Title
Statics and dynamics of cerebral blood flow is mediated through Hypoxia-inducible factor 2-alpha in the tumor suppressor von Hippel-Lindau knock-out mice

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Chair

University of California, San Diego

2009
Dedication

This thesis is dedicated to my community, both new and old, and forever changing.
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List of Abbreviations

VHL — von Hippel Lindau
HIF — hypoxia-inducible factor
PHD — prolyl hydroxylase domain containing
ARNT — aryl hydrocarbon receptor translocator
EPO — erythropoietin
GFAP — glial fibrillary acidic protein
CNS — central nervous system
BBB — blood-brain barrier
VEGF — vascular endothelial growth factor.
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Figures and the materials and methods of generating transgenic mice, in part, has been submitted for publication of the material as it may appear in
Hypoxia-inducible transcription factors (HIF) mediate the transcription of a wide range of physiologically important genes including erythropoietin (EPO) and vascular endothelial growth factor (VEGF) to maintain oxygen homeostasis during development, (patho)-physiological adaptation, and ischemia. Previous studies have elucidated the molecular mechanisms underlying HIF activity with
EPO and VEGF induction, but little has been done to understand the affects of these hypoxia-inducible genes with the physiology of blood flow in vivo. In an attempt to explain this, two complementary measures, vascular lumen diameter and red blood cell velocity, are used to characterize the vascular dynamics of individual capillaries in mice with conditional deletions in astrocytes of the major factors that mediate hypoxic transcriptional response and regulate EPO and VEGF, i.e., HIF-1α, HIF-2α and their negative regulator VHL. In this thesis I show that vascular dynamics are altered primarily through a HIF-2α dependent pathway with respect to cerebral blood flow.
INTRODUCTION

That a soluble factor might exist that controls angiogenesis is a concept that dates back almost 40 years ago to Folkman’s work in the 1970s[1]. It was later found that the vascular endothelial growth factor (VEGF) would serve as the chief angiogenic mitogen in mammals, and both its expression and regulation depend deeply on oxygen tension [2,3]. Specifically, it was found that hypoxia causes stabilization of VEGF mRNA. Moreover, subsequent investigations aiming to understand the molecular pathways of VEGF regulation led to the same set of transcription factors responsible for inducing the erythropoietin hormone during hypoxia — hypoxia inducible transcription factors (HIF) [4-6].

HIF is a heterodimeric transcription factor that is composed of two basic helix-loop-helix proteins — the nuclearly located, constitutive, non-oxygen-responsive subunit HIF-1β (also termed ARNT) and one of either the hypoxia-inducible α-subunits, HIF-1α or HIF-2α: all of which are members of the PAS family. HIF-α subunits are highly inducible by hypoxia, but are rapidly degraded in normoxia [7]. The interface between oxygen and the HIF-α subunits in normoxia is in part due to the oxygen-dependent prolyl hydroxylases that hydroxylate two proline residues located in the oxygen-dependent degradation domain of the α-subunits. These hydroxylated residues act as the docking site for the von Hippel-Lindau tumor suppressor protein (pVHL), which targets HIF-α for proteolysis by the ubiquitin-proteasome pathway [8].

Inactivating mutations of the VHL tumor suppressor gene can lead to von
Hippel-Lindau syndrome, a familial cancer disorder that results in constitutive stabilization of HIF-1α and HIF-2α in the presence of oxygen [9]. As a result, patients suffering from VHL syndrome are predisposed to highly vascularized tumors such as hemangioblastomas in the central nervous system and retina, and clear cell renal carcinomas [10]. Furthermore, it has been reported that VHL mutations linked to clear cell renal carcinomas and hemangioblastomas constitutively overproduce VEGF and platelet-derived growth factor B (PDGFβ), implicated in angiogenesis; and several other mitogenic compounds such as transforming growth factor alpha (TGFα) and erythropoietin (EPO) — all of which are accompanied by an increase in their cognate receptors [11-14].

