Published in final edited form as:

Brain Res. 2014 June 20; 1569: 41–47. doi:10.1016/j.brainres.2014.04.035.

HIF-1 α /COX-2 expression and mouse brain capillary remodeling during prolonged moderate hypoxia and subsequent re-oxygenation

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Abstract

Dynamic microvascular remodeling maintains an optimal continuous supply of oxygen and nutrients to the brain to account for prolonged environmental variations. The objective of this study was to determine the relative time course of capillary regression during re-oxygenation after exposure to prolonged moderate hypoxia and expression of the primary signaling factors involved in the process. Four-month old male C57BL/6 mice were housed and maintained in a hypobaric chamber at 290 torr (0.4 ATM) for 21 days and allowed to recover at normoxia (room air) for up to 21 days. The mice were either decapitated or perfused in-situ and brain samples collected were either homogenized for Western blot analysis or fixed and embedded in paraffin for immunohistochemistry. Hypoxia inducible factor-1 α (HIF-1 α), vascular endothelial growth factor (VEGF) and erythropoietin (EPO) expression were increased during hypoxic exposure and diminished during subsequent re-oxygenation. However, cyclooxygenase-2 (COX-2) and angiopoietin-2 (Ang-2) were both elevated during hypoxia as well as subsequent re-oxygenation. Significantly increased capillary density at the end of the 3rd week of hypoxia regressed back toward normoxic baseline as the duration of re-oxygenation continued. In conclusion, elevated COX-2 and Ang-2 expression during hypoxia where angiogenesis occurs and re-oxygenation, when microvessels regress, identifies these proteins as vascular remodeling molecules crucial for angioplasticity.

Keywords

VEGF; EPO; Ang-2; brain capillary remodeling

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1. INTRODUCTION

The structural and functional integrity of the brain profoundly depends on a continuous and controlled supply of oxygen and glucose. The brain cannot tolerate extended periods of hypoxia or hyperoxia due to an insufficient energy supply to the brain by anaerobic glycolysis during hypoxia and production of excessive reactive oxygen specie (ROS) which causes damage to the genome, cellular contents, and membranes during hyperoxia (Bitterman, 2004 and Fong, 2008). Therefore, transient vascular remodeling to regulate oxygen supply seems to be one of the major acclimatization mechanisms of the brain to fluctuations in tissue oxygen partial pressure (Dore-Duffy and LaManna, 2007; Pichiule and LaManna, 2002).

Prolonged exposure to moderate hypoxia produces a significant increase in cerebral capillary density in rats and mice alike (Benderro and Lamanna, 2011; Boero et al., 1999; Ndubizu et al., 2010; Pichiule and LaManna, 2003; LaManna et al., 1992). The involvement of Ang-2 has been implicated in the reversibility of the angiogenic process, in the rat brain, when the animal was returned to normoxia (Pichiule and LaManna, 2003). However, whether HIF-1 α and COX-2 protein levels were affected during re-oxygenation has not been recorded.

HIF is a critical mediator of endothelial growth factors and signaling proteins (such as VEGF, EPO, glycolytic enzymes and glucose transporters) during periods of metabolic stress and vascular remodeling (angiogenesis and regression) (Lum et al., 2007; Masson and Ratcliffe, 2003; Pichiule and LaManna, 2003; Semenza, 2004). Among the HIF isoforms, HIF-1 α is thought to be the primary responder and main regulator of angiogenic changes during hypoxia due to its inductive effect on transcriptional activity of pro-angiogenic proteins such as VEGF (Semenza, 2004; Sharp and Bernaudin, 2004). Hypoxia is the main inducer of HIF-1 α accumulation (Puchowicz et al., 2008; Semenza, 2007; Sharp and Bernaudin, 2004; Webb et al., 2009). Nevertheless, it was also shown that various metabolic and environmental stressors, which can disturb cellular homeostasis, can induce accumulation of HIF-1 α under normoxic conditions (Benderro et al., 2012; Lu et al., 2002; Mekhail et al., 2004; Puchowicz et al., 2008).

On the other hand, in vitro and vivo results indicate COX-2 to be the main regulator of Ang-2 expression (LaManna et al., 2006; Pichiule et al., 2004). Recently, we reported HIF-1 α and HIF-2 α independent synergistic expression of COX-2 and Ang-2 during chronic moderate hypobaric hypoxia and chronic moderate normobaric hyperoxia, in the mouse brain (Benderro and Lamanna, 2011; Benderro et al., 2012). Although increased expression of Ang-2 during re-oxygenation after chronic hypoxia was reported in the rat brain (Pichiule and LaManna, 2002), simultaneous relative expression of COX-2 was not determined.

