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Regular Article

# Defining the critical hypoxic threshold that promotes vascular remodeling in the brain

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ABSTRACT

In animal models, hypoxic pre-conditioning confers protection against subsequent neurological insults, mediated in part through an extensive vascular remodeling response. In light of the therapeutic potential of this effect, the goal of this study was to establish the dose–response relationship between level of hypoxia and the extent of cerebrovascular modeling, and to define the mildest level of hypoxia that promotes remodeling. Mice were exposed to different levels of continuous hypoxia (8–21% $O_2$) for seven days before several aspects of vascular remodeling were evaluated, including endothelial proliferation, total vascular area, arteriogenesis, and fibronectin/α5β1 integrin expression. For most events, the threshold level of hypoxia that stimulated remodeling was 12–13% $O_2$. Interestingly, many parameters displayed a biphasic dose–response curve, with peak levels attained at 10% $O_2$, but declined thereafter. Further analysis in the 12–13% $O_2$ range revealed that vascular remodeling occurs by two separate mechanisms: (i) endothelial hyperplasia, triggered by a hypoxic threshold of 13% $O_2$, which leads to increased capillary growth, and (ii) endothelial hypertrophy, triggered by a more severe hypoxic threshold of 12% $O_2$, which leads to expansion of large vessels and arteriogenesis. Taken together, these results define the hypoxic thresholds for vascular remodeling in the brain, and point to two separate mechanisms mediating this process.



## Introduction

Many neurological diseases have vascular dysfunction either as the root or as the central part of the pathogenic process. These include ischemic stroke (del Zoppo and Hallenbeck, 2000; Dirnagl et al., 1999), multiple sclerosis (MS) (Gay and Esiri, 1991; Kirk et al., 2003) and vascular dementia (Brown et al., 2009; Zlokovic, 2011). Despite all the intensive research efforts in these different conditions, no current drugs or therapies have been identified that target the vascular origin.

Interestingly, over the last decade, a number of studies have demonstrated the protective potential of hypoxic pre-conditioning, in which a period of training at a sub-clinical hypoxic level protects against subsequent neurological sequelae (Dirnagl et al., 2003). This has been most effectively shown in ischemic stroke in which a short (2–4 h) exposure to 8–10% hypoxia reduces the size of ischemic lesion and subsequent inflammation if ischemia occurs within 2–3 days of pre-conditioning (Dunn et al., 2012; Miller et al., 2001). In addition, a recent study suggests that more long-term hypoxic pre-conditioning might also protect against inflammatory demyelinating disease in the mouse model of MS, experimental autoimmune encephalomyelitis (EAE), in part by limiting leukocyte infiltration (Dore-Duffy et al., 2011).

Interestingly, chronic mild hypoxia (CMH) induces beneficial physiological adaptations in cerebral vessels that work in the opposite direction to age-related deterioration, by promoting angiogenic and arteriogenic remodeling, thus increasing vessel density, blood–brain barrier (BBB) integrity and cerebral blood flow (LaManna et al., 1992, 2004; Li et al., 2010). It is well established that mice exposed to CMH (8% $O_2$ over a two week period), show greater than 50% increased vascular density in all areas of the brain. We have shown that this response involves active angiogenic remodeling underscored by endothelial cell proliferation driven by upregulation of the fibronectin-α5β1 integrin axis (Li et al., 2012; Milner et al., 2008). Significantly, CMH-induced vascular remodeling is not just limited to capillaries; it also involves robust generation of new arterioles (arteriogenesis), consistent with the idea that mild hypoxia stimulates remodeling at all stages of the vascular tree (Boroujerdi et al., 2012). Furthermore, cerebral blood vessels in hypoxic-exposed mice show strong upregulation of tight junction proteins, including claudin-5 and zonula occludens-1 (ZO-1) (Li et al., 2010), suggesting that CMH also promotes integrity of the BBB, the cellular barrier that protects the sensitive neuropil from the potentially hazardous components of blood (Ballabh et al., 2004; Pardridge, 2003).