Moreover, it has been found that prolonged exposure to hypoxia, acting through a HIF-1 dependent manner, increases VEGF mRNA in the brain (15–16), promotes the formation of new cerebral blood vessels (17–18), and induces blood brain barrier (BBB) permeability (19). Therefore, to test how the impact of these changes, knockout mice were used with conditional deletions in astrocytes of the major factors which mediate hypoxic transcriptional response and regulate VEGF, i.e., HIF-1α, HIF-2α and VHL. Here, I show through loss of function mutations that hypoxic response of the CNS leads to an increase in capillary lumen diameter and a significant reduction of red blood cell velocity within the same vessels, both of which are mediated by overexpression of HIF-2α in astrocytes.
RESULTS

A. Astrocytic knockdown of the von Hippel-Lindau tumor suppressor results in a decrease in red blood cell velocity and an increase in capillary lumen diameter

To test whether VHL had any affect on vascular dynamics in vivo, GFAPcre+/VHL$^{+/+}$ and GFAPcre-negative mice were analyzed for two complementary measures, capillary lumen diameter and red blood cell (RBC) velocity. Capillaries were surveyed between 100 and 300 µm below cortex, and animals with a deletion of VHL in astrocytes were found to have an increase in capillary lumen diameter from $3.501 \pm 0.0616$ µm in wild type controls, to $4.7756 \pm 0.1477$ µm in GFAPcre+/VHL$^{+/+}$ mice (Fig. 1). In addition, animals with a deletion of VHL in astrocytes were found to have a significant decrease in capillary RBC velocity from $2.929 \pm 0.2076$ mm/sec in wild type controls, to $1.1057 \pm 0.0790$ mm/sec in GFAPcre+/VHL$^{+/+}$ mice (Fig. 2). Taken together, this data shows that deletion of VHL in astrocytes induces a severe pathology, which manifests itself as an enlargement in capillary lumen diameter and a reduction in capillary RBC velocity — ultimately altering vascular dynamics in vivo.

B. Changes in vascular dynamics is dependent on HIF-2α overexpression in astrocytes

A number of studies have shown that in the context of loss of VHL function, HIF-2α becomes the dominant HIF-α isoform [20–23]. To investigate
which HIF-\(\alpha\) isoform is the major determinant of phenotypic changes in the
tissue-specific deletion of VHL in astrocytes, deletions of VHL and HIF-1\(\alpha\)
(GFAPcre+/VHL\(^{+/-}\)/HIF-1\(\alpha^{+/-}\)) and VHL and HIF-2\(\alpha\) (GFAPcre+/VHL\(^{+/-}\)/HIF-2\(\alpha^{+/-}\)) were generated. Using the same measures as before, mice with a deletion
of VHL and HIF-1\(\alpha\) in astrocytes were found to have an increase in capillary
lumen diameter from 3.501 ± 0.0616 \(\mu\)m in wild type controls, to 4.6019 ± 0.0929
\(\mu\)m in GFAPcre+/VHL\(^{+/-}\)/HIF-1\(\alpha^{+/-}\). This increase in lumen diameter is almost
indistinguishable from the 4.7756 ± 0.1477 \(\mu\)m found in GFAPcre+/VHL\(^{+/-}\) mice
(Fig. 1). Interestingly, mice with a deletion of VHL and HIF-2\(\alpha\) in astrocytes had a
luminal diameter that was almost indistinguishable from wild type controls,
3.8638 ± 0.1098 \(\mu\)m vs. 3.501 ± 0.0616 \(\mu\)m in GFAPcre-negative mice (Fig. 1).

