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Neuronal deficiency of HIF prolyl 4-hydroxylase 2 in mice improves ischemic stroke recovery in an HIF dependent manner

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ABSTRACT

Hypoxia inducible factors (HIFs) mediate the endogenous adaptive responses to hypoxia. HIF prolyl 4-hydroxylase domain proteins (PHD) are important suppressors of the HIF pathway. Recently, we demonstrated that neuron-specific deletion of *Phd2* reduces cerebral tissue damage in the very acute phase of ischemic stroke. In the present study, we investigated whether neuronal *Phd2* ablation is likewise beneficial for stroke recovery, and aimed to identify underlying cellular mechanisms. Mice underwent permanent occlusion of the distal middle cerebral artery (pdMCAO) for either 7 days (sub-acute stage) or 30 days (chronic stage). One week after pdMCAO the infarct size of *Phd2*-deficient mice was significantly reduced as compared to wild-type (WT) mice. Accordingly, *Phd2*-deficient animals showed less impaired sensorimotor function. Neuronal loss of *Phd2* upregulated vascular endothelial growth factor (VEGF) and significantly increased microvascular density along the infarct border in the sub-acute stage of stroke. *Phd2*-deficient mice showed reduced expression of pro-inflammatory cytokines and increased numbers of resting microglia/macrophages and reactive astrocytes within peri-infarct regions in comparison to WT littermates. Finally, brain tissue protection and increased angiogenesis upon sub-acute ischemic stroke was completely absent in *Phd2* knockout mice that were additionally deficient for both *Hif1a* and *Hif2a*. Our findings suggest that lack of PHD2 in neurons improves histological and functional long-term outcome from ischemic stroke at least partly by amplifying endogenous adaptive neovascularization through activation of the HIF-VEGF axis.

1. Introduction

Cerebral tissue hypoxia is one of the primary mediators of stroke pathophysiology. Nevertheless, animals exposed to mild hypoxia are found to be more resistant to subsequent cerebral ischemia (Bernaudin et al., 2002; Miller et al., 2001; Poinsatte et al., 2015; Ratan et al., 2007). This preconditioning effect indicates that activation of the endogenous hypoxia response has beneficial protective functions in the organism. Hypoxic conditioning also works when applied after the onset of stroke (postconditioning) (Leconte et al., 2009). It has been known for a while that preconditioning also works with hypoxia-mimetic agents like desferrioxamine and cobalt chloride (Bergeron et al., 2000; Jones et al., 2008; Sharp et al., 2001; Zhao and Rempe, 2011). Both pre- and postconditioning activate the same oxygen sensing machinery (Bernaudin et al., 2002; Leconte et al., 2009), and thus, hypoxia-inducible transcription factors (HIFs) and their regulators as well as hypoxia-inducible target gene expression came into focus of attention.

HIFs are heterodimers consisting of an α and a β subunit. HIF-1α/HIF-1β and HIF-2α/HIF-1β dimers are the primary factors regulating hypoxic transcriptional responses in most mammalian cells (Kaelin and Ratcliffe, 2008). HIFs are primarily regulated by changes in protein stability which controls their transcriptional activity in an oxygen-dependent manner. Under normoxic conditions, both α subunits are hydroxylated on conserved proline and asparagine residues by the family of prolyl-4-hydroxylase domain (PHD) proteins and factor inhibiting HIF (FIH), respectively, whose activity is dependent on molecular oxygen, ferrous iron, 2-oxoglutarate and reducing compounds like ascorbate. While prolyl-4-hydroxylation results in recruitment of the von Hippel-Lindau protein E3 ubiquitin ligase and immediate proteasomal degradation of HIF-1α and -2α, asparaginyl hydroxylation blocks interaction with the transcriptional co-activators p300 and CBP, reducing its transcriptional activity (Kaelin and Ratcliffe, 2008). By contrast, when oxygenation declines during hypoxia or ischemia, PHDs and FIH are less active. As a result, hypohydroxylated HIF-α accumulates and translocates into the nucleus to form the HIF complex by dimerization with HIF-1β (also known as aryl hydrocarbon receptor nuclear translocator, ARNT). Subsequently, HIF specifically binds to

Abbreviations: BBB, blood-brain barrier; GFAP, glial fibrillary acidic protein; HIF, hypoxia inducible factor; KO, knockout; MCAO, middle cerebral artery occlusion; PHD, prolyl 4-hydroxylase domain protein; ROI, region of interest; WT, wild-type; SVZ, subventricular zone; VEGF, vascular endothelial growth factor.

