 2000; Shibuya, 2001). In the brain, HIF-1 α is known to be involved in angiogenesis through induction of VEGF, though VEGF expression is also known to be induced by PGC-1 α , independent of HIF-1 α (Arany et al., 2008; Benderro and LaManna, 2011; Chinsomboon et al., 2009; Ndubizu et al., 2010). In adults, systemic EPO is mainly produced by the kidneys and in small amounts by the liver, and is essential for maintenance of tissue oxygen homeostasis by stimulating red blood cell production (Fisher, 2003). There is also paracrine EPO production in the brain parenchyma (Bartesaghi et al., 2005; Rabie and Marti, 2008). It has been demonstrated that HIF-2 α is the main regulator of brain erythropoietin production, which is known to be a key neuroprotective factor in central nervous system (Chavez et al., 2006; Ponce et al., 2012; Rabie and Marti, 2008; Xiong et al., 2011). As a neuroprotective agent, EPO antagonizes glutamate cytotoxic action, enhances antioxidant enzyme expression and reduces the free radical production rate (Bartesaghi et al., 2005).

The COX-2/Ang-2 pathway is also essential during stress responses and angiogenic remodeling (Liang and Jiang, 2009; Wilkinson-Berka et al., 2003). Ang-2 induction is known to occur mainly by COX-2 enzyme activities, independent of the HIF induction

pathway (Pichiule et al., 2004). Ang-2 appears to be an important signaling molecule during vascular remodeling, both increases and decreases in microvascular density (Dore-Duffy and LaManna, 2007). During vascular remodeling Ang-2 causes destabilization of microvessels, and if a sufficient amount of VEGF is present subsequent endothelial cell growth, proliferation, and formation of capillary sprouts leads to angiogenesis. However, with an insufficient amount or absence of VEGF, the activities of Ang-2 lead capillaries to undergo apoptotic regression (Pichiule and LaManna, 2002). Ang-2 is expressed at low levels in most normal adult brain, but is strongly upregulated during hypoxia, re-oxygenation after hypoxia and at sites of active vessel remodeling, such as ovarian and tumor tissues (Carmeliet, 2003). It is upregulated at times of both growth and regression, suggesting that it plays an active role in angioplasticity (Koh et al., 2002). Also there are reports showing an increase in COX-2 and Ang-2 expression in rodent lungs, retina and cerebral cortex in response to oxidative stress in acute hyperoxia (Bhandari et al., 2006; Liang and Jiang, 2009; Perez-Polo et al., 2011). However, influence of chronic hyperoxia on COX-2 and Ang-2 expression and comparative effects through the HIF/VEGF pathway, and microvascular density in the brain have not been defined.

There are numerous reports on the effects of short term hyperbaric and normobaric hyperoxia on the brain, but effects of chronic hyperoxia on brain acclimatization changes (molecular, structural or angiogenic) have not been established. Hence, in this study we investigated the effects of chronic moderate normobaric hyperoxia (50% O₂) on expression of the main angiogenic growth factors and their activities on brain vascular remodeling. Increasing oxygen partial pressure tends to produce an increased vasoconstrictive tone which somewhat limits the overall levels to which brain tissue oxygen tension rises, but does not reverse it. Measurements of tissue oxygen partial pressure demonstrated increased oxygen and increased hemoglobin oxygen saturation (Bulte et al., 2007; Demchenko et al., 2000; Duong et al., 2001; LaManna, 2007).

We previously showed that lowering oxygen in the inspired air led to angiogenesis, suggesting a modulatory role for oxygen in determining capillary density (Benderro and LaManna, 2011; Ndubizu et al., 2010; Pichiule and LaManna, 2002). These observations led to the question whether capillary density was proportional to oxygen delivery and whether the main mechanism linking tissue oxygen partial pressure and capillary density was the HIF/VEGF pathway. The results demonstrate that hyperoxia does indeed result in decreased capillary density consistent with a fall in VEGF, but also showed a seemingly paradoxical increase in HIF-1 and 2 α accumulation. HIF-1 α mRNA levels were not affected, but PHD-2 expression was diminished in chronic hyperoxia suggesting that HIF- α accumulation, leading to upregulation of EPO, was due to reduced posttranslational degradation. Decreased VEGF expression could have been due to a decrease in PGC-1 α , implying the importance of this molecule in the VEGF induction pathway. The remodeling signaling, COX-2 and Ang-2, protein levels were also upregulated.

2. Results

2.1. Change in body weight, hematocrit and arterial blood oxygen saturation during hyperoxia

All mice kept in 50% oxygen normobaric hyperoxia for up to 3 weeks survived throughout hyperoxic exposure. Compared to their normoxic littermates the mice in hyperoxia tended toward a stunted body weight increase. But the difference in body weight between normoxic and hyperoxic mice throughout 3 week exposure did not reach statistical significance (Fig. 1A and 1B). Hematocrit was significantly decreased ($p < 0.05$) at day 4 and beyond, of hyperoxic exposure (Fig. 1C). The hematocrit values decreased from $46.4 \pm 1.3\%$ in normoxia to $35.3 \pm 1.7\%$ by the end of the 3rd week of hyperoxia. The arterial oxygen

saturation was significantly increased ($p < 0.05$) from about 96% under normoxic condition to 99% under all hyperoxic durations (Fig. 1D).

