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Cellular oxygen-sensing through HIF-1α and NF-κB: A therapeutic target for ischemia.

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A therapeutic target for ischemia

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B.S., Biology, University of Virginia, January 2004

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Abstract

**Cellular oxygen-sensing through HIF-1α and NF-κB:
A therapeutic target for ischemia**

By Molly Elizabeth Ogle

Stroke is the fourth leading cause of death and the leading cause of severe disability in the United States and yet few effective treatments are available to reduce ischemic brain damage. The brain has an evolutionarily conserved adaptive response to low oxygen which is a potent protective signaling pathway and a novel target for post-ischemic stroke therapy. Oxygen deprivation inhibits prolyl hydroxylase (PHD) enzyme activity and stimulates a protective oxygen-sensing response in part through the stabilization and activation of the Hypoxia Inducible Factor (HIF) 1α transcription factor and stimulation of the NF-κB transcription factor family. This dissertation tested the therapeutic potential of enhanced activation of oxygen-sensing pathways by pharmacologic PHD inhibition after stroke, hypothesizing that post-ischemic PHD inhibition would reduce neuronal cell death and require the activation of HIF-1α and/or the NF-κB family. The PHD inhibitor dimethyloxaloylglycine (DMOG) enhanced the stabilization of HIF-1α protein and HIF-1-responsive adaptive genes in neural tissues. DMOG also increased the activation of the NF-κB family in cortical neurons. Post-ischemic treatment with DMOG reduced ischemic damage including peri-infarct apoptosis, maintained cerebral blood flow in regions of the ischemic territory that were at risk for infarction, and improved functional outcome after MCAO. The beneficial effects of PHD inhibition after ischemia required HIF-1α and an intact NF-κB pathway. Investigation of the NF-κB family activation through PHD inhibition in neurons suggests that there is an interaction between NF-κB and the expression of HIF-1α mRNA and protein. Taken together, the data presented in this dissertation suggest that supplemental activation of oxygen-sensing pathways after stroke may provide a clinically applicable acute therapeutic intervention for the promotion of neuronal cell survival after ischemia.

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List of Abbreviations

2-OG	2-oxoglutarate
ACA	anterior cerebral artery
CCA	common carotid artery
DMOG	Dimethyloxaloylglycine
EPO	erythropoietin
FADD	Fas associated death receptor
HDAC	histone deacetylase complex
HIF	Hypoxia-inducible factor
HRE	Hypoxia-responsive element
IkB	Inhibitor of kB
IKK	Inhibitor of kB Kinase
MCA	middle cerebral artery
NMDA	N-methyl-D-aspartate
OGD	oxygen-glucose deprivation
PC	preconditioning
PDH	Pyruvate dehydrogenase
PDK1	Pyruvate dehydrogenase kinase 1
PHD	Prolyl Hydroxylase
RHD	Rel homology domain
TCA	tri-carboxylic acid
tPA	tissue plasminogen activator
VEGF	vascular endothelial growth factor

VHL Von Hippel Lindau

Chapter I

Background: Ischemic Stroke

A. Stroke:

1. Prevalence, incidence, and cost

Stroke is the fourth leading cause of death in the United States, contributing to one out of every eighteen deaths. Each year, 795,000 individuals experience a stroke and over 610,000 of those are new attacks. Despite the staggering mortality, approximately seven million Americans over the age of 20 are living in the aftermath of this devastating disease. This statistic makes stroke the leading cause of disability in the United States (Roger, V.L. et al., 2012). Of these surviving patients, half experience hemi-paresis, a quarter are dependent in activities of daily life, and a quarter require long-term care in a nursing facility (Carmichael, S.T., 2005). The annual direct and indirect costs of stroke in the United States for 2010 were estimated to exceed 53.9 billion dollars. With the burgeoning aging population and expense of health care, total cardiovascular disease-related direct medical costs are projected to triple in the next 20 years (Heidenreich, P.A. et al., 2011). Clearly, stroke is a major public health issue that must be further addressed in preclinical research in an attempt to produce new therapeutic advances to limit the number of patients affected by this devastating condition.

2. Etiology

Of all strokes, 87% are ischemic, 10% are intracerebral hemorrhage, and 3% are subarachnoid hemorrhage (Roger, V.L. et al., 2012). The pathology of each type of stroke is distinct; however, this dissertation will focus on ischemic attacks since these constitute the majority of cases. Ischemic stroke is caused by the obstruction of a cerebral artery supplying the brain, resulting in the loss of oxygen and metabolic substrates to the affected region. The most common risk factors are high blood pressure,

high cholesterol, atrial fibrillation, diabetes mellitus, and cigarette smoking (Roger, V.L. et al., 2012). The disability and mortality rendered by stroke is associated with neuronal death, loss of neural circuitry, and consequently regions of diminished brain function.

FDA-approved treatment for stroke is limited to the thrombolytic agent tissue plasminogen activator (tPA) that reduces the injury by disrupting the arterial clot and allowing the return of blood flow to the brain. The therapeutic treatment window is a critical issue in stroke due to the rapid nature of cell death. Current clinical treatment guidelines for vessel recanalization therapy by tPA require the patient to be within 3-4.5 hours of ischemic onset for inclusion (Grunwald, 2011). Administration of the drug beyond that window produces increased risk of hemorrhagic transformation. Because of the limited time-line for administration and the negative consequences of drug delivery beyond the target window, only a small percentage of patients are eligible to receive tPA. As an increasing number of preclinical neuroprotectants have failed in human clinical trials (reviewed in (Lee, J.-M. et al., 2000)), the search for new ways to maximize endogenous neuroprotective pathways is an urgent issue in the field of stroke research. Novel therapeutic approaches must be developed to reduce cell death in the ischemic brain area, thereby preserving neuronal function and decreasing the mortality and disability of stroke victims.

B. Animal models of ischemic stroke.

Experimental models of ischemic stroke provide a reproducible and relatively high-throughput system for analysis of the pathophysiology of ischemic stroke, as well as investigation of new therapeutic approaches. A good model system is one that: a)

reliably recapitulates the human anatomy and pathology; b) produces stable and consistent results so that variables may be introduced; and c) is cost effective. Rodent models provide a mammalian brain and circulatory system that produces cerebral injury that is similar to human stroke in many important ways (Carmichael, S.T., 2005; Demetrius, L., 2005). Notably, the evolving and expanding nature of ischemic damage after stroke is well modeled in rodent stroke. In addition, rodent stroke models can be adapted to produce many variables of human stroke such as size, location, and extent of reperfusion. Some stroke models are more consistent than others. Reproducibility depends on factors such as surgical method and skill, and control of environmental factors such as anesthesia, animal temperature, housing conditions, and time of day. Animal research comes with its costs, but with a consistent stroke model that allows for good reproducibility, the monetary considerations can be kept to a minimum. Smaller rodents are the most cost effective animal model for high-throughput testing. Mouse model systems provide an invaluable tool of genetic manipulation for deciphering mechanistic questions about physiology, pathology, or experimental therapeutics.

Animal models are an essential part of stroke research; however, there are some limitations. For example, mice are not just a miniature version of humans, even though we share an overwhelming conservation in gene and protein expression. The processes of metabolism and ageing can occur much more rapidly in mice compared to humans (Demetrius, L., 2005). Additionally, stroke model systems frequently lack important variables of the underlying cardiovascular disease process that occurs in humans, including the pathologies of high blood pressure, diabetes, obesity, and age. Despite some limitations, rodent stroke provides a powerful tool to investigate physiology,

pathology, and therapeutics. Careful acknowledgement and understanding of the pitfalls of the system should always be considered when extrapolating results from mouse to human pathology.

1. Focal ischemia.

The middle cerebral artery (MCA) is a central target of surgical focal stroke models systems. The MCA is a major artery supplying blood to essential regions of the cerebral cortex. Two major variables contribute to the type of injury that is produced in an MCA occlusion model: location and duration of occlusion. The artery may be occluded at positions either proximal or distal to the origination point of the vessel from the circle of Willis. Proximal occlusion will disrupt the blood flow to a greater vascular bed than distal occlusion and therefore generate a more expansive affected area. The duration of occlusion and whether or not the vessel is reperfused also contributes to the extent of injury. Reperfusion is a prevalent element of human stroke, contributing greatly to the pathology of cell damage and is essential to consider in model systems. Typically, the longer the loss of blood flow, the more damage ensues.

a. Distal MCA occlusion: Somatosensory Barrel Cortex Model.

Distal occlusion of the MCA can be accomplished by unilateral occlusion of one or many distal branch(es) of the MCA (Carmichael, S.T., 2005; Wei, L. et al., 1995). This procedure requires opening the skull by craniotomy to expose the MCA at the surface of the brain (Fig. 1.1A). In the mouse somatosensory barrel cortex stroke model, a major MCA branch that supplies the right cortex is ligated (Fig. 1.1). The somatosensory barrel

cortex is the brain region is responsible for sensorimotor function in the contralateral whiskers, forepaw, and lower jaw (Nunn, P.B. et al., 1991).

MCA occlusion is achieved by sliding a curved suture needle through the dural membrane, behind the vessel, and then out of the brain where suture is tied in a surgical knot. Occlusion at this site is permanent. Due to extensive end-to-end anastomoses between the MCA and the anterior cerebral artery (ACA) (Fig. 1.1, A-B arrows), occlusion of the MCA is compensated by collateral flow from the anterior and posterior communicating arteries (ACA and PCA, respectively) (Wei, L. et al., 2001). If only the MCA is occluded there is little to no damage in the cortex because of this compensatory collateral circulation. To circumvent this issue and produce a focal stroke model with a reperfusion element, the bilateral common carotid arteries (CCAs) are occluded for 7-15 minutes depending on the size of stroke desired and the strain of mice (Majid, A. et al., 2000). CCA occlusion is accomplished by tying a sterile suture around each artery. The suture is then gently removed at the end of the desired occlusion time (7-15 mins). When the CCAs are occluded, there is not a severe reduction of blood flow to the majority of the brain because oxygenated blood continues to be supplied through the vertebral arteries. Since the MCA is occluded distally, the combination of the bilateral CCA occlusion reduces collateral flow to the somatosensory cortex such that the flow in the barrel cortex is reduced to between 10-20% of normal. This insult produces a small focal ischemic infarct in the targeted region (Fig. 1.1, B-C). Release of the CCAs produces reperfusion, which is an important element of the pathology of ischemic stroke in humans and is important to model in the surgical stroke (Carmichael, S.T., 2005). After distal MCA + CCA occlusion, primary ischemic damage is prevalent in the somatosensory

barrel cortex. There is no apparent insult to regions aside from the cortex with 5-15 minutes of CCA occlusion. Cell death expands to the peri-infarct region over the hours and days following occlusion. The mechanisms of ischemic cell death will be discussed in further detail below.

Size and location of a stroke determines the amount and type of damage. According to analysis of population studies in clinical trials, a majority of human strokes are small in size with an approximate hemispheric percentage of 4.5 to 14% (Carmichael, S.T., 2005). These smaller strokes are the target group for neuroprotective therapies in preclinical investigations. Larger strokes typically produce so much rapid damage, there is little hope for post-ischemic intervention aside from rehabilitation therapy. Distal MCA ligation in the mouse barrel cortex stroke produces an infarct of 6-9% of the hemispheric volume. One advantage of this stroke model is that the size and progression of injury is comparable to the highly prevalent small human stroke. The damage produced is very consistent, especially when the same surgeon performs the procedure each time. The focal nature of the stroke produces damage to a restricted region of the cortex in the somatosensory barrel region. Behavioral testing of somatosensory function can, therefore, provide insight into the way that the stroke damage correlates with normal function. The body of this dissertation is based on the distal focal stroke model of MCA occlusion with 7-minute CCA occlusion unless otherwise stated.

b. Proximal MCA occlusion.

Proximal occlusion can be produced by insertion of a silicone-coated filament into the internal carotid artery through the circle of Willis and blocking the bifurcation of the

ACA and MCA (Carmichael, S.T., 2005). This model is also referred to as the intra-luminal

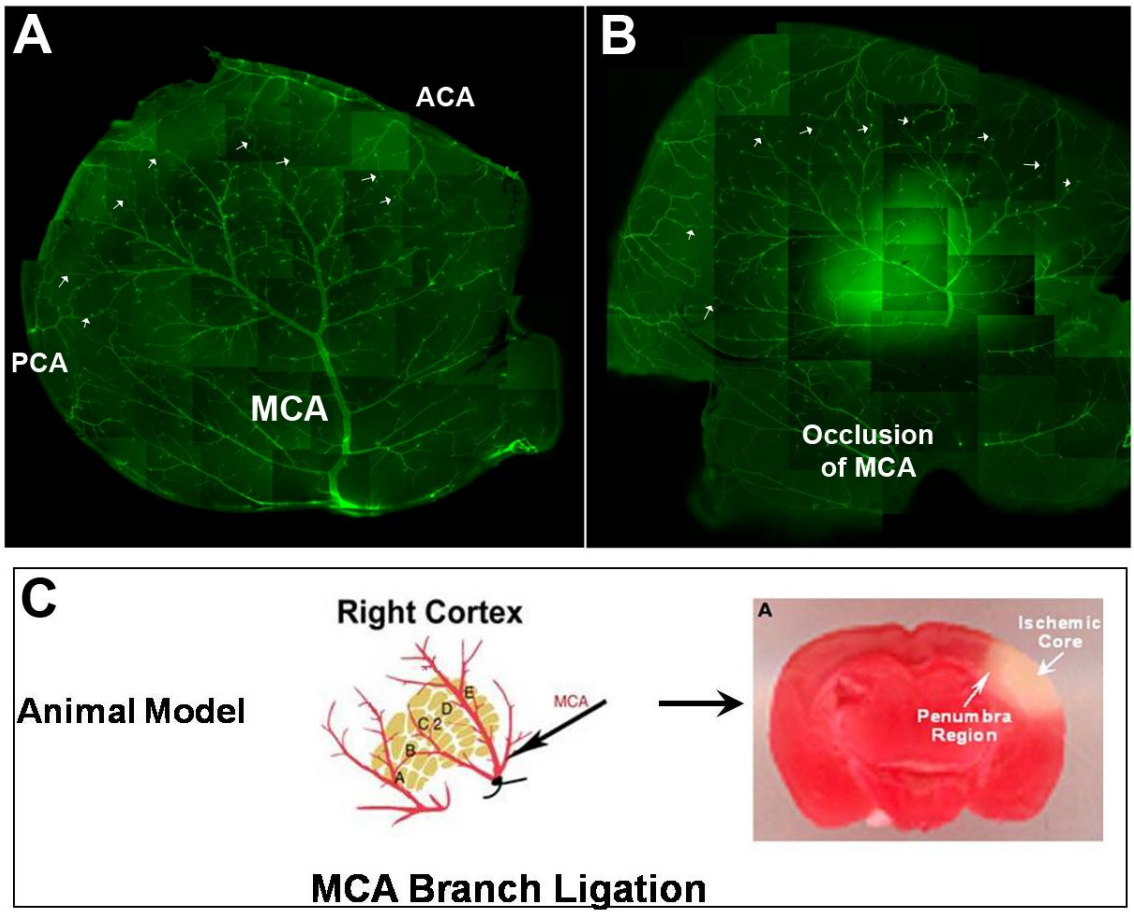


Figure 1.1. Mouse cortical vessel anatomy and distal focal MCA model.

(A) Middle cerebral artery in the mouse cortex is visualized in a transgenic mouse expressing a GFP-labeled arterial marker (smooth muscle α-actin). Small collateral connections between the ACA and the MCA are visible at the top of the cortex. (B) GFP-smooth muscle alpha-actin animal one day after distal focal ischemic stroke. Infarct area appears bright green due to auto-fluorescence of the ischemic core. (C) (left) Diagram of ligation of the MCA in distal MCA occlusion and correlation with neuronal barrel structures that correlate to whisker, forelimb, and lower jaw sensorimotor function. (right) Coronal brain section 3 days after stroke stained with a vital dye (triphenyltetrazolium chloride, TTC). Live tissue is pink and dead tissue is white. Location of infarct within the cortex is evident from the white tissue.

filament model, since the vessel is occluded from the inside. For reperfusion, the filament is withdrawn after 30-120 minutes or the filament can be left in place for permanent occlusion. This model precludes the need for craniotomy; although it requires damage to the tissue surrounding the external carotid arteries, which can affect animal eating and drinking activities. The natural differences in vascular architecture from animal to animal and variation in silicone-coated filaments contribute to a variable range in the size and location of damage in this model. Variability may lower the statistical power in tests of therapeutics and therefore may increase the cost and number of animals required.

This experimental stroke is a large, nearly half-hemisphere stroke and models a human occlusion of a large arterial branch. Initial ischemic cell death can be found in the striatum, frontal, parietal, temporal, and occipital cortices. In a subset of animals, proximal MCA occlusion also damages the thalamus, cervico-medullary junction, substantia nigra and hypothalamus (Carmichael, S.T., 2005). Human stroke does not commonly affect all these regions, particularly the hypothalamus. Damage to the hypothalamus can cause a dysregulation in body temperature, which can interfere with experimental results due to the effect of temperature on metabolic processes. The large size of this stroke model and the many areas affected cause animals to manifest many functional deficits. The damage and functional output is complex due to the wide range of affected neural circuitry. These factors can make analysis of recovery in animal models difficult to interpret. The size of this stroke is much larger than typical human stroke aside from those with malignant infarction (Carmichael, S.T., 2005). One advantage of this model is that it requires less surgical skill for successful stroke. With

careful control of environmental variables and monitoring of blood flow during vessel occlusion, optimization of a more consistent stroke can be achieved.

2. Global ischemia.

Global ischemia is experienced in a clinical situation of cardiac arrest where the brain is deprived of all oxygenated blood. Depending on the duration of ischemia, it leads to bilateral damage in the hippocampus, cerebellum, strial, and cortical regions. In rodent models, global ischemia is most commonly produced by the four-vessel occlusion model targeting the vertebral and carotid arteries (Pulsinelli, W.A. and Brierley, J.B., 1979). The stroke is accomplished by first occluding the vertebral arteries and allowing the animals to recover for at least 24h; then, the bilateral CCAs are occluded reversibly for the desired amount of time for a full four-vessel occlusion. Global ischemia only represents a small fraction of human stroke incidence.

3. *In vitro* ischemia.

In vitro ischemia can be accomplished by removing oxygen and glucose simultaneously from cultured cells/tissue; this process is known as oxygen-glucose deprivation (OGD). Media is exchanged with a basal physiologic balanced salt solution lacking glucose. Cells are then subjected to hypoxic or anoxic environment for varying lengths of time depending on the desired pathology. Mixed culture neurons and glia, or slice cultures may be used for this type of study. Primary cortical neurons must be maintained for more than 10 days in culture to allow maturation of glutamate receptors. A typical model for ischemic cell death is cortical neuron cultures exchanged with a

physiological balanced salt solution and incubated at 0.2% O_2 for 2 hours. Neurons are then returned to normal oxygen and nutrient conditions for 24 hours of "reperfusion".

C. Cerebral ischemic injury

In order to treat ischemia, it is essential to appreciate the underlying physiological and pathological processes and the logical points at which therapeutic interventions would produce a beneficial effect. Ischemia is a complex pathology that embodies multiple combined stresses for neurons and surrounding brain parenchyma incurred by reduced blood flow, oxygen, and metabolic substrates. Neuronal death in the ischemic core occurs within minutes of vessel occlusion. Cell death continues and expands in the "penumbra" or peri-infarct region for several days beyond the original ischemic event through a spectrum of necrotic and apoptotic mechanisms. Initial injury of cortical neurons is mediated through reduced ability to produce ATP and impaired ionic homeostasis. Acute cell death is typically un-programmed necrotic death by cellular swelling and bursting of the plasma membrane secondary to the ionic imbalances. Such acute injury occurs in the core or focus of the ischemia where the reduction of blood flow is most severe. In the brain regions adjacent to the core, the ischemic penumbra, the reduction in blood flow is not as severe due to supply of collateral blood flow from the surrounding arterial tree (Erecinska, M. and Silver, I.A., 1994).

1. Primary injury: the ischemic core.

   a. Oxygen and energy.

Deprivation of oxygen, known as hypoxia, is a major component of several pathologies, including stroke, heart attack, and cancer. Oxygen is physiologically necessary as the terminal electron acceptor of the electron transport chain during aerobic cellular respiration. Processing of glucose through aerobic metabolism involves breakdown of glucose to pyruvate through glycolysis, then pyruvate is processed in the mitochondria through the tricarboxcylic acid (TCA) cycle and the electron transport chain ultimately producing ATP. In the absence of oxygen, cells may breakdown glucose through glycolytic anaerobic metabolism, producing significantly less ATP per glucose molecule. Side effects of anaerobic metabolism include an increase in cellular lactate and decreases in pH. In the absence of oxygen, the reduced capacity to generate energy (ATP) causes neuronal tissues to rapidly lose the ability to control energy-requiring processes such as membrane potential and ionic homeostasis.

Interestingly, mammals have a conserved mechanism to sense fluctuations in oxygen in their environment and respond through modulation of important adaptive pathways, including anaerobic metabolism, glucose uptake, inflammation, angiogenesis and vessel architecture. By understanding the molecular mechanism of oxygen-sensing pathways in brain cells, we may better understand how to prevent cell death during oxygen deprivation. The oxygen-sensing pathways will be discussed in detail in subsequent chapters.

b. Energy demands of neurons and ion homeostasis.

Neuronal tissues are physiologically highly susceptible to injury by ischemia due to their high metabolic demands and relatively low storage supply of glucose or glycogen metabolic substrates (Lee, J.-M. et al., 2000). The rate of cell death varies among

regional populations of neurons; however, typically neurons undergo rapid cell death at the focus of ischemia within minutes (Lipton, P., 1999).

The electrochemical ionic gradients maintained by neurons are essential to their function in the initiation and exchange of signaling impulses/action potentials as well as maintenance of homeostasis. The gradients are characterized by the unequal distribution of Na^+, K^+, and Ca^{2+} between the extracellular and intracellular spaces. Approximately 50-60% of the total ATP produced in the brain is devoted to maintaining the gradients through voltage-gated and receptor-operated channels, ion exchangers, ion pumps, ion transporters, and ATPases (Erecinska, M. and Silver, I.A., 1994). Normal neuronal signaling leads to redistribution of ions coupled to oxygen- and glucose-mediated energy production. Brain pathologies, such as ischemia, hypoxia or hypoglycemia disable energy-supplying processes, resulting in a rapid loss of glucose, ATP and the ability to support ionic gradient homeostasis. During ischemia, glucose concentration in the brain can go from 1.93 µmol/g wet weight of brain tissue to 0.11 µmol/g wet weight in only 2 minutes. In parallel, ATP is depleted from a resting concentration of 2.79 µmol/g to 0.35 µmol/g wet weight (Reviewed by (Erecinska, M. and Silver, I.A., 1994)). These dramatic losses of glucose and ATP lead to the pathological consequences of ischemia.

c. Consequences of ATP depletion.

Ion dysregulation and membrane depolarization. Without a viable mechanism to sustain ATP concentrations, neurons experience an undesirable Na^+ and Ca^{2+} influx, and K^+ efflux. The Na^+/K^+ ATPase is a major transporter that contributes to the normal ionic gradients and is unable to perform in ischemic conditions. Some ion transporters (Na^+/H^+ and $Na^+/K^+/Cl^-$) also require small amounts of ATP for their function (Goss, G.G. et al.,

1994) and may be disturbed in the ischemic core where there is a drastic loss of ATP (Jayakumar, A.R. and Norenberg, M.D., 2010). Since the characteristic unequal ion distribution of neurons normally support the negative membrane polarization, loss of ionic distribution results in depolarization of the membrane and a loss of signaling capacity.

Excitotoxic amino acid release. Ischemic membrane depolarization can cause the unintended release of neurotransmitters such as glutamate at excitatory synapses (Rothman, S., 1984). Lack of energy impairs the function of endogenous excitatory amino acid re-uptake mechanisms. Taken together, increased release and impaired uptake leads to high parenchymal concentrations of the excitatory neurotransmitter glutamate. The specifics of glutamate-induced death will be discussed in detail below. Briefly, glutamate leads to further Na^+ and Ca^{2+} influx which energy depleted neurons cannot rectify.

Cell swelling and explosion. Ionic imbalance leads to the passive entry of water and Cl^- ions into the cell causing swelling. The mitochondria and other organelles swell as well leading to further cellular dysfunction. Eventually, with lack of energy to restore ion gradients or water content, the cells swell to the point where the plasma membrane and organelle membranes are ruptured.

d. The role of excitatory amino acids in ischemic neuronal death.

Glutamate is the major excitatory neurotransmitter in the central nervous system and binds to three types of receptors: N-methyl-D-aspartate (NMDA), DL-amino-3-hydroxy-5- methylisoxazole-propionic acid (AMPA), and kainate receptors. The NMDA receptor is associated with a membrane channel that is selectively permeable to Na^+ and Ca^{2+}

(Mayer, M.L. and Westbrook, G.L., 1987). In pathological situations such as ischemia, extracellular glutamate increases massively through synaptic release after loss of ionic homeostasis and membrane depolarization. During sustained ischemic insult, astrocytes can also contribute to the increased extracellular pool of glutamate (Mitani, A. et al., 1994). Exposure of neurons to excess extracellular glutamate leads to excitotoxic cell death (Mitani, A. et al., 1994). Excitotoxicity by glutamate primarily proceeds through necrotic cellular swelling and bursting (Choi, D.W., 1996; Gwag, B.J. et al., 1997; Olney, J.W., 1986).

Glutamate binds to the NMDA receptor and the receptor-associated channels open and allow influx of extracellular Na^+ and Ca^{2+}. Since ischemia impairs the ability of cells to generate sufficient energy to maintain ion gradients, the internal concentration of Ca^{2+} is increased. Acute cellular swelling following glutamate-stimulated Ca^{2+} and Na^+ influx may cause cells to burst, releasing stores of intracellular glutamate into the extracellular space, further contributing to extracellular glutamate. Exacerbating the problem, elevated intracellular Ca^{2+} stimulates the release of internal Ca^{2+} stores from the endoplasmic reticulum (Bouchelouche, P. et al., 1989). Influx of calcium ions not only produces an imbalance in the membrane potential, but also, calcium is an important signaling molecule for many proteins. Ultimately, glutamate-induced intracellular Ca^{2+} dysregulation contributes to neuronal death in part through activation of catabolic enzymes phospholipase A_2 and C and calpains that disassemble proteins, nucleic acids, and lipids. Genetic or pharmacologic blockade of phospholipases or calpains reduces the damage incurred by stroke, demonstrating that these pathways are important in

contributing to damage (Bonventre, J.V. et al., 1997; Choi, D.W., 1988a; Markgraf, C.G. et al., 1998; Umemura, A. et al., 1992).

NMDA receptor antagonists attenuate anoxic/ischemic neuronal cell death suggesting that NMDA and excitatory amino acids play a role in propagating neuronal injury (Choi, D.W. et al., 1988; Lee, K. et al., 2009b; Rothman, S., 1984). Some neuronal cell types are more vulnerable to excitotoxicity than others, such as the neurons of the CA1 and CA3 regions of the hippocampus (Choi, D.W., 1988b). These regions not surprisingly are also much more sensitive to ischemic insult than cortical regions. The CA1 neurons exhibit much greater NMDA receptor-dependent Ca^{2+} influx, higher concentration of reactive oxygen species, and mitochondrial dysfunction than CA3 neurons under *in vitro* ischemia. Susceptibility to glutamate through different channel compositions is one mechanism for differential neuronal subtype sensitivity to ischemia.

2. Secondary cell death: the ischemic penumbra.

The neurons adjacent to the forming infarct make up what is known as the penumbra region. These cells do not exhibit normal function after stroke; however, they also do not undergo immediate cell death (Astrup, J. et al., 1977). Secondary injury in the peri-infarct/penumbra region occurs through contact with excitatory amino acids released from depolarized neurons, contents of burst cells, including excitatory amino acids and lysosomal proteases, reactive oxygen species and diminished capacity to maintain ATP supplies (Lipton, P., 1999). Further injury is propagated during the progression of inflammation, weakening of the blood brain barrier, and edema (Iadecola, C. and Alexander, M., 2001). Secondary injury in the penumbra is more programmed than the

primary ischemic injury in the core. Since cell death in the penumbra occurs with a slight delay and partly through an active process of signaling, this region may be salvageable through post-ischemic targeted therapies. The delay in cell death provides a window of opportunity to treat patients after stroke, and the active process of apoptosis provides a signaling pathway upon which to target therapeutics.