In addition, mice with a loss of VHL and HIF-1\(\alpha\) in astrocytes were found
to have a significant decrease in capillary RBC velocity from 2.929 ± 0.2076
mm/sec in wild type controls to 1.0175 ± 0.1107 mm/sec in
GFAPcre+/VHL\(^{+/-}\)/HIF-1\(\alpha^{+/-}\) mice (Fig. 2). Moreover, this decrease in capillary
RBC velocity is nearly identical to that of GFAPcre+/VHL\(^{+/-}\) mice, 1.1057 ±
0.0790 mm/sec (Fig. 2). Likewise, mice with a loss of VHL and HIF-2\(\alpha\) in
astrocytes had an RBC velocity that was almost indistinguishable from wild type
controls, 2.8056 ± 0.2129 vs. 2.929 ± 0.2076 mm/sec in GFAPcre-negative mice
(Fig. 2). Taken together, this data suggests that HIF-2\(\alpha\) is indeed the dominant
the dominant isoform that mediates the severe pathology seen in VHL syndrome.
FIGURE 1: Capillary lumen diameter is enlarged in GFAPcre+/VHL^{+/+} and GFAPcre+/VHL^{+/+}/HIF-1α^{+/+} mice. In GFAPcre+/VHL^{+/+} knockout animals (n = 4 animals, 39 vessels), capillary lumen diameter is increased compared to GFAPcre-negative mice (n= 4 animals, 76 vessels; p <0.001), however no statistical difference was found when compared to GFAPcre+/VHL^{+/+}/HIF-1α^{+/+} double knockout mice (n = 4 animals, 65 vessels; p = 0.6966). Whereas the lumen diameter of capillaries in GFAPcre+/VHL^{+/+}/HIF-2α^{+/+} mice (n = 4 animals, 66 vessels) had no statistical difference between GFAPcre-negative mice (n = 4 animals, 76 vessels; p = 0.8474), but had a significant reduction in size compared to GFAPcre+/VHL^{+/+} (p <0.001) and GFAPcre+/VHL^{+/+}/HIF-1α^{+/+} mice (p <0.001).
FIGURE 2: Capillary red blood cell velocity is decreased in GFAPcre+/VHL^{+/+} and GFAPcre+/VHL^{+/+}/HIF-1α^{+/+} mice. In GFAPcre+/VHL^{+/+} knockout animals (n = 4 animals, 39 vessels), red blood cell (RBC) velocity is decreased compared to GFAPcre-negative mice (n=4 animals, 76 vessels; p <0.001), however no statistical difference was found when compared to GFAPcre+/VHL^{+/+}/HIF-1α^{+/+} double knockout mice (n = 4 animals, 65 vessels; p = 0.1334). Whereas the RBC velocity in capillaries of GFAPcre+/VHL^{+/+}/HIF-2α^{+/+} mice (n = 4 animals, 66 vessels) had no statistical difference between GFAPcre-negative mice (n = 4 animals, 76 vessels; p = 0.6323), but had a significant reduction in velocity compared to GFAPcre+/VHL^{+/+} (p <0.001) and GFAPcre+/VHL^{+/+}/HIF-1α^{+/+} mice (p <0.001).
FIGURE 3: Two-photon laser scanning microscopy of Fluorescently labeled cortical vasculature in vivo. Maximal projection of a high-magnification (40x) TPLSM image stack of fluorescently labeled brain vasculature in GFAPcre-negative (A), GFAPcre+/VHL<sup>+/−</sup> (B), GFAPcre+/VHL<sup>+/−</sup>/HIF-2α<sup>+/−</sup> (C), and GFAPcre+/VHL<sup>+/−</sup>/HIF-1α<sup>+/−</sup> (D) mice. In the inset is a low-magnification maximal projection of TPLSM image stacks (20x) of somatosensory cortex, with a box that indicates the approximate location of the high-magnification image. Bar, 50 µm; inset bar, 200 µm.
DISCUSSION

I describe here evidence that tissue specific deletion of the von Hippel-Lindau tumor suppressor in astrocytes contributes to an increase in cerebral capillary lumen diameter and a decrease in the velocity of red blood cells passing through capillaries; and that this response occurs in a HIF-2α dependent fashion. It has been widely established that mutations in VHL protein function (as in von Hippel-Lindau syndrome) are known to affect the cell’s response to hypoxia via upregulation of HIF-1α and HIF-2α with subsequent induction of VEGF and EPO even under normoxic conditions Maxwell et al., (1999). Work done by Ogunshola et al., (2000) show that neuronal VEGF expression correlates with angiogenesis in postnatal developing rat brain and observed a 40-51% increase in cerebral microvascular lumen diameter. In addition, work done by Weidemann et al., (unpublished) show that mice harboring deletions of VHL in astrocytes have a significant increase in VEGF mRNA relative to control animals, and that double knockout of VHL and HIF-1α does not change VEGF expression significantly, whereas double knockout of VHL and HIF-2α significantly reduce VEGF upregulation to wild type levels. In agreement with this, my work shows an increase in cerebral microvascular lumen diameter in GFAPcre+/VHL^{+/+} mice and GFAPcre+/VHL^{+/+}/HIF-1α^{+/+} mice, which is correlated to an increase in brain VEGF mRNA reported by Weidemann et al., and which is also regulated in a HIF-2α dependent manner in vivo (Fig. 3).
Moreover, work done by Weidemann et al., (unpublished) show that deletion of the VHL gene in astrocytes leads to a HIF-2α driven pathologic erythrocytosis correlated with an endogeneous overproduction of EPO in the CNS. The affects of which are now speculated to be a decrease in RBC velocity in cerebral microvascular in GFAPcre+/VHL<sup>+/−</sup> mice and GFAPcre+/VHL<sup>+/−</sup>/HIF-1α<sup>+/−</sup> mice, indicating that HIF-2α is also responsible for the changes in vascular dynamics in vivo. Indeed, double knockout of VHL and HIF-2α abolished the high hematocrit seen in these mutant mice, and in doing so, also increases RBC velocity back to wild type conditions. Taken together with the results of this study, in vivo vascular dynamics of cerebral microvasculature appears to be regulated through HIF-2α when its negative regulator, pVHL, is not functioning.
MATERIALS AND METHODS