2.2. Arterial blood gas content during hyperoxia

For mice placed in 50% O₂ hyperoxia, arterial blood pH and CO₂ content did not show significant change but the arterial blood O₂ content was significantly increased ($P < 0.05$) compared to normoxic controls (Table 1).

2.3. HIF-1 α and HIF-2 α protein accumulation in cerebral cortex

Since HIF-1 and 2 α are early responding transcription factors for the induction of many genes involved in oxidative stress, adaptive responses and angiogenic remodeling (Fong, 2008; Mole and Ratcliffe, 2008; Semenza, 2011), we examined the response of these transcription factors during hyperoxia. Immunoblot analysis demonstrated a significant ($p < 0.05$) accumulation of both HIF-1 and 2 α (at about 120 kDa) in the 1st week of hyperoxia which declined with prolonged hyperoxic durations (Fig. 2A and 2B).

2.4. VEGF and Flk-1 expression in the cerebral cortex

VEGF and its receptor (Flk-1) are critical factors during new vessel formation (angiogenesis) or vascular remodeling (Ferrara et al., 2003; Mizukami et al., 2007). Western blot analysis showed diminished VEGF and Flk-1 protein expression, at 23 kDa and about 180 – 200 kDa respectively, throughout hyperoxic periods (Fig. 3A). These decreases in VEGF and Flk-1 levels in chronic moderate hyperoxia were statistically significant ($p < 0.05$) relative to their normoxic expression (3B).

2.5. HIF-1 α and VEGF RT-PCR, PHD-2 and VHL expressions in cerebral cortex

In order to determine whether the increase in HIF-1 α or the decrease in VEGF protein levels were due to the effect of hyperoxia on their transcriptional activation, we measured their mRNA levels in the first week of hyperoxic exposure (Fig. 4A). RT-PCR analysis indicated that HIF-1 α did not show significant change in m-RNA content in hyperoxia relative to its normoxic expression. However, VEGF m-RNA expression tended to be decreased in hyperoxia. Since the RT-PCR result revealed that HIF-1 α accumulation was not due to increase in its synthesis, we explored if hyperoxia has effects on the posttranslational degradation system of HIF- α . In this regard PHD-2 and VHL are vital components of HIF- α degradation (Ivan et al., 2001; Jaakkola et al., 2001). Hence, we probed for the level of expression of both these molecules in hyperoxia. The results indicated significantly ($p < 0.05$) blunted expression of PHD-2 (protein bands at about 46 kDa), mainly in the first week of hyperoxia, and no significant change in VHL expression (bands at about 18 kDa) in hyperoxia compared to normoxia (Fig. 4B and 4C).

2.6. PGC-1 α and EPO protein expression in the cerebral cortex

PGC-1 protein level (characteristic band at about 91 kDa) was significantly decreased ($p < 0.05$) in chronic hyperoxia, compared to its relative expression in normoxia. Of note, PGC-1 α , which is known to be a HIF independent inducer of VEGF (Arany et al., 2008; Chinsomboon et al., 2009), showed a similar trend of expression with VEGF in hyperoxia (Fig. 3). Conversely, EPO protein level (bands at about 34 kDa) was significantly increased ($p < 0.05$) in all durations of chronic hyperoxia (Fig. 5A and 5B). It was demonstrated that EPO expression in brain was mainly regulated by HIF-2 α (Chavez et al., 2006). This may explain the similar trend in expression of HIF-2 α (Fig. 2) and EPO (Fig. 5) in cerebral cortex of the mouse during hyperoxia.

2.7. COX-2 and Ang-2 expression in the cerebral cortex

HIF independent induction of Ang-2 by COX-2 was well established (Pichiule and LaManna, 2002). Western blot analysis showed a similar and strong expression of COX-2 (band at about 72 kDa) and Ang-2 (band at about 70 kDa) in hyperoxia (Fig. 6A). Both COX-2 and Ang-2 protein expressions were significantly increased ($p < 0.05$) in all durations of chronic moderate hyperoxia of 3 weeks (Fig. 6B).

2.8. Microvascular density in the cerebral cortex

The vascular network of the mature rodent brain is relatively stable with quiescent endothelial membrane, but metabolic stressors are known to induce microvascular remodeling (Dore-Duffy and LaManna, 2007; Sharp and Bernaudin, 2004). In this study cerebral capillaries were identified by GLUT-1 immunostaining and their density was quantified by counting the number of positively stained capillaries per mm^2 . Mice exposed to chronic moderate hyperoxia showed a moderate decrease in microvascular density in the 1st and 2nd weeks, which became statistically significant ($p < 0.05$) at the end of the 3rd week of exposure (Fig. 7A and 7B). The microvascular density decreased from $435 \pm 22/\text{mm}^2$ in normoxia to $426 \pm 34/\text{mm}^2$ in the 1st week, $408 \pm 30/\text{mm}^2$ in the 2nd week and $390 \pm 23/\text{mm}^2$ (about 9% decrease) in the 3rd week of chronic moderate hyperoxia.

3. Discussion

The primary finding in this study was that chronic hyperoxic exposure leads to brain capillary regression and decreased microvascular density. Considered with the previous finding of angiogenesis and increased capillary density during chronic hypoxic exposure, this study strongly implies that brain capillary density is proportional to stable ambient oxygen availability.