3. Mechanisms and features of cell death.

c. *Necrosis* is predominantly triggered in severe cell injury such as at the focus of an ischemic stroke. As discussed above, the sudden, overwhelming failure of energy, leads to a passive ionic dysregulation, swelling of cellular compartments and organelles, and bursting of membranes. The release of cellular contents can negatively impact surrounding cells and induce inflammatory responses. Many early observations about ischemic necrosis suggested that it is an un-programmed, cell signaling-independent response to energy failure, since ATP, gene expression and protein synthesis are negligible (Olney, J.W. et al., 1986). There is, however, some evidence that necrosis shares some common features with apoptosis in the terminal cell death processes. These features include the release of cytochrome *c* from the mitochondria leading to activation of caspase-9 and caspase-3 (Niquet, J. et al., 2003).

b. Apoptosis, or programmed cell death, occurs as both a normal physiological and a pathological process. For example, physiologically, apoptosis is involved during development in the pruning of neurons in the brain as proper neural connections are established (Cowan, W.M. et al., 1984). Pathologically, when environmental conditions do not favor cell survival, apoptosis is a controlled mechanism for a cell to "commit

suicide". Apoptosis is morphologically characterized by cell shrinkage, nuclear condensation, membrane blebbing, DNA laddering, and organized cell fragmentation. It is an orderly process of cell death that breaks down macromolecules and parcels cell contents in a way that reduces detrimental effects on neighboring cells and likelihood of an inflammatory response. Apoptosis is a protein synthesis-dependent process and can be blocked by application of protein synthesis inhibitors such as cyclohexamide (Choi, D.W., 1996; Linnik, M.D. et al., 1993). The apoptotic pathway can be roughly divided into inducing and executing signals. Caspases are a family of cysteine-aspartate proteases necessary for both initiation and execution of apoptosis. The protease activity of caspases is selective to specific target sequences and therefore specific target proteins which are involved in processes such as the ordered breakdown of DNA, the cytoskeleton, the nucleus and the plasma membrane (Reviewed by Hengartner, M.O., 2000). Caspases are evolutionarily conserved and were originally identified from work in *C. elegans* (Reviewed by Fuchs, Y. and Steller, H., 2011). Caspase pro-enzymes are widely expressed and have little to no basal enzymatic activity. Activation occurs through auto-catalytic or hetero-catalytic cleavage at conserved sequences by another caspase family member (Thornberry, N.A. et al., 1997). The proteins are typically activated in a particular sequence or cascade, which allows for amplification and integration of different cell death promoting signals. The cascade also provides a regulatory structure for the apoptotic pathway such that many regulatory steps must be met in order for the reaction to continue towards cell death (Hengartner, M.O., 2000).

Caspase-3 is one of the major executioner proteases; activation of caspase-3 is considered committed to the path of apoptosis. Caspase-3 is involved in activating many

of the processes that contribute to the classical morphology of apoptosis. For example, DNA degradation is initiated through cleaving the inhibitor of the caspase-activated deoxyribonuclease (CAD) (Enari, M. et al., 1998; Mukae, N. et al., 1998; Sakahira, H. et al., 1998). Membrane blebbing is due to the caspase-3 mediated activation of ROCK1 (Coleman, M.L. et al., 2001). Chromatin condensation is regulated by caspase-mediated activation of MST-1 (Ura, S. et al., 2001). Pharmacological inhibition of caspase activation can abolish programmed cell death.

While the classifications of apoptosis and necrosis are helpful in distinguishing pathological processes, the death that occurs during ischemia in the brain appears to be somewhat of a continuum, with true necrosis in the core, hybrid death occurring at the border regions of the stroke and apoptosis in the penumbra (Wei, L. et al., 2006a).

c. Evidence for apoptosis in ischemia

The hallmarks of apoptosis are evident in the brain after ischemic stroke, including coarse chromatin aggregation, DNA fragmentation, cell membrane blebbing and the creation of apoptotic bodies, shrinking cell volume, and mitochondrial depolarization (Wei, L. et al., 2006b). Pro-caspases are constitutively expressed in the brain and therefore are readily available for apoptotic signaling in response to damage (Namura, S. et al., 1998). During ischemic injury, caspases, particularly caspase-3, participate in executing neuronal apoptotic death (Chen, J. et al., 1998). Multiple studies have shown that caspases are cleaved and activated in the penumbra region after stroke (Ferrer, I. et al., 2003; Fink, K. et al., 1998; Linnik, M.D. et al., 1993; Namura, S. et al., 1998). The timeline of activation of apoptosis in the penumbra suggests that active caspase-3 is not

expressed after 1h of ischemia, but after 4 hours, caspase-3 and other mediators of apoptosis are prevalent. This delay provides a potential window of opportunity to salvage the tissue surrounding the ischemic core. In fact, interventions that block processing of caspases are neuroprotective in ischemic models, even when delivered up to one hour after stroke (Chen, J. et al., 1998; Fink, K. et al., 1998; Namura, S. et al., 1998).

D. <u>Penumbra: the target of neuroprotective therapies.</u>

The profile of ATP loss during ischemia varies greatly depending on the proximity to the blocked vessel. The core of ischemic stroke is considered to have less than 15% of normal blood flow where the penumbra maintains around 40% flow (Lipton, P., 1999). As discussed above, since neurons are such highly metabolic cells, loss of oxygen and glucose severely impairs their survival and is the primary step in ischemic death. In the intra-luminal filament proximal MCAO, the ischemic core experiences losses of 74 to 93% of normal ATP concentrations from 5 minutes to 4 hours (Folbergrova, J. et al., 1995; Sun, G.Y. et al., 1995). In contrast, penumbral tissues experience approximately 50% loss in ATP (Folbergrova, J. et al., 1995). After 2 hour proximal MCA occlusion, core tissue has negligible remaining supplies of glucose and glycogen, while the penumbra maintains approximately 65% of normal levels (Folbergrova, J. et al., 1995). Penumbral cells are functionally silent, suggesting they may not have enough energy to support neuronal signaling, but the region maintains enough metabolic activity to support limited survival (Broughton, B.R. et al., 2009). The cells in the penumbra receive limited oxygen and metabolic substrates through the blood flow from collateral vessels, allowing

them to sustain viability longer than the core. The differences in blood flow and sustained ATP concentrations between core and penumbra cells partially underlie the distinct pathological processes between the two regions of the tissue.

E. Summary and conclusions

In summary, ischemic stroke is a devastating condition affecting thousands of Americans each year and yet treatment options remain limited to recanalization therapy by tPA and long-term rehabilitation (Grunwald, I.Q. et al., 2011). The development of appropriate animal models for ischemia has contributed to a greater understanding of the underlying pathology of cell death that occurs after stroke. The delayed and programmed nature of cell death in the ischemic penumbra provides a rational target for time windows and mechanisms of therapeutic interventions. With this foundation, the next section will further explore the endogenous protective signaling pathway that is regulated by environmental oxygen as a therapeutic target for minimizing the progression of cell death after ischemic stroke.

Chapter II

Oxygen-sensitive signaling:
Prolyl hydroxylases and Hypoxia inducible factor

A. Oxygen

Oxygen is physiologically essential for aerobic generation of cellular energy through oxidative phosphorylation; however, excessive oxygen can produce damage to proteins and DNA through the formation of reactive oxygen species. Tight regulation and homeostatic control is necessary to balance these oxygen-mediated life sustaining and detrimental effects. In order to regulate oxygen, cells must be able to "sense" the relative level of oxygen in their environment and react to changes in the concentration at a certain critical level. Demand for oxygen varies based on tissue/organism size and metabolic requirements; however, elements of the oxygen-sensing mechanisms are evolutionarily conserved in animals, including the metazoan vertebrate and invertebrate lineages (Kaelin, W.G., Jr. and Ratcliffe, P.J., 2008; Rytkonen, K.T. and Storz, J.F., 2011). Invertebrate model systems such as *C. elegans* and *D. melanogaster* express primitive versions of the key protein families involved in sensing oxygen (Jiang, H. et al., 2001; Lavista-Llanos, S. et al., 2002; Nambu, J.R. et al., 1996; Rytkonen, K.T. and Storz, J.F., 2011). Examination of the evolutionary border between the unicellular protists and simple multi-cellular animals demonstrates that the simplest animal *T. adhaerens* expresses the hypoxia-inducible factor (HIF) transcription factor, but choanoflagellate *M. brevicollis* (unicellular species closest to animals) does not (Loenarz, C. et al., 2011). Single celled organisms attain their oxygen by diffusion from the environment, where multi-cellular organisms evolved circulatory systems to distribute oxygen around tissues. The branch between unicellular and multi-cellular species is a great evolutionary step not only in body organization, but also in regulatory systems for maintaining homeostasis

such as oxygen-sensing. The conservation of this pathway suggests its importance in animals.

The mammalian brain is a highly metabolic tissue, and requires considerable oxygen in order to generate sufficient energy to fuel basal activity. Due to the vital importance of oxygen and exquisite sensitivity of neurons to variations in environmental oxygen levels, neuronal tissues are an excellent system to study the mechanisms controlling reaction to varying oxygen levels. The mechanisms of signaling in these natural homeostatic pathways may provide insight for the development of therapeutic interventions for stroke.

B. Prolyl hydroxylases: Cellular oxygen-sensors

The prolyl hydroxylase (PHD) enzyme family is a central component of the oxygen-sensing machinery (Bruick, R.K. and McKnight, S.L., 2001). PHDs are part of a super-family of 2-oxoglutarate (2-OG) and iron-dependent dioxygenases. PHDs require molecular oxygen, 2-OG, iron, and ascorbate for the hydroxylation of proline residues within conserved sequences of target proteins (Schofield, C.J. and Zhang, Z., 1999). In the hydroxylation reaction, the enzyme uses one molecule of oxygen to hydroxylate a proline residue and another to couple the reaction to the decarboxylation of 2-OG into succinate (Reviewed by Semenza, G.L., 2007) (Fig. 2.1).

One key property of PHD family enzymes is that they require oxygen as a co-substrate. Therefore, low environmental oxygen causes a substrate-level reduction in activity of the enzymes (Jaakkola, P. et al., 2001). PHD enzyme activity is dependent on oxygen and signaling events are mediated by the local environmental oxygen concentrations. In this way, PHDs allow the cell to "sense" and react to varying oxygen

levels. The activity of PHDs is inhibited or greatly reduced during hypoxia or ischemia in the brain, which plays a role in regulating the pathophysiological response to these events.

1. PHD isoform expression, localization, regulation.

The PHD family is composed of three major enzymes (1-3) that are highly conserved in mammals. The active sites of the three isoenzymes also maintain considerable conservation. Differences in the isoenzyme specificity may lie in their more variable N- and C-terminal regions (McDonough, M.A. et al., 2006). The PHDs are expressed throughout most tissues, including the brain, but each isoform is expressed at varying levels. PHD-1 is most highly expressed in the adult brain, placenta, lung, and kidney. At least three splice variants of PHD-2 are expressed, one full length (exons 1-4), and two additional versions lacking either exon 3 or 4. Full-length PHD 2 mRNA is most highly expressed in the adult heart, brain, lung, and liver and in fetal brain, heart, spleen, and skeletal muscle. PHD 3 is also alternatively spliced with two major mRNA variants that are expressed equally. The expression of full-length PHD-3 is highest in the heart, brain, placenta, lung, and skeletal muscle and fetally in the heart, spleen and skeletal muscle. Splice variants of PHDs do not have measurable activity, possibly due to lack of one of the iron-binding sites; therefore, the physiological significance of the alternatively spliced mRNAs and resultant proteins is unclear (Hirsila, M. et al., 2003). The sub-cellular localization differs between the three isoforms. PHD-1 is primarily localized in the nucleus, PHD-2 in the cytoplasm, and PHD-3 is fairly heterogeneous in cellular

localization (Gout, O. et al., 1999). The location of each protein on the tissue and cellular level may provide insight into their individual functions.

2. Pathophysiologic inhibition of PHDs

PHDs use oxygen to catalyze the oxidative decarboxylation of 2-OG yielding succinate and carbon dioxide while hydroxylating a proline residue. PHDs require oxygen and 2-OG as substrates to perform the proline hydroxylation so the enzymes are inhibited by the reduced availability of either oxygen or 2-OG. Depletion of the co-factors ascorbate and iron also inhibit or reduce the activity. Over-abundance of succinate, a product of the hydroxylation reaction, reduces the activity of PHDs. Succinate is a Kreb cycle intermediate, so disruptions in normal mitochondrial metabolism can contribute to PHD inhibition despite oxygen levels (Abdel-Fattah, M. et al., 2011). Excess succinate can be caused by the mutation of succinate dehydrogenase (Lee, S. et al., 2005; Selak, M.A. et al., 2005).

Many groups have attempted to measure the affinity of the PHD enzymes for oxygen in many systems. The projected affinity of the PHD enzymes for oxygen is relatively low with K_m values in the range of 230-250uM (Hirsila, M. et al., 2003). These values are higher than typical tissue oxygen concentrations; arterial oxygen is approximately 120μM, and oxygen concentration in capillary and interstitial tissue is less than 40μM (Ward, J.P., 2008). Tissue responses to oxygen changes are highly non-linear, suggesting a combination effect of oxygen and other factors.

Very few proteins have been identified as PHD-target proteins. The proline residues in the HIF-1α oxygen-dependent degradation domain are the best-characterized substrate for PHD hydroxylation (Bruick, R.K. and McKnight, S.L., 2001; Epstein, A.C. et al.,

2001). More recently, studies have demonstrated that the hypoxic activation of the NF-κB family of transcription factors is in part regulated by PHD inhibition and proline hydroxylation of upstream kinases in the NF-κB pathway, but these findings have not been directly characterized in a neuronal system (Cummins, E.P. et al., 2006).

3. Pharmacological inhibition of PHDs.

Competitive inhibition of the 2-OG binding site inhibits PHDs activity if the molecule occupying the binding site cannot undergo the oxidative decarboxylation reaction. Dimethyloxaloylglycine (DMOG) and dihydroxybenzoate are 2-OG analogs that bind to PHDs and competitively inhibit their activity (Fig. 2.1). Iron is essential for PHD activity, so iron chelators such as cobalt-chloride and desferrioxamine can also be utilized to chemically inhibit PHDs and thereby activate oxygen-sensing signaling (Prass, K. et al., 2002). Gene arrays have shown that there is a concordant regulation of genes in both the positive and negative direction between hypoxia and DMOG treatment of cells, suggesting that hypoxia and PHD inhibitors activate the same transcriptional pathway or pathways (Elvidge, G.P. et al., 2006).

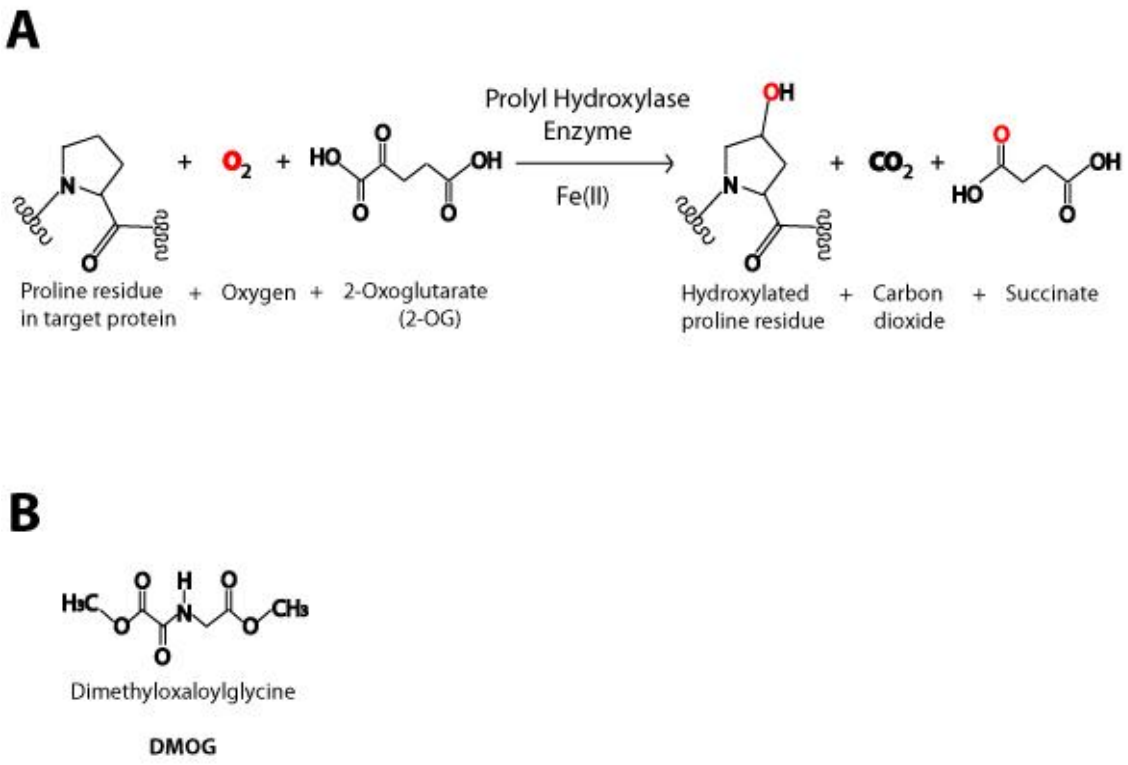


Figure 2.1. Prolyl hydroxylase enzymes

(A) PHDs require molecular oxygen, 2-oxoglutarate (2-OG), and iron to hydroxylate a target proline residue. One molecule of oxygen is used to hydroxylate the proline. The hydroxylation is coupled to the decarboxylation of 2-OG into succinate. Iron is a co-factor for both parts of this reaction.

(B) The 2-OG analog DMOG competitively inhibits the binding site for 2-OG in all PHD enzymes. DMOG cannot participate in the decarboxylation reaction and therefore inhibits the activity of the enzymes.

C. <u>The Hypoxia-Inducible Factor (HIF)</u>.

The HIF transcription factor plays an essential role both in development as well as the cellular response to hypoxia. Knockout of the HIF-1α gene in mice is embryonic lethal due to defects in embryonic vascularization (Iyer, N.V. et al., 1998). HIF-1 is a hetero-dimeric transcription factor with an oxygen-sensitive α-subunit and a constitutively expressed ß-subunit. HIF-1 is a member of the basic helix-loop-helix per-ARNT-sim family of transcription factors (Wang, G.L. and Semenza, G.L., 1995). HIF-1α protein is nearly absent or undetectable in normoxic cells and tissues, although it is constitutively transcribed (Semenza, G.L., 2001). The protein is degraded under normal oxygen conditions through E3-ubiquitin ligase-targeted proteasomal degradation but is robustly stabilized under low oxygen tension (Jiang, B.H. et al., 1996; Yeager, M. et al., 2009).

PHDs post-translationally regulate the expression of HIF-1α by hydroxylating two proline residues in the protein that promote interaction with the E3-ubiquitin ligase Von Hippel Lindau (VHL). PHD enzymes recognize the proline residues in the consensus motif of *Leu-X-X-Leu-Ala-Pro* in HIF-1α's oxygen dependent degradation domain (X is any amino acid) (Ivan, M. et al., 2001; Jaakkola, P. et al., 2001; Yu, F. et al., 2001a, b). Hydroxylation of HIF-1α on Pro-402 and Pro-564 by PHDs under normoxic conditions enhances binding of HIF-1α to VHL and proteasomal targeting (Jaakkola, P. et al., 2001) (Fig. 2.2).

All three PHD enzymes can hydroxylate HIF-1α, however, PHD-2 hydroxylates HIF-1α with faster kinetics than the other two isoenzymes and is suggested to be the major regulator of HIF-1α (Kitchen, C.M. et al., 2009). PHD-2 is the isoenzyme that is most commonly located in the cytoplasm, which corresponds to the localization of inactive

HIF-1α protein. PHD-3 effectively hydroxylates Pro-564 but displays slower kinetics than PHD-2 and has low or undetectable hydroxylation at the Pro-402 site. PHD-1 has very little hydroxylation activity on either of the conserved proline hydroxylation sites in HIF-1α. The two prolines in HIF are differentially regulated. PHD-2 hydroxylates Pro-564 more rapidly than Pro-402, suggesting that the hydroxylation may be a sequential event, although the biological significance is not clear (Chan, D.A. et al., 2005; Pappalardi, M.B. et al., 2011).

Hydroxylated HIF-1α is poly-ubiquitinated and targeted for proteasomal degradation, thereby keeping steady-state levels of HIF-1α protein low (Ahmad, A. et al., 2005). In hypoxia, PHD activity is limited by low substrate availability of oxygen and thus HIF-1α is not degraded and protein accumulates in the cell. HIF-1α hetero-dimerizes with the non-oxygen sensitive HIF-1β subunit. Together, the hetero-dimer translocates to the nucleus, and binds to hypoxia responsive DNA elements in target genes to regulate hypoxia-adaptive gene expression. The expression of PHD-2 and -3 mRNA and protein increases during hypoxia, perpetuating a negative feedback loop to turn off HIF-1 activity (Aprelikova, O. et al., 2004; Francis, K. et al., 2009; Wei, E.P. and Kontos, H.A., 1999).

1. Oxygen-sensing and ischemic stroke.

HIF-1α is upregulated within hours after stroke and in some models exhibits a biphasic response with an early 1-24 h stabilization followed by a drop in protein and then a longer term upregulation of protein between two and seven days (Baranova, O. et al., 2007). HIF-1 activation in neurons contributes to the positive and negative regulation of hundreds of genes. Neuron-specific (CaMKII-Cre-driven) knockout of HIF-1α

increases ischemic damage after MCAO, implying that HIF-1α is a protective factor in the endogenous response to ischemia (Baranova, O. et al., 2007).

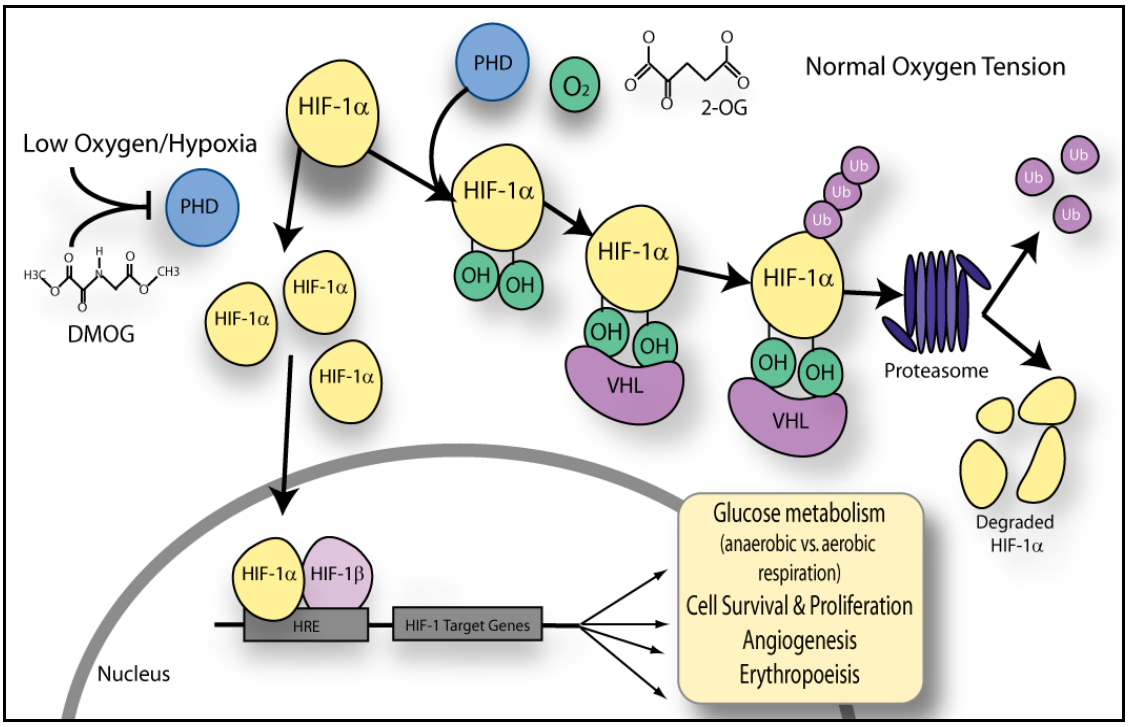


Figure 2.2. Regulation of HIF-1α by PHDs.

Under normal oxygen tension, in the presence of oxygen, 2-OG, iron, and ascorbate, PHDs hydroxylate the two consensus proline residue on HIF-1α. The hydroxylated-prolines enhance the interaction between HIF-1α and VHL E3-Ubiquitin ligase. HIF is poly-ubiquitinated and degraded through the proteasome. In low oxygen, hypoxia, or in the presence of the competitive inhibitor/2-OG analog, DMOG, PHD enzyme activity is blocked. HIF-1α proline residues are not hydroxylated and therefore the protein is stabilized. HIF-1α and HIF-1β heterodimerize and translocate to the nucleus. The HIF-1 transcription factor binds to hypoxia responsive elements, in conjuction with other regulatory transcription factors. Genes involved in the adaptive response to hypoxia are regulated.

D. Adaptive gene regulation in hypoxia.

As discussed previously, cells have a vested interest in monitoring the level of oxygen in their environment. Too much oxygen can cause oxidative damage, while too little oxygen can cause energy failure. In order to compensate for the latter, when cells "sense" low oxygen with the PHD enzymes, HIF-1 activity is increased. The HIF-1 transcription factor responds to low oxygen by stabilization of the α-subunit and activation of an adaptive transcriptional response. HIF-1 is activated by hypoxia, ischemia, and pharmacologic PHD inhibition and is an important mediator of endogenous neuroprotective response to ischemia in neurons (Baranova, O. et al., 2007). HIF-1 targets a variety of genes with a central theme of adaptation to lower oxygen conditions. These genes are involved in anaerobic energy metabolism, glucose transport, cell proliferation, cell survival/death, angiogenesis, and erythropoiesis.

1. Adaptation: Metabolism.

The reduction of cellular oxidative respiration during hypoxia or ischemia is not only due to the passive decline of environmental oxygen, but also through active signaling events. Active suppression of oxidative respiration manifests as attenuated pyruvate catabolism and mitochondrial oxygen consumption with a promotion of glycolytic metabolism mechanisms (Kim, J.W. et al., 2006; Papandreou, I. et al., 2006). The glycolytic enzyme pyruvate dehydrogenase kinase 1 (PDK1) is a HIF-1 target gene, which negatively regulates pyruvate dehydrogenase (PDH). PDH is responsible for the conversion of pyruvate into acetyl-CoA for entry into the TCA cycle and aerobic metabolism. Increase in PDK thus prevents pyruvate entry into the TCA cycle by

inhibiting PDH activity. The expression of PDK1 is inversely related to oxygen consumption (Kim, J.W. et al., 2006; Papandreou, I. et al., 2006). Cardiomyocytes actively suppress oxygen consumption after treatment with PHD inhibitors and activation of HIF-1α, even in normoxic conditions (Sridharan, V. et al., 2008). The increased expression of HIF-1 and PDK1 in cancer cells and fibroblasts leads to the suppression of oxygen consumption (Papandreou, I. et al., 2006).

Transcription of some glycolytic enzymes is also enhanced by activation of HIF-1 (Iyer, N.V. et al., 1998; Zaman, K. et al., 1999a). Exposure of human cell cultures to 1% hypoxia leads to an increase in glycolytic enzyme mRNA for pyruvate kinase M, phosphoglycerate kinase 1, and aldolase A. The promoters of these genes all possess a hypoxia-responsive element (HRE) consensus sequence of 5'-(C/G/T)ACGTGC(G/T)-3' suggesting regulation by HIF-1 (Semenza, G.L. et al., 1994). Increase in glycolytic enzyme production suggests an increase in utilization of anaerobic metabolic mechanisms.

Cells that activate HIF-1 under hypoxia are able to conserve oxygen by reducing oxidative phosphorylation and maintaining some production of ATP through increased glycolytic metabolism. In the brain, since storage of metabolic substrates is low compared to other tissue types (Lee, J.-M. et al., 2000), this mechanism may only sustain the cells for a short period. Hypoxia also signals the enhanced expression of glucose transporters 1 and 3, which helps to maximize the uptake of available environmental glucose, however in the ischemic core, there is extremely limited environmental glucose (Bergeron, M. et al., 1999). One side effect of glycolytic metabolism is the increased

production of lactate and decreased pH, so this system may sustain cell viability for short term, but a long-term switch to glycolytic metabolism could contribute to further damage.