Microvascular remodeling is one of the main mechanisms by which the brain acclimatizes to oxidative and metabolic stresses (Boero et al., 1999; Dore-Duffy and LaManna, 2007). In response to hypoxia, the increased capillary density results in decreased intercapillary diffusion distance thereby maintaining tissue oxygen tensions near normoxic levels (Boero et al., 1999; Fong, 2008; Harik et al., 1995). During prolonged hyperoxia appropriate

avoidance response mechanisms are activated in which vascularity is diminished to regulate oxygen partial pressure in brain parenchyma, presumably to avoid excessive ROS (Benderro et al., 2012). Recently, we have deduced that brain capillary density is a continuously adjusted variable with tissue oxygen availability as one of the controlling modulators in this dynamic model of angioplasticity (Benderro et al., 2012).

In this study we have quantified changes in capillary density and relative time course of its regression during prolonged re-oxygenation after chronic moderate hypobaric hypoxia. We have also determined, for the first time, expression of HIF-1 α , COX-2 and EPO proteins during re-oxygenation after acclimatization to chronic moderate hypoxia. Expression of VEGF and Ang-2 were also determined and time-course changes in some physiological variables such as body weight, hematocrit and arterial oxygen saturation (SaO₂) were also measured.

Results indicate that decreased body weight and SaO₂, and increased hematocrit during hypoxia tended to return toward normoxic baseline during re-oxygenation. HIF-1 α , VEGF and EPO protein expression, which were increased in hypoxia, diminished during re-oxygenation. However, COX-2 and Ang-2 expression remained elevated during hypoxia as well as during re-oxygenation. Significantly increased capillary density at the end of the 3rd week of hypoxia regressed toward normoxic baseline when re-oxygenation period was prolonged.

2. RESULTS

2.1. Change in body weight during hypoxia and subsequent re-oxygenation

All mice exposed to chronic hypoxia showed the expected rapid and significant ($p < 0.05$) body weight loss during hypoxic exposure (Fig. 1). Average normoxic body weight (29.2 ± 3.9 g) decreased to 22.9 ± 3.3 g at 4th day of hypoxia. Then, a slight recovery was observed on the 7th, 14th, and 21st (24.4 ± 3.8 g) day of hypoxia. The lost body weight was regained rapidly after re-oxygenation. The average body weight during re-oxygenation increased from 26.6 ± 3.4 g on the 1st day to 29.9 ± 3.7 g on the 21st day of re-oxygenation. Littermates kept in room air (normoxia) and maintained on the same schedule during the period of the experimental exposure showed an average body weight gain from 29.3 ± 2.6 g at the start of the experiment to 30.9 ± 2.2 g at day 21 and 32.4 ± 2.3 g at day 42 (Fig. 1).

2.2. Hematocrit and SaO₂ during chronic hypoxia and subsequent re-oxygenation

Polycythemia (increase in hematocrit or packed red cell volume) is one of the systemic adaptive mechanisms to chronic hypoxia. We recorded progressively increased hematocrit during prolonged hypoxia. Hematocrit values increased from $46 \pm 1\%$ at baseline to $81 \pm 3\%$ at day 21 of hypoxia and progressively decreased during re-oxygenation and approached to $51 \pm 2\%$ after 21 days of re-oxygenation (Fig. 2A). As expected, the arterial oxygen saturation was significantly decreased ($p < 0.05$) during hypoxia (from about 96% at baseline to about 70% in hypoxia) (Fig. 2B). However, when the mice were returned to normoxia SaO₂ returned back to baseline levels.

2.3. HIF-1 α accumulation & COX-2 expression in cerebral cortex

HIF-1 α accumulation during chronic hypoxia was significant ($p < 0.05$) compared to normoxia but it returned back to normoxic level during re-oxygenation (Fig. 3A and 3B). The trend in COX-2 expression was similar to HIF-1 α during hypoxia, but its expression remained significantly higher ($p < 0.05$) during re-oxygenation periods, compared to its normoxic level (Fig. 3A and 3B).