Vascular remodeling is a fundamental physiological process during development and acclimatization to adverse environments (Carmeliet, 2003; Ferrara et al., 2003). This complex process requires coordinated production and signaling interaction of multiple growth factors involved in angioplasticity (Dore-Duffy and LaManna, 2007; Tham et al., 2002). Among these factors, the balance between VEGF and Ang-2 may be the primary determinant of the balance between capillary growth and capillary regression, and is known to play a major role in physiological and pathological conditions such as hypoxia and ischemia (Pichiule and LaManna, 2002). In this study we report progressive vascular regression in chronic moderate hyperoxia, which was correlated with an imbalance between VEGF and Ang-2 protein expression throughout hyperoxic exposure. We also documented significantly increased transient accumulation of HIF-1 and 2 α during hyperoxic exposure.

Due to its importance as a transcriptional activation factor favoring the encoding of numerous genes responsible for angiogenic, neuroprotective and metabolic functions such as VEGF, EPO, glucose transporters and glycolytic enzymes, for almost two decades research has focused on HIF- α as a key regulator of the cellular oxygen response system (Fong, 2008; Semenza, 2011). Accordingly, if oxygen tension were the controlling signal for HIF- α accumulation, it would have been expected that HIF-1 and 2 α accumulation should be decreased in hyperoxia. We have instead recorded paradoxical accumulations of HIF-1 and 2 α during hyperoxia, and the main factor for their accumulation seems to be reduction in level of essential molecule (PHD-2) in their degradation pathway. PHDs and VHL are the main regulators of HIF- α degradation (Ivan et al., 2001; Jaakkola et al., 2001). It was shown that among the PHDs, PHD-2 is the major isoform in controlling HIF-1 and 2 α stability with PHD-1 and 3 having very little or no effect on HIF- α degradation (Berra et al., 2003; Mole and Ratcliffe, 2008). Oxidative stress by an increase or decrease in the cellular oxygen

concentration (hyperoxia and hypoxia respectively) results in generation of excess ROS at complex III of the mitochondrial respiratory chain which inhibits PHD-2 activity (Guzy and Schumacker, 2006; Semenza, 2007). Thus, decreased PHD-2 expression, with its reduced activity as demonstrated before (Guzy and Schumacker, 2006; Semenza, 2007), may have resulted in reduced HIF-1 and 2 α degradation, explaining their accumulation in hyperoxia.

Consistent with the increased HIF-1 and 2 α accumulation was the upregulation of EPO which has an HRE and whose induction is dependent on HIF activation (Chavez et al., 2006; Vazquez-Valls et al., 2011). EPO is an endogenous mediator of neuroprotection in the CNS (Ponce et al., 2012; Rabie and Marti, 2008; Xiong et al., 2011), and it may be that the stabilization of HIF-1 and 2 α during hyperoxia is essential for the neuroprotective response to the toxicity of excessive free radicals of O₂ which are produced during hyperoxia.

VEGF also has an HRE, but was not upregulated in hyperoxia. Though HIF-1 α is known to be a transcriptional activator of VEGF, there are other regulatory signaling pathways that are involved in VEGF expression as co-activators or independent of HIF regulators (Arany et al., 2008; Fong, 2008; Mizukami et al., 2007; Ndubizu et al., 2010). Among these, the importance of PGC-1 α in VEGF transcriptional activation was previously demonstrated (Arany et al., 2008; Chinsomboon et al., 2009). Reduced transcriptional activation of VEGF gene and protein expression could be explained by these alternative mechanisms. In this study we report attenuated protein levels of PGC-1 α during hyperoxia. Association of diminished VEGF m-RNA as well as protein expression to diminished PGC-1 α protein level in chronic hyperoxia implies a significant role of this component in the VEGF induction pathway during hyperoxia.

A multitude of factors are involved in the angiogenic response during vascular remodeling, but the VEGF and the angiopoietin families of proteins have more endothelial specific control effects (Tham et al., 2002). Among the angiopoietin family proteins (Ang1 – 4), Ang-2 plays a major role in vascular angioplasticity (Holash et al., 1999; Pichiule and LaManna, 2002). Ang-1 is constitutively expressed and is responsible for tight mechanical integrity of the brain endothelial cell membrane junctions through activation of the Tie-2 endothelial cell receptor (Hori et al., 2004; Sundberg et al., 2002). Ang-2 is induced during conditions that favor angioplasticity, and acts to block the Tie-2 receptor, preventing Ang-1 activation and de-stabilizing the capillary (Eklund and Olsen, 2006; Hori et al., 2004). Ang-2 expression is mainly regulated by COX-2 (Pichiule et al., 2004). Here we observed a robust increase in expression of both COX-2 and Ang-2 during the hyperoxic periods. Hyperoxic induction of Ang-2 expression in the lungs and its mediation of acute lung injury and endothelial regression has been previously reported in rodent and human (Bhandari et al., 2006; Bhandari and Elias, 2007). Ang-2 is pro-angiogenic in the presence of VEGF, and acts as an anti-angiogenic factor during vascular remodeling in the reduction or absence of VEGF (Dore-Duffy and LaManna, 2007; Pichiule and LaManna, 2002). Hence, increased Ang-2 levels, together with decreased VEGF may have compromised the integrity of some of the microvessels, leading to capillary regression and reduced microvascular density. Decreased Flk-1, the main angiogenic VEGF receptor, may be a reflection of the decreased numbers of capillaries, assuming no change in the receptor density.