2. Adaptation: Cell survival

A number of mechanisms may be involved in the promotion of cell survival by the oxygen-sensing pathway. HIF-1 can promote the expression of neurotrophic growth factors and hormones such as the vascular endothelial growth factor (VEGF) and erythropoietin (EPO) (Forsythe, J.A. et al., 1996; Wang, G.L. and Semenza, G.L., 1993b). VEGF and EPO can both function as neuroprotective molecules after ischemia by reducing cell death and caspase-3 activation in neuronal ischemic model systems (Jin, K.L. et al., 2000; Keogh, C.L. et al., 2007; Kilic, E. et al., 2005; Sun, Y. et al., 2003). Both proteins are well-established promoters of cell survival in neuronal systems. The HIF-1-mediated activation of EPO is implicated in supporting protective responses to hypoxia (Acler, M. et al., 2009a). Administration of EPO protein before or after ischemia in mice attenuates brain damage from focal ischemia (Li, Y. et al., 2007).

Exogenous stimulation of HIF-1 in a TNF-α mediated model of intestinal inflammatory death reduces TNF receptor mediated apoptosis through a HIF-1-dependent transcriptional repression of the Fas-associated death receptor (FADD) (Hindryckx, P. et al., 2010). These data suggest that HIF-1 regulated genes contribute to cell survival in many ways, including suppression of death associated genes and activation of neurotrophic growth factors and hormones.

3. Adaptation: Vascular system

HIF-1 also promotes adaptive changes in the vasculature by regulation of genes that promote angiogenesis such as VEGF and its receptors, Flt and Flk. New vessel growth over longer periods of ischemia contributes to revascularization of the damaged region. HIF-1 contributes to the production of more red blood cells through the increased expression of EPO. This factor is not only a neuroprotective factor, but also can participate in paracrine signaling to induce erythropoeisis (Wang, G.L. and Semenza, G.L., 1993b). Production of more red blood cells during low oxygen may help to deliver more oxygenated blood to the remaining perfused tissue after ischemia. HIF-1 also targets genes involved in regulation of vascular tone, thereby influencing the control of reperfusion after ischemia. These targets include the endothelial nitric oxide synthase (eNOS) (Coulet, F. et al., 2003; Palmer, L.A. et al., 1998; Samdani, A.F. et al., 1997) and ecto-5'-nucleotidase (CD73) (Hart, M.L. et al., 2011).

Beyond the specific targets discussed here, hypoxia mediates the up- and down-regulation of hundreds of genes. HIF-1 is a major contributor to the expression of these hypoxia-sensitive genes since neuron-specific knockout of HIF-1α reduces the ischemia-mediated induction of these genes (Baranova, O. et al., 2007). The oxygen-sensing response provides a cellular mechanism to adapt to environmental changes. The potent protective nature and broad coordinated efforts of theses oxygen-sensitive transcriptional responses make this pathway an optimal target to enhance cell survival after stroke.

E. Therapeutic use of oxygen-sensing pathways

1. Preconditioning

Preconditioning (PC) or ischemic tolerance is the paradoxical phenomenon by which a brief exposure to a sub-lethal priming event induces an enhanced tolerance to a subsequent severe and potentially lethal ischemic episode. The priming event induces endogenous protective signaling mechanisms and the tissue is acutely protected from ischemic injury. There are many different kinds of stimuli that induce an ischemic tolerance phenotype in the brain. These include ischemia (Kitagawa, K. et al., 1990; Liu, Y. et al., 1992; Murry, C.E. et al., 1986), hypoxia (Gidday, J.M. et al., 1994), PHD inhibitors (Siddiq et al., 2008; Siddiq, A. et al., 2005), iron chelators (Siddiq, A. et al., 2005), hypothermia (Chen, P. et al., 2000), hyperthermia (Chen, P. et al., 2002), hyperbaric oxygen (Chen, W. et al., 2008; Wei, L. et al., 1995), inflammatory mediators (Lee, D.W. et al., 2009), and oxidative stress (Murphy, T.H. and Corbett, D., 2009). PC does not make a tissue completely invincible to subsequent ischemic damage; the amount of protection is linked to the strength and duration of ultimate ischemic insult. For example, in cardiac ischemia, an ischemic PC paradigm protects against a 40 minute ischemic challenge, but not a 3h ischemic challenge (Murry, C.E. et al., 1986). Mechanisms by which the PC stimuli induce tolerance to subsequent ischemia are the subject of a vast body of research and a great number of mechanistic questions still linger.

Hypoxic and ischemic PC studies lead to the initial understanding of the presence of an oxygen-mediated endogenous protective signaling pathway. Sub-lethal hypoxia stimulates endogenous cellular oxygen-sensing pathways, which are necessary in the PC-mediated protection. PC with PHD inhibitors 6 hours prior to ischemia leads to substantial reduction in infarct formation and better neurological recovery scores. HIF-

1α is implicated in the development of the protection since the brain-specific HIF-1α knockout mouse (CamKII-Cre driven) had a reduced response to 2 different PHD inhibitors (Baranova, O. et al., 2007). These data suggest that HIF-1α plays a role in PC-mediated protection, but is not the only factor contributing to the beneficial effects (Baranova, O. et al., 2007).

This potent natural protective response is an attractive target for treatment of ischemic stroke; however, the clinical use of PC is limited by the unpredictable nature of ischemic stroke. Encouragingly, various forms of PC are currently in clinical trials for use in patients undergoing elective heart or brain surgery (www.clinicaltrials.gov).

PHD inhibitor therapy differs from both hypoxic and ischemic PC because it activates the oxygen-sensing pathways without actually depriving the cells of oxygen or glucose. Additionally, PHD inhibition can be effected pharmacologically without oxygen deprivation or invasive manipulation of the vasculature. The PC model provides an invaluable research tool to further understand the complex mechanisms of ischemic cell death and mechanisms of endogenous neuroprotection that may be targeted for post-ischemic therapies.

2. Post-conditioning.

Interestingly and contrary to the original model that a delayed time window was necessary for ischemic tolerance, many new studies have demonstrated that post-ischemic conditioning interventions manifest similar protective functions as PC. Mechanical postconditioning, also known as “modified reperfusion” is accomplished by repeated cycles of vessel occlusion and release during reperfusion. In rat proximal MCA

occlusion, postconditioning of multiple cycles of reperfusion and re-occlusion immediately after transient intra-luminal MCA occlusion or global ischemia improves the behavioral neurological score, and reduces infarct volume compared to MCAO alone (Chen, L. et al., 2005; Chen, Y.F. et al., 2006; Ferrer, I. et al., 2003; Iadecola, C. and Alexander, M., 2001; Pignataro, G. et al., 2008; Zhao, H. et al., 2006). Reduced infarct volume correlated with reduced incidence of TUNEL-positive dead cells in the penumbra region, but not the ischemic core (Zhao, H. et al., 2006). The effectiveness of the postconditioning treatments vary based on duration and interval of occlusions as well as the delay of treatment after reperfusion. Treatment was more significantly effective in smaller stroke models (Zhao, H. et al., 2006). Similar to PC, the amount of protection seems to be linked to the severity of the insult.

Encouraging results from a human clinical trial for myocardial postconditioning suggests a promising translation of postconditioning to patients (Greijer, A.E. et al., 2005). While the demonstration of the feasibility of ischemic postconditioning is encouraging, the mechanisms remain unclear. The protection mediated by postconditioning is not additive with PC since application of both PC prior to ischemia and postconditioning after ischemic challenge did not increase the amount of protection over PC or postconditioning alone (Pignataro, G. et al., 2008). These findings suggest that the pre- and post- ischemic targeting of tolerance may occur through similar pathways that are already maximally stimulated. There are currently no reports on whether oxygen-sensing mechanisms may play a role in the phenomenon of postconditioning.

Two studies indicate that postconditioning stimulates activation of Akt, a protein invoked in survival processes (Hoyte, L.C. et al., 2010; Pignataro, G. et al., 2008). Addition of a specific inhibitor of Akt, LY294002, reduced the infarct-sparing property of postconditioning (Hoyte, L.C. et al., 2010; Pignataro, G. et al., 2008). Active Akt has previously been indentified as a positive regulator of HIF-1α (Harada, H. et al., 2009); however the correlation between Akt and HIF-1α in postconditioning remains untested.

PC studies implicated NF-κB as essential for development of ischemic tolerance (Blondeau, N. et al., 2001). The NF-κB family is another target of the PHD enzymes (Cummins, E.P. et al., 2006) and this regulation will be discussed in detail in the next chapter. In postconditioning, inhibition of NF-κB with Diethyl-dithiocarbamic acid, blocks the majority of postconditioning mediated attenuation of infarct volume (Chen, Y.F. et al., 2006). The downstream effectors of NF-κB activity in postconditioning have not yet been investigated.

Postconditioning provides a novel potential post-ischemic intervention for ischemic stroke patients and suggests that there are signaling mechanisms inducible after ischemia that can mediate a reduction in damage. The role of oxygen-sensing pathways in postconditioning remains unclear. The fact that PC and postconditioning responses are not additive may indicate that the same pathways are maximally activated by each conditioning stimulus. No direct evidence for the role of HIF-1 in postconditioning has been described, however, the Akt pathway, which is known to stimulate HIF-1α, is required for neuroprotection. Additionally, NF- κB, which is a PHD-regulated pathway, is required for postconditioning-mediated protection. Taken together, this evidence

suggests that the oxygen-sensing response may be involved in postconditioning-mediated protection.

The invasive manipulation of brain vessels is impractical for human emergency situations; however, post-ischemic tolerance may be initiated pharmacologically by targeting the cellular and molecular pathways that are activated by pre- and post-conditioning. Modulation of PHDs, a major preconditioning target (Siddiq, A. et al., 2005), provides a method of triggering endogenous protective mechanisms without applying hypoxic or invasive ischemic treatments.

F. Summary and conclusions

In summary, oxygen-sensing is a powerful evolutionarily-conserved cellular defense against the negative consequences of low oxygen. The PHD enzyme family works as a sensor for the availability of oxygen and metabolic substrates. Activity of PHD enzymes through signaling events to downstream propagators of the hypoxic response is determined by the relative availability of oxygen and metabolic intermediates (eg. succinate). Activation of the HIF-1 transcription factor mediates a coordinated stress response that stimulates adaptive changes for survival under low oxygen. Therapeutic oxygen deprivation or ischemia before or after stroke leads to a paradoxical protective effect against tissue damage. The PHD and HIF-1 signaling axis are clear regulators of the PC response, but the activity of this pathway in postconditioning awaits further elucidation. The following studies in this dissertation explore whether globally enhancing oxygen-sensor signaling after ischemia with the PHD inhibitor DMOG provides neuroprotection after ischemic stroke. The roles of two hypoxia-regulated

signaling pathways, HIF-1α and NF-κB, will be analyzed for their contribution to protection and signaling in the pathology and therapy of ischemic stroke.

Chapter III

NF-κB
Interaction of oxygen-sensing and inflammatory pathways

NF-κB is a ubiquitous and pleiotropic transcription factor family with an evolutionarily conserved role in a broad range of biological processes including inflammation, development, apoptotic cell death, and cell survival. NF-κBs respond to a diverse range of activating signals to produce an equally broad spectrum of cellular responses (Li, X. and Stark, G.R., 2002). The NF-κB family is expressed, organized and regulated in a way that allows for rapid activation of transcription factor units by a broad array of stimuli and therefore plays an important role in many early stress responses. NF-κB family members are expressed in the brain and participate in signaling during homeostasis, development, and pathological processes (O'Neill, L.A. and Kaltschmidt, C., 1997; Saleh, A. et al., 2011). The NF-κB pathway can be activated through the inhibition of oxygen-sensing PHD enzymes (Cummins, E.P. et al., 2006). Several studies have discussed reciprocal interactions between the regulators of innate immunity (NF-κB) and the hypoxic response. The current understanding of the NF-κB signaling network, the model of activation of NF-κBs by PHD inhibition, and the interaction between the inflammatory and HIF-1α pathways will be reviewed in this chapter.

A. NF-κB transcription factor family.

1. <u>NF-κB transcription factor family members</u>.

There are five main constituents of the NF-κB family: p105/p50 (NF-κB1), p100/p52 (NF-κB2), p65 (RelA), RelB, and c-Rel. These proteins are characterized by a highly conserved Rel homology domain (RHD) which allows for subunit dimerization, DNA binding, nuclear translocation, and interaction with inhibitory proteins (Li, X. and Stark, G.R., 2002). The C-terminal domains of the five NF-κB proteins are less conserved than

the RHD and therefore the proteins can be divided into two sub-families based on the C-terminal structure.

The Rel sub-family members (p65 (RelA), RelB, and c-Rel) have a C-terminal transactivation domain for activation of transcription, whereas the NF-κB sub-family members (*NFKB1*-p105/p50 and *NFKB2*-p100/p52) do not have transactivation domains (Li, X. and Stark, G.R., 2002). The NF-κB sub-family proteins are synthesized as inactive 105 and 100 kDa proteins respectively. Upon the appropriate upstream signals, p100 is proteolytically cleaved to produce p52 (Lin, L. and Ghosh, S., 1996). Stimulus-specific processing of p105 produces p50 by proteolysis (Lang, V. et al., 2003). The p50 protein can also be liberated from p105 co-translationally in the 20S proteasome where it undergoes immediate dimerization with p105 protein produced from the same transcript (Moorthy, A.K. et al., 2006).

2. Dimerization and DNA binding specificity.

A functional transcription factor unit is made up of a homodimer or heterdimer of NF-κB subunits. All of the subunits can homo- or hetero- dimerize except for RelB, which is only known to participate in heterodimers (Sun, S.C., 2011). Many of the possible dimer pairs have been described in the literature; however, the p50/p65 heterodimer is the most ubiquitous of all the dimers and therefore tends to be the most commonly studied. The composition of dimer pairs determines their specificity in DNA binding and transcriptional regulation (Hoffmann, A. and Baltimore, D., 2006; Perkins, N.D. et al., 1992).

The NF-κB subfamily proteins p50 and p52 do not have a C-terminal transactivation domain so these two sub-units cannot activate transcription as homodimers, but are able to participate in heterodimers with Rel-sub-family proteins to contribute to transcriptional activation. The homodimeric p50-p50 NF-κB can bind to -κB sites and block transcription (Driessler, F. et al., 2004). This basal inactivation of NF-κB responsive promoters occurs in part through association with histone deacetylase complex-1 (HDAC) (Zhong, H. et al., 2002). The p50-p50 dimer also can respond to specific stress or signaling inputs as a stimuli-specific transcriptional repressor (Tong, X. et al., 2004).

Each different NF-κB dimer has a slightly different preference for κB motif sequences and interacts with distinct co-activators (Kunsch, C. et al., 1992; Perkins, N.D. et al., 1992; Zhong, H. et al., 2002). This produces a great diversity in the sets of genes that can be regulated by NF-κB transcription factors. NF-κB p50-p65 binds with high affinity to the consensus "κB" site: 5'-GGGPuNNPyPyCC-3' (Chen, F.E. and Ghosh, G., 1999). Crystal structure of the p50-p65 heterodimer provides evidence that the p50 protein makes contact with the DNA through the 5'-GGGPuN-3' half site, where p65 interacts with DNA at the 5'-PyPyCC-3' half site (Chen, F.E. and Ghosh, G., 1999; Kunsch, C. et al., 1992). The dimer composition is an important aspect of the specificity of -κB binding sites and regulation of transcriptional activity through the NF-κB pathway (Perkins, N.D. et al., 1992). The pleiotropic nature of the NF-κB pathway is explained by the capacity of these proteins to mix-and-match partners to create distinct functional units that are able to respond to different signals and activate/repress different genes.

3. <u>Traditional upstream NF-κB signaling network: Activation of NF-κBs</u>

a. *Canonical NF-κB pathway*. When the NF-κB pathway is not active, NF-κB dimers are sequestered in the cytoplasm by inhibitor of κB (IκB) proteins. There are at least four IκB proteins (α, β, γ, and ε) that have varying affinities for each of the NF-κB dimers. The C-terminal domains of p105 and p100 consist of a series of long ankyrin repeats that bind to and inhibit DNA binding of other RHD proteins (Liou, H.C. et al., 1992; Moorthy, A.K. and Ghosh, G., 2003). Interestingly, the NF-κB1 gene not only encodes p105/p50, but also generates the p70 IκB-γ transcript through an alternate promoter. The p70 protein is an IκB protein homologous to the C-terminal portion of p105 (Gerondakis, S. et al., 1993; Inoue, J. et al., 1992). Specific roles for p70 versus p105 IκB activity are not clear. NF-κBs interact with IκB proteins through multiple contacts, including masking the nuclear localization signal and the DNA binding region (Ghosh, S. and Karin, M., 2002).

In the canonical signaling pathway, IκB proteins are regulated by the heteromeric IκB kinase (IKK). IKK is made up of two catalytic components, IKK-α and IKK-β, as well as the regulatory subunit IKKγ/NEMO. Many extracellular and intracellular stimuli activate IKK, which in turn phosphorylates IκB at consensus serine residues. Phosphorylated IκB proteins are targeted for K-48 linked poly-ubiquitination and proteasomal degradation. When IκB is degraded, the nuclear localization sequence of NF-κB is unmasked and the protein dimer translocates to the nucleus to bind to consensus κB sites in target gene enhancer and promoter sequences (Barkett, M. and Gilmore, T., 1999; Karin, M., 1999; Li, X. and Stark, G.R., 2002; Schwaninger, M. et al., 2006).

b. Non-canonical pathway.

In the non-canonical pathway, activation of p100 proceeds through proteolytic processing to p52 rather than degradation of IκB. Activation of this process occurs through the NF-κB inducing kinase (NIK) and IKKα and requires *de novo* protein synthesis. The upstream signals that activate NIK and/or IKKα homodimer belong to a specific subset of the TNFR super-family. Non-canonical signaling liberates p52-containing dimers, typically p52/RelB, for translocation to the nucleus and further downstream signaling. Due to the unique requirement of *de novo* protein synthesis, activation of the non-canonical pathway is slower and more persistent than the canonical pathway (Reviewed by (Sun, S.C., 2011)).

4. Additional modulators of NF-κB transcriptional activity

The regulation of NF-κB transcriptional activity is highly complex. There are many further layers of NF-κB regulation including additional regulatory proteins; co-activators and repressors that associate with various complexes to further modulate activity (Zhong, H. et al., 2002). Post-translational modification of the NF-κB subunits can play a role in modifying the transcriptional activity and binding partners (Zhong, H. et al., 2002).

a. Co-activators. The p50-p50 repressive complex can bind to HDACs but not the co-activator complex CBP/p300 (Sheppard, K.A. et al., 1999; Zhong, H. et al., 2002). HDACs deacetylate chromatin for a more closed DNA conformation and reduced transcription. The p65 protein can interact with both HDACs and the co-activator CBP/p300 to either repress or promote transcription at κB sites (Zhong, H. et al., 2002).

b. Post-translational modification of NF-κB. Following release of the NF-κB subunits from their inhibitory IκB binding partners, there are even further regulatory

signals that may occur through post-translational modification of the proteins. There are many characterized phosphorylation and acetylation sites that play a role in the activity of NF-κB. For example, DNA binding of the repressive p50-p50 dimer is influenced by Serine 337 phosphorylation by protein kinase C (Guan, H. et al., 2005; Hou, S. et al., 2003). Additionally, phosphorylation of p65 controls its association with either HDAC repressive proteins or its co-activator CBP/p300 (Zhong, H. et al., 2002).

B. Activation of NF-κB through hypoxia

NF-κB is activated by many different stress conditions or environmental changes as an early response transcription factor. A number of studies have demonstrated hypoxia-inducible activation of the NF-κB family in many different cancer, immortalized fibroblast, and immune system cells (Jurkat T-cells, HeLa, CaCo-2, THP-1, osteosarcoma, breast cancer, embryonic kidney, and mouse fibroblast cell lines) (Culver, C. et al., 2010; Cummins, E.P. et al., 2006; Koong, A.C. et al., 1994). The signaling pathways controlling NF-κB activation through hypoxia are less elucidated than inflammatory and developmental activation. Direct stimulation of NF-κB by hypoxia in neurons is untested.

Both IKK-α and IKK-β contain an evolutionarily conserved sequence *Leu-X-X-Leu-Ala-Pro* that is the same as the hydroxylation sites in the HIF-1α oxygen dependent degradation domain. These IKK proline motifs are potential targets for prolyl hydroxylation by the PHD enzymes (Cummins, E.P. et al., 2006). IKK-α and -β both co-immunoprecipitate with FLAG-tagged PHD-1 in normoxia, suggesting an interaction between the kinases and the hydroxylase (Cummins, E.P. et al., 2006). Hypoxia leads to

an increase in the relative abundance of IKK-β protein and hypoxia-sensitivity is dependent on the consensus proline residue in the LXXLAP motif (Cummins, E.P. et al., 2006). These data support a model where in normal cellular oxygen concentration active PHD enzymes hydroxylate IKK-β on the consensus proline residue to negatively regulate the kinase activity, favoring an inactive kinase. During hypoxia when PHDs are inhibited, IKK-β in not hydroxylated. The lack of hydroxylation favors a more active kinase, but is not sufficient for activation (Fig. 3.1). Proline hydroxylation is considered to be a modulator, but not sufficient for activation or inactivation of the IKK proteins (Cummins, E.P. et al., 2006).

When IKK is activated, it phosphorylates the substrate IκB proteins. Phospho-IκBs are degraded by the proteasome. NF-κB dimers are released and trafficked to the nucleus, where they can bind to κB target DNA sites to activate transcription. The specific NF-κB dimers that are activated by hypoxia have not been characterized, nor have the transcriptome of hypoxia-activated NF-κB, however it is clear that hypoxia-stimulated NF-κB can both activate and repress transcription (Culver, C. et al., 2010).

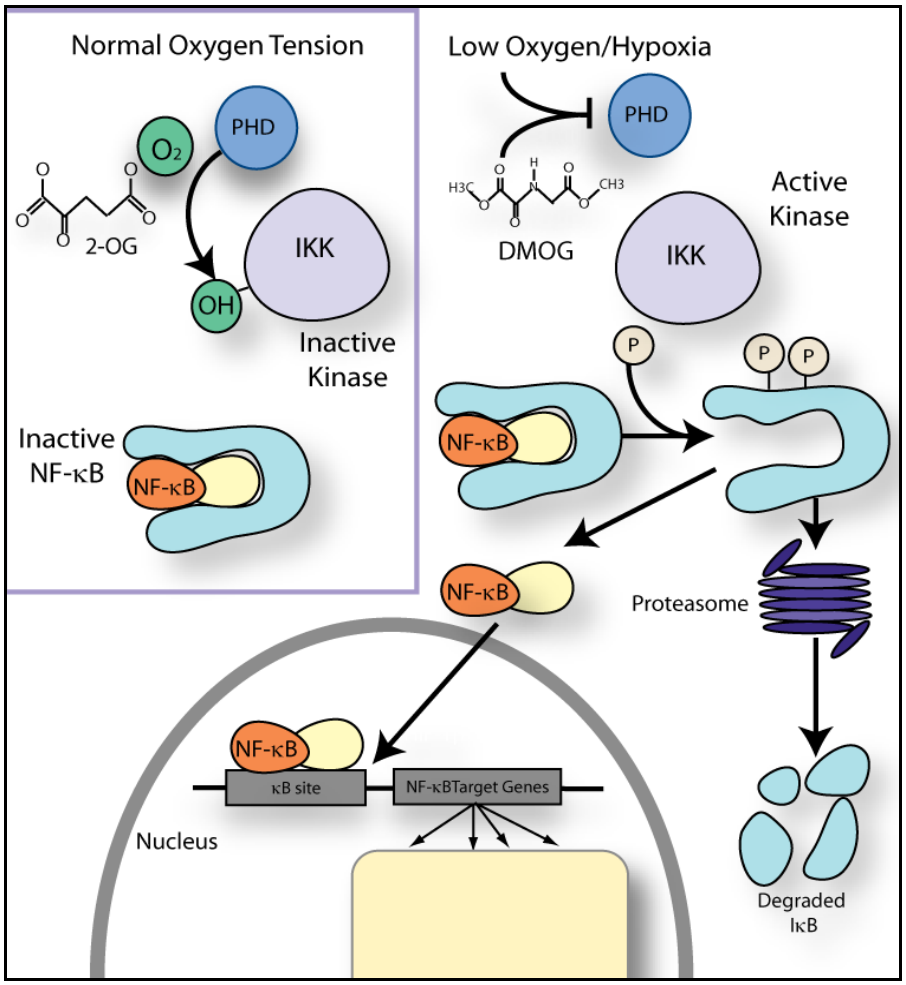


Figure 3.1 PHD mediated signaling to NF-κB

PHDs regulate the NF-κB family at the level of the upstream IKK complex. Under normoxia, PHD enzymes interact with IKK-β and hydroxylate a consensus proline residue. This modification favors an inactive kinase, but is not sufficient to control inactivity. In hypoxia, or when PHDs are inhibited, the IKK-β protein is not hydroxylated. Lack of hydroxylation favors an active kinase that phosphorylates IκBα, targeting it for degradation. This process frees NF-κB dimers for translocation to the nucleus and DNA binding for target gene regulation. The specific dimer composition and population of PHD-inhibitor regulated NF-κB targets is currently unknown.

C. Inflammation induces HIF-1α

Inflammatory cytokines such as IL-1B and TNF-α can induce HIF-1α expression in a time and dose dependent manner under normoxia in transformed and immune system cell lines (Jung, Y.J. et al., 2003; van Uden, P. et al., 2008). Similarly, exposure of bone marrow derived macrophages to either Gram-positive or Gram-negative bacteria induces expression of HIF-1α and requiring IKK-ß (Rius, J. et al., 2008). The increase in HIF-1α expression takes 2-4 hours of inflammatory stimulus exposure, where induction of HIF-1α in hypoxia is more rapid, easily detectible in 30 minutes. In hypoxia, HIF-1 protein is stabilized by loss of hydroxylation by the PHD enzymes and reduced interaction with VHL (E3-ubiquitin ligase). The time difference in the inflammatory pathway may suggest an additional level of regulation, such as transcription and translation rather than just protein stabilization. The role of posttranslational control of HIF-1 during inflammation is not clear. These pathways have not been confirmed in neuronal cell types. NF-κB proteins do have brain-specific functions and therefore, signaling in the brain is not always the same as in other cell lines.

D. Basal regulation of the HIF-1 promoter by NF-κBs

NF-κB transcription factor subunits are regulated by oxygen levels through the PHDs. NF-κB signaling can transcriptionally regulate both HIF-1α and -β (van Uden, P. et al., 2008). In human embryonic kidney cells all of the NF-κB subunits, RelB, c-Rel, p65, p50, and p52, were found at the HIF-1α promoter by electrophoretic mobility shift assay (EMSA) (van Uden, P. et al., 2008). Analysis of the human HIF-1α promoter by previous studies shows a cluster of a number of putative regulatory sites in the 5'

promoter region of the gene. In HEK-293 cells, over expression of different NF-κB subunits in normoxia, showed that p50, p65, c-Rel, and p52 can bind to the -197/188 site in the promoter. Relative strength of binding is not clear since the proteins were expressed at different levels. RelA, c-Rel, and p52 all bound more strongly to a canonical κB site than the putative HIF-1α κB site. When the RelA, RelB, and p52 were knocked down one at a time with siRNA, the basal level of HIF-1α was reduced. HIF-1α mRNA was not statistically reduced in the absence of p50 or c-Rel. These data suggest that one or more of the NF-κB subunits directly bind and regulate the basal expression of HIF-1α. RelA and c-Rel over expression lead to enhanced HIF-1α protein (van Uden, P. et al., 2008).

E. Summary and Conclusions

The NF-κB family of transcription factors is a highly complex signaling mechanism for many types of early stress responses and basal gene regulation. NF-κB is regulated and plays distinct roles in neurons compared to other cell types. Regulation of NF-κB by hypoxia and PHD inhibition has been shown in many cell types, but is untested in neurons. NF-κB may bind to the HIF-1α promoter in fibroblast cells and regulate basal levels of the gene transcription. Since hypoxia can mediate regulatory events in the NF-κB pathway, this may be one mechanism for transcriptional regulation of HIF-1α. The role of NF-κB at the HIF-1α promoter during hypoxia has not been determined. The link between PHDs, NF-κB, and HIF-1α lead us to hypothesize that NF-κB may participate in mediating oxygen-sensitive endogenous protective signaling through the regulation of HIF-1α and other important stress response genes.