Our studies over the last five years have defined the time-course of cerebrovascular remodeling events in response to a standard hypoxic regimen of 8% $O_2$ (Boroujerdi et al., 2012; Li et al., 2010). To our

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knowledge, no studies have examined the dose–response relationship between level of hypoxia and extent of vascular remodeling within the brain. More importantly, the threshold level of hypoxia that induces these changes has not yet been defined. This is an important issue because most animal studies to date have employed fairly severe levels of hypoxia, e.g.: 8% $O_2$, which is equivalent to an altitude of 23,500 ft, conditions that are not practical or tolerable for most humans (Kupper et al., 2011). So before considering the clinical potential of this approach in human patients, it is important to define the mildest level of hypoxia that promotes beneficial remodeling. With this in mind, the goal of this study was to define the dose–response relationship between level of hypoxia and vascular remodeling in the mouse brain, and specifically to identify the hypoxic threshold at which vascular remodeling changes occur. As vascular remodeling encompasses a large number of distinct events, we focused specifically on: (i) endothelial cell proliferation, (ii) increase in total vascular area, (iii) vessel size distribution, (iv) arteriogenesis, (v) upregulated expression of the remodeling proteins fibronectin and its receptor α5β1 integrin, and (vi) upregulated expression of the tight junction protein claudin-5.

## Materials and methods

### *Animals*

The studies described have been reviewed and approved by The Scripps Research Institute Institutional Animal Care and Use Committee. Wild-type C57Bl/6 mice were maintained under pathogen-free conditions in the closed breeding colony of The Scripps Research Institute (TSRI).

### *Chronic hypoxia model*

Wild-type C57Bl/6 littermate mice, 8–10 weeks of age, were housed 4 to a cage, and placed into a hypoxic chamber (Biospherix, Redfield, NY) for 7 days maintained at different oxygen levels of 8, 10, 12, 14, and 16% $O_2$. Control mice were kept in the same room under similar conditions except that they were kept at ambient sea-level oxygen levels (normoxia, approximately 21% $O_2$ at sea-level) for the duration of the experiment. In subsequent experiments, mice were maintained at 8% and 13% $O_2$ for periods of 7 and 14 days. Every few days, the chamber was briefly opened for cage cleaning and food and water replacement as needed.

### *Immunohistochemistry and antibodies*

Immunohistochemistry was performed as described previously (Milner and Campbell, 2002) on 10 μm frozen sections of cold phosphate buffer saline (PBS) perfused brains taken from mice subject to either normoxia (control) or hypoxic conditions. The following monoclonal antibodies were obtained from BD Pharmingen (La Jolla, CA): rat monoclonal antibodies reactive for: CD31 (clone MEC13.3), CD105 (clone MJ7/18), and the integrin subunit α5 (clone 5H10-27 (MFR5)). Other antibodies used included: rabbit anti-fibronectin (Sigma, St. Louis, MO), mouse anti-α-SMA-Cy3 conjugate (Sigma, clone 1A4), rabbit anti-claudin-5 (Invitrogen, Carlsbad, CA), and rabbit anti-Ki67 (Vector laboratories, Burlingame, CA). Secondary antibodies used included goat anti-rabbit Cy3 (Jackson Immunoresearch, Baltimore, PA) and anti-rat Alexa Fluor 488 (Invitrogen).