Although various studies on hyperoxic cell death in lung tissue in vitro and vivo demonstrated involvement of both apoptotic and necrotic pathways (Ahmad et al., 2005; Mantell et al., 1999; Bhandari and Elias, 2007), the mechanism involved in the mouse brain was not examined in this study. However, we have previously shown under analogous conditions in the rat brain that capillary regression during re-oxygenation after 3 weeks of hypoxic acclimatization was associated with TUNEL (terminal transferase deoxyribonucleotidyl dUTP nuclear end labeling) and caspase-3 positivity in endothelial

cells and pericytes (Pichiule and LaManna, 2002). Also the decrease in capillary density reported here is similar to what was observed during re-oxygenation after chronic hypoxia (Pichiule and LaManna, 2002). No evidence for wide spread blood brain barrier disturbance was noted in that study. It is likely that capillary regression occurred over an extended period with no significant leak at the blood brain barrier. This acclimatization change is apparently a functional, or physiological angioplasticity, and not a pathological degradation, at least in the short term.

It remains unknown if the change in capillary density is accompanied by a change in cerebral blood flow. In hypoxic acclimatization, cerebral blood flow is initially and transiently elevated prior to any increase in capillary density. By the time that acclimatization process is complete (3 weeks) the blood flow has returned to baseline. Hence, increased capillary density is not necessarily associated with increased perfusion, though it does affect overall cerebral circulation by increasing the capillary mean transit time (Xu and LaManna, 2006). It is possible that the same relationship between capillary density and cerebral perfusion will be found in hyperoxia, but this remains to be determined.

In summary, our results suggest that the adaptive response during chronic moderate hyperoxia includes 1) increased expression of the potent neuroprotective molecule, EPO; 2) diminished expression of the potent angiogenic factor, VEGF; and 3) decreased microvascular density. We can, therefore conclude that brain microvascular density can be controlled by HIF-independent mechanisms, and that brain capillary density is a continuously adjusted variable with tissue oxygen availability as one of the controlling modulators.

4. Experimental procedures

4.1. Exposure to chronic hyperoxia

Male 4-month old C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and maintained at the Case Western Reserve University Animal Care Facility for at least a week before experiments. The experimental protocol was approved by the Institutional Animal Care and Use Committee. Mice that were exposed to hyperoxia were placed in a normobaric chamber (Oxycycler™; BioSpherix Ltd., Lacona, NY), to which computer regulated 50% O₂ was supplied for up to 3 weeks. The littermate, normoxic control mice were housed in the same room next to the chamber to match ambient conditions.

4.2. Measurement of physiological variables

Arterial oxygen saturation (SaO₂), which is hemoglobin oxygen saturation in arterial blood, was recorded in a subgroup of mice kept in normoxia or hyperoxia using a MouseOx™ Pulse Oximeter (STARR Life Sciences Corp., Oakmount, PA), as described earlier (Benderro and LaManna, 2011). For arterial blood gas content measurement, in a separate group of mice, anesthesia was induced by 2.5% isoflurane in medical air and maintained with 1-2% isoflurane through a nasal cone in normoxia or hyperoxia. A cannula was placed in common carotid artery using polyethylene tubing (PE-10, 0.011" i.d., 0.024" o.d.). The mice were allowed to recover for at least 1 hour after surgery while in normoxia or hyperoxia. After an hour blood samples were obtained from the carotid artery using a Natelson blood collecting tube (Fisher Scientific, Pittsburgh, PA) and arterial blood pH, O₂, and CO₂ values were recorded by ABL5 blood gas analyzer (Radiometer Medical, Copenhagen, Denmark).

Mice were weighed daily during the experiment and blood samples were obtained for hematocrit determination before the animals were sacrificed under anesthesia. Mice were

either sacrificed for collection of fresh samples or perfused transcardially with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA), for in vivo fixation of the tissues.

4.3. Preparation of whole cell lysates and Western blot analysis

Tissue lysate preparation and Western blotting was done as described previously (Benderro and LaManna, 2011). Briefly, fresh cerebral cortex was dissected and immediately frozen in liquid nitrogen and stored at -80 °C until further processing. Frozen brain cortex was homogenized in ice-cold RIPA lysis buffer with protease inhibitor (Complete Mini, Roche Diagnostics, Indianapolis, IN). Homogenates were centrifuged and protein contents in the supernatants were determined by a Bradford protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as a standard. Proteins were separated by using SDS gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were blocked in 5% skimmed milk in tris-buffered saline with 0.1% tween (TBS-T), and incubated in the same blocking solution with the primary antibodies. The specific primary antibodies used were: HIF-1 and HIF-2 α (1:500; R&D Systems, Minneapolis, MN), VEGF-A, FLK-1 and EPO (1:750; Santa Cruz Biotechnology, Santa Cruz, CA), Ang-2 (1:200; Millipore Co., Billerica, MA), COX-2 (1:150, Cayman, Ann Arbor, MI), PGC-1 (1:750; Novus, Littleton, CO), PHD-2 (1:500; Novus, Littleton, CO), VHL (1:500; Santa Cruz, CA), β -actin (1:2000; Santa Cruz, CA) and β -Tubulin (1:3000, Cell Signaling Tech., Beverly, MA). The membranes were washed with TBS-T, followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies (Millipore Co., Billerica, MA). Immunoreactive protein bands were visualized using an enhanced chemiluminescence detection system (Super Signal ECL Kit, Thermo Scientific, IL) and subsequent exposure of the membrane to Hyperfilm (Thermo Scientific, IL). Western blots were scanned and densitometry of protein bands were measured and normalized to that of β -actin (optical density ratio) using ImageJ, an open-source Java image-processing software from the NIH (<http://rsb.info.nih.gov/ij/>).