Chapter IV

Rationale, Aims, and Experimental Methods

A. Rationale and significance.

Although stroke is the third leading cause of death and primary source of chronic disability in the US, FDA approved clinical treatments after stroke are extremely limited. Disrupted oxygen/nutrient delivery during ischemia triggers brain tissue death and manifests as cognitive and sensory/motor dysfunction in stroke patients. The development of therapeutic strategies to treat ischemic cell death requires a greater understanding of endogenous cellular protective mechanisms that can be enhanced to prevent neuronal degeneration. A major endogenous pathway enabling cells/tissues to tolerate ischemic stress is the evolutionarily conserved oxygen-sensing signaling pathway, which is normally engaged during stroke. Exogenous activation of this protective pathway prior to ischemia (preconditioning) results in increased neuronal viability and reduced stroke volume. Inhibition of PHD enzymes is an early stage of oxygen-sensing signal transduction, linking molecular oxygen availability to signaling events.

Since stroke is not a predictable condition in humans, the application of pre-ischemic therapy will not translate clinically; however, elucidation of the underlying mechanisms of such neuroprotection is critical to maximize therapeutic strategies and limit negative side effects. Mechanistic investigation of neuroprotective therapies can also provide valuable insight into the mechanism governing the physiological and pathological process of ischemia. Due to the delayed nature of cell death in the ischemic penumbra region, interventions after stroke may be able to limit the spread of infarct into the penumbra.

The current study hypothesized that: 1. Application of the PHD inhibitor DMOG *after* ischemic stroke would rescue the peri-infarct region from ischemic damage; and 2.

This protection would be mediated through oxygen-sensitive signaling pathways HIF-1α and NF-κB.

In pursuing these questions, this study also examines the physiological and pathological link between oxygen-sensing pathways and the NF-κB family in the brain, which is currently unknown. This dissertation not only contributes to the understanding of a potentially clinically relevant therapeutic for stroke, but also, to understanding the endogenous mechanisms of adaptation pathways regulated by availability of oxygen.

B. Specific aims.

<u>Aim 1</u>: To examine the acute effects of PHD inhibitor post-treatment on neuronal cell death and oxygen sensor signaling.

<u>Hypothesis</u>: PHD inhibition after stroke reduces neuronal apoptotic cell death and requires HIF-1α activation.

1.1. To test the protective effect of PHD inhibitor post-treatment on gross ischemic damage, we assessed 72h infarct volume by TTC staining in animals post-treated with PHD inhibitor DMOG 30, 60, or 120 minutes after stroke.

1.2. To test the effect PHD inhibitor post-treatment on oxygen sensor signaling, we analyzed protein expression of HIF-1α, a marker of active oxygen-sensing after post-treatment *in vivo* and apoptotic and ischemic models *in vitro*.

1.3. To test the impact of PHD inhibitor post-treatment on apoptotic and excitotoxic cell death we assessed caspase-3 expression in the peri-infarct region by immunostaining after stroke or DMOG post-treatment *in vivo* and assessed cell death with or without DMOG post-treatment in apoptotic and ischemic *in vitro* models.

1.4. To determine the necessity for HIF-1α for the post-treatment mediated protection from excitotoxic cell death, we knocked down HIF-1α by lentiviral mediated shRNA delivery in the ischemic cell death model and pharmacologically inhibited HIF-1α by two functionally distinct inhibitors in the *in vivo* ischemia model.

Aim 2: To examine the role of NF-κB transcription factors in PHD inhibitor-mediated neuroprotection and the interaction of NF-κB and HIF-1α.

Hypothesis: NF-κB transcription factors are activated after PHD inhibition and are required for PHD-inhibitor mediated neuroprotection.

2.1. To test the hypoxia/PHD regulated activity of NF-κB in neurons, we assessed protein nuclear localization of p50 and p65 *in vitro* using mouse cortical neurons.

2.2. To test the requirement of NF-κB for neuronal protection we performed *in vitro* apopototic and ischemic cell death models in NF-κB KO mice +/- PHD inhibitor treatment, and *in vivo* ischemia +/- PHD inhibitor post-treatment in NF-κB p50-/- mice.

2.3. To study the interaction between the NF-κB and HIF-1α transcription factors, expression of HIF-1 and NF-κB family members was assessed *in vitro* and *in vivo* in WT and p50 KO animals. The promoter of HIF-1 was analyzed for NF-κB binding sites, and the binding of proteins to putative κB sites after hypoxia in WT vs p50 KO animals was assessed.

C. Materials and Methods.

1. Primary Cortical Neuron Culture

a. Dissection and culture. Primary neuronal and glial cell cultures were isolated from Swiss Webster fetal mice (E14-16) by dissection of the cerebral cortex as previously described (Semenza, G., 2002). Cortical mantel of the embryonic brain was isolated in dissection media (Hank's balanced salt solution + glucose), and then incubated in 0.1% trypsin-EDTA at 37C for 30 minutes. Cortex was dissociated by pipetting with a glass pipet in Minimal essential media (Gibco) + 10% horse serum (Gibco). Cells were plated in 35mm dishes at a density of $0.8x\ 10^6$. Cells were maintained in Neurobasal media with B-27 supplement (50X) and L-glutamine (400X) (Invitrogen) until time of experiments.

b. Apoptotic cell death model. For apoptosis studies, primary cultures were maintained as nearly purely neuronal by addition of cytosine arabinoside (ARA-C, 5 µM) after three days of culture *in vitro* to halt proliferation of glial cells. Apoptosis was induced after 7 days *in vitro* cells by withdrawal of B-27 serum-free culture supplement and maintained in basal media for 24h (Farinelli, S.E. et al., 1998; Zhang, S.X. et al., 2003). B-27 contains a mix of necessary anti-oxidant and trophic support components for *in vitro* neuronal cell survival. After 24h, cell death was assessed by trypan blue staining and microscopy.

c. In vitro ischemia (Oxygen-glucose deprivation (OGD)). For ischemic cell death model, primary cortical cells were cultured as mixed neuronal and glial population for 12-13 days. In the OGD group, media was exchanged for a physiological buffer solution lacking glucose (120mM NaCl, 25mM Tris-HCl, 5.4mM KCl, 1.8 mM $CaCl_2$, pH to 7.4

with NaOH) and then cells were incubated in a calibrated hypoxia chamber (BioSpherix) perfused with 5% CO_2 and balanced nitrogen for a final ambient oxygen level of 0.2% for 2h. Oxygen level was established, maintained and monitored by the ProOx 360 sensor (Biospherix, NY). After 2h, cells were returned to the normal 5% CO_2 incubator and the existing OGD media was diluted by half with normal oxygenated complete neuronal culture media. After 24h, cell death was assessed by trypan blue staining and microscopy.

d. Trypan blue staining for cell death. Trypan blue solution was added directly to cell culture media and incubated for 5 mins at 37C. Cells were imaged immediately for trypan blue uptake. At least 6 randomly chosed 20X microscope fields were captured for each 35mm dish for each condition. Two to three culture dishes per treatment group per experiment were assessed. Total data was collected from at least three separate experiments from different primary cultures.

2. Animal Experiments

All *in vivo* experimental procedures were approved by the Institutional Animal Care and Use Committee at Emory University.

a. Focal cerebral ischemia- Middle cerebral artery occlusion (MCAO) was conducted as previously described (Krishnamachary, B. et al., 2003; Treins, C. et al., 2002) with some modifications. Briefly, adult male B6;129PF2/J (wild-type (WT)) or B6;129PNFκB1^{tm1Bal}/J mice (NF-κB KO) (Jackson Labs) weighing 20-25g were anesthetized with 4% chloral hydrate. Craniotomy was performed over the M3 branch of the right middle cerebral artery (MCA) supplying the barrel cortex. The right MCA was

permanently ligated by 10-0 suture, and the common carotid arteries (CCA) were occluded for 7-min and then reperfused.

b. PHD inhibitor administration- Dimethyloxaloylglycine (DMOG) (Frontier Scientific) was dissolved in DMSO at a concentration of 1M. For animal administration, DMOG solution was diluted further in sterile saline and injected at a final concentration of 50mg/kg i.p. Control animals received the same volume of injection of saline-diluted DMSO vehicle in sterile saline (final dose of DMSO $1x10^{-5}$mL/kg). Animals were i.p. injected with DMOG or Saline/DMSO vehicle 30 or 60 min after reperfusion of the CCAs.

c. HIF inhibitor administration- Digoxin (DIG) and Acriflavine Hydrochloride (ACF) (Sigma) were dissolved in sterile phosphate buffered saline. In order to inhibit HIF-1α, DIG or ACF were administered at 2mg/kg i.p. 24h prior to surgical stroke and daily thereafter until sacrifice (Akita, M. et al., 2004; Yoshida, T. et al., 2010; Zhang, H. et al., 2008).

d. Assessment of ischemic infarct volume- Ischemic infarct size was assessed 72h following stroke. The brain was sliced into 1-mm coronal sections using a mouse brain matrix (Harvard Bioscience, South Natick, MA), and incubated in 2% Triphenyl Tetrazolium Chloride (TTC) at 37^{o}C for 5 min. Brain sections were scanned and the unstained vs. stained TTC area was determined using NIH ImageJ on the six brain slices per animal. The infarct volume (mm^3) was determined by multiplying the area of staining in each slice by the slice thickness (1mm) and summing the volume of each slice for each animal.

e. Measurement of Local cerebral blood flow- Laser Doppler scanner imaging of cortical cerebral blood flow above the territory of the right MCA was conducted as previously described (Krishnamachary, B. et al., 2003). Briefly, animals were anesthetized with injection of 4% chloral hydrate solution at (400 mg/kg) and an incision was made to expose the skull above the territory of the right MCA. The laser was centered over the right coronal suture as indicated by the square in the diagram of Fig. 5.7. A 3x3mm square area around the initial spot was scanned by the Periscan Laser Doppler perfusion imaging system and analyzed by the LDPI Win 2 software (Perimed AB, Stolkholm Sweden). This technique is based on the principle that photons from the laser interact and are Doppler shifted only by moving red blood cells. Tissue perfusion is calculated by the LDPI program as the mean and amplitude of the Doppler shift. These parameters translate to average velocity and the concentration of the moving blood cells (Semenza, G.L., 2002). During the laser Doppler study, we measured blood flow in the exact same location for each animal at time points immediately before stroke, during the MCA and CCA occlusions, and then 12, 24, 48, or 72h after stroke. Blood flow values are presented as percent of baseline (before stroke) flow for each animal.

f. Behavioral Testing- Forelimb sensorimotor deficits after stroke were analyzed by a modified adhesive removal test (Bouet, V. et al., 2009; Freret, T. et al., 2009). Briefly, the time to remove sticker was recorded one day prior to stroke, 7 days after stroke, and 14 days after stroke. Animals were trained with one trial per day for three days prior to testing. To reduce anxiety, which could interfere with the performance of the task, animals were habituated for 1 minute in a testing cage identical to their home cage with a small amount of home cage bedding sprinkled in the four corners. An adhesive sticker

(Tough spots, 9.5mm diameter, cut into quarters) was applied to the hair-free area (three pads, thenar, hypothenar) of either the left or right forepaw. The mouse was then placed back into the test cage and the time to remove the sticker was recorded with a maximum of 120s. Left and right paw order was alternated between trials, however all mice were tested in the same order. In each testing session, there were four trials (four left, four right). The first trial was considered training; the last three were averaged and analyzed for each animal. The same blinded experimenter conducted all tests so the pressure of sticker application was consistent. Data was analyzed as fold change compared to before stroke for each group.

3. Immunohistochemistry

a. Immunohistochemistry in vivo- Brains were frozen in optimal cutting temperature media (Sakura Finetek) at -80^{o}C. Frozen sections were cut (10µm) on a cryostat microtome. For active caspase-3 staining, sections were fixed 2 min in 10% buffered formalin, washed in phosphate buffered saline (PBS) three times, then incubated 10min in -20^{o}C methanol. Sections were air-dried, then rehydrated in PBS, incubated in 0.02% Triton X-100 for 15 min. Slides were washed and blocked in fish gelatin (Sigma). Active Caspase-3 antibody (R&D systems) and neuronal nuclei (NeuN, Millipore) were incubated overnight at 4^{o}C. Slides were washed and incubated with donkey anti-rabbit 488-conjugated secondary antibody (Invitrogen) and donkey anti-mouse Cy3 (Jackson Immunological).

b. In vitro staining of cortical neurons- Cortical neurons were fixed in 4% Paraformaldehyde in phosphate buffer for 10 minutes and the washed three times in PBS.

Cells were incubated in blocking/permeabilization solution (1% fish gelatin/0.2% TritonX-100) for 45 minutes. Primary antibodies for HIF-1α was applied overnight at 4C. Secondary staining was as described above with donkey anti-rabbit Cy3. Nuclear staining was accomplished by Hoescht treatment for 5 minutes at 1:50,000 dilution prior to coverslipping and imaging.

c. Cell counting using design based stereology- For design based stereology with systematic random sampling, every 20th brain slice across the region of interest were imaged and counted (6 sections per animal, with sections more than 200μm apart) as previously described (Treins, C. et al., 2002). For multistage random sampling, six 40X images from the cortical region supplied by the right MCA were captured in each sampled section. The region of interest was defined as a 1.2 mm region within the perfusion territory of the MCA. Data is represented as the total cells per animal in the region of interest. Cells were counted in a double blind manner by two investigators and results were averaged.

4. Protein Analysis-

a. Protein isolation. Total protein was isolated from cells or tissue by homogenization in a modified RIPA lysis buffer (50mM Tris pH 7.8, 150mM NaCl, 0.1% SDS, 0.5% NaDeoxycholate , 1% NP40 detergent, 1mM PMSF, 1X Sigma protease inhibitor cocktail, $NaVO_4$). Tissue homogenate was incubated on ice for 30 minutes in lysis buffer and then centrifuged at >14,000 x g at 4C for 15 minutes. Supernatant containing total protein was collected. Protein concentration was determined by standard Bicinchronic Acid (BCA) assay.

b. Nuclear/Cytoplasmic protein separation. Nuclear and cytoplasmic enriched protein fractions were isolated by a low salt/high salt buffer extraction. First, cells were collected in a low salt buffer (10mM Hepes ph7.9, 10mM KCl, 2mM $MgCl_2$, 0.1mM EDTA, 1mM DTT, and protease inhibitors) for 10 minutes on ice. Then NP-40 detergent was added at a final concentration of 0.55%. Cells were vortexed, incubated on ice for one minute, vortexed again, and then centrifuged at 16,000x g for 5 minutes. The supernatant was collected as the cytoplasmic enriched fraction. The pellet was washed in low salt lysis buffer and then resuspended in high salt lysis buffer (20mM Hepes pH 7.4, 0.4 M NaCl, 10mM KCl, 1mM MgCl2, 0.5mM DTT, 20% glycerol). Samples are incubated in this buffer for 40 minutes with vigorous vortexing every 10 minutes and then centrifuged at 16,000 x g for 10 minutes. Supernatant is the nuclear enriched fraction.

c. Electrophoresis- Equal amounts of protein were mixed with a 5X SDS sample loading dye and separated by electrophoresis on a 10% polyacrylamide gel. Proteins were transferred to a PVDF membrane by overnight transfer at 4C.

d. Immuno-blotting- PVDF membranes were blocked with either 10% milk proteins in tris-buffered saline with 1% tween-20 (TBST) or 10% bovine serum albumin (BSA) in TBST for at least 1 hour. HIF-1α blots were always blocked in milk. Primary antibody solutions were prepared in 5% blocking solution and applied to membranes for 1 to 24 hours with agitation. Longer incubations were done at 4C. Blots were then washed 5 x 5 minutes in TBST, and then appropriate secondary antibodies linked to horseradish peroxidase (HRP) were applied for 45 minutes to 1 hour at a dilution of 1:2000-1:5000.

After secondary antibody, blots were washed 5 x 5 minutes and then developed with chemiluminescence and imaged to film.

e. Anti-bodies-

Target Protein	Company	Cat number	App	MW
Active (Cleaved) Caspase 3	R&D systems	af835	IHC	N/A
Hypoxia Inducible Factor-1a	NeoMarkers	MS-701-P0	IHC	N/A
Neuronal Nuclei	Millipore	mab377	IHC	N/A
Active (Cleaved) Caspase 3	millipore	ab3623	WB	17
B-Actin	Sigma		WB	42
Hypoxia Inducible Factor-1a	Novus Biologicals	100-479	WB	115
NF-κB p105/p50	abcam	ab7971	WB	105/48-50
NF-κB RelA or p65	cell signalling	8242	WB	65
α Tubulin	Santa Cruz	sc8035	WB	50
Lamin A/C	Sigma	SAB4200236	WB	65/74

5. RNA Analysis

a. RNA isolation- Total RNA was isolated from cortical neurons at 24h after DMOG treatment with Trizol (Invitrogen).

b. Reverse transcription- cDNA was generated from 0.5-1µg of total RNA and random primers with the High capacity cDNA kit (Applied Biosystems).

c. Quantitative real-time Polymerase Chain Reaction (qRT-PCR) – SYBR green qRT-PCR was used to assess relative levels of the genes of interest using Applied Biosystems StepOnePlus machine. RNA with no reverse transcriptase in the RT reaction was used as a negative control for contaminating genomic DNA. Amplification of 18S RNA was used as an internal control for relative amounts of starting RNA. Fold change was calculated by the $\Delta\Delta C_T$ method. Briefly, threshold cycle of amplification was identified by the StepOne software for each gene of interest in each sample. C_T of the 18S control was subtracted from the C_T the gene of interest for each sample (ΔC_T). Then

average of control ΔC_T for each gene was subtracted from the C_T for the experimental samples. Fold change was calculated by the formula $2^{-\Delta\Delta Ct}$.

d. Primers

Gene name	Primer Sequence	
HIF-1α	F	TGGTCAGCTGTGGAATCCA
	R	GCAGCAGGAATTGAACATT
VEGF	F	CTCACCAAAGCCAGCACATA
	R	AAATGCTTTCTCCGCTCTGA
EPO	F	ACCACCCCACCTGCTCCACTC
	R	GTTCGTCGGTCCACCACGGT
eNOS	F	GGCTGGGTTTAGGGCTGT
	R	GCTGTGGTCTGGTGCTGGT
PDK-1	F	TTCACGTCACGCTGGGCGAG
	R	GGGCACAGCACGGGACGTTT
PDK-4	F	GATGAAGGCAGCCCGCTTCG
	R	TGCTTCATGGACAGCGGGGA
18S	F	GACTCAACACGGGAAACCTC
	R	ATGCCAGAGTCTCGTTCGTT

6. <u>DNA binding assay</u>-

DNA binding to kB site in the HIF-1 promoter was assessed by EMSA assay. Nuclear protein was isolated and 40ug was incubated with biotin labeled duplex of the HIF-1a kB site (5'-biotin-GCAACGTGGGCTGGGGT**GGGGCCTGGCC**GCCTGCGT CCTTC-3') in the presence of NP-40 and glycerol for 60 mins. Competition reaction was preformed with 100X unlabeled oligo pre-incubation for 30 minutes. Samples were separated on a 6% Tris-Borate-EDTA acrylamide gel and transferred to a Hybond membrane. Protein-DNA complexes were crosslinked to the membrane with UV light for 15 minutes. Membranes were blocked for 15 minutes and then incubated with HRP-

streptavidin solution. Protein-DNA complexes were visualized by enhanced chemiluminescence.

7. Statistical analyses-

All analyses were performed using Graphpad Prism 4.0 statistical software (GraphPad Software, Inc., La Jolla, CA). Multiple comparisons were performed by one-way or two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc analysis. Single comparisons were performed using Student-T's test. Changes were identified as significant if the p-value was less than 0.05. Mean values are reported with the standard error of the mean (SEM).

Chapter V

Inhibition of prolyl hydroxylases by dimethyloxaloylglycine after stroke reduces ischemic brain injury and requires HIF-1α

Adapted from
Ogle ME, Gu X, Espinera AR, Wei L.
Neurobiology of Disease. (2012) 45(2):733-74

A. Introduction

Stroke is the fourth leading cause of human death and the primary source of disability in the United States (Demchuk, A.M. et al., 1999). Characterized by a vascular occlusion in the brain, stroke reduces brain tissue perfusion and rapidly starves neurons of nutrients and oxygen supply. This initiates a cascade of events that leads to necrotic, apoptotic, and excitotoxic cell death (Lipton, P., 1999). Acute necrotic death is difficult to target therapeutically; however, directing treatments towards preventing the spread of damage into the peri-infarct region may be a viable avenue for novel targeted therapies. Very few successful post-stroke interventions are available for clinical use; thus, development of an effective therapy for acute ischemic CNS/brain attack is an urgent research issue.

The evolutionarily conserved cellular oxygen-sensing pathway that detects and reacts to varying tissue oxygen availability is a potent endogenous protective mechanism against hypoxic injury and other cellular stresses. Key elements in this homeostatic pathway are the PHD oxygen-sensing enzymes and the HIF transcription factor (Jiang, B.H. et al., 1996; Wang, G.L. and Semenza, G.L., 1993c, 1995). The mechanisms of this pathway's activation are discussed in detail in Chapter 2. Briefly, under normal oxygen availability (normoxia), PHDs constitutively hydroxylate HIF-1α on two conserved proline residues (Epstein, A.C. et al., 2001), mediating the interaction of HIF-1α with the E-3 ubiquitin ligase VHL (Ivan, M. et al., 2001; Jaakkola, P. et al., 2001). Following hydroxylation, HIF-1α is poly-ubiquitinated and targeted for proteasomal degradation (Sutter, C.H. et al., 2000). During hypoxia or PHD inhibition, HIF-1α is unhydroxylated and therefore not degraded by the VHL-ubiquitin-proteasome pathway. The protein is stabilized and in conjunction with its heterodimeric partner HIF-1ß activates gene

transcription at hypoxia-responsive elements. Targeted pathways include adaptive pathways that are involved in the promotion of cell survival (Zaman, K. et al., 1999b), angiogenesis (Forsythe, J.A. et al., 1996; Palmer, L.A. et al., 1998), and anaerobic metabolism (Semenza, G.L., 1994). Among the HIF-1-responsive genes are the vascular endothelial growth factor (VEGF) (Forsythe, J.A. et al., 1996), endothelial nitric oxide synthase (eNOS) (Palmer, L.A. et al., 1998), erythropoietin (EPO) (Wang, G.L. and Semenza, G.L., 1993b), and pyruvate dehydrogenase kinases (PDK) 1 and 4 (Almehdar, H. et al., 2011; Aragones, J. et al., 2008; Kim, J.W. et al., 2006).

PHDs require oxygen, iron, 2-oxoglutarate, and ascorbate for the HIF hydroxylation reaction to proceed. PHD activation, therefore, may be inhibited by depletion or competition of these factors, leading to HIF-1α stabilization (Bruick, R.K. and McKnight, S.L., 2001; Siddiq, A. et al., 2005; Wang, G.L. and Semenza, G.L., 1993b). The 2-oxoglutarate analog dimethyloxaloylglycine (DMOG) is a pan-PHD inhibitor that is cell permeable and well-tolerated in animal models (Kasiganesan, H. et al., 2007). Previous studies have indicated that preconditioning of neuronal cultures with pharmacological PHD inhibitors induces HIF-1α and a corresponding protective response that renders cells resistant to subsequent oxidative cell stress (Siddiq, A. et al., 2005) or nerve growth factor withdrawal (Lomb, D.J. et al., 2007). Furthermore, in vivo studies have demonstrated that administration of hypoxia, ischemia or small molecule PHD inhibitors prior to stroke or immediately upon reperfusion reduces infarct severity (Gidday, J.M. et al., 1994; Kitagawa, K. et al., 1990; Liu, Y. et al., 1992; Siddiq, A. et al., 2005).

Manipulation of the robust endogenous protective pathway of oxygen-sensing has been widely discussed in the literature as a potential therapeutic intervention for

neuroprotection in stroke. Unfortunately, pre-treatment is not a clinically relevant therapeutic paradigm because ischemic events in humans are hardly predictable. The present study investigated the therapeutic potential of globally enhancing the oxygen-sensing pathways by delayed pharmacologic inhibition of PHD enzymes after ischemic stroke and sought to determine whether the mechanisms of PHD inhibitor mediated protection require HIF-1α.

B. Results

1. DMOG induces stabilization of HIF-1α protein and HIF-1α-responsive genes in cortical neurons.

The concentration at which PHD inhibitor DMOG induced oxygen-sensing signaling in primary mouse cortical neurons was investigated using HIF-1α protein as a marker. HIF-1α is highly stabilized in low oxygen (Wang, G.L. and Semenza, G.L., 1993a) or by PHD inhibition (Wang, G.L. and Semenza, G.L., 1993b). In normoxic cortical neurons, expression of HIF-1α was stimulated by treatment with 50-500μM of DMOG for 24h (Fig. 5.5.1A). DMOG was dissolved in dimethyl sulfoxide (DMSO); so, control cultures were treated with highest equivalent concentration of DMSO vehicle. HIF-1α increased dose-dependently up to 250μM but waned at the 500μM dose. Cell death was observed in the 500μM condition (data not shown), possibly explaining the reduced HIF-1α signaling. HIF-1α western blots frequently exhibit multiple bands around the expected molecular weight of 115 kDa, representing multiple post-translational modifications of the HIF-1α protein. A non-hypoxia-inducible band was present in all cortical neuron protein samples at approximately 150 kDa and is suspected to be a non-specific band.

Expression of VEGF mRNA, a HIF-1α target gene, also increased after 24h exposure to DMOG (Fig. 5.5.1B), demonstrating that the PHD inhibitor DMOG induces hypoxia-sensitive gene transcription under normal oxygen conditions in cortical neuron cultures.

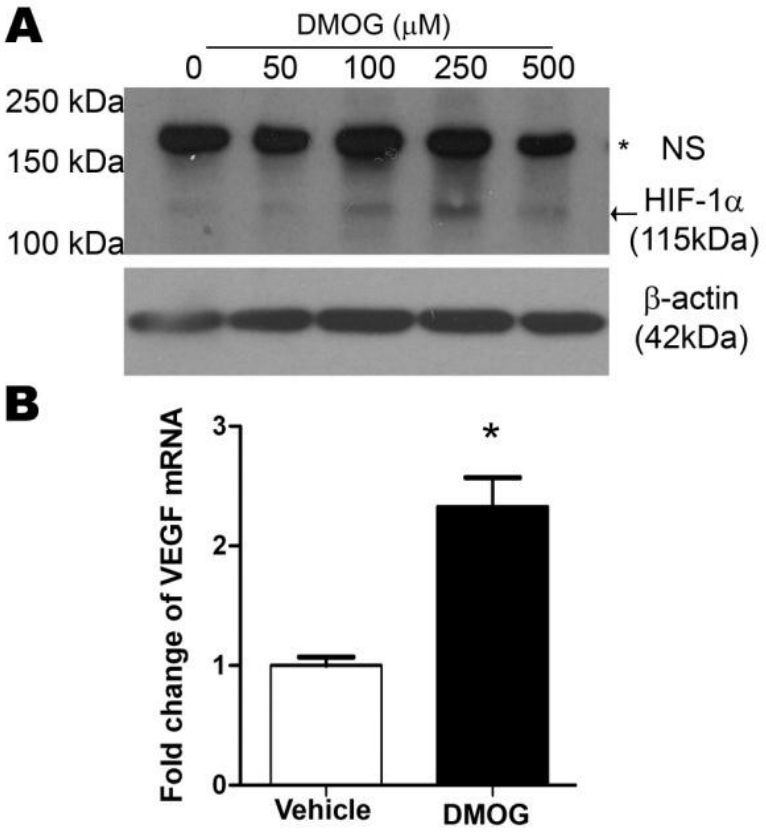


Figure 5.1. DMOG induces normoxic HIF-1α expression in cortical neurons.

A. Normoxic primary cortical neuronal cultures were treated with indicated concentration of DMOG for 24h. Western blot demonstrates an increase in HIF-1α (115 kDa) protein expression with DMOG treatment. β-actin is a loading control. B. Neurons were treated for 24h with vehicle or 250μM DMOG. Quantitative RT-PCR was used to determine the expression of HIF-1-dependent gene VEGF. (n=3, Mean ± SEM, * $p<0.05$).

2. PHD inhibitor pre-treatment or post-treatment attenuates ischemic cortical neuron cell death *in vitro*.

Previous literature has demonstrated that preconditioning the brain with sub-lethal hypoxia or ischemia attenuates cell death when administered prior to an otherwise lethal ischemic challenge (Gidday, J.M. et al., 1994; Kitagawa, K. et al., 1990). PHD inhibition prior to *in vitro* ischemia is previously untested. *In vitro* models of ischemia are useful to dissect the complex aspects of signaling and insult that occur during and after ischemia in the animal model. *In vitro* ischemia isolates the signaling events to two primary cell types, neurons and astrocytes and avoids confounding signaling through blood flow, vasculature-associated cells and infiltrating immune cells.