A. Transgenic mice

All procedures involving animals were approved by the UCSD Animal Care Committee, which serves to ensure that all federal guidelines concerning animal experimentation are met. Generation of mice carrying the loxP-flanked conditional alleles of HIF-1α, HIF-2α, VEGF and VHL was described previously [24-26]. Astrocyte-specific inactivation was achieved by crossbreeding those mice to GFAP-Cre transgenic mice (generously provided by D. Guttmann [27]) in C57BL/6 background or the respective double knockouts (VHL/HIF-1α and VHL/HIF-2α) in mixed background. Cre-negative homozygous littermates for the conditional alleles were used as controls.

B. Surgery

In total, 16 male and female mice were used in this study, 4 animals per each genotype, 19 to 38g in mass, that were anesthetized by 1 to 2% (v/v) isoflurane (Baxter Healthcare, Deerfield, IL, USA) in 30% oxygen and 70% nitrous oxide. Body temperature was maintained at 37˚C with a feedback-regulated head pad (21052-00; Fine Science Tools, Foster City, CA, USA). A subcutaneous scalp injection of 2% lidocaine (Hospira INC., Lake Forest, IL, USA) was administered into the scalp prior to exposing the skull through a midline incision. Cranial windows, 2 x 2 mm in size and centered at 3 mm medial-
lateral and -1.0 mm anterior-posterior were constructed over the somatosensory cortex. This region was covered with 1.5% low-melting point agarose (Type 3-A; Sigma-Aldrich; A9793) dissolved in artificial cerebral spinal fluid (ACSF, composition in mM: NaCl 125, KCl 5, Glucose 10, HEPES 10, CaCl$_2$ 3.1, MgCl$_2$ 1.3; Ph 7.4) and sealed with a glass cover (no. 1, cut to size). To minimize movement artifact, the skull was fastened with cyanoacrylate glue and dental cement to a stainless-steel frame that was directly affixed to the optical apparatus. Intraperitoneal injections of 5% (w/v) glucose in 1 mL saline were given every 2 h for re-hydration.

C. Two-Photon Microscopy

Images were collected using a two-photon laser scanning microscope of custom design [28], that was controlled by MPScope software [29]. The blood serum was labeled using 0.1 mL of 2 MDa fluorescein dextran (FD2000S; Sigma, St Louis, MO, USA) prepared at a concentration of 2.5% (w/v) in physiological saline, and delivered through a tail vein injection with 0.1 mL supplements as required. A 0.5 numerical aperture (NA), 20-times magnification water-dipping objective (Olympus, Center Valley, PA, USA) was used to collect large-scale image stacks of surface vasculature, whereas a 0.8-NA, 40-times magnification water-dipping objective (Olympus, Center Valley, PA, USA) was used to obtain high-resolution line scan and planar data. The line scans were collected along the centerline of each vessel over a
length of 500 pixels at a scan rate of 1.6 kHz/line. Red blood cell velocity was
determined using a method based on the Radon transform [30]. Planar image
stacks, 256 x 256 pixels, were acquired to establish the diameter of the vessel. A
median filter (radius, 1 pixel) was applied to each image to reduce background
noise.

D. Statistical Analyses

Data are presented as mean ± s.e.m. If not otherwise noted, a
nonparametric Kruskal-Wallis one-way analysis of variance was used, as the in
vivo imaging data were not always normally distributed.

Figures and the materials and methods of generating transgenic mice, in
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