4.4. Real-time PCR analysis

At the indicated time points, brains were harvested and total RNA was extracted using the RNA extraction kit (Qiagen). Single-stranded cDNA was synthesized from a total of 2 μ g RNA using the High Capacity cDNA, Reverse Transcription Kits (Applied Biosystems, Foster City, CA). Real-time PCR analysis was performed with 0.5 μ l of the final cDNA synthesis mix using mouse-specific Taq-Man-based gene expression assays (Applied Biosystems, Foster City, CA). The following assays were used: HIF-1 α (catalog # Mm00468869_m1), VEGF-A (catalog # Mm00437304_m1), β -actin (catalog # Mn00607939_s1). The PCR reaction was performed in an ABI 7500 real time PCR thermocycler (Applied Biosystems). All reactions were performed in triplicate using β -actin as an endogenous control.

4.5. Immunohistochemistry and determination of the capillary density

Tissue collection and immunohistochemistry were done as described previously (Benderro and LaManna, 2011). Briefly, the brain samples were dehydrated, embedded in paraffin, and coronal serial sections (5 μ m) were cut with a microtome. The sections on slides were deparaffinized, subjected to antigen retrieval at 90 °C for 10 minutes in 0.1M sodium citrate buffer, and endogenous peroxidase were quenched with 3% H₂O₂. The sections were washed with 1X PBS, blocked in 10% normal horse serum, incubated with goat polyclonal primary antibody against glucose transporter-1 (GLUT-1) (1:200; Santa Cruz, CA), washed again and covered with biotinylated horse anti-goat secondary antibody (Vector Labs, Burlingame, CA). Then the sections were covered with ABC solution for (Vector Labs, Burlingame, CA), washed, incubated in DAB solution (Vector Labs, Burlingame, CA),

dehydrated with ethanol and cleared with xylene. The slides were cover-slipped and visualized using a brightfield microscope. A photo montage spanning the full depth of the parietal cortex was created using a Q imaging digital camera connected to a Nikon E600 Eclipse microscope with a 20X objective. Adobe Photoshop CS5 and ImageJ were used to count positively stained microvessels, less than 20 μm in diameter, to determine the capillary density (number per mm^2 of brain tissue).

4.6. Statistical analysis

Quantitative data were expressed as mean $\pm$ standard deviation (SD). Statistical analysis was carried out using Origin Lab Data Analysis and Graphing software version 8.1. Statistical comparisons were performed by one-way ANOVA followed by Tukey's comparison. In all cases, $P < 0.05$ was considered statistically significant.

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REFERENCES

- Ahmad A, Ahmad S, Chang LY, Schaack J, White CW. Endothelial Akt activation by hyperoxia: role in cell survival. *Free Radic. Biol. Med.* 2006; 40:1108–1118. [PubMed: 16545678]
- Allen BW, Demchenko IT, Piantadosi CA. Two faces of nitric oxide: implications for cellular mechanisms of oxygen toxicity. *J. Appl. Physiol.* 2009; 106:662–667. [PubMed: 18845774]
- Arany Z, Foo SY, Ma Y, Ruas JL, Bommi-Reddy A, Girmun G, Cooper M, Laznik D, Chinsomboon J, Rangwala SM, Baek KH, Rosenzweig A, Spiegelman BM. HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1 α . *Nature.* 2008; 451:1008–1012. [PubMed: 18288196]
- Archibald F. Oxygen toxicity and the health and survival of eukaryote cells: a new piece is added to the puzzle. *Proc. Natl. Acad. Sci. U.S.A.* 2003; 100:10141–10143. [PubMed: 12939409]
- Bartese S, Marinovich M, Corsini E, Galli CL, Viviani B. Erythropoietin: a novel neuroprotective cytokine. *Neurotoxicology.* 2005; 26:923–928. [PubMed: 15927257]
- Benderro GF, LaManna JC. Hypoxia-induced angiogenesis is delayed in aging mouse brain. *Brain Res.* 2011; 1389:50–60. [PubMed: 21402058]
- Berra E, Benizri E, Ginouves A, Volmat V, Roux D, Pouyssegur J. HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1 α in normoxia. *EMBO J.* 2003; 22:4082–4090. [PubMed: 12912907]
- Bhandari V, Choo-Wing R, Lee CG, Zhu Z, Nedrelov JH, Chupp GL, Zhang X, Matthay MA, Ware LB, Homer RJ, Lee PJ, Geick A, De Fougères AR, Elias JA. Hyperoxia causes angiopoietin 2-mediated acute lung injury and necrotic cell death. *Nat. Med.* 2006; 12:1286–1293. [PubMed: 17086189]
- Bhandari V, Elias JA. The role of angiopoietin 2 in hyperoxia-induced acute lung injury. *Cell Cycle.* 2007; 6:1049–1052. [PubMed: 17438375]
- Bitterman N. CNS oxygen toxicity. *Undersea Hyperb. Med.* 2004; 31:63–72. [PubMed: 15233161]
- Boero JA, Ascher J, Arregui A, Rovainen C, Woolsey TA. Increased brain capillaries in chronic hypoxia. *J. Appl. Physiol.* 1999; 86:1211–1219. [PubMed: 10194205]
- Brekken RA, Overholser JP, Stastny VA, Waltenberger J, Minna JD, Thorpe PE. Selective inhibition of vascular endothelial growth factor (VEGF) receptor 2 (KDR/Flk-1) activity by a monoclonal anti-VEGF antibody blocks tumor growth in mice. *Cancer Res.* 2000; 60:5117–5124. [PubMed: 11016638]
- Bulte DP, Chiarelli PA, Wise RG, Jezard P. Cerebral perfusion response to hyperoxia. *J. Cereb. Blood Flow Metab.* 2007; 27:69–75. [PubMed: 16670698]
- Carmeliet P. Angiogenesis in health and disease. *Nat. Med.* 2003; 9:653–660. [PubMed: 12778163]