2.4. Expression of VEGF & Ang-2 in cerebral cortex

Both VEGF and Ang-2 showed significantly increased expression during hypoxic exposure ($p < 0.05$). However, during re-oxygenation, Ang-2 was still upregulated but VEGF expression returned back to a normoxic level (Fig. 4A and 4B). Expression of VEGF during hypoxia and re-oxygenation was similar to that of HIF-1 α whereas Ang-2 induction during hypoxia and re-oxygenation was similar to that of COX-2 expression (Fig 3).

2.5. Expression of EPO in cerebral cortex

Cerebral EPO expression during chronic hypoxia was significantly increased ($p < 0.05$) compared to normoxia but it returned back toward normoxic baseline level during re-oxygenation (Fig. 5A and 5B). The trend of EPO expression was similar to that of HIF-1 α and VEGF during chronic hypoxia and re-oxygenation.

2.6. Capillary density in cerebral cortex

Cerebral capillary density was determined by immunostaining for Glucose Transporter-1 (GLUT-1) and counting the number of GLUT-1 positive capillaries per unit area (N/mm²). In agreement with our previous report (Benderro and LaManna, 2011), quantitative analysis showed that capillary density in the mouse cerebral cortex was significantly increased ($p < 0.05$) during chronic moderate hypoxia of 21 days. Capillary density increased from 411 ± 38 at baseline to 628 ± 45 after 21 days of hypoxia. Re-oxygenation following chronic hypoxia was accompanied by vascular regression. During re-oxygenation, microvascular density decreased to 580 ± 34 after 1 week, 516 ± 53 after the 2nd week and 445 ± 64 by the 3rd week of exposure to room air. Hence, by the end of 3rd week of re-oxygenation, microvascular density was no longer significantly different from the normoxic control level (Fig. 6B).

3. DISCUSSION

Brain vascular remodeling requires complex signaling interactions of angiogenic proteins, and is an essential physiological adaptation response to fluctuation in tissue oxygen supply (Dore-Duffy and LaManna, 2007). In this study, we have demonstrated the time-course in expression of the main angiogenic transcription factor, HIF-1 α , and an enzyme involved in inflammation and angiogenesis, COX-2, during hypoxia and subsequent re-oxygenation in mouse cerebral cortex. We observed that, as previously shown, HIF-1 α protein level was elevated during hypoxia and diminished during re-oxygenation in the mouse brain. Conversely, COX-2 protein expression was elevated during hypoxia and remained elevated throughout the course of re-oxygenation as well. Using the mouse model, we also confirmed the previous report in the rat brain (Pichiule and LaManna, 2002) indicating decreased

VEGF and increased Ang-2 expression, and progressive return of microvascular density toward pre-hypoxic value during re-oxygenation. We also observed increased EPO protein expression during hypoxia and its return toward constitutive baseline level during re-oxygenation in the mouse brain.

As expected, mice successfully acclimatized to the hypoxic challenge with the usual mammalian responses (for review see Xu and LaManna, 2006; LaManna, 2004) regained the lost body weight during re-oxygenation after chronic moderate hypoxia. This is consistent with the report from rats exposed to similar conditions (Pichiule and LaManna, 2002). Re-oxygenation also resulted in a reduction of hematocrit from the elevated hypoxic value. At a molecular level, decrease in hematocrit during re-oxygenation after hypoxia may imply a decrease in HIF-1 α which leads to decreased EPO expression with subsequent decrease in RBC production (Fisher, 2003; Kendall, 2001).

Consistent with the reports from our lab and others (Benderro and LaManna, 2011; LaManna, 2006; Masson and Ratcliffe, 2003; Semenza, 2004; Wilkinson-Berka, et al., 2003), HIF-1 α and COX-2 protein levels were observed to increase during chronic hypoxia in the mouse brain. Increased levels of both HIF-1 α and COX-2 during hypoxia suggest a synergistic function of these proteins during hypoxia induced cerebral physiological adaptive responses, such as angiogenesis (Boero et al., 1999; LaManna et al., 2006; Semenza, 2011; Wilkinson-Berka et al., 2003). However, diminished HIF-1 α level during re-oxygenation may indicate that HIF-1 α is not actively involved in the vascular regression process. Meanwhile, maintained upregulation of COX-2 during re-oxygenation implies that COX-2 may serve a dual function in the angioplasticity model, i.e. its functional activity in both angiogenesis and regression alike.