### *Image analysis*

Images were taken using a 20× objective on a Zeiss Imager M1.m. All analysis was performed in the frontal lobe region of the brain. For each antigen, three images were taken per region at 10× or 20× magnification and the mean calculated for each subject over three different sections. All data analyses were performed using the Perkin Elmer Volocity software (Waltham, MA). This includes quantification of total vessel (CD31-positive) area, numbers of CD31/Ki67 dual-positive cells, α-SMA-positive vessels, α5 integrin-positive vessels, low intensity CD105 vessels and the size distribution of vessels. To quantify the mean expression levels of vascular fibronectin, α5 integrin, CD105, and claudin-5, Volocity software was used to measure the fluorescent intensity of each vessel, and thereby calculate the mean vessel intensity per field of view for each hypoxic condition. Each experiment was performed with four different animals per condition, and the results were expressed as the mean ± SEM. Statistical significance was assessed by using the Student's *t* test, in which $p < 0.05$ was defined as statistically significant.

## Results

### *The threshold level of hypoxia that stimulates vascular remodeling in the brain lies between 12 and 14% $O_2$*

To define the dose–response relationship between hypoxia and cerebrovascular remodeling, mice were incubated for seven days either under control (normoxic) conditions or at different levels of hypoxia including 16, 14, 12, 10 and 8% $O_2$. In previous studies we have shown that hypoxic-induced vascular remodeling employing a hypoxic level of 8% $O_2$ occurs across all areas of the brain (Milner et al., 2008). In the current study we focused our attention specifically on the frontal lobe as this is a large structure with well-defined vascular architecture. We chose the seven day period for two reasons. First, this is the time-point at which many of the remodeling parameters show peak levels, including fibronectin and α5β1 integrin expression, endothelial proliferation, and the number of low-CD105 vessels as a marker of arteriogenesis (Boroujerdi et al., 2012; Li et al., 2010). Second, seven days is sufficient time to detect significant differences in total vascular area. All changes in vascular remodeling were evaluated by immunofluorescence (IF) and digital software analysis using several well-defined measures that are altered during hypoxia-induced cerebrovascular remodeling. To start with, we examined three parameters of vascular remodeling that show clear changes in response to hypoxia: endothelial cell proliferation (CD31/Ki67), total vessel area (CD31), and the number of large area vessels (>500 $\mu m^2$).

As shown in Fig. 1, significant increases in endothelial cell proliferation (identified by CD31/Ki67 dual-positive cells) were not observed until ambient $O_2$ levels reached 12% or lower. Compared with normoxic (control) conditions (0.15 ± 0.1 proliferating cells per field of view), endothelial cell proliferation was significantly increased at $O_2$ levels of 12% (4.4 ± 0.5 proliferating cells per field of view, $p < 0.005$), 10% (5.1 ± 0.9 proliferating cells per field of view, $p < 0.005$), and 8% (3.2 ± 0.3 proliferating cells per field of view, $p < 0.01$) (Fig. 1B). In a similar manner, alterations in total vascular area were not observed until ambient $O_2$ levels were 12% or lower (Fig. 1C). Compared with normoxic (control) conditions, total vascular area was significantly higher at $O_2$ levels of 12% (125 ± 9.5%, $p < 0.05$), 10% (141.3 ± 15.1%, $p < 0.05$), and 8% (134.5 ± 2.3%, $p < 0.005$).

In previous studies our lab and the Moreno lab independently demonstrated that one of the most striking effects of CMH is an increase in the number of large area vessels (>500 $\mu m^2$) (Boroujerdi et al., 2012; Freitas-Andrade et al., 2011). When we examined the dose–response of this effect, we found that hypoxic-induced increases in the number of large area vessels were not observed until ambient $O_2$ levels were 12% or lower (Fig. 1D). Compared with normoxic (control) conditions, the number of large area vessels was significantly higher at $O_2$ levels of 12% (135.8 ± 10.9%, $p < 0.05$), 10% (187 ± 35.5%, $p < 0.05$), and 8% (156.5 ± 19.2%, $p < 0.05$). Thus, for all three parameters examined, the threshold hypoxic level that stimulated vascular remodeling appeared to lie somewhere in the range of 12–14% $O_2$. Surprisingly, in all of the parameters examined, the extent of remodeling at 8% $O_2$ appeared to be less than that at 10% $O_2$, suggesting the presence of a