- Chavez JC, Baranova O, Lin J, Pichile P. The transcriptional activator hypoxia inducible factor 2 (HIF-2/EPAS-1) regulates the oxygen-dependent expression of erythropoietin in cortical astrocytes. *J. Neurosci.* 2006; 26:9471–9481. [PubMed: 16971531]
- Chinsomboon J, Ruas J, Gupta RK, Thom R, Shoag J, Rowe GC, Sawada N, Raghuram S, Arany Z. The transcriptional coactivator PGC-1 α mediates exercise-induced angiogenesis in skeletal muscle. *Proc. Natl. Acad. Sci. U.S.A.* 2009; 106:21401–21406. [PubMed: 19966219]
- Demchenko IT, Boso AE, O'Neill TJ, Bennett PB, Piantadosi CA. Nitric oxide and cerebral blood flow responses to hyperbaric oxygen. *J. Appl. Physiol.* 2000; 88:1381–1389. [PubMed: 10749833]
- Diringer MN. Hyperoxia: good or bad for the injured brain? *Curr. Opin. Crit. Care.* 2008; 14:167–171. [PubMed: 18388679]
- Dore-Duffy P, LaManna JC. Physiologic angiodynamics in the brain. *Antioxid. Redox Signal.* 2007; 9:1363–1371. [PubMed: 17627476]
- Duong TQ, Iadecola C, Kim SG. Effect of hyperoxia, hypercapnia, and hypoxia on cerebral interstitial oxygen tension and cerebral blood flow. *Magn. Reson. Med.* 2001; 45:61–70. [PubMed: 11146487]
- Eklund L, Olsen BR. Tie receptors and their angiopoietin ligands are context-dependent regulators of vascular remodeling. *Exp. Cell Res.* 2006; 312:630–641. [PubMed: 16225862]
- Ferrara N, Gerber HP, Lecouter J. The biology of VEGF and its receptors. *Nat. Med.* 2003; 9:669–676. [PubMed: 12778165]
- Fisher JW. Erythropoietin: physiology and pharmacology update. *Exp. Biol. Med.* (Maywood). 2003; 228:1–14. [PubMed: 12524467]
- Fong GH. Mechanisms of adaptive angiogenesis to tissue hypoxia. *Angiogenesis.* 2008; 11:121–140. [PubMed: 18327686]
- Guzy RD, Schumacker PT. Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia. *Exp. Physiol.* 2006; 91:807–819. [PubMed: 16857720]
- Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR, Zagzag D, Yancopoulos GD, Wiegand SJ. Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. *Science.* 1999; 284:1994–1998. [PubMed: 10373119]
- Hori S, Ohtsuki S, Hosoya K, Nakashima E, Terasaki T. A pericyte-derived angiopoietin-1 multimeric complex induces occludin gene expression in brain capillary endothelial cells through Tie-2 activation in vitro. *J. Neurochem.* 2004; 89:503–513. [PubMed: 15056293]
- Hosford GE, Olson DM. Effects of hyperoxia on VEGF, its receptors, and HIF-2 α in the newborn rat lung. *Am. J. Physiol. Lung Cell Mol. Physiol.* 2003; 285:L161–L168. [PubMed: 12626331]
- Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohm M, Salic A, Asara JM, Lane WS, Kaelin WG JR. HIF α targeted for VHL-mediated destruction by proline hydroxylation: implications for O₂ sensing. *Science.* 2001; 292:464–468. [PubMed: 11292862]
- Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ. Targeting of HIF- α to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation. *Science.* 2001; 292:468–472. [PubMed: 11292861]
- Koh GY, Kim I, Kwak HJ, Yun MJ, Leem JC. Biomedical significance of endothelial cell specific growth factor, angiopoietin. *Exp. Mol. Med.* 2002; 34:1–11. [PubMed: 11989972]
- LaManna JC. In situ measurements of brain tissue hemoglobin saturation and blood volume by reflectance spectrophotometry in the visible spectrum. *J. Biomed. Opt.* 2007; 12:062103. [PubMed: 18163806]
- Lee ES, Smith WE, Quach HT, Jones BD, Santilli SM, Vatassery GT. Moderate hyperoxia (40%) increases antioxidant levels in mouse tissue. *J. Surg. Res.* 2005; 127:80–84. [PubMed: 15921701]
- Liang WY, Jiang L. [Relationship between expression of angiopoietin-2 and retinal vascular development in hyperoxic rats]. *Zhonghua Er. Ke. Za. Zhi.* 2009; 47:204–208. [PubMed: 19573435]
- Lopez-Lazaro M. HIF-1: hypoxia-inducible factor or dysoxia-inducible factor? *FASEB J.* 2006; 20:828–832. [PubMed: 16675839]
- Lu H, Forbes RA, Verma A. Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis. *J. Biol. Chem.* 2002; 277:23111–23115. [PubMed: 11943784]