Our result showing upregulation of VEGF and Ang-2 in chronic hypoxia affirmed our previous report of increased expression of these proteins in young mice (Benderro and LaManna, 2011) and rats (Ndubizu et al., 2010) brain in hypoxia. Synergistic expression of VEGF and Ang-2 during hypoxia was known to enhance the hypoxia induced angiogenic process (Dore-Duffy and LaManna, 2007; Pichiule and LaManna, 2002). Similar to the report on rats (Pichiule and LaManna, 2002), we also observed decreased VEGF and increased Ang-2 expression in the cerebral cortex of the mouse during re-oxygenation after chronic moderate hypoxia. Upregulation of Ang-2 during the time when there is reduced VEGF expression may have led to vascular apoptotic regression during re-oxygenation (Bhandari and Elias, 2007; LaManna et al., 2006; Maisonpierre et al., 1997).

Brain microvessels exhibit constant remodeling during normal physiological adjustment and under certain stressful conditions (Anderson et al., 2009; Wei et al., 2001). In this study we observed increased capillary density in mouse cerebral cortex after 3 weeks of chronic moderate hypoxia and progressive regression toward normoxic baseline in 3 weeks of re-oxygenation. This result is consistent with the report from cerebral cortex of rats (Pichiule and LaManna, 2002; Pichiule and LaManna, 2003), but showed a longer time to reach a normoxic level than that of rats. Modulation of capillary density according to fluctuation of tissue oxygen partial pressure and cellular oxygen demand explains the presence of brain

angioplasticity at the microvascular level as acclimatization to a wide range of oxygen partial pressures (Dore-Duffy and LaManna, 2007).

In conclusion, for the first time, we have demonstrated decreased accumulation of HIF-1 α and increased expression of COX-2 during re-oxygenation after chronic moderate hypoxia in the mouse brain. We also confirmed diminished VEGF and increased Ang-2 expression in the mouse, which may have contributed to vascular regression during re-oxygenation. Increased COX-2 and Ang-2 expression during hypoxia where angiogenesis occurs and re-oxygenation, when microvessels regress, indicates the involvement of these proteins in a wide range of brain angioplasticity.

4. EXPERIMENTAL PROCEDURES

4.1. Exposure to chronic hypoxia, re-oxygenation and SaO₂ measurement

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. Animal housing and handling met Institutional Animal Care and Use Committee (IACUC) standards. Mice that were exposed to hypoxia were placed in hypobaric chambers for 3 weeks at a constant pressure of 0.4 ATM (290 Torr) and brought back to room air (normoxia) for up to 3 weeks. The littermate normoxic control mice were housed in the same room next to the hypobaric chamber under identical ambient conditions. SaO₂ was assessed by using a Mouse-OxTM pulse oximeter (STARR Life Sciences Corp., Oakmount, PA), as described earlier (Benderro and Lamanna, 2011). Pulse Oximetry data were recorded in awake mice after an hour of adaptation in room air or in hypoxia.

4.2. Tissue Collection

Mice were weighed, anesthetized and tail blood samples were obtained for hematocrit determination before the animals were sacrificed. For Western blot analysis, cerebral cortex was dissected and immediately frozen in liquid nitrogen and stored at -80 °C until further processing. To collect tissues for immuno-staining, animals from normoxia or hypoxia were perfused transcardially with about 30 – 50 ml of ice cold PBS, pH 7.2, followed by 4% paraformaldehyde in PBS (about 100 ml). Brain and other organs were dissected and postfixed in 4% paraformaldehyde overnight and then stored at 4 °C in Millonig's buffer (NaH₂PO₄-H₂O, NaOH, Sodium Azide and ddH₂O), for cytoprotection, until embedded in paraffin wax.

4.3. Preparation of cell lysate and Western blot analysis

Tissue lysate preparation and Western blot analyses were done as described previously (Benderro and Lamanna, 2011). Briefly, proteins from the whole cell lysates 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 of interest were: HIF-1 α (1:500; R&D Systems, Minneapolis, MN), VEGF-A (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), Ang-2

(1:200; Millipore Co., Billerica, MA), COX-2 (1:150, Cayman, Ann Arbor, MI), EPO (1:750; Santa Cruz Biotechnology, Santa Cruz, CA) and β -actin (1:2000; Santa Cruz, CA). The membranes were washed three times with TBS-T, followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies (Millipore Co., Billerica, MA). After a series of three washes with TBS-T, immunoreactive protein bands were visualized using an enhanced chemiluminescence detection system (SuperSignal 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 β -actin (optical density ratio) using NIH ImageJ.