To determine whether PHD inhibition is sufficient to reduce neuronal ischemic cell death, an *in vitro* ischemia-reperfusion model was generated by exposing cells to combined oxygen and glucose deprivation (OGD), followed by reperfusion with normal oxygen and media. After 2h of OGD and 24h reperfusion, 29.72 ± 2.88% of neuronal cells die. Preconditioning with DMOG (100μM) for 24h prior to OGD induces protein stabilization of HIF-1α (Fig. 5.1A) and significantly reduces OGD-induced cell death to 9.05 ± 2.75% (Fig. 5.2A). Post-treatment with the PHD inhibitor 2h after initiation of OGD, at the start of the reperfusion phase, significantly attenuated cell death to 13.69 ± 1.77% (Fig. 5.2A). Post-treatment protection did not statistically differ from DMOG pre-treatment. Western blotting demonstrates that HIF-1α protein is mildly enhanced 3h after OGD in the DMOG post-treatment group compared to OGD-control (Fig. 5.2B). HIF-1α protein is not enhanced as much as in control conditions presumably due to limitations on protein synthesis after OGD. These results indicate that enhancing oxygen-sensitive

cellular signaling by PHD inhibition, either before or after an ischemic insult, reduces cell death of cortical neurons.

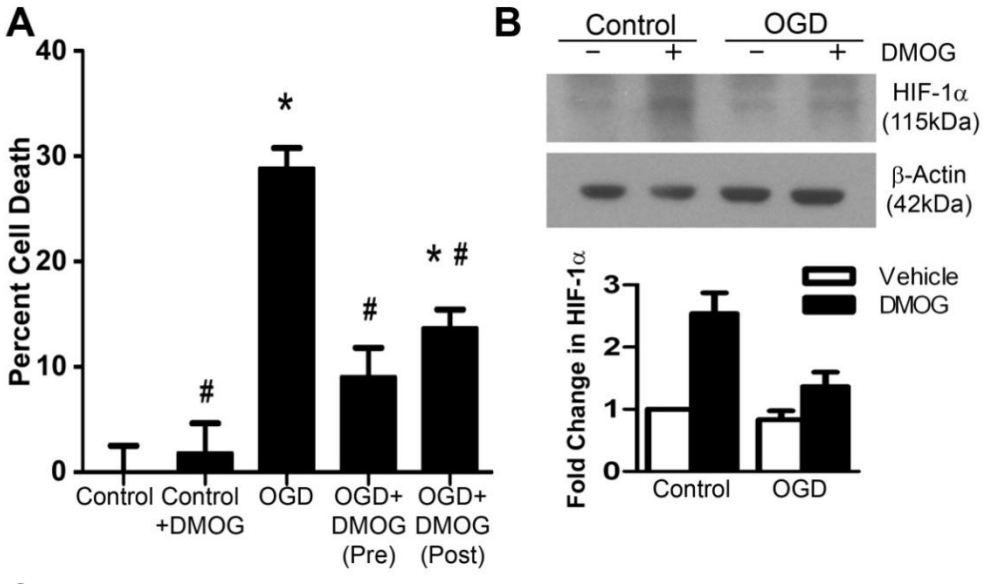


Figure 5.2. PHD inhibitor attenuates OGD-induced cell death and induces HIF-1α.

Cortical neurons were subjected to OGD for 2h with 24h reperfusion. Cell death was assessed by trypan blue exclusion 24h after OGD. Cortical neurons were pre-treated 24h prior to OGD or post-treated after OGD with 100μM DMOG. (A) DMOG reduced cell death in both pre- and post- treatment. (1-way ANOVA, *$p<0.05$ compared to control; #$p<0.05$ compared to OGD; n=4). (B) Western blot of protein collected from cortical neurons 3h after OGD or OGD + DMOG demonstrate that HIF-1α is enhanced after OGD in the DMOG treatment group (n=3). Data are the mean ± SEM from at least three independent experiments.

3. *In vitro* ischemic neuroprotection by PHD inhibitor requires HIF-1α.

To determine whether HIF-1α signaling plays a role in the PHD inhibitor mediated protection observed during and after OGD, HIF-1α expression was knocked-down in primary cortical neurons with lentiviral HIF-1α-targeted shRNA. Neurons were incubated with a virus containing a HIF-1α shRNA sequence or control empty virus for 48h prior to OGD with or without PHD inhibitor DMOG. Success of the HIF-1α knockdown was assessed by western blotting after 24h treatment with 250μM DMOG to stabilize HIF-1α. DMOG-treated cultures had enhanced HIF-1α protein expression over control, while HIF-shRNA pretreated/DMOG-treated cultures had markedly reduced expression of HIF-1α (Fig. 5.3A). HIF-1α knockdown cultures exhibited higher cell death compared to parallel control virus. Cortical cultures with HIF-1α knockdown displayed no protection against OGD with DMOG pre- or post- treatment (Fig. 5.3B) suggesting that HIF-1α is necessary for the PHD inhibitor mediated protection from ischemic cell death.

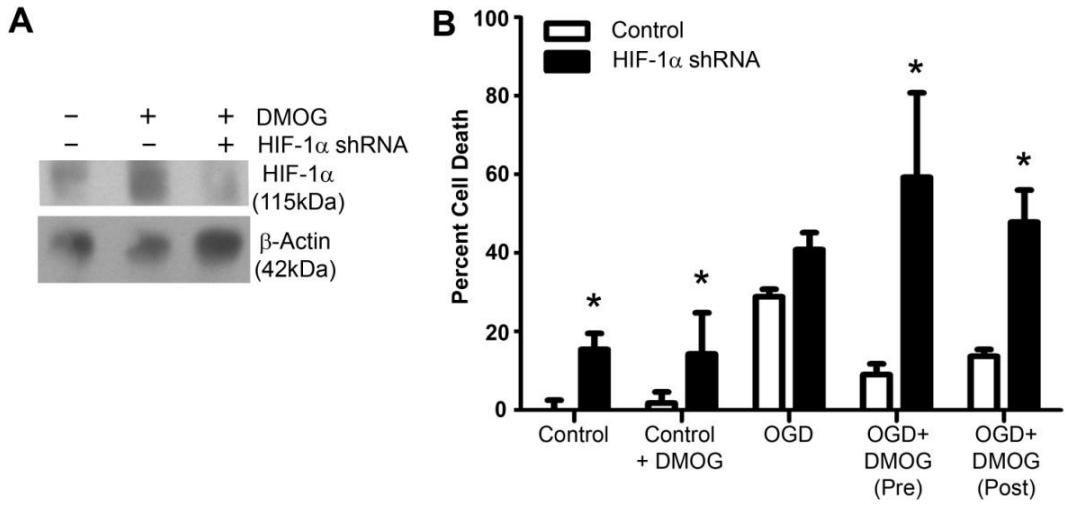


Figure 5.3. PHD inhibitor requires HIF-1α to attenuate OGD-induced cell death.

(A) HIF-1α was knocked down in neuronal cultures by infection with lentiviral shRNA directed against HIF-1α for 48h. Parallel control empty vector (pLKO.1) cells and HIF-1α -shRNA cells were treated with 250μM DMOG for 24h to induce HIF-1α protein stabilization. Control cultures but not HIF-1-shRNA treated cultures induce HIF-1α protein expression in response to DMOG. (B) Cortical neurons were subjected to OGD for 2h with 24h reperfusion as in Fig. 5.2. Cell death was assessed by trypan blue exclusion 24h after OGD. Cortical neurons were pre-treated 24h prior to OGD or post-treated after OGD with 100μM DMOG. DMOG did not reduce cell death from OGD in cells with HIF-1α knockdown. Data are the mean ± SEM from at least three independent experiments. (*compared to parallel control cultures by 2-way ANOVA, $p<0.05$, n=3).

4. PHD inhibitor DMOG attenuates apoptotic cell death in cortical neurons.

Ischemic stroke induces a multitude of combined cellular stresses that lead to the death of neurons (Lipton, P., 1999). In order to model programmed cell death, neuronal cell cultures were subjected to withdrawal of B-27 culture supplement and maintained in only basal media (Farinelli, S.E. et al., 1998; Zhang, S.X. et al., 2003) in the presence of the PHD inhibitor DMOG (250 μM) or equal volume of DMSO vehicle for 24h. In the absence of B-27 supplement, 35.3±2.2% of the cortical neurons died in 24h as assessed by trypan blue nuclear staining (Fig. 5.4A). B-27 withdrawal induced a time-dependent increase in activated caspase-3, a key step in the apoptotic process (Fig. 5.4C). When PHD inhibitor DMOG was co-applied to the cells during B-27 withdrawal, there was significantly less cell death (21.3±2.9%) and markedly reduced activation of caspase-3, compared to vehicle treated controls (Fig. 5.4A, C). HIF-1α stabilized protein was detected in the DMOG/B-27 withdrawal group but not in control or withdrawal conditions (Fig. 5.4B). These data suggest that stimulation of oxygen-sensing signaling pathways during apoptosis-inducing stress reduces apoptotic neuronal cell death.

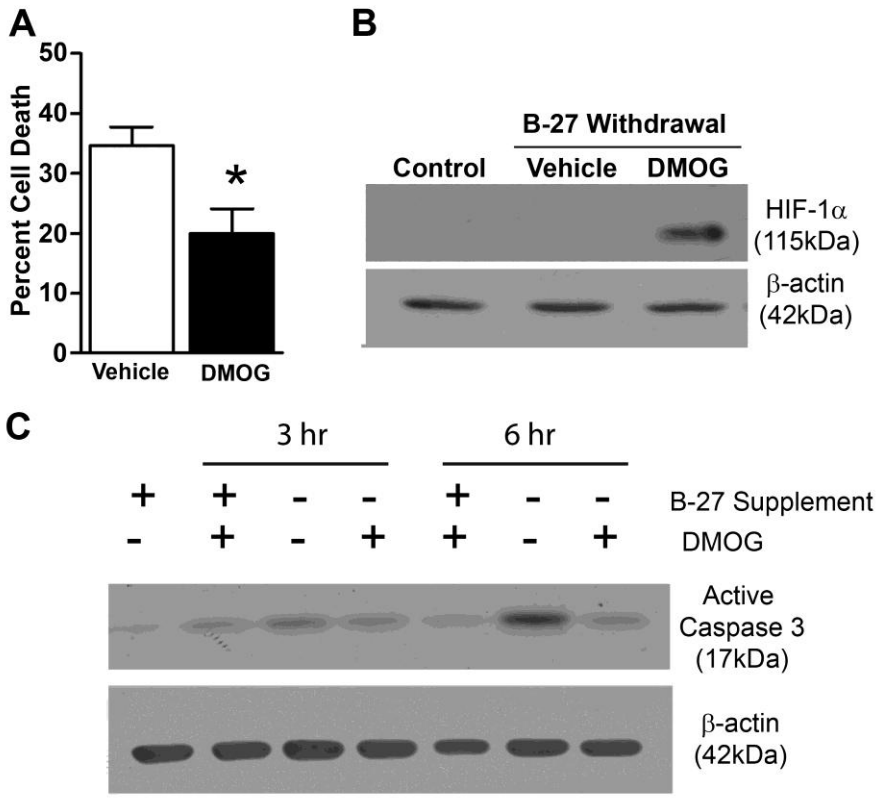


Figure 5.4. PHD inhibitor DMOG attenuates apoptotic cell death in cortical neurons.

(A) Pure neuronal cultures were subjected to an apoptotic insult of B-27 supplement withdrawal for 24h, and dead cells were counted by trypan blue staining. Data are the mean ± SEM from at least three independent experiments. (Student's *t* test, *$p<0.05$; n=3). (B) Neuronal lysate was collected after 24h and immunoblotted for HIF-1α and β-actin loading control. (C) Western blot for the caspase-3 active fragment 3 and 6h after initiation of B-27 withdrawal. Representative western blots from three independent experiments are shown.

5. Cell permeable PHD inhibitor DMOG induces stabilization of HIF-1α protein in vivo.

HIF-1α protein expression is stabilized in normal oxygen tension by DMOG (Fig. 5.1A, 5.4B). In order to test whether DMOG reaches the brain cortex and stimulates oxygen-sensing pathways after i.p. injection, HIF-1α protein levels were assessed in the cortex as a reporter of PHD inhibition. According to previous reports, injection of 100mg/kg i.p. DMOG enhances the expression of HIF-1α in the liver and mediates a whole-body hypoxia tolerance (Kasiganesan, H. et al., 2007). Two doses of DMOG (50 and 500mg/kg) were initially tested in a time course experiment to determine whether HIF-1α expression was enhanced in the brain cortex. DMOG injection (50 mg/kg, i.p.) resulted in a time-dependent increase in protein levels of HIF-1α in the mouse brain cortex (Fig. 5.5 A, B). Similar results were obtained with 500mg/kg (data not shown); however, the lower dose was selected for the post-conditioning trials in order to minimize any possible unknown off-target effects of the higher dose. HIF-1α began to increase as early as 3h after DMOG injection and reached statistical significance 12 and 24h after injection with an increase of more than 3-fold, compared to the vehicle-treated control. HIF-1α protein declined to nearly basal levels between 72 and 96h after a single injection (Fig. 5.5). The drop in HIF-1 protein could stem from either clearance or breakdown of DMOG and shut down of the oxygen-sensing pathways or could reflect the stimulation of a negative feedback loop to shut off HIF-1α. The pharmacokinetics of DMOG were not addressed in this study.

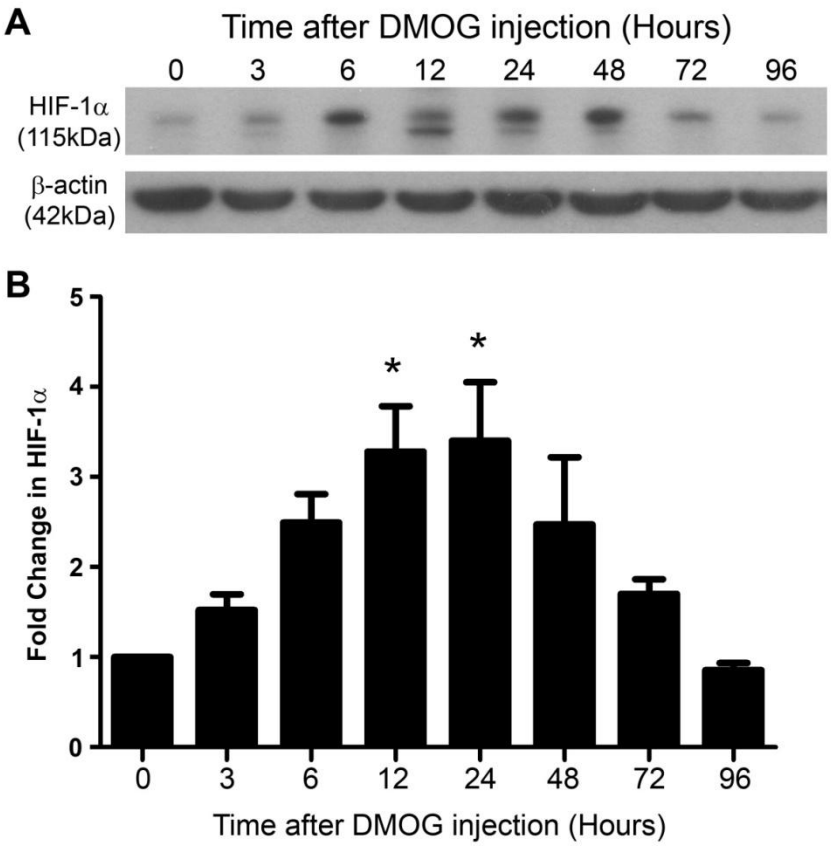


Figure 5.5. DMOG intraperitoneal injection stabilizes HIF-1α protein in the brain. (A) Adult male mice were injected with 50mg/kg DMOG and sacrificed at the indicated time intervals (hours). Cortical tissue was harvested and immunoblotted for HIF-1α or β-actin. Representative western is shown. (B) Summary of densitometry analysis of western blots for HIF-1α relative to loading control β-actin. (n=3-4 animals per time-point; mean ± SEM, *compared to vehicle injected animal by 1-way ANOVA, $p<0.05$).

6. PHD inhibitor post-ischemic treatment reduces focal ischemic infarct formation.

To determine whether delayed PHD inhibition after stroke could impact the gross ischemic infarct volume, animals were treated with DMOG (50 mg/kg, i.p.) 30 or 60 min after ischemic stroke. Small focal ischemic stroke was surgically implemented by combined permanent distal occlusion of the right MCA and 7 minute transient bi-lateral ligation of the CCAs in adult mice as previously described (Acler, M. et al., 2009b). Cerebral ischemia was confirmed by laser Doppler blood flow scanner before and during the surgery. MCA and CCA occlusions reduced cortical blood flow in the targeted region by more than 80% (Fig. 5.7). Ischemic infarct volume was assessed 72h after stroke surgery because the bulk of cell death in the ischemic region occurs within the first 72h (Lipton, P., 1999). The infarct was primarily cortical and located within the vascular bed supplied by the right MCA with no detectible damage to the contra-lateral hemisphere. Control stroke animals had an infarct volume of 12.44±1.25 mm^3 (n=12). A single DMOG administration 30 or 60 min after ischemia significantly attenuated infarct volume to 7.04±1.07 or 7.15±1.22 mm^3 respectively (n = 10 each), indicating that delayed DMOG administration after stroke reduces infarct volume (Fig. 5.6).

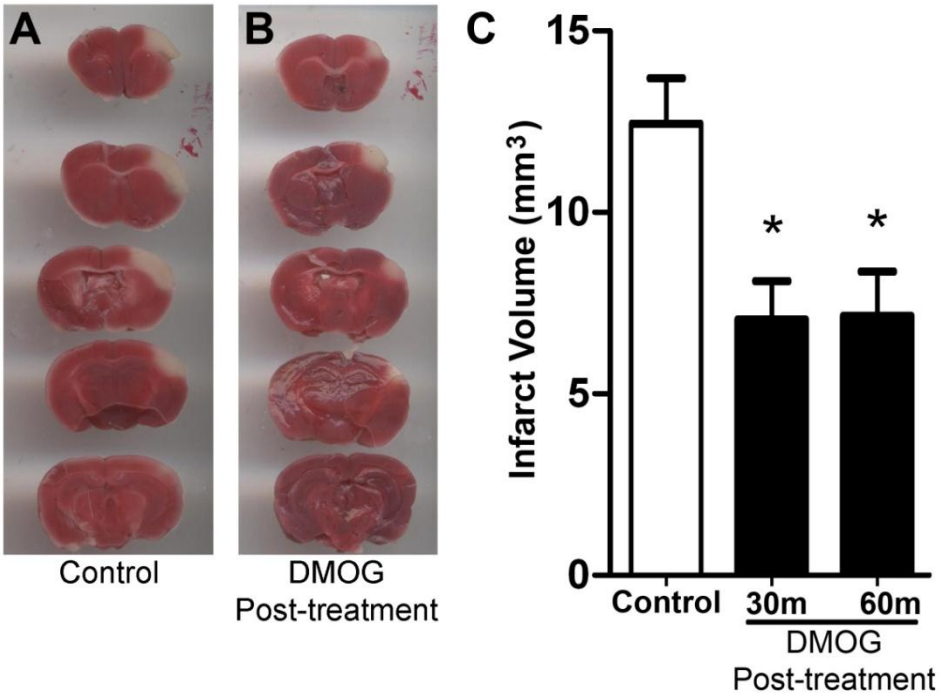


Figure 5.6. PHD inhibitor post-ischemic treatment reduces ischemic infarct formation.

Representative TTC staining of ischemic infarct 72h after stroke in (A) control and (B) DMOG (50mg/kg) 60min post-treatment animals. (C) Quantification of TTC infarct volume (mm^3) 72h after stroke demonstrates the protective effect of PHD inhibitor post-ischemic treatment. (n=10-12 animals per group, 1-way ANOVA, *$p<0.05$).

7. PHD inhibitor post-ischemic treatment reduces loss of local cerebral blood flow.

Laser Doppler perfusion imaging was used to analyze the change in perfusion of the MCA territory before, during and after surgical ischemia. The mean perfusion was analyzed in six measurements of a 3 x 3mm area overlying the occluded branch of the right MCA before, during, and 12, 24, 48 or 72h after stroke in each animal (Fig. 5.7). The change in blood flow was recorded as a ratio to the initial baseline value for each mouse. During stroke (MCA and CCA occlusion), blood flow to the MCA territory was reduced by 80-90% in all animals (Fig. 5.7C). Upon release of the bi-lateral CCAs, the area surrounding the occluded MCA was gradually reperfused. Animals were treated with DMOG or vehicle 1h after release of the CCA, and blood flow was measured 12, 24, 48, or 72h later. The reperfusion blood flow in the DMOG treatment group was significantly higher than control groups 24-72h after stroke (Fig. 5. 7). Blood flow was restored to 77.9% of initial flow after 72h (n=8), significantly higher than the vehicle treatment group (53.1% of initial flow, n=8, $p<0.05$) (Fig. 5.7).

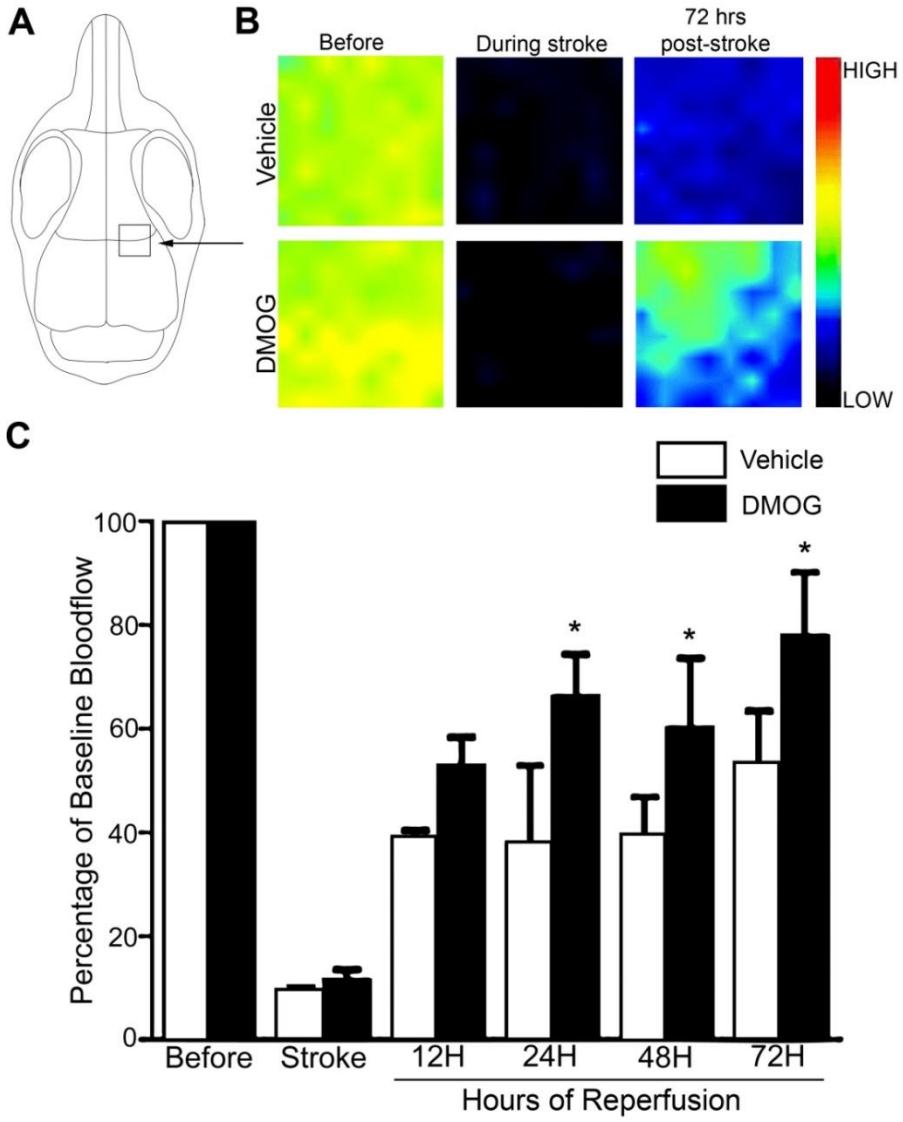


Figure 5.7. PHD inhibitor post-ischemic treatment attenuates peri-infarct loss of cerebral perfusion.

(A) Local cerebral blood flow (CBF) was examined over the territory of the right MCA covering the ischemic border region in the distal focal ischemic stroke model. (B) Pseudo-colored representation of intensity of CBF before, during, and 72h after stroke in control or DMOG post-treated animals. (C) Quantification of CBF relative to initial flow. (n=8, 2-way ANOVA, *$p<0.05$).

8. PHD inhibitor treatment after stroke reduced activation of caspase-3 in the ischemic cortex.

DMOG treatment was hypothesized to limit ischemic infarct formation by reducing programmed cell death and caspase-3 activation in the peri-infarct region after stroke. In vehicle-treated control stroke animals, there was extensive active caspase-3 immunostaining 24h after stroke consistent with previous reports of apoptosis in peri-infarct neurons (Ferrer, I. et al., 2003). Immunohistochemistry showed cells positively stained for activated caspase-3 within and adjacent to the ischemic core. Caspase-3-positive cells had neuronal morphology and co-stained with the neuronal marker NeuN. Caspase-3 cells also displayed a characteristic apoptotic phenotype of shrunken/condensed nuclei and reduced cell volume. DMOG post-treatment significantly reduced the number of caspase-3-positive cortical neurons in the penumbra 24h after stroke (Fig. 5.8).

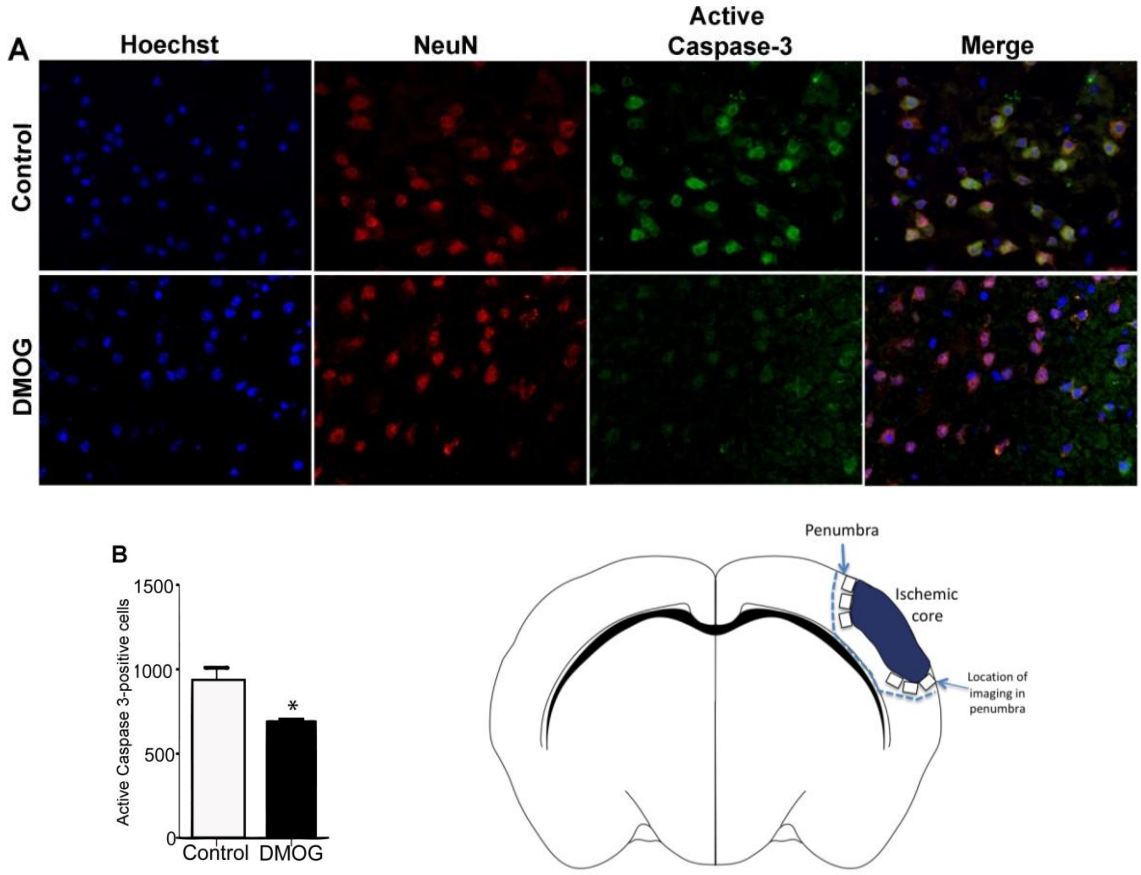


Figure 5.8. PHD inhibitor treatment reduces activation of caspase-3 in the ischemic cortex.