- Lum JJ, Bui T, Gruber M, Gordan JD, Deberardinis RJ, Covello KL, Simon MC, Thompson CB. The transcription factor HIF-1 α plays a critical role in the growth factor-dependent regulation of both aerobic and anaerobic glycolysis. *Genes Dev.* 2007; 21:1037–1049. [PubMed: 17437992]
- Mantell LL, Horowitz S, Davis JM, Kazzaz JA. Hyperoxia-induced cell death in lung-the correlation of apoptosis, necrosis and inflammation. *Ann. NY. Acad. Sci.* 1999; 887:171–180. [PubMed: 10668473]
- Mekhail K, Gunaratnam L, Bonicalzi ME, Lee S. HIF activation by pH-dependent nucleolar sequestration of VHL. *Nat. Cell Biol.* 2004; 6:642–647. [PubMed: 15181450]
- Miro-Murillo M, Elorza A, Soro-Arnaiz I, Albacete-Albacete L, Ordonez A, Balsa E, Vara-Vega A, Vazquez S, Fuertes E, Fernandez-Criado C, Landazuri MO, Aragonés J. Acute Vhl gene inactivation induces cardiac HIF-dependent erythropoietin gene expression. *PLoS. One.* 2011; 6:e22589. [PubMed: 21811636]
- Mizukami Y, Kohgo Y, Chung DC. Hypoxia inducible factor-1 independent pathways in tumor angiogenesis. *Clin. Cancer Res.* 2007; 13:5670–5674. [PubMed: 17908955]
- Mole DR, Ratcliffe PJ. Cellular oxygen sensing in health and disease. *Pediatr. Nephrol.* 2008; 23:681–694. [PubMed: 17955264]
- Ndubuizi OI, Tsipis CP, Li A, LaManna JC. Hypoxia-inducible factor-1 (HIF-1)-independent microvascular angiogenesis in the aged rat brain. *Brain Res.* 2010; 1366:101–109. [PubMed: 20875806]
- Perez-Polo JR, Reilly CB, Rea HC. Oxygen resuscitation after hypoxia ischemia stimulates prostaglandin pathway in rat cortex. *Int. J. Dev. Neurosci.* 2011; 29:639–644. [PubMed: 21514373]
- Pichiule P, Chavez JC, LaManna JC. Hypoxic regulation of angiopoietin-2 expression in endothelial cells. *J. Biol. Chem.* 2004; 279:12171–12180. [PubMed: 14702352]
- Pichiule P, LaManna JC. Angiopoietin-2 and rat brain capillary remodeling during adaptation and deadaptation to prolonged mild hypoxia. *J. Appl. Physiol.* 2002; 93:1131–1139. [PubMed: 12183511]
- Ponce LL, Navarro JC, Ahmed O, Robertson CS. Erythropoietin neuroprotection with traumatic brain injury. *Pathophysiology.* 2012 In press.
- Puchowicz MA, Zechel JL, Valerio J, Emancipator DS, Xu K, Pundik S, LaManna JC, Lust WD. Neuroprotection in diet-induced ketotic rat brain after focal ischemia. *J. Cereb. Blood Flow Metab.* 2008; 28:1907–1916. [PubMed: 18648382]
- Rabie T, Marti HH. Brain protection by erythropoietin: a manifold task. *Physiology (Bethesda).* 2008; 23:263–274. [PubMed: 18927202]
- Semenza GL. Life with oxygen. *Science.* 2007; 318:62–64. [PubMed: 17916722]
- Semenza GL. Regulation of Metabolism by Hypoxia-Inducible Factor 1. *Cold Spring Harb. Symp. Quant. Biol.* 2011; 1–7. [PubMed: 22194359]
- Sharp FR, Bernaudin M. HIF1 and oxygen sensing in the brain. *Nat. Rev. Neurosci.* 2004; 5:437–448. [PubMed: 15152194]
- Shibuya M. Structure and dual function of vascular endothelial growth factor receptor-1 (Flt-1). *Int. J. Biochem. Cell Biol.* 2001; 33:409–420. [PubMed: 11312109]
- Sundberg C, Kowanetz M, Brown LF, Detmar M, Dvorak HF. Stable expression of angiopoietin-1 and other markers by cultured pericytes: phenotypic similarities to a subpopulation of cells in maturing vessels during later stages of angiogenesis in vivo. *Lab. Invest.* 2002; 82:387–401. [PubMed: 11950897]
- Tham E, Wang J, Piehl F, Weber G. Upregulation of VEGF-A without angiogenesis in a mouse model of dilated cardiomyopathy caused by mitochondrial dysfunction. *J. Histochem. Cytochem.* 2002; 50:935–944. [PubMed: 12070272]
- Vazquez-Valls E, Flores-Soto ME, Chaparro-Huerta V, Torres-Mendoza BM, Gudino-Cabrera G, Rivera-Cervantes MC, Pallas M, Camins A, Armendariz-Borunda J, Beas-Zarate C. HIF-1 α expression in the hippocampus and peripheral macrophages after glutamate-induced excitotoxicity. *J. Neuroimmunol.* 2011; 238:12–18. [PubMed: 21807419]