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hypoxia response elements (HREs) in the promoters and enhancers of numerous genes followed by recruitment of p300/CBP resulting in enhanced transcription of HIF-responsive genes (Kaelin and Ratcliffe, 2008).

As several HIF downstream targets are able to induce post-stroke neuroprotection, neurogenesis, and angiogenesis, PHDs as HIF regulating enzymes have been considered as potential therapeutic targets. Indeed, newly developed small molecule PHD inhibitors turned out to be neuroprotective in rodent stroke models. For example, a cell permeable peptide inhibitor targeting all three PHD isoforms prevented oxidative death of cortical neurons (Siddiq et al., 2005). Furthermore, the 2-oxoglutarate antagonists dimethyloxallyl glycine (DMOG) or dihydroxybenzoic acid administrated at the time of reperfusion or after the onset of ischemia reduced infarct size, alleviated neurological deficits and increased cerebral blood flow in the compromised hemisphere (Nagel et al., 2011; Ogle et al., 2012; Siddiq et al., 2005). We recently showed using both *in vitro* and *in vivo* ischemia models that the administration of the novel PHD inhibitor FG-4497 promoted neuronal survival, reduced infarct size in both acute and sub-acute stages and stabilized the blood-brain barrier (BBB) (Reischl et al., 2014). Our group also demonstrated that neuron-specific PHD2 deletion significantly reduced the infarct size, activated HIF signaling and declined cell death of hippocampal CA1 neurons during the acute phase after transient cerebral ischemia (Kunze et al., 2012).

These results indicate that PHD2 inhibition might have a predominantly protective role in the central nervous system (CNS). Indeed, among the three isoforms, PHD2 is the key oxygen sensor, setting low steady-state levels of HIF-1α in normoxia (Appelhoff et al., 2004; Berra et al., 2003), and has been demonstrated to be the most abundant isoform in the brain (Rabie et al., 2011). However, it is still unknown whether neuronal PHD2 deficiency also improves stroke recovery in the long term. Furthermore, the underlying cellular mechanisms leading to post-stroke recovery are yet to be characterized in more detail. Finally, since HIF-α is only one of several substrates regulated by PHDs (Myllyharju and Koivunen, 2013; Wong et al., 2013), it remains to be established whether PHD2-dependent neuroprotection requires HIF activation. In this study, we therefore intended to address the unsettled role of neuronal PHD2 implicated in the sub-acute and chronic stages after ischemic stroke by analyzing the cellular response of endothelial cells, microglia and astrocytes. We further characterized the effects exerted by PHD2 inactivation in the absence of neuronal HIF-α.

2. Materials and methods

2.1. Animal experiments

All animal experiments were approved by the local animal welfare committee (Regierungspräsidium Karlsruhe, Germany, approval number: 35-9185.81/G-103/12, 35-9185.81/G-210/14), conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, and were performed in accordance with the recently published Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines (http://www.nc3rs.org/ARRIVE). All mice used in the animal experiments were randomized. The operator or investigator was blinded for the respective mouse genotypes throughout the study. Evaluation of all read-out parameters were done independently and in a blinded fashion.

Table 1
List of primers used to genotype mice.

Gene	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
CaMKIIα:cre	GGTTCTCCGTTTGCACTCAGGA	GCTTGCAGGTACAGGAGGTAGT
Hif1a	GCAGTTAAGAGCACTAGTTG	GGAGCTATCTCTCTAGACC
Hif2a	GAGAGCAGCTTCTCCTGGAA	TGTAGGCAAGGAAACCAAGG
Phd2	CTCACTGACCTACGCCGTGT	CGCATCTTCCATCTCCATTT