(A) Immuno-staining in the peri-infarct region for active caspase-3 and neuronal nuclei (NeuN) 24h after ischemia. (B) Quantification of the number of active caspase-3 positive cells in the peri-infarct region. DMOG post-treatment reduced the number of apoptotic neurons. (n=4, *$p<0.05$). Diagram shows the imaging location for 6 pictures per section in the peri-infarct penumbra region in the cortex.

9. PHD inhibitor post-ischemic treatment reduces behavioral deficits after stroke.

The distal MCA focal stroke model targets the barrel somatosensory field that controls whisker and forelimb function (Murphy, T.H. and Corbett, D., 2009; Sigler, A. et al., 2009; Wei, L. et al., 1995). The adhesive removal task has been previously described as a sensitive measure of sensorimotor function in mouse focal ischemic stroke (Bouet, V. et al., 2009; Bouet, V. et al., 2007; Freret, T. et al., 2009). After stroke and treatment with either vehicle or DMOG, animals were assessed on days 1-3, 7 and 14 for deficits in sensorimotor function, using the adhesive removal task. The control stroke animals displayed significantly impaired function of the left forelimb, contra-lateral to the ischemic injury, as measured by the time required to remove the adhesive sticker from the forepaw (Fig. 5.9 A). Right forepaw function was not affected (Fig 5.9 B). Performance after stroke was compared to baseline assessment before surgery and between treatment groups by 2-way ANOVA. DMOG-treated animals were not significantly impaired 7 or 14 days after stroke (Fig. 5.9). The DMOG animals performed the adhesive removal task significantly faster than vehicle-treated animals at 7 days and maintained the same trend at 14 days (Fig. 5.9). DMOG animals were not statistically different from baseline performance at any timepoint after stroke. These less severe functional deficits are consistent with the smaller stroke volume in the DMOG group 72h after stroke.

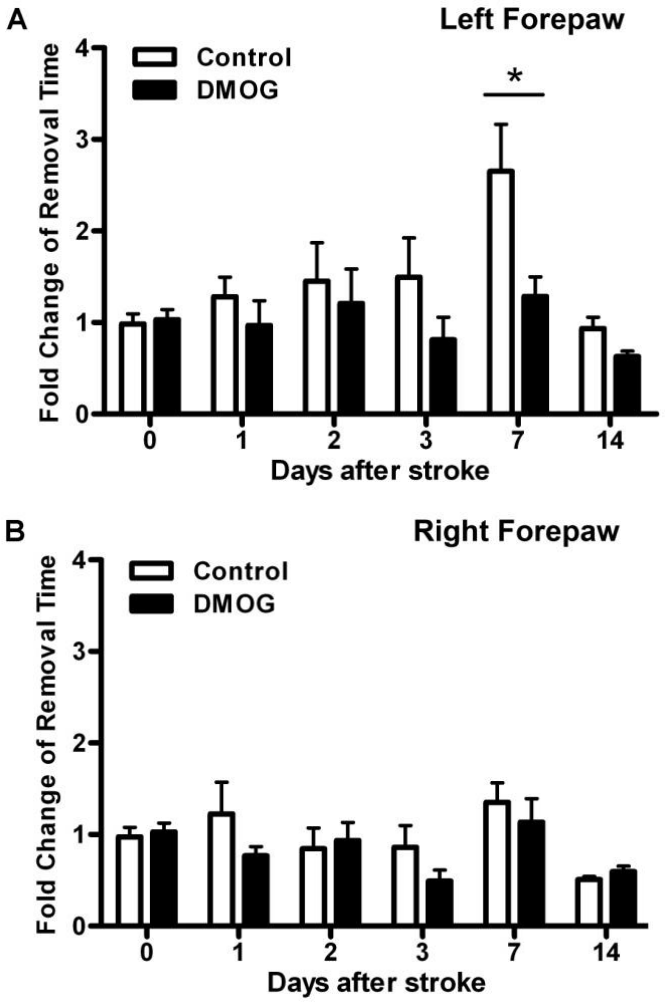


Figure 5.9. PHD inhibitor post-ischemic treatment reduces sensorimotor behavioral deficits after stroke.

Adhesive removal test for sensorimotor forelimb function before, 1-3, 7 and 14 days after stroke. Deficits are measured as increased amount of time to remove the adhesive dot in the affected left forepaw (A) and the unaffected right forepaw (B). Control animals had a significant deficit 7 days after stroke, but DMOG-treated animals did not.

(2-way ANOVA comparing treatment over time, *$p<0.05$, n=10-12 animals per group)

10. HIF-1α protein and HIF-1-regulated gene transcription is enhanced after stroke with DMOG post-ischemic treatment.

Ischemia increased HIF-1α protein in the peri-infarct cortex 3h after stroke, compared to control. DMOG post-treatment further enhanced HIF-1α protein levels in the peri-infarct region, compared to stroke with vehicle post-treatment (Fig. 5.10 A). While HIF-1α is only slightly elevated 3h after DMOG injection alone (Fig. 5.5) or 3h after stroke, the combination of stroke + DMOG injection contributes to a more robust stabilization of HIF-1α protein.

Peri-infarct mRNA expression of known HIF-1α-regulated genes, HIF-1α, VEGF, EPO, eNOS, and PDK1, is enhanced 12h after stroke in animals that received DMOG injection versus vehicle control (Fig. 5.10 B). PDK4 was not enhanced by DMOG treatment.

11. Digoxin and Acriflavine Hydrochloride inhibit HIF-1α in the mouse brain and abrogate DMOG-mediated protective gene expression.

To determine whether HIF-1α is responsible for the beneficial outcome after DMOG post-ischemic treatment, two different HIF-1α inhibitors, digoxin (DIG) and acriflavine hydrochloride (ACF) were tested in combination with DMOG post-treatment. To evaluate the effectiveness of HIF-1 inhibition by DIG and ACF in the brain, adult male mice were injected with vehicle, DIG (2mg/kg i.p.), or ACF (2mg/kg i.p.). DMOG (50mg/kg) or vehicle was injected 6h later to induce HIF-1α stabilization in the brain. After 24h, a time sufficient for DMOG to induce HIF-1α and HIF-1 downstream gene transcription, cortical brain tissue was harvested for protein and mRNA analysis. DMOG stabilized HIF-1α protein expression, compared to vehicle control. As expected, DIG

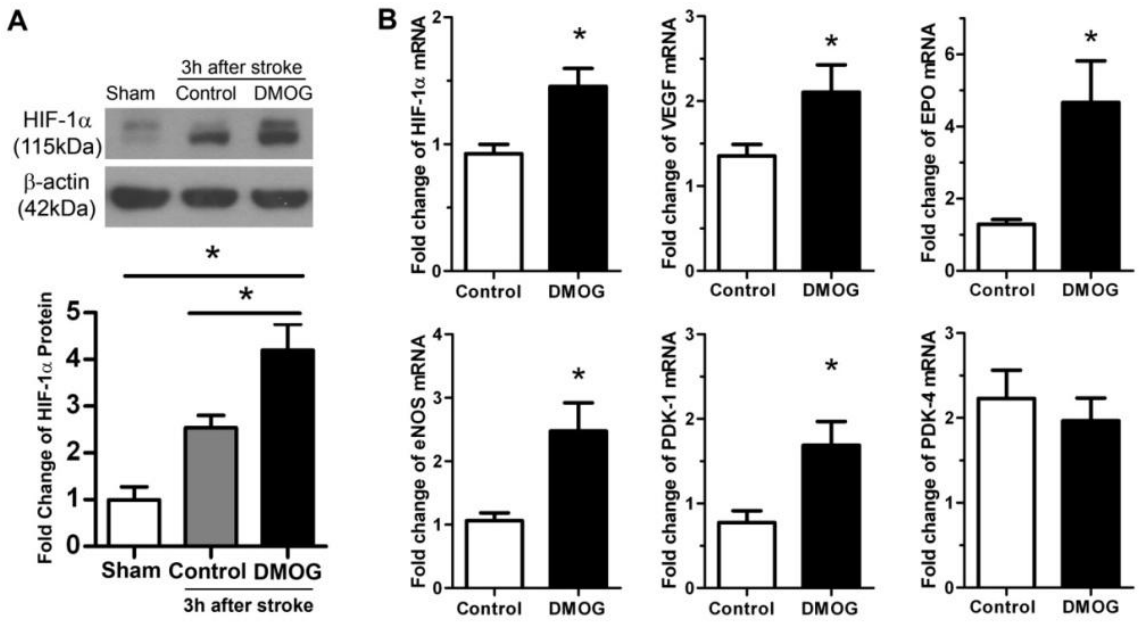


Figure 5.10. Post-ischemic DMOG therapy enhances HIF-1α expression and HIF-1-responsive gene expression.

(A) Expression of HIF-1α 3h after stroke with vehicle or DMOG 30 min post-treatment (1-way ANOVA, Bonferroni post-test, * $p<0.05$, n=4). (B) mRNA expression of HIF-1 responsive genes in control stroke animals or DMOG-treated animals 12h after stroke. (Student's t-test, * $p<0.05$, n=3-4)

pre-treatment tissue did not undergo an increase in HIF-1α with DMOG treatment, since DIG inhibits HIF-1α transcription. ACF pre-treated animals did have a small increase in HIF-1α upon DMOG treatment compared to ACF alone; however, it was not as substantial an increase as control animals and was not statistically significant (Fig. 5.11A). Since ACF inhibits HIF-1 activity, but not protein expression (Lee, K. et al., 2009b), HIF-1α target gene VEGF expression was next analyzed after DMOG treatment with or without the HIF inhibitors. DMOG significantly enhanced the expression of VEGF mRNA in control cortical tissues; however, there was no change in VEGF expression in the groups pre-treated with HIF-1α inhibitors DIG or ACF (Fig. 5.11B). These results suggest that DIG and ACF block the DMOG-induced activity HIF-1α in the normal (non-ischemic) mouse cortex.

Having established that DIG and ACF inhibit HIF-1α in the brain, the effect of HIF-1α inhibitors on the post-ischemic DMOG-mediated increase in HIF-1 and downstream genes VEGF and EPO were next assessed. Animals were pretreated for 24h with vehicle control, DIG, or ACF (2mg/kg). MCAO was performed as in previous experiments, and DMOG was administered 30 min after stroke. Either DIG or ACF were administered again at the time of stroke and every 24h until sacrifice. To assess the effect of DIG and ACF on ischemic HIF-1□, one group of animals was sacrificed 12h after stroke and peri-infarct cortical tissue was harvested. HIF-1□ protein was enhanced in ischemia and ischemia + DMOG treatment groups; although, only the DMOG group reached significance compared to sham. DIG/DMOG and ACF/DMOG animals had significantly less HIF-1α protein than DMOG treated controls (Fig. 5.11C). mRNA expression of VEGF and EPO were increased 12h after stroke by DMOG; however, HIF-1α inhibitors

DIG and ACF abrogated this increase (Fig. 5.11D). These findings validate that DIG and ACF block the DMOG targeted HIF-1α increase and subsequent HIF-1-responsive gene expression increase.

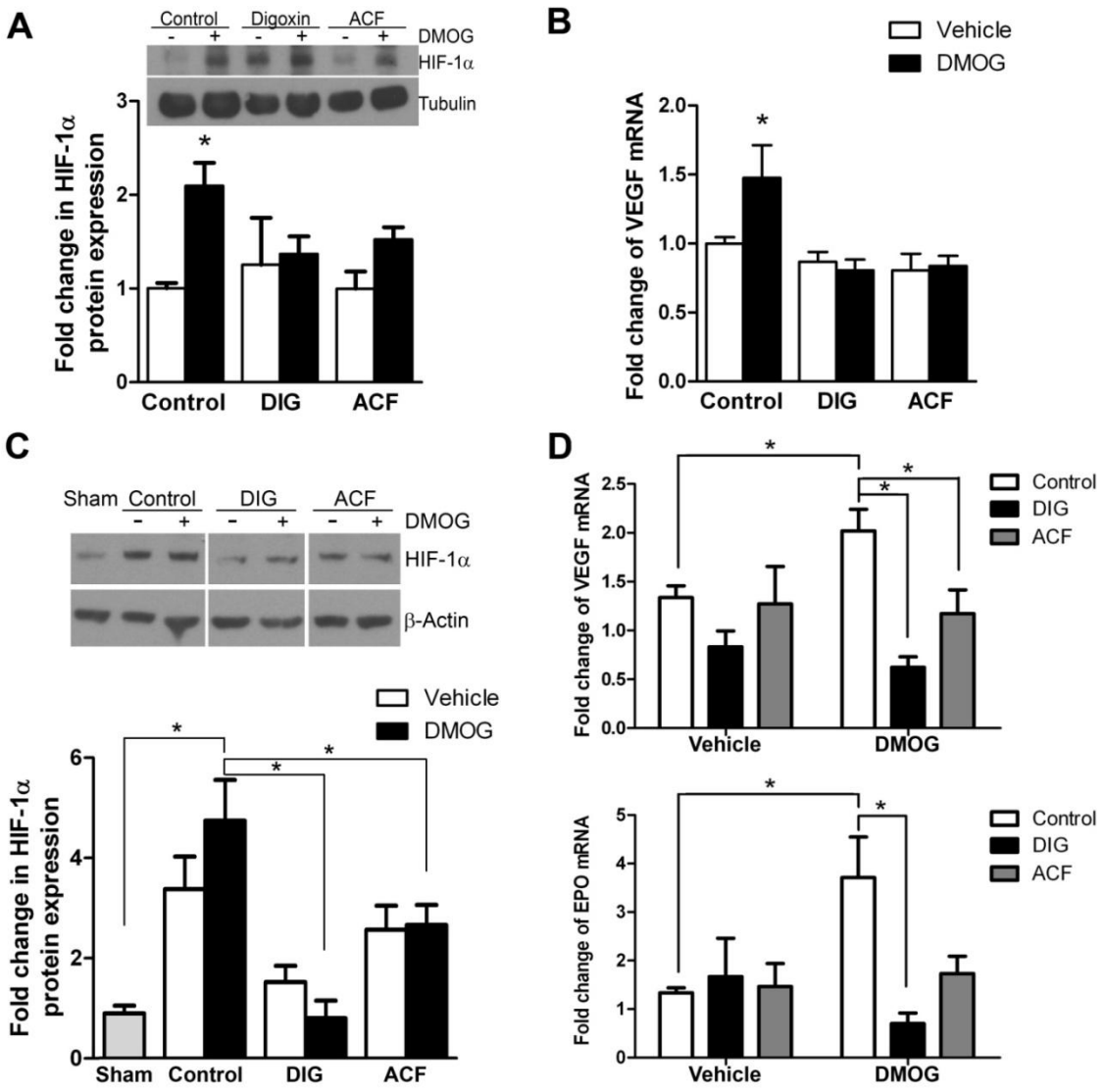


Figure 5.11. Digoxin and Acriflavine Hydrochloride inhibit HIF-1α in the mouse brain and reduce DMOG-mediated protective gene expression.

(A) Mice were pre-treated for 6h with saline or HIF-1α inhibitors 2mg/kg DIG, or 2mg/kg ACF and then treated with DMOG (50mg/kg) or vehicle. After 24h, cortical tissues were immunoblotted for HIF-1α. Densitometry is represented as fold change to vehicle for each condition. DMOG enhances HIF-1α expression compared to vehicle in control conditions, but not under DIG treatment. ACF shows a trend for enhanced

expression of HIF-1α with DMOG, but it is not statistically significant. (n=3 animals per group). (B) Quantitative RT-PCR for the HIF-1α-dependent gene VEGF (using 18s as the housekeeping gene) illustrates that DIG and ACF inhibit the DMOG-induced transcription of HIF-1 dependent VEGF expression. (C) Mice were pre-treated for 24h with saline, DIG, or ACF and then underwent surgical dMCAO. Mice in each group were treated 30 m after stroke with either DMOG or vehicle. After 12h, peri-infarct cortical tissue was harvested and immunoblotted for HIF-1α and β-actin loading control. DMOG enhances HIF-1α expression compared to sham in control conditions; DIG and ACF reduce the DMOG-mediated increase in HIF-1α. Quantification of densitometry is fold change compared to sham control. (n=3-4, $p<0.05$) (D) Quantitative RT-PCR analysis of VEGF and EPO from peri-infarct cortical tissue 12h after stroke (using 18s as the housekeeping gene) shows that DIG and ACF reduce the DMOG-mediated increase in expression. All data are fold change relative to sham control. (n=3-4 animals per group, $p<0.05$)

12. Inhibition of HIF-1α abrogates the post-ischemic DMOG-mediated neuroprotection.

Infarct volume analysis by TTC demonstrated that both HIF-1α inhibitors, DIG and ACF block the infarct-sparing effects of DMOG post-ischemic treatment (Fig. 5.5.12). The infarct of the animals treated with HIF-1α inhibitors trended towards larger than control but did not reach statistical significance. These data suggest that HIF-1α is an integral part of the mechanism of PHD inhibitor mediated post-ischemic neuroprotection.

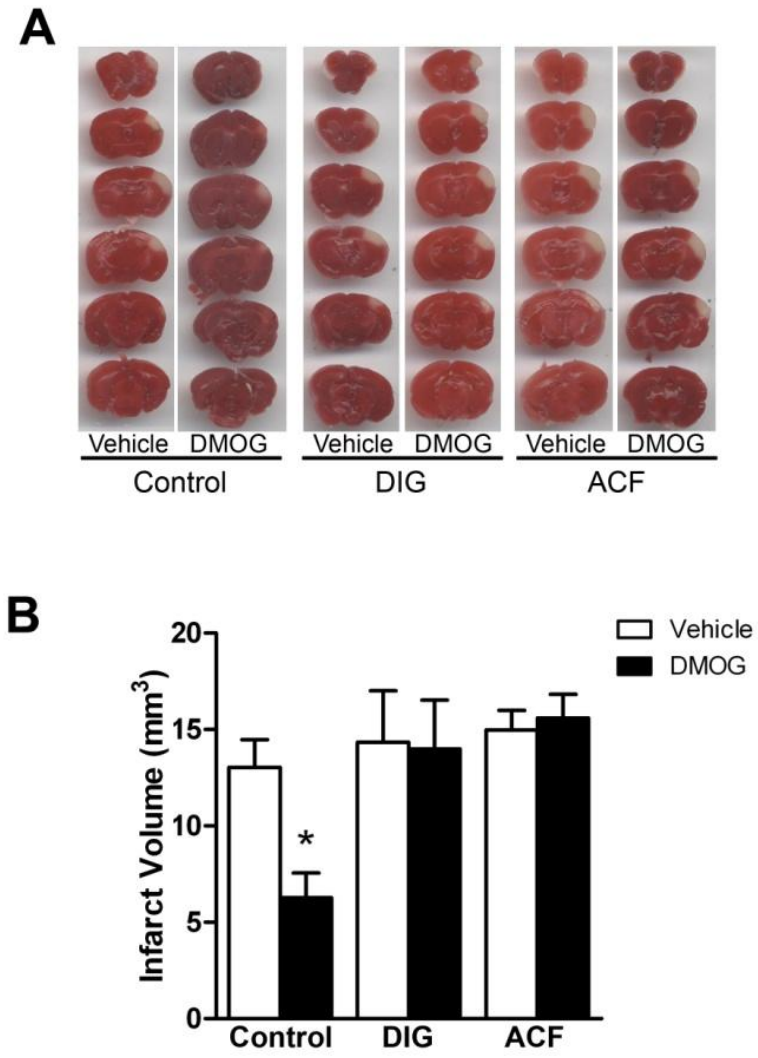


Figure 5.12. Inhibition of HIF-1α abrogates the post-ischemic DMOG-mediated neuroprotection.

(A) Mice were pre-treated for 24h with HIF inhibitors DIG or ACF. TTC staining of ischemic infarct 72h after stroke with or without DMOG post-treatment shows no DMOG-mediated protection in conjunction with either of the HIF-1α inhibitors. (B) Quantification of infarct volume. (n=8-10 in each group, * $p<0.05$).

C. Discussion

Inhibition of PHDs, a major preconditioning target (Siddiq, A. et al., 2005), provides a method of triggering endogenous cellular protective mechanisms without actually depriving cells of oxygen or nutrients through hypoxic or invasive ischemic treatments. The present investigation explored whether globally enhancing oxygen-sensor signaling after ischemia with the PHD inhibitor DMOG could reduce cell death after ischemia and improve overall stroke outcome. The central findings of this study suggest that administration of DMOG after focal cortical ischemia reduces ischemic damage, including apoptotic neural death. The protection is associated with the enhanced expression of HIF-1α protein and HIF-1-target gene expression in peri-infarct cortical tissue. Multiple lines of evidence suggest that the enhanced activation of HIF-1α by PHD inhibition after ischemia is necessary for the protective signaling and the reduction of cortical cell death.

Penumbra: the therapeutic target

In a focal ischemic stroke, a small ischemic core is acutely affected by restricted oxygen and nutrients; however, over time the infarct expands due energy failure, loss of ionic homeostasis, excitatory amino acid release, decreased pH, inflammation, edema causing expanding damage and apoptosis in the surrounding areas of the brain parenchyma (Erecinska, M. et al., 1994; Jayakumar, A.R. and Norenberg, M.D., 2010). One strategy for post-ischemic neuroprotective therapies is to target survival mechanisms in the peri-infarct (or penumbra) region to rescue peri-infarct cells from the spreading damage of the initial injury. Primary ischemic damage occurs very rapidly in the brain,

and secondary damage continues for days after insult. Cell death in the core can occur within minutes; whereas peri-infarct tissues begin to display molecular markers of the apoptotic process within 3-6 hours following reperfusion (Ferrer, I. et al., 2003; Fink, K. et al., 1998). Apoptotic indicators include upstream mitochondrial events such as increase in mitochondrial associated Bax and cytochrome *c* release from the mitochondria, as well as terminal downstream activation of caspase- 3 by four hours after stroke (Ferrer, I. et al., 2003). These events suggest irreversible cell death occurs in the penumbra by 4h after stroke. The speed of irreversible cell death process activation and the lack of robust cell replacement mechanisms in the brain underscores the extreme difficulty in producing effective drugs or therapeutic methods for stroke.

Window of intervention

In the current study, we have targeted the natural oxygen-sensing pathway that is already stimulated by ischemia. By enhancing a cellular signaling process that is already partially active, we attempt to hasten and broaden the innate protective response. Treatment with DMOG by i.p. injection in non-stroke animals begins to increase hypoxia sensitive proteins by 3 hours but did not reach a significant level until 12 hours; therefore, injection 30-60 minutes after stroke should produce an effect within 3.5-12.5 hours after ischemia in mice. DMOG treatment either 30 or 60 minutes after reperfusion of the CCAs provides significant reduction of infarct size. No meaningful or statistical difference was found between the level of protection seen between 30 or 60 minutes, suggesting that treatment within the first hour of reperfusion is functionally equivalent. Unfortunately, an attempt to extend the time window of treatment to two hours after

stroke did not produce favorable results in our model, suggesting that treatment must occur prior to 2h (data not shown).

Translation to human stroke

The therapeutic treatment window is a critical issue in stroke due to the rapid nature of cell death. Current clinical treatment guidelines for vessel recanalization therapy by tPA require the patient to be within 3-4.5 hours of ischemic onset for inclusion (Grunwald, 2011). Administration of the drug beyond that window produces increased risk of hemorrhagic transformation. Because of the limited time-line for administration and the negative consequences for drug delivery beyond the target window, only a small percentage of patients are eligible to receive the drug. If the current study were to translate to human stroke, a 30-60 minute treatment window would be extremely challenging for clinical use, unless a patient suffered a stroke while already in the hospital.

In order to lengthen the time window of PHD inhibitor treatment, more extensive studies are needed on the pharmacokinetic profile of the drug and delivery methods to enhance its action. For example, the current study used i.p. injection, which is an effective method for drug delivery but may not be as fast as an intra-venous route. Delivering DMOG through the circulatory system may accelerate the onset of activity in the brain. Another consideration in examining time windows for treatment in a rodent model is that mice are not simply small humans; mouse basal metabolic rate is faster than human metabolism and the kinetics of cell death are faster (Demetrius, L., 2005). Therefore, in extrapolation of therapeutic interval to human clinical use the treatment

window would likely be longer than in the mouse model. There are many ways to target PHD enzymes, and many companies, such as FibroGen, are working on developing more specific compounds with varying pharmacokinetic and pharmacodynamic profiles. With the knowledge gained from this data set, perhaps other PHD inhibitors may be developed with faster kinetics, longer half lives, and higher specificity for translation to human stroke therapy.

HIF-1α activity during stroke and DMOG treatment

HIF-1 is pathologically stimulated in the ischemic region as part of the endogenous protective response to low oxygen. In the HIF-1α brain-specific conditional knockout mouse ischemic damage is greatly increased, suggesting that HIF-1α mediates a role in the natural protection of the brain from ischemic damage (Baranova, O. et al., 2007). Thus, if endogenous oxygen-sensing pathways including HIF-1 are already stimulated by ischemia and necessary for survival, how does post-ischemic stimulation of these pathways provide protection?

To address this issue, we focused our attention on the "salvageable" area of the brain, the peri-infarct region. Blood circulation from collateral vessels supplies partial flow to the penumbra, and therefore, it does not experience total energy failure (as in the ischemic core). The peri-infarct region can be visibly distinguished from the core by color of the tissue as early as 3h after stroke and can be dissected from core tissue for analysis. HIF-1α protein is expressed in the peri-infarct region within 3 hours after ischemia. The increase in detectable protein is likely due to stabilization of the existing pool of HIF-1 protein due to reduced activity of the PHD enzymes in ischemia; HIF-1α

mRNA expression is not increased 3 hours after stroke (Data not shown). Since the peri-infarct region maintains some blood flow and is partially oxygenated, HIF-1α may not be maximally activated. DMOG injection (i.p.) globally inhibits PHDs in the brain and whole body (Kasiganesan, H. et al., 2007), allowing enhanced activation of the oxygen-sensing pathways in the cells surrounding the infarct. DMOG treatment augmented early HIF-1α expression after stroke in the penumbra.

HIF-1-responsive genes are activated by DMOG post-ischemic treatment

Global pharmacologic PHD inhibition may act like a preconditioning signal in the non-ischemic penumbra region, stimulating the tissue to initiate HIF-1 protein and downstream HIF-mediated anti-apoptotic, vascular, and glycolytic metabolic changes before the area is enveloped by the spreading ischemic core. Supporting this model, DMOG enhanced peri-infarct HIF-1α protein and transcription of VEGF, EPO, eNOS, and PDK-1 12h after ischemia. Each of these genes enhanced by DMOG may contribute to the neuroprotective phenotype. The transcripts fall into three categories of the adaptive response to low oxygen: adaptations in survival pathways (VEGF, EPO), metabolism (PDK 1) and vasculature (eNOS, VEGF, EPO).

Cell survival and cell death pathways

VEGF and EPO can both function as neuroprotective molecules after ischemia by reducing cell death and caspase-3 activation in neuronal ischemic model systems (Jin, K.L. et al., 2000; Keogh, C.L. et al., 2007; Kilic, E. et al., 2005; Sun, Y. et al., 2003). Both proteins are well- established promoters of cell survival in neuronal systems.

DMOG post-treated animals had significantly reduced caspase-3 activation in the peri-infarct region 24h after MCAO, resulting in a significantly smaller infarct at 72h after stroke. Additionally, DMOG reduced *in vitro* activation of caspase-3 protein and cell death by trophic withdrawal in cortical neurons in normoxic conditions. Because caspase-3 activation is a hallmark for apoptosis induction, these data suggest that DMOG rescues neurons from terminal apoptosis. The reduction in caspase-3 mediated cell death could be related to the increase in neuroprotective factors VEGF and EPO.