4.4. Determination of the capillary density

Immunohistochemistry and capillary counts 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 peroxidases 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 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 (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 μ m in diameter, to determine the capillary density (number per mm² of brain tissue).

4.5. 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.

Acknowledgments

This study was supported by National Institutes of Health grant NS-38632.

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Research highlights

- HIF-1 α expression is increased during hypoxia but decreased during re-oxygenation
- VEGF and EPO expression is increased in hypoxia but decreased during re-oxygenation
- COX-2 and Ang-2 expression is increased in both hypoxia and re-oxygenation
- Increased capillary density during chronic hypoxia regressed during re-oxygenation

Fig. 1. Change in body weight in 4-month old mice during hypoxia and recovery from hypoxia (re-oxygenation)

Solid lines indicate change in body weight during 21 days of hypoxia followed by 21 days of re-oxygenation. Dashed lines indicate weight change in normoxic littermate control during the experimental periods. * $P < 0.05$ compared with initial normoxic value. ♣ $P < 0.05$ compared with corresponding normoxic value. Values are mean $\pm$ SD; $n = 16$ for normoxia; 10 for 1, 4, 7, 14, and 21 days of hypoxia and day 1 re-oxygenation; 9 for 4, 7, 14 days of re-oxygenation and 5 for 21 days of re-oxygenation.

Fig. 2. Hematocrit and arterial oxygen saturation during normoxia (N), chronic hypoxia (H), and re-oxygenation (R) in 4-month old mice

A. Change in hematocrit value: * $P < 0.05$ compared with normoxia; $\clubsuit P < 0.05$ compared to 21 days of hypoxia. Values are mean $\pm$ SD; $n = 16$ for normoxia, 8 for 21 day hypoxia, 6 for 1 and 4 day recovery, 9 for 7, 14 and 21 day recovery. B. Arterial oxygen saturation: Values are mean $\pm$ SD; $n = 5$ mice in each case. * $P < 0.05$ compared with normoxic value. Error bars indicated at normoxia, and 1, 4, 7, 14, and 21 days of hypoxia and re-oxygenation.

Fig. 3. HIF-1 α and COX-2 expression in mouse cerebral cortex during hypoxia and reoxygenation

A: HIF-1 α and COX-2 protein expression in normoxia, hypoxia and reoxygenation. B: Optical density ratio in normoxia (0), and 1, 4, 7, 14, and 21 days of hypoxia and consecutive 21 days (22 – 42 days) of re-oxygenation at room air. *P < 0.05 compared with normoxic control values of each category. Values are mean $\pm$ SD. n = 3 mice per time point.

Fig. 4. VEGF and Ang-2 expression in mouse cerebral cortex during hypoxia and reoxygenation
 A: VEGF and Ang-2 protein expression in normoxia, hypoxia and re-oxygenation. B: Optical density ratio in normoxia (0), and 1, 4, 7, 14, and 21 days of hypoxia and consecutive 21 days (22 – 42 days) of re-oxygenation. * $P < 0.05$ compared with normoxic control values of each category. Values are mean $\pm$ SD. $n = 4$ mice per time point.

Fig. 5. EPO protein expression in mouse cerebral cortex during hypoxia and reoxygenation
 A: EPO protein expression in normoxia, hypoxia and re-oxygenation. B: Optical density ratio in normoxia (0), and 1, 4, 7, 14, and 21 days of hypoxia and consecutive 21 days (22 – 42 days) of re-oxygenation. * $P < 0.05$ compared with normoxic baseline value. Values are mean $\pm$ SD. $n = 3$ mice per time point.

Fig. 6. Microvascular density in mice cerebral cortex during re-oxygenation after 3 weeks of hypoxia

A: composite photomicrograph of GLUT-1-stained sections spanning part of the parietal cortex of mice at normoxia, 21 days of hypoxia and 21 days of re-oxygenation B: Capillary density (N/mm²) of GLUT-1-stained sections of the mice during normoxia (0), 21 days of hypoxia (21H), and 7, 14 and 21 days of re-oxygenation in room air (R). * $p < 0.05$ compared with corresponding normoxic control. Values are mean $\pm$ SD, $n = 5$ mice per time point in each group.