2.2. Generation of neuron-specific Phd2 and Phd2/Hif1a/Hif2a knockout mice

All mice were maintained at the animal facility of the University of Heidelberg under specific pathogen-free conditions, a controlled 12:12 hour light-dark cycle, constant room temperature (22 ± 2 °C) and relative humidity (50–55%) with food and water *ad libitum*. Neuron-specific *Phd2* knockout mice ($nPhd2^{\Delta/\Delta}$) were generated as described recently (Kunze et al., 2012). $Hif1a^{flox/flox}$ (Ryan et al., 2000) and $Hif2a^{flox/flox}$ mice (Gruber et al., 2007) were initially intercrossed, and resulting $Hif1a/Hif2a^{fl/fl}$ mice were mated with $nPhd2^{\Delta/\Delta}$ mice to obtain homozygous neuron-restricted knockout mice ($nPhd2/Hif1a/Hif2a^{\Delta\Delta\Delta/\Delta\Delta\Delta}$). Mice were genotyped using primers (Eurofins Genomics, Ebersberg, Germany) described in Table 1. The mean body weight of $nPhd2^{\Delta/\Delta}$ and $nPhd2/Hif1a/Hif2a^{\Delta\Delta\Delta/\Delta\Delta\Delta}$ mice was not significantly different as compared to $Phd2^{flox/flox}$ and $Phd2/Hif1a/Hif2a^{fl/fl}$ animals, respectively (Fig. S1).

2.3. Experimental stroke model

12–14 week old male mice (22–28 g body weight) were anesthetized by the inhalation of a mixture containing 3% isoflurane, 70% N_2O and remainder O_2, and were maintained by reducing the isoflurane concentration to 1.0–1.5%. Core body temperature was maintained at 37 °C throughout surgery by using a feedback-controlled heating pad. Focal cerebral ischemia was induced by permanent transcranial electrocoagulation of the left middle cerebral artery distal to the lenticulostriate arteries (pdMCAO) as described previously (Llovera et al., 2014). A laser-Doppler flowmetry (LDF) probe (Perimed Instruments, Barsinghausen, Germany) was positioned and firmly attached to the skull (1 mm posterior, 3 mm lateral from bregma), and the regional cerebral blood flow (rCBF) was measured before and after electrocoagulation of the distal middle cerebral artery. Animals were sacrificed after 7 or 30 days by cervical dislocation, and brains were harvested and embedded into Tissue-Tek (Sakura Finetek, Staufen, Germany) for histological analyses. From each brain, 24 coronal sections (10 μm thickness; 0.4 mm distance) were prepared using a Leica CM1520 cryostat (Leica Biosystems, Wetzlar, Germany) at a constant temperature of −15 °C, and submitted to Nissl staining (Kunze et al., 2012; Rabie et al., 2011; Reischl et al., 2014). Healthy tissue appears dark while infarcted tissue appears light (cresyl violet-deficient). From each animal series of brain slices were digitized, and the total infarct volume was calculated as the summation of the total infarct area of each section multiplied by the distance between each section. The area of each hemisphere was measured using the ImageJ software. To calculate the infarct area of each section the following equation was applied: $I = (CD + CT - IT)$, where I = infarct area in mm^2, CD = cresyl violet-deficient area in mm^2, CT = total area of the contralateral hemisphere in mm^2, IT = total area of the ipsilateral hemisphere in mm^2. Thus, the total infarct volume in $mm^3 = \Sigma I * 0.4$, where 0.4 = the distance between each section in mm. This equation was used to correct for the increase in volume of the ipsilateral hemisphere due to edema-induced swelling. Accordingly, the following equation was used to

Table 2
Overview of animals that met defined exclusion criteria. Animals that met the following criteria were excluded from end-point analyses: (i) the brain surface was visibly damaged during the surgery, (ii) the MCA bled owing to incomplete artery coagulation during the surgery, (iii) death after induction of MCAO and (iv) intracerebral hemorrhage.

Mouse line	Number of mice met exclusion criteria (% of total animals)				
	i	ii	iii	iv	total
$Phd2^{flox/flox}$	3	1	1	–	5/41 (12%)
$nPhd2^{\Delta/\Delta}$	1	3	1	–	5/50 (10%)
$nPhd2/Hif1a/Hif2a^{fl/fl}$	–	–	–	–	0/15
$nPhd2/Hif1a/Hif2a^{\Delta\Delta\Delta/\Delta\Delta\Delta}$	–	–	–	–	0/15