Metabolic adaptation

During hypoxia, cells stimulate an active suppression of pyruvate catabolism and oxygen consumption by mitochondria while promoting glycolytic mechanisms (Kim, J.W. et al., 2006; Papandreou, I. et al., 2006). PDK-1 is the kinase responsible for phosphorylating and inactivating the pyruvate dehydrogenase (PDH) enzyme complex. PDH controls the switch between aerobic metabolism and anaerobic glycolysis by controlling the entry of pyruvate into the TCA cycle (Martin, E. et al., 2005). PDK-1 is a HIF-1 target gene (Almehdar, H. et al., 2011; Aragones, J. et al., 2008; Kim, J.W. et al., 2006). Increased activation of PDKs in the PHD-1 knockout mouse leads to an anaerobic metabolic switch (Aragones, J. et al., 2008). Increased expression of PDK-1 in the peri-infarct region of DMOG-treated animals may support an ability of the neurons to continue to generate energy even under reduced perfusion. Since energy failure is a major cause of primary and secondary cell death after ischemia, increase of this mechanism may help to explain how DMOG treatment reduces apoptosis and infarct formation.

Effects on the vasculature

Oxygen-sensitive signaling targets the vasculature in ways that enhance blood flow to the deprived ischemic region. This targeting can be through changes in vascular tone to direct more blood to a certain location, changes in the vascular architecture by angiogenesis or opening of collateral vessels or through the circulatory system by enhancing the number of red blood cells. The eNOS is important for regulation of vascular tone and increase in protein expression leads to increases in perfusion. The gene can be expressed in both endothelial cells and neurons (Samdani, A.F. et al., 1997). Studies involving eNOS knockout mice show reduced cortical reperfusion following stroke and greater ischemic damage, suggesting that eNOS plays a protective function in the normal response to ischemia (Huang, Z. et al., 1996; Samdani, A.F. et al., 1997). Additionally, the beneficial effects of ischemic preconditioning require eNOS in both heart and liver models (Cao, Z. et al., 2008; Talukder, M.A. et al., 2010), further supporting the notion that enhanced expression of eNOS may contribute to the protective responses observed in DMOG-treated animals.

VEGF and EPO may also provide some support for the vascular system, since VEGF is a major angiogenic factor and EPO stimulates the production of red blood cells. These effects are typically more prevalent in long-term stimulation of the pathways, and the present study only focused on an acute timeframe. The previously mentioned increase in blood flow in the DMOG treatment relative to control stroke is unlikely to reflect new vessel growth at these early time points. One possible explanation for these findings is enhanced opening of collateral vessels between the MCA and the ACA, which does not

require formation of new vessels, but the opening of small existing arterioles to shunt blood to blocked regions of a major artery (Wei, L. et al., 2001). Constitutively active HIF-1α enhances arteriogenic responses in a hind-limb ischemia model (Patel, T.H. et al., 2005). In addition, HIF-1α activates the expression of eNOS, an enzyme associated with vasodilating properties (Palmer, L.A. et al., 1998). We found HIF-1α and eNOS to be elevated in the penumbra compared to control stroke and HIF-1 was necessary for the protective effect.

Effects on Blood Flow

The distal stroke model is a permanent occlusion of the right MCA combined with transient occlusion of the bi-lateral CCAs for seven minutes (Li, Y. et al., 2007). Blood flow in the core is reduced to 10-20% during combined occlusion. Blood flow was measured using laser Doppler scanning at the location of the brain, corresponding to the ischemic border region or the region at risk for infarction. Although the MCA is occluded, total reduction in flow is not seen because the area receives some collateral flow from the anterior cerebral artery (Wei, L. et al., 2001). Following release of CCA ligation, the affected whisker barrel cortex is gradually and moderately reperfused. After initial loss of blood flow in the core, the microvasculature in the peri-infarct region displays a delayed secondary drop in micro-perfusion between one and four hours after MCA occlusion (Dawson, D.A. et al., 1997). This drop in perfusion may be caused by swelling of the microvascular pericytes, leading to vessel constriction, or by blockage of the luminal space of the vessels by infiltrating inflammatory cells. Preconditioning stimuli can prevent this secondary drop and maintain micro-vascular patency in the peri-

infarct region after stroke in association with prevention of tissue death (Dawson, D.A. et al., 1999). DMOG treatment attenuated loss of perfusion in the peri-ischemic area 24 to 72 hours after stroke; however, the direct mechanism of this finding is unknown. The measurement of more flow in DMOG treated animals is consistent with the fact that the infarct area is smaller.

Behavioral functional testing

The adhesive removal task is a functional measure of the sensorimotor circuitry in the mouse cortex (Bouet, V. et al., 2009; Bouet, V. et al., 2007; Freret, T. et al., 2009). The distal focal stroke model targets the barrel sensorimotor cortex, which controls whisker, forelimb, and lower jaw sensorimotor ability. The brain region that correlates with forelimb function is at the top, medial side of the normal infarct; thus, when the size of the infarct is reduced by DMOG, this region is spared from terminal damage. The data from the adhesive removal task demonstrated that control stroke animals develop impairment in the function of the task by 7 days after stroke as the infarct expands and envelops the penumbra and the forelimb sensorimotor region. DMOG-treated animals, however, do not develop significant impairment in function. These data may be explained by the fact that the DMOG stroke animals have a smaller overall stroke area and protection of apoptosis in the penumbra that prevents the spreading of ischemic damage into the region that controls sensorimotor function.

Role of HIF-1α in DMOG-mediated neuroprotection

Oxygen-sensitive pathways clearly activate HIF-1 transcription factor, but the specific role of HIF-1 in pre- and post-conditioning remains inconclusive. HIF-1α has both pro-survival and pro-death roles in the ischemic brain depending on the injury model, time point, or cell type assessed (Aminova, L.R. et al., 2005; Baranova, O. et al., 2007). Previous *in vitro* studies of the role of HIF in a model of oxidative stress suggest that PHD inhibitors do not require HIF-1 or HIF-2α for neuroprotection (Siddiq, A. et al., 2009); whereas our findings suggest that HIF-1α is required for neuroprotection from OGD. *In vitro* knockdown of HIF-1α in cortical neuron cultures showed that DMOG-mediated protection against ischemic challenge is abrogated in the absence of HIF-1α and basal cell death is increased between 10 and 20%. Siddiq, et al (2009) studied the role of HIF-1 in a glutathione depletion/ oxidative stress model. While oxidative stress is an essential component of ischemic injury, the glutathione depletion and OGD insults are vastly different in cellular pathology. Moreover, the concentration of DMOG used against the oxidative stress model (Siddiq, A. et al., 2009) was 10-fold higher than the current study. Perhaps the increased concentration of PHD inhibitor causes an activation of other additional pathways that are not activated at the micromolar concentrations used in our study.

Given that total knockout of HIF-1α is embryonic lethal (Iyer, N.V. et al., 1998) and that tissue specific knockouts have given conflicting results on the role of HIF during ischemia (Baranova, O. et al., 2007; Helton, R. et al., 2005), a pharmacologic approach was chosen to study the role of HIF-1α in the DMOG-mediated post-conditioning effect. DIG and ACF were identified as HIF inhibitors in cancer therapeutic drug screens (Lee, K. et al., 2009b; Zhang, H. et al., 2008). DIG inhibits HIF by inhibiting its transcription

and since the protein is rapidly turned over in normoxic conditions, the net effect is to reduce protein expression of HIF-1α (Zhang, H. et al., 2008). ACF inhibits HIF by interfering with dimerization to HIF-1β, thereby inhibiting transcriptional activity of HIF-1α (Lee, K. et al., 2009b). The dose of the HIF-1α inhibitors was chosen based on previous reports that 2mg/kg i.p. injection of these compounds reduced tumor derived HIF-1α and suppressed tumor growth in animal models (Lee, K. et al., 2009a; Lee, K. et al., 2009b; Zhang, H. et al., 2008).

Both of these compounds inhibited DMOG-induced HIF-1 function, as measured by transcription of HIF-1 responsive gene VEGF and blocked the protective capacity of DMOG against ischemic damage. ACF and DIG (2mg/kg daily) did not strongly suppress endogenous HIF-1α in the peri-infarct cortex, but they did abrogate the DMOG-mediated increase in HIF-1α. Animals that were co-treated with DMOG and either DIG or ACF did not increase expression of HIF-1α or HIF-1 sensitive genes VEGF or EPO compared to DMOG/vehicle treated animals. The fact that there was little modulation of the endogenous HIF-1 response explains why there is little exacerbation of infarct volume with the inhibitors *in vivo*.

The DIG and ACF inhibitors are not highly specific pharmacologic tools for HIF-1 and are bound to have undetected off target effects. However, we feel confident in the conclusion that HIF contributes to the DMOG-mediated protection based on the multiple approaches that were taken. First, the two inhibitors have distinct mechanisms of action: one inhibits protein synthesis of HIF, and the other inhibits its transcriptional activity. And second, *in vitro* knockdown of HIF-1α in cortical neuron cultures by shRNA showed that DMOG-mediated protection against ischemic challenge is abrogated in the absence

of HIF-1α. Although HIF-1α is necessary for PHD inhibitor-mediated protection in this study and HIFs are the best-characterized targets for PHD inhibition, HIF-1 may not be sufficient for the protection. Other pathways that are activated by PHD inhibition such as NF-κB (Cummins, E.P. et al., 2006) may be involved as well.

Conclusion

Taken together, the findings reported here demonstrate that PHD inhibition after ischemia: 1. Enhances the activation of the HIF-1α and HIF-1-responsive adaptive genes, 2. Reduces ischemic damage including peri-infarct apoptosis, 3. Maintains cerebral blood flow in regions of the ischemic territory that are at risk for infarction, and 4. Improves functional outcome after MCAO. The beneficial effects of PHD inhibition after ischemia require the activity of HIF-1α. These data suggest that supplemental activation of oxygen-sensing pathways after stroke may provide a clinically applicable acute therapeutic intervention for the promotion of neuronal cell survival after ischemia.

Chapter VI

Homozygous knockout of NF-κB p105/p50 disrupts PHD inhibitor-mediated postconditiong and HIF-1α expression after hypoxia/ischemia

A. Introduction

The enhanced stimulation of oxygen-sensing pathways through therapeutic PHD inhibition after ischemia reduces ischemic damage and apoptotic cell death, maintains cerebral blood flow in regions of the ischemic territory that are at risk for infarction, and improves functional outcome after MCAO (Ogle, M.E. et al., 2012). The beneficial effects of PHD inhibition therapy after ischemia require the activity of HIF-1α; however, the role of other PHD-regulated pathways has not been tested. PHD inhibition stimulates the NF-κB pathway through the regulation of the upstream inhibitory kinase complex IKK (Cummins, E.P. et al., 2006), but this regulation has not specifically been tested in brain tissue. Previous studies in models of ischemic pre- and post- conditioning suggest that an intact NF-κB signaling module is required for the generation of a protective response (Blondeau, N. et al., 2001; Rehni, A.K. et al., 2009).

NF-κB is a ubiquitous and pleiotropic transcription factor family with an evolutionarily conserved role in a broad range of biological processes including inflammation, development, apoptotic cell death, cell survival, and many early stress responses (Li, X. and Stark, G.R., 2002). NF-κB family members are expressed in the brain and participate in signaling during homeostasis, development, and pathological processes including ischemic stroke (O'Neill, L.A. and Kaltschmidt, C., 1997; Saleh, A. et al., 2011). There are five NF-κB family transcription factor subunits: p105/p50, p100/p52, p65 (RelA), RelB, and c-Rel. A functional transcription factor is made up of a homodimer or heterdimer of these NF-κB subunits. All of the subunits can homo- or hetero- dimerize except for RelB, which is only known to participate in heterodimers (Sun, S.C., 2011). The composition of dimer pairs determines their specificity in DNA

binding and transcriptional regulation (Hoffmann, A. and Baltimore, D., 2006; Perkins, N.D. et al., 1992).

The Rel sub-family members (p65 (RelA), RelB, and c-Rel) have a C-terminal transactivation domain for activation of transcription, whereas the NF-κB sub-family members (*NFKB1*-p105/p50 and *NFKB2*-p100/p52) do not have transactivation domains (Li, X. and Stark, G.R., 2002). The NF-κB sub-family proteins are synthesized as inactive 105 and 100 kDa proteins, which function as inhibitory IκB proteins. Upon the appropriate upstream signals, p105 or p100 are proteolytically cleaved to produce p50 and p52, respectively (Lang, V. et al., 2003; Lin, L. and Ghosh, S., 1996). Without a C-terminal transactivation domain neither p50 nor p52 can activate transcription as a homodimer, but are able to participate in heterodimers with Rel-sub-family proteins to contribute to transcriptional activation. The homodimeric p50-p50 NF-κB can bind to -κB sites and actively block basal transcription and also can respond to certain stress or signaling inputs as a stimuli-specific transcriptional repressor (Driessler, F. et al., 2004; Tong, X. et al., 2004).

The NF-κB pathway can be activated through the inhibition of oxygen-sensing PHD enzymes indicating an interaction between the regulators of innate immunity (NF-κB) and the hypoxic response (Cummins, E.P. et al., 2006). Hypoxia induces activation of the NF-κB family in many different cancer, immortalized fibroblast, and immune system cells (Jurkat T-cells, HeLa, CaCo-2, THP-1, osteosarcoma, breast cancer, embryonic kidney, and mouse fibroblast cell lines) (Culver, C. et al., 2010; Cummins, E.P. et al., 2006; Koong, A.C. et al., 1994). The signaling pathways controlling NF-κB activation

through hypoxia are less elucidated than inflammatory and developmental activation and the link between NF-κB and HIF-1α during stroke or hypoxia in the brain is unknown.

Both IKK-α and IKK-β contain an evolutionarily conserved sequence *Leu-X-X-Leu-Ala-Pro* that is the same as the hydroxylation sites in the HIF-1α oxygen dependent degradation domain. These IKK proline motifs are potential targets for prolyl hydroxylation by the PHD enzymes (Cummins, E.P. et al., 2006). Under normoxia, PHD enzymes hydroxylate IKK-β on the consensus proline residue to negatively regulate the kinase activity, favoring an inactive kinase. During hypoxia when PHDs are inhibited, IKK-β is not hydroxylated. The lack of hydroxylation favors a more active kinase (Fig. 3.1). Active IKK complex phosphorylates the downstream IκB inhibitory proteins that maintain NF-κB DNA-binding subunits latent in the cytoplasm. Phosphorylation of the IκB proteins targets their proteasomal degradation and NF-κB dimers are released and trafficked to the nucleus to modulate target gene transcription. Proline hydroxylation is considered to be a modulator, but neither necessary nor sufficient for activation or inactivation of the IKK proteins (Cummins, E.P. et al., 2006). The specific NF-κB dimers that are activated by hypoxia have not been characterized, nor have the transcriptome of hypoxia-activated NF-κB, however it is clear that oxygen-regulated NF-κB can both activate and repress transcription (Culver, C. et al., 2010).

Since NF-κB family transcriptional activity can be modulated by PHDs, the current study sought to determine whether there is a role for NF-κB signaling in the PHD inhibitor-mediated neuroprotection by DMOG after stroke. The goals of this research are not only to understand the mechanisms by which PHD-inhibitor therapy produces neuroprotection after stroke, but also to gain insight into the interaction of the NF-κB and

oxygen-sensing pathways after ischemic stroke. We hypothesized that NF-κB may play a role in PHD inhibitor-mediated neuroprotection. In order to test this hypothesis, mice lacking expression of p105/p50 (NF-κB p50 KO) were examined for responsiveness to PHD inhibitor protection in vitro and in vivo in parallel with WT background strain mice. In the absence of NFκB p105/p50, DMOG did not rescue neurons from apoptotic or ischemic insult, suggesting that this protein is necessary for PHD-inhibitor mediated protection. Since HIF-1 is necessary for the action of DMOG (Ogle, M.E. et al., 2012) and previous studies have reported a transcriptional link between NF-κB and HIF, the basal and hypoxic/ischemic expression of HIF-1 was analyzed in the absence of p105/p50. Interestingly, loss of p105/p50 caused a dysregulation of HIF-1α mRNA and protein expression both at resting and hypoxic/ischemic conditions indicating that the NF-κB family is an important regulator of the HIF-1 response in the brain.

B. Results

6.1. *Hypoxia and PHD inhibition induce nuclear translocation of NF-κB.*

Hypoxia and PHD inhibition enhance nuclear localization and DNA binding activity of NF-κB in many cancer, immune system, and fibroblast cell lines (Culver, C. et al., 2010; Cummins, E.P. et al., 2006); however the activation of NF-κB in brain tissue remains untested. NF-κB has distinct signaling roles in the central nervous system (O'Neill, L.A. and Kaltschmidt, C., 1997). Considering that neurons are exquisitely sensitive to hypoxia, it is important to characterize the regulation of NF-κB in primary cortical neuron cultures under hypoxia or pharmacologic PHD inhibition. Cortical

neurons were treated with either hypoxia (0.5% O_2) or the PHD inhibitor/hypoxia mimetic DMOG (250µM) for 1

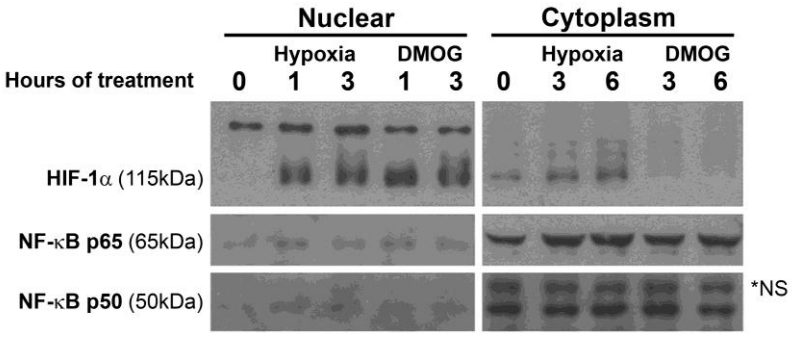


Figure 6.1. Hypoxia and PHD inhibition induce nuclear localization of NF-κB in cortical neurons.

Representative western blots show an increase in HIF-1α, p50, and p65 nuclear localization after either hypoxia (0.5% O_2) or DMOG.

or 3 hours. Nuclear protein lysates were analyzed by immunoblotting for expression of NF-κB p50 and p65 subunits and HIF-1α. The protein stabilization and nuclear translocation of HIF-1α was a positive control for effective activation of the oxygen-sensing pathways. Hypoxia and DMOG both modestly increased NF-κB p50 and p65 nuclear translocation concurrent with HIF-1α increased protein stabilization during 1 and 3 hours of treatment (Fig. 6.1).

6.2. *Homozygous knockout of NF-κB p105/p50 impairs DMOG-mediated protection against apoptosis in cortical neurons.*

WT cortical neuron cultures co-treated with the PHD inhibitor DMOG during an apoptotic insult experience significant attenuation of cell death and caspase-3 activation (Ogle, M.E. et al., 2012). In order to test the role of the NF-κB p50 protein in DMOG-mediated neuroprotection, cell death was assessed in homozygous NF-κB p105/p50 knockout cortical neurons. To induce apoptosis, parallel neuronal cultures from WT and p105/p50 KO mice were subjected to withdrawal of B-27 culture supplement and maintained in only basal media for 24h (Farinelli, S.E. et al., 1998; Ogle, M.E. et al., 2012; Zhang, S.X. et al., 2003). Cultures were co-treated with either the PHD inhibitor DMOG (250 μM) or equal volume of DMSO vehicle. Cell death was quantified by trypan blue nuclear staining. After 24h in basal media with no B27 culture supplement, WT and p105/p50 KO neurons had a similar percentage of neurons undergoing cell death, 34.6±3.2% and 35.66±0.9% respectively (Fig. 6.2). While cell death was attenuated in the WT cultures with DMOG treatment, the p105/p50 KO cultures did not

respond to DMOG. There was a slight trend, but not statistically significant increase in cell death in DMOG treated p105/p50

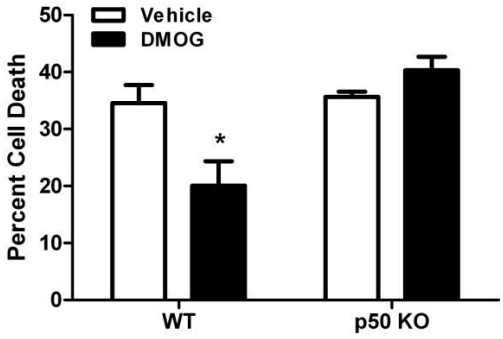


Figure 6.2. Homozygous knockout of NF-κB p50 abrogates DMOG-mediated protection against apoptosis in cortical neurons.

Pure neuronal cultures from either WT or $p50^{-/-}$ embryos were cultured for 7 days in vitro and then subjected to an apoptotic insult of B-27 supplement withdrawal for 24h and dead cells were counted by trypan blue staining. (*$p<0.05$; n=3-4).

KO neurons. These data suggest that lack of the p50 NF-κB subunit impairs the PHD inhibitor-mediated protection against apoptosis.

6.3. *Homozygous knockout of NF-κB p105/p50 impairs the pre and post-conditioning response of cortical neurons during OGD.*

To further test the role of p105/p50 in pre- and post-conditioning mediated protection, WT and p105/p50 KO neurons were subjected to OGD in vitro ischemia. Parallel cultures of WT and p105/p50 KO neurons were maintained for 12-13 days *in vitro* to allow for the expression of mature neuronal receptor phenotypes. Ischemia was modeled in vitro by incubating cells in a glucose-free balanced salt solution for 2 hours at 0.2% oxygen and then returning cells to normal media and incubator conditions for 24 hours of reperfusion. For preconditioning, DMOG (100μM) was added to the cultures 24 hours prior to induction of OGD. For post-conditioning, DMOG was added to the media 2 hours after the initiation of OGD, at the beginning of the reperfusion phase. Control cultures were exposed to vehicle or DMOG for 24 hours. DMOG induced a significant level of cell death in the p105/p50 KO control neurons, and did not reduce the death of neurons from OGD in either the pre or post-conditioning models (Fig. 6.3).

6.4. *DMOG post-ischemic treatment reduces focal ischemic infarct volume in WT but not homozygous p105/p50 KO mice.*

To test whether PHD inhibition requires NF-κB p105/p50 for in vivo ischemic neuroprotection after stroke, age matched WT and p105/p50 KO male mice underwent

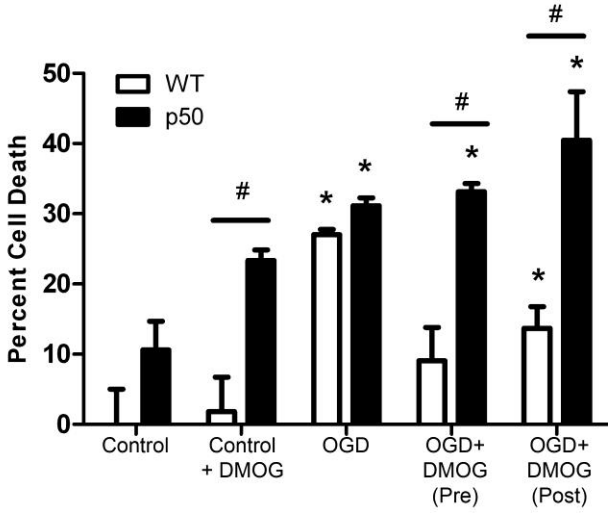


Figure 6.3. Homozygous knockout of NF-κB p50 impairs the pre and post-conditioning response of cortical neurons during OGD.

Parallel primary cortical neurons from WT and NF-κB p50 KO embryos were subjected to OGD for 2h with 24h reperfusion. Cell death was assessed by trypan blue exclusion 24h after OGD. Cortical neurons were either pre-treated 24h prior to OGD or post-treated after OGD with 100μM DMOG. While DMOG reduced cell death in WT cultures in both pre- and post- treatment conditions, p50 KO neurons were not spared by either pre- or post-conditioning. Data are the mean ± SEM from at least three independent experiments. (*$p<0.05$ compared to control culture; #$p<0.05$ compared between genotypes; n=3-4 independent cultures)

surgical stroke by distal occlusion of the MCA and 7 minute bilateral CCA occlusion. Mice were treated 30 minutes after stroke with either vehicle or DMOG (50mg/kg). Infarct volume was assessed 72 hours after stroke by TTC staining. Data is reported as ratio of infarct volume to total brain volume in 6 x 1mm coronal slices. The p105/p50 KO mice trended toward a larger stroke volume as previously reported (Li, J. et al., 2008). DMOG post-treatment significantly reduced stroke volume in the WT mice, but not in the KO mice (Fig. 6.4). Taken together, the findings in the apoptotic and the OGD *in vitro* models, and the *in vivo* stroke model suggest that mice lacking the p105/p50 NF-κB protein do not respond to PHD inhibitor mediated neuroprotection.

6.5. *Knockout of p105/p50 causes disregulation of HIF-1α in cortical tissue.*

There is a clear link between pathways of innate immunity such as the NF-κB family and the oxygen-sensing pathways (Rius, J. et al., 2008; van Uden, P. et al., 2008). Activation of NF-κB in some cell lines induces the expression of HIF-1α (Jung, Y.J. et al., 2003; Rius, J. et al., 2008; van Uden, P. et al., 2008). In order to determine if the loss of responsiveness to DMOG in the absence of p105/p50 is associated with changes in HIF-1α expression, cortical tissue from WT and p105/p50 animals before and after stroke were analyzed for HIF-1α mRNA and protein.

HIF-1α mRNA was enhanced in the p105/p50 KO mouse brain under basal conditions (Fig. 6.5A) suggesting that p105/p50 might play a role in negatively regulating HIF-1α expression. Corresponding with the basal increase in expression of HIF-1α mRNA, protein expression of HIF-1α was also elevated in p105/p50 KO cortical tissue (Figure 6.5 B-C). After stroke, mRNA and protein for HIF-1α drop dramatically in the

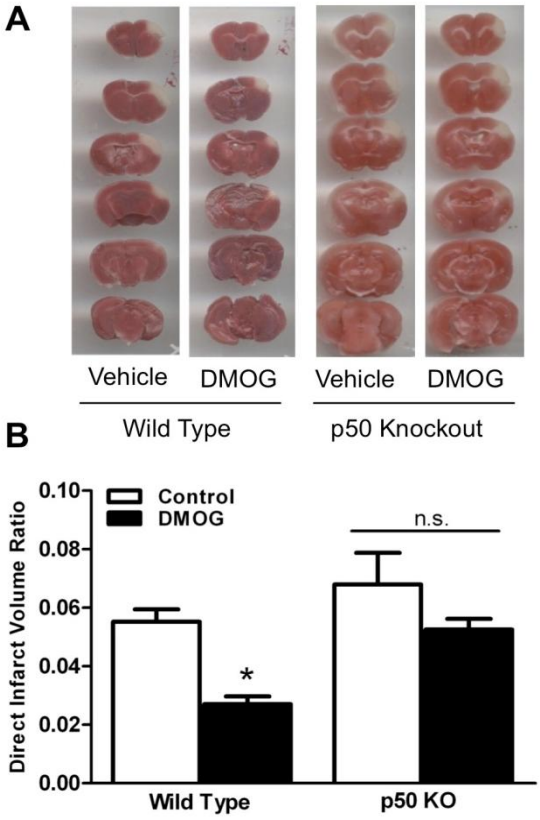


Figure 6.4. DMOG post-ischemic treatment reduces focal ischemic infarct volume in WT but not homozygous p50 KO mice.

(A) Representative TTC staining of ischemic infarct 72h after stroke or stroke with 30-minute DMOG (50mg/kg) post-treatment in WT or NF-κB p50 KO mice. (B) Quantification of TTC ratio of infarct volume to total brain volume in 6 x 1mm coronal brain sections 72h after stroke. DMOG attenuates stroke volume in WT but not p50 KO mice. (n=10-15 animals per group, *$p<0.05$).

p105/p50 KO mouse. The mRNA expression of HIF-1α in the p105/p50 mice after stroke is not significantly different from WT levels. The interpretation of this finding is difficult since the drop in mRNA could either be the result of a signaling event or could be the result of the overall drop in transcription in the peri-infarct region due to ischemic energy-failure. The protein expression of HIF-1α in p105/p50 KO mice after stroke does not follow the same activation kinetics as in the WT animals.

6.6. *Knockout of p105/p50 causes disruption of normal NF-κB signaling and hypoxia responsiveness.*

Total protein from peri-infarct brain tissue was analyzed for expression of members of the NF-κB family after stroke. In WT peri-infarct tissue, there is a time dependent increase in the expression of NF-κB p50 after stoke. The protein continues to increase at least through 72h following ischemia (Fig 6.6). The NF-κB p65 protein also increases after stroke (Fig. 6.6). Loss of the p50 protein is evident in the knockout mice by lack of immunoreactivity for the 50 kDa band. Two additional bands were observed surrounding p50 in both the WT and KO protein at approximately 40 and 55kDa that may be non-specific. The p105/p50 KO mice expressed a higher basal level of the p65 subunit and displayed a different pattern of regulation of p65 after stroke. The p105/p50 cortical tissue has an initial decrease of p65 protein at 24h after stroke and then an increase that returns to above basal expression levels by 72h.