- Wilkinson-Berka JL, Alousis NS, Kelly DJ, Gilbert RE. COX-2 inhibition and retinal angiogenesis in a mouse model of retinopathy of prematurity. *Invest Ophthalmol. Vis. Sci.* 2003; 44:974–979. [PubMed: 12601017]
- Xiong M, Chen S, Yu H, Liu Z, Zeng Y, Li F. Neuroprotection of erythropoietin and methylprednisolone against spinal cord ischemia-reperfusion injury. *J. Huazhong Univ. Sci. Technolog. Med. Sci.* 2011; 31:652–656. [PubMed: 22038355]
- Xu K, LaManna JC. Chronic hypoxia and the cerebral circulation. *J. Appl. Physiol.* 2006; 100:725–730. [PubMed: 16421279]

Research highlights

- Diminished hematocrit in chronic moderate hyperoxia in mice
- VEGF, Flk-1 and PGC-1 α expression was decreased in hyperoxia
- HIF- α , EPO, COX-2 and Ang-2 expression was increased in hyperoxia
- Brain microvasculature was decreased in prolonged moderate hyperoxia

Fig. 1. Change in body weight, hematocrit and arterial oxygen saturation during chronic moderate hyperoxia

Error bars (SD) are indicated at normoxia (0), and 1, 4, 7, 14, and 21 days of hyperoxia. A, change in gross body weight during hyperoxia; and B, relative change in % of body weight during hyperoxic exposure; $n = 10$ for normoxia and 15 for all hyperoxic durations. C, hematocrit values; $n = 11$ in normoxia (0) and 4 day of hyperoxia, and $n = 12$ in 7, 14 and 21 day of hyperoxia. D, arterial oxygen saturation; $n = 6$ in all cases. Values are mean $\pm$ SD; * $P < 0.05$ compared to normoxic value of each category.

Fig. 2. HIF-1 α and HIF-2 α accumulation in cerebral cortex of mouse in chronic hyperoxia
 A, HIF-1 and 2 α accumulation. B, optical density (OD) ratio of HIF-1 and 2 α normalized to β -actin in normoxia (0), and 1, 4, 7, 14, and 21 days of hyperoxia. * $p < 0.05$ compared with normoxic control. Each value represents the mean $\pm$ SD from 5 mice.

Fig. 3. Expression of VEGF and Flk-1 in mouse cerebral cortex during hyperoxia

A, VEGF and Flk-1 protein expression in normoxic control and hyperoxia. B, optical density ratio in normoxia (0), and 1, 4, 7, 14, and 21 days of hyperoxia. * $P < 0.05$ compared with normoxic control values of each category. Values are mean $\pm$ SD. $n = 5$ mice per time point.

Fig. 4. HIF-1 α and VEGF RT-PCR, PHD-2 and VHL protein expressions in mouse cerebral cortex during hyperoxia

A, transcriptional activation of HIF-1 α and VEGF m-RNA in normoxia and hyperoxia normalized to β -actin m-RNA. n = 4 in normoxia (0), 1 and 4 days of hyperoxia and 3 in 7 days of hyperoxia. B, PHD-2 and VHL protein expression in normoxic control and hyperoxia. C, optical density ratio of PHD-2 and VHL in normoxia (0), and 1, 4, 7, 14, and 21 days of hyperoxia. n = 3 mice per time point. Values are mean $\pm$ SD. *P < 0.05 from normoxic control values of each category.

Fig. 5. Expression of PGC-1 α and EPO in mouse cerebral cortex during hyperoxia

A, PGC-1 α and EPO protein expression in normoxic control and hyperoxia. B, Optical density ratio in normoxia (0), and 1, 4, 7, 14, and 21 days of hyperoxia. * $P < 0.05$ compared with normoxic control values of each category. Values are mean $\pm$ SD. $n = 5$ mice per time point.

Fig.6. Expression of COX-2 and Ang-2 in mouse cerebral cortex during hyperoxia

A, COX-2 and Ang-2 protein expression in normoxic control and hyperoxia. B, optical density ratio in normoxia (0), and 1, 4, 7, 14, and 21 days of hyperoxia. * $P < 0.05$ compared with normoxic control values of each category. Values are mean $\pm$ SD. $n = 5$ mice per time point.

Fig.7. Microvascular density in mouse cerebral cortex in prolonged hyperoxia

A, composite photomicrograph of GLUT-1–stained sections spanning part of the parietal cortex of a mouse in normoxia and 21 days of hyperoxia. B, capillary density (number per mm²) of GLUT-1-stained sections at normoxia (0), and 7, 14 and 21 days of hyperoxia. Values are mean ± SD, *p < 0.05 compared with normoxic control. n = 6 mice per time point.

Table 1

Arterial blood gas content during normobaric hyperoxia (50% O₂).

Variable	Normoxia	Hyperoxia				
		1 day	4 day	7 day	14 day	21day
pH	7.35 ± 0.06	7.40 ± 0.04	7.36 ± 0.05	7.35 ± 0.09	7.35 ± 0.10	7.40 ± 0.06
PO ₂ (mmHg)	105 ± 12	313 ± 19 [*]	309 ± 21 [*]	314 ± 18 [*]	320 ± 20 [*]	313 ± 27 [*]
PCO ₂ (mmHg)	37 ± 4	40 ± 2	38 ± 4	37 ± 4	38 ± 4	39 ± 3

Values are mean ± SD

* P < 0.05 compared to normoxic value of each category; n = 7 for normoxia and 5 for all hyperoxic cases.