The expression of the p105 precursor of p50 was also examined using the same p50 antibody, which recognizes both proteins through a common epitope. NF-κB p105 plays an important role in generating p50 by proteolytic cleavage in the 20S proteasome, as

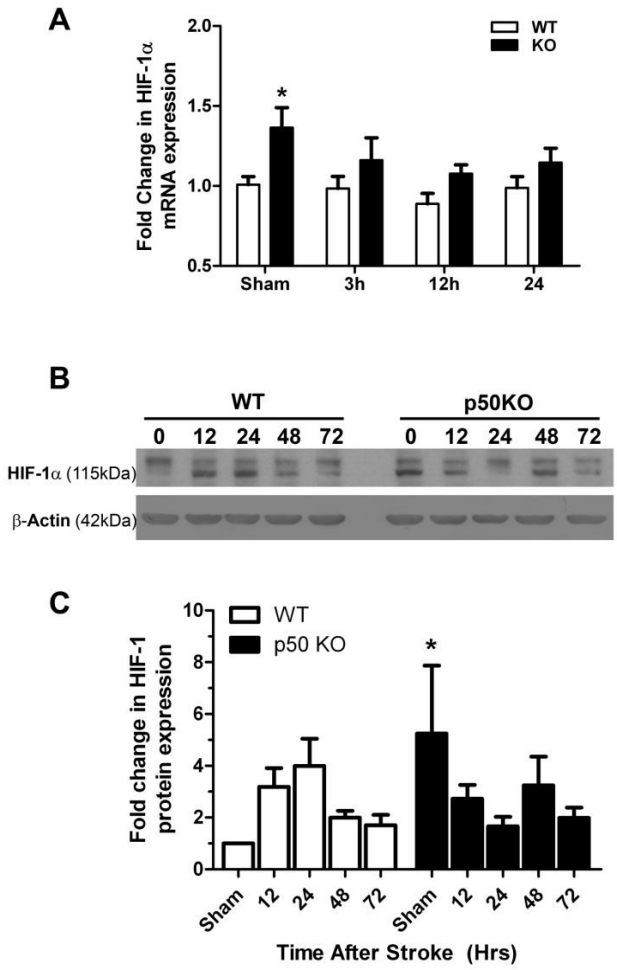


Figure 6.5 Knockout of p50 causes disregulation of HIF-1α basally and after stroke in cortical tissue.

(A) HIF-1α mRNA expression is basally increased in the cortex of p50 KO mice compared to WT mice. The mRNA expression of HIF-1α in the peri-infarct cortex is reduced after ischemic stroke from 3-24h after ischemia. (B) Representative expression of HIF-1α protein before and after stroke in WT and p50 KO peri-infarct cortex. (C) The expression of HIF-1α is enhanced in normal brain tissue from p50 KO mice compared to WT. After ischemia, p50 KO mice dysregulate the protein expression of HIF-1α compared to WT response to ischemia. (n=5; *$p<0.05$)

well as inhibiting the nuclear translocation and activity of some NF-κB dimers (Liou, H.C. et al., 1992; Moorthy, A.K. and Ghosh, G., 2003; Moorthy, A.K. et al., 2006). In order to visualize p105 an increased exposure of the immunoblot was required. WT mice had increased expression of p105 after stroke concordant with the upregulation of p50; however, KO mice did not express p105 (Fig. 6.6 B). These finding suggest that the expression of NF-κB p65 is mis-regulated basally and during ischemia in the absence of p105 and p50 proteins.

When the immunoblot exposure was increased to visualize 105, an approximately 70 kD protein band was apparent in only the p50 KO mouse protein samples. The 70 kD protein was increased 12h after stroke and decayed rapidly thereafter (Fig. 6.6 B). The NF-κB1 gene encodes both p105 and the 70kD IκB-γ protein from different promoters. The proteins share a great deal of the same sequence, including part of the epitope recognized by the p105/p50 polyclonal antibody (Abcam 7971). IκB-γ shares the inhibitory functions of p105 through the multiple ankryin repeat domains. Further studies would be necessary to confirm that this protein band is the p70 IκB-γ.

6.7. *HIF-1α promoter has 2 -κB sites.*

Analysis of the human HIF-1α promoter by previous groups has suggested the presence of possible NF-κB binding sites. Since the current studies are in a mouse model sequence alignment was performed for the promoter region of human HIF-1α and mouse HIF-1α genes from -238bp to +300bp relative to the site of transcription initiation. Base pair numbering corresponds to the human promoter. The mouse and human promoter

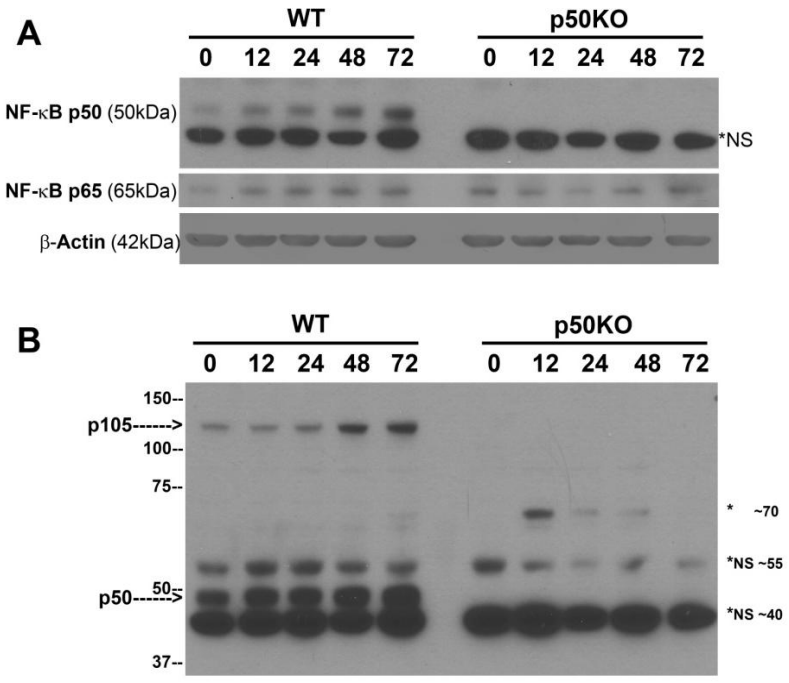


Figure 6.6. Knockout of p50 causes disruption of normal NF-κB signaling and hypoxia responsiveness.

(A) Peri-infarct cortex protein expression of NF-κB subunits p50 and p65 in WT and KO mice before and after stroke. B-actin is a loading control. (B) Increased-exposure immunoblot with Abcam 7971 detects p105, p50, and other proteins approximately 70, 55, and 40 kD.

region that was analyzed maintained nearly 70% identity in alignment (Fig. 6.7). As previously reported, three putative κB sites were found in the promoter, one upstream of the transcriptional start site at -197/188 (κB1) and the other two between the transcriptional initiation site and the site of translation initiation at +150/159 (κB2) and +183/192 (κB3) (Minet, E. et al., 1999; van Uden, P. et al., 2008). In κB1 and κB2 the putative 10 bp binding sites were conserved in 8 out of 10 bases between mouse and human (Table 6.1). The unconserved residues were in the internal region of the κB site, in which sequence specificity is less stringent for NF-κB protein binding (Chen, F.E. and Ghosh, G., 1999; Kunsch, C. et al., 1992). In the κB3 site only 6 out of the 10 bases were conserved between mouse and human and the 3' half site was less conserved (Fig 6.7). The degree of conservation in the promoter varied among different regions; however the sequence around the κB1 site was well conserved suggesting it may be an important regulatory site.

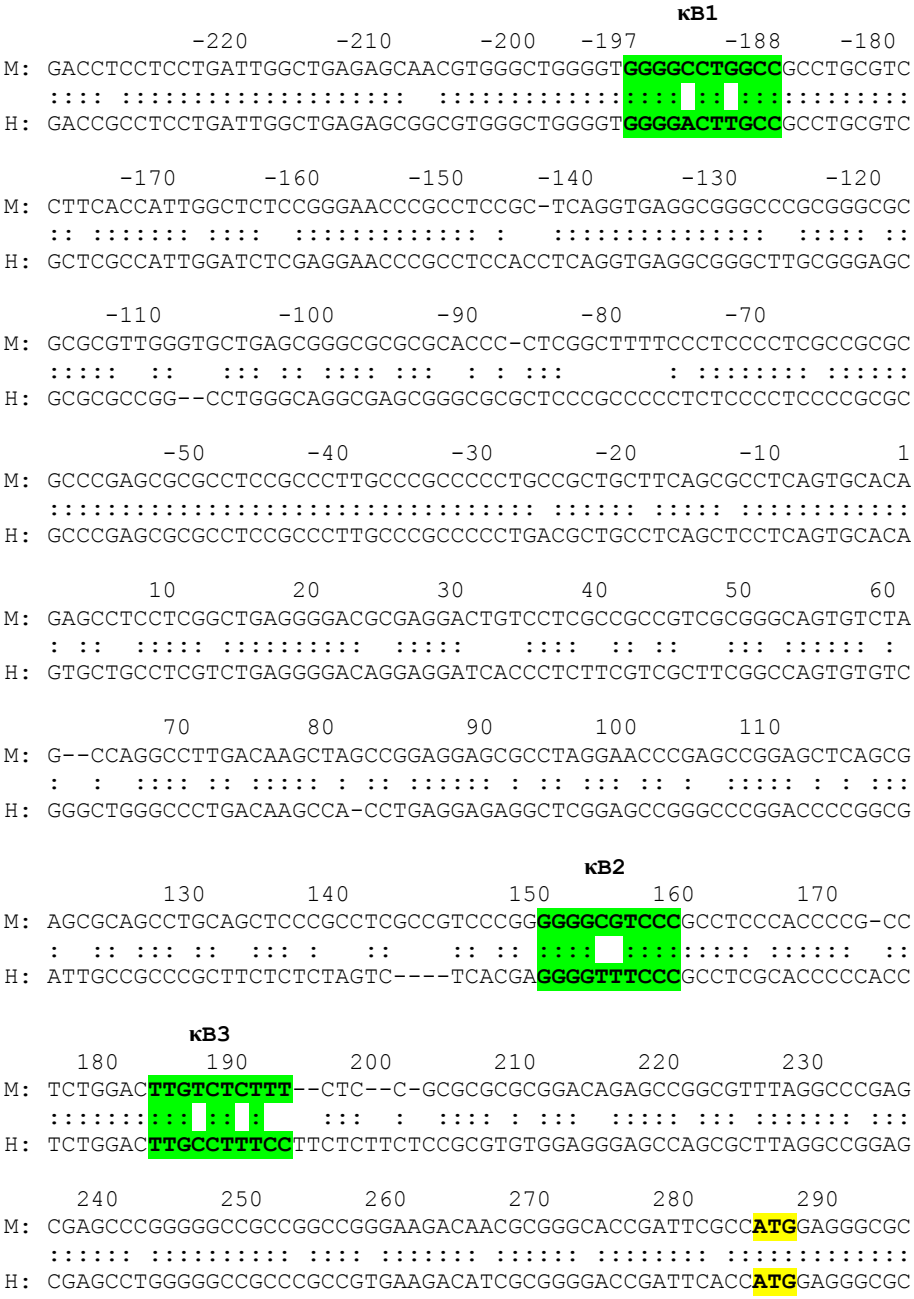
Table 6.1

κB site	Species	Sequence
κB1	M	GGGGcCTgGCC
	H	GGGGACTTGCC
κB2	M	GGGGcgTCCC
	H	GGGGTTTCCC
κB3	M	TTGtCTcTtt
	H	TTGCCTCTCC

Comparison of the sequences of the putative κB sites in the HIF-1α promoter between human and mouse. Non-conserved residues are highlighted in yellow and lower case

letters.

Figure 6.7. HIF-1α promoter analysis



Sequence alignment of the mouse and human HIF-1α promoter regions. Numbering corresponds to the human gene base pairs relative to the site of transcription initiation. Putative κB sites are bolded and highlighted in green. Translational start site is highlighted in yellow.

6.8. *Protein binding to the κB site -197/188 of the HIF-1α promoter under normoxia/hypoxia is dysregulated in KO neurons*

In order to test the whether NF-κB binds to the κB1 site in the HIF-1α promoter under normoxic or hypoxic conditions EMSA analysis was performed on WT and p50 KO cortical neurons. Nuclear lysates were tested for gel retardation of a biotinylated oligonucleotide duplex corresponding to the mouse HIF-1α κB1 site (5'-biotin-GCAACGTGGGCTGGGGT**GGGGCCTGGCC**GCCTGCGTCCTTC-3'). In WT cortical neurons, binding was low under normoxic conditions and increased during 1 and 3 hours of 0.5% O_2 treatment (Fig. 6.8A). This result suggests that there is some basal protein binding to the κB1 site in the HIF-1α promoter under normoxic conditions and binding is enhanced by hypoxia. In p105/p50 KO neurons, the reverse is true. Under basal conditions, a high level of protein was bound to the κB1 oligonucleotide and this binding was reduced by hypoxia treatment. Consistent with these findings, HIF-1α immunostaining in WT cortical neurons was faint but was enhanced by treatment with 0.5% O_2 for 3h. In contrast, p105/p50 KO cortical neurons strongly immunostain for HIF-1α in normoxia and have diminished expression after 3h of hypoxia (Fig. 6.8B).

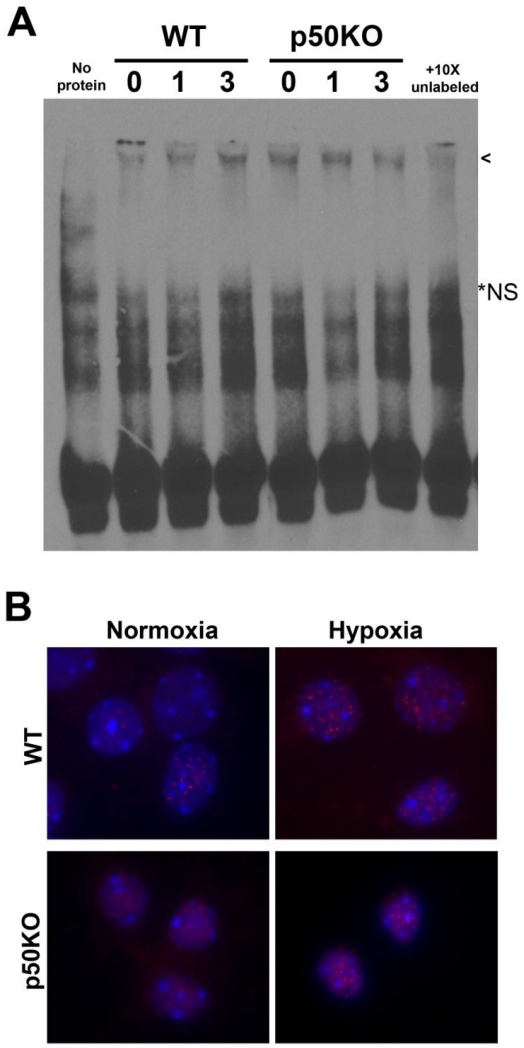


Figure 6.8. Protein binding to the κB site -197/188 of the HIF-1α promoter under normoxia/hypoxia is disregulated in KO neurons

EMSA gel shift assay with biotinylated κB1 oligo and nuclear protein from either WT or KO cortical neurons treated with hypoxia (0.5% O_2) for the indicated amount of time. The first lane has oligo with no protein to demonstrate the non-specific bands, and the last lane has 10X the concentration of unlabeled oligo to compete with the specific binding. (B) Immunocytochemistry for HIF-1α in cortical neuron cultures treated with 3 h of hypoxia. Red=HIF-1, Blue=Hoechst nuclear dye. 100X

C. Discussion

Stimulation of the oxygen sensing pathways after stroke with PHD inhibitors reduces stroke volume by enhancing the activation of HIF-1α; however, other pathways contributing to this oxygen-sensor mediated protection have not been elucidated. The current study found that hypoxia and PHD inhibition stimulate the activation of NF-κB transcription factor family in neurons. Interestingly, the beneficial effects of PHD inhibitor ischemic post-treatment were abolished in animals lacking the p105/p50 NF-κB proteins. In addition, the p105/p50 KO animals displayed a hypoxia-sensitive dysregulation of HIF-1α mRNA and protein. The data presented here show that an intact NF-κB pathway is necessary for the protective capacity of the PHD inhibitor DMOG against apoptotic and ischemic cell death. Disruption of the NF-κB pathway by deletion of p105/p50 interferes with the normal regulation of HIF-1α in the brain, which is necessary for PHD-mediated protection. While interference with other downstream NF-κB sensitive pathways may also contribute to the loss of PHD inhibitor-mediated protection, the current study supports a model where disruption of NF-κB signaling impedes normal HIF-1α oxygen-regulated expression, thereby attenuating the postconditioning response after stroke.

Loss of p105/p50 abolishes PHD inhibitor mediated protection

Ischemic tolerance (preconditioning) can be accomplished by a number of different stress stimuli. While the classical preconditioning signals activate the oxygen-sensing machinery, inflammatory stimuli such as LPS can also confer neuroprotection in a preconditioning model suggesting that activation of inflammatory cascades such as NF-

κB can also produce ischemic tolerance (Dawson, D.A. et al., 1999). Recent studies have demonstrated that NF-κB is an oxygen-sensitive pathway; therefore, the role of NF-κB in PHD-mediated protection was assessed. In apoptotic and ischemic assays in vitro, NF-κB p105/p50 cultures were not protected from damage by DMOG. Interestingly, DMOG treatment alone for 24h increased cell death suggesting that disruption of the normal DMOG-stimulated pathways leads to cell death. Similarly, the p105/p50 KO mice did not display protection in the in vivo post-ischemic DMOG therapy model. Taken together, these data suggest that the loss of p105/p50, which leads to dysregulation of the NF-κB signaling network, abrogates the ability of cells to respond to the PHD inhibitor DMOG to induce protective signaling against stress.

Activation of NF-κB by hypoxia and PHD inhibition.

NF-κB is an important transcription factor that regulates a diverse set of genes in response to an array of biological processes. Recent evidence suggests a signaling link between hypoxia, PHD inhibition, and NF-κB (Culver, C. et al., 2010; Cummins, E.P. et al., 2006). NF-κB subunit nuclear localization was enhanced by both hypoxia and DMOG in cortical neurons (Fig.6.1) consistent with other cell types (Culver, C. et al., 2010; Cummins, E.P. et al., 2006). The regulation of the NF-κB family by PHD inhibition is proposed to occur in a protein-synthesis independent manner. Therefore an acute time-course of nuclear localization was assessed during exposure to hypoxia/PHD inhibition. The duration of the response and dose dependence was not assessed, but may provide further insight into the effects that oxygen-sensitive activation of NF-κB may have on other signaling networks.

Evidence for a link between NF-κB and HIF-1α regulation

NF-κB family members bind to the HIF promoter under normoxic conditions in cancer cells suggesting an NF-κB mediated transcriptional regulation of HIF-1α in normoxia (van Uden, P. et al., 2008). One caveat of the previous study is that many of the assays involved over-expression of the NF-κB family members, which may not replicate the physiological or pathological conditions of the NF-κB-HIF-1α promoter interaction. Since HIF-1 is required for PHD-mediated protection (Ogle, M.E. et al., 2012), the regulation of HIF-1α in p105/p50 KO brain tissue was further assessed under normoxic and hypoxic/ischemic conditions for physiological and pathological models of NF-κB expression, respectively. In addition, we compared WT samples to p105/p50 KO samples that display disruption in many elements of the NF-κB signaling network (Rajendrasozhan, S. et al., 2010).

Alignment of the HIF-1α promoter between mouse and human reveals that two out of three previously identified putative κB sites are well conserved in the mouse promoter. Therefore, NF-κB has two or three possible binding sites for HIF-1α promoter regulation. The κB1 site was chosen for further gel shift analysis since it was within the better-conserved region of the promoter. Hypoxia enhanced protein binding to the putative HIF-1α promoter κB1 site in WT cortical neurons suggesting that there is an increase in regulation of HIF-1α through this κB site during hypoxia. Further studies such as EMSA supershift, oligonucleotide pull-down, and chromatin immunoprecipitation are necessary to determine the exact NF-κB protein/s that bind to the HIF-1 promoter consensus κB sequence.

In the absence of p105/p50, HIF-1α protein expression was significantly increased in brain tissue and cortical neuron cultures. Previous studies have not directly addressed the effect of p105/p50 KO on the expression of HIF-1α; however, indirect evidence for enhanced HIF-1 activity exists. Proteomic analysis of the brains of p105/p50 KO mice showed a higher expression level of many glycolytic proteins in the brain, including Aldolase C (Owen, J.B. et al., 2008) which can be directly regulated by HIF-1α (Jean, J.C. et al., 2006).

Gel shift assay showed that p105/p50 KO cortical neuron lysate had an increased interaction with the HIF-1α κB1 promoter oligonucleotide in normoxia compared to WT. The binding was reduced in hypoxia suggesting that loss of p105/p50 may lead to the increased activation of another protein that can bind to this κB1 oligo under normoxia and the regulation is oxygen-sensitive. Alternatively, loss of p105/p50 may lead to chromatin remodeling that would allow enhanced binding of HIF-1α promoter regulators.

In p105/p50 KO mice, p65 expression is enhanced in basal conditions and dysregulated after stroke. The enhanced basal expression of p65 is consistent with previous studies in the p105/p50 KO mouse (Rajendrasozhan, S. et al., 2010) that showed not only enhanced activation of p65, but also altered chromatin modifications such as increased acetylation and phosphorylation of the histones H3 and H4. Such modifications suggest remodeling to a more open DNA configuration and may occur in part through interaction of activated p65 with co-activators such as p300 (Zhong, H. et al., 2002). The overall result of these modifications would increase the transcription of NF-κB targeted genes.

After hypoxia or ischemia, the expression of NF-κB family members was increased in WT cortical neurons or brain tissue, respectively. Additionally, NF-κB binding to the κB1 site of the HIF-1α promoter was increased in WT neurons. Ischemia dramatically increases HIF-1α in WT mice, but this increase is through posttranslational mechanisms more than transcriptional regulation since the transcription of HIF-1α mRNA did not increase. KO animals reduce the expression of HIF-1α after stroke compared to sham, but the expression is not statistically different from WT post-ischemic HIF-1α expression.

Binding to the HIF-1α promoter oligonucleotide κB1 in KO mice is reduced after exposure to hypoxia. Similarly, mRNA and protein expression of HIF-1α is reduced by hypoxia and ischemia compared to normoxia in KO mice. These data support model of oxygen-sensitive regulation of the HIF-1α promoter by members of the NF-κB family that is disrupted by loss of the p105/p50 protein. The exact mechanism by which this regulation occurs remains unclear.

Complexities of the NF-κB p105/p50 KO model system.

The NF-κB1 p105/p50 KO mouse was utilized to assess the effect of PHD inhibitor-mediated protection in the absence of a key member of the NF-κB signaling network. The NF-κB1 knockout mouse was produced by recombination of exon 6 of the NF-κB1 gene with a neomycin cassette to selectively block the production of functional p105/p50 mRNA and protein (Sha, W.C. et al., 1995). The NF-κB1 gene produces at least two mRNA products from separate promoters, either a 4.0kb p105 product or a 2.6kb product through an alternate promoter. The 2.6kb product encodes a 70kD IκB protein, also

known as IκB- γ (Inoue, J. et al., 1992). The NFKB1 KO mouse was designed to specifically block the production of the full-length p105 transcript that produces p105 and p50 proteins, but not the p70/IκB-γ mRNA sequence (Sha, W.C. et al., 1995).

The homozygous NFKB1 KO mouse does not express p105 or p50 proteins (Fig. 6.6) (Sha, W.C. et al., 1995). Many studies of this mouse model fail to recognize that the mouse is not a pure p50 KO, but also results in the obligate loss of the precursor protein p105 which may have functions independent of p50 that contribute to changes in phenotype in the KO mouse. The full-length p105 protein does not have DNA binding activity, but does have an important function in inhibiting the nuclear translocation of NF-κB p50 through its IκB-like ankryin repeat domains (Liou, H.C. et al., 1992). Although originally thought to only bind and inhibit p50, recent studies have suggested a role for p105 in inhibiting the nuclear translocation of other NF-κB family members as well. Therefore, the NF-κB p105/p50 KO mouse may affect the regulation of NF-κB signaling beyond the loss of p50 and extending to other proteins that may be regulated by p105 as well.

In the p105/p50 KO mouse, p50 and p105 are not expressed as assessed by western blot, but a protein species was recognized in p50 KO mice with the p105/p50 antibody at 70 kD and around 40 and 55kD. The 40 and 55kD species are expressed in both the WT and p105/p50 KO tissue samples and are suspected to be non-specific. The epitope for this antibody (Abcam, ab7971) is amino acid 330-433 of the human p50 protein. After proteolytic cleavage at a glycine rich repeat in the p105 protein, p50 is liberated and is the N-terminal 1-433 amino acids of the p105 protein (Lin, L. and Ghosh, S., 1996).

IκB-γ is produced as a separate transcript from the NF-κB1 gene and the protein product overlaps with the ankyrin repeat domains in the C-terminal region of the p105 protein. The p70/IκB-γ protein begins sequence homology with the p105 protein from amino acid 364 (Inoue, J. et al., 1992; Lin, L. and Ghosh, S., 1996), and therefore overlaps with the C-terminal end of the p50 protein (aa364-433). Since this polyclonal antibody recognizes the 330-433aa sequence of p50, it could also recognize this domain in the p70/IκB protein, although this has not been previously characterized.

Interestingly, the p70/IκB-γ 2.6kB mRNA is expressed at low levels in the brain and other tissues and the protein product can inhibit p65 and c-Rel transactivation (Davis, N. et al., 1991; Gerondakis, S. et al., 1993). While the 70 kDa band in the p50 KO mice has not been confirmed to be p70, we can speculate that if this is the p70/IκB-γ protein, increased expression of this inhibitory factor after stroke in the p105/p50 KO mice might contribute to a down-regulation of p65 and c-Rel transactivation. This inhibitory function would explain the reduction in protein complexes at the HIF-1α κB site during hypoxia and the concordant down regulation in HIF-1α protein in KO mice.

Previous studies in the NF-κB KO mouse have documented dysregulation of other components of the NF-κB pathway. For example, in a model of lung inflammation, loss of p105/p50 lead to increased activation of RelA and decreased levels of RelB under both basal and inflammatory induced conditions (Rajendrasozhan, S. et al., 2010). Corresponding to that finding, protein samples from un-stimulated NF-κB p105/p50 KO mice displayed enhanced binding to an NF-κB consensus oligonucleotide in a DNA binding assay. These data suggest that interpretation of data from the p105/p50 KO mouse must consider the dysregulation of the NF-κB signaling network as a whole.

There are many possible models for how disruption of the p105/p50 protein could effect changes in HIF-1α. 1) p50-p50 homodimer is a negative regulator of the HIF-1 promoter under basal conditions and therefore loss of p50 removes the negative regulatory pathway. 2) p105 is a negative regulator of a HIF-1 activating complex, and loss of this inhibitor frees the activating complex to translocate to the nucleus and induce HIF expression. 3) Loss of p105/p50 leads to chromatin remodeling, allowing for increased binding of NF-κB subunits to the HIF-1α promoter. 4) Aberrant expression of p70, in the absence of p50 and p105 causes dysregulation of the NF-κB signaling network.

Beyond testing these different models, other unanswered questions remain. For example, since HIF-1α protein is heavily post-translationally regulated, what causes the normoxic stabilization of HIF-1α protein in NF-κB KO mice. Many cancer cells are able to stimulate the expression of normoxic HIF-1 in order to promote tumor growth, vascularization, and metastasis (Minet, E. et al., 2000; Zhong, H. et al., 1998). Many cancer cells also have mis-regulated NF-κB pathways. However, the mechanism of normoxic HIF expression is not clear and could be due to unknown effects of NF-κB signaling on PHD expression or the ubiquitination and proteasomal degradation of HIF-1α.

In summary, loss of NF-κB p105/p50 impairs the protective effects of therapeutic PHD inhibition after ischemic stroke. PHD inhibition-mediated protection requires HIF-1α and loss of the intact NF-κB signaling network leads to aberrant expression of HIF-1α

basally and during hypoxia/ischemia conditions. While there is clearly a link between the NF-κB and HIF-1α pathways, there are many levels of complexity that have not been addressed in the current study. These will be the subject of future investigation.

Chapter VII

Summary and Conclusions

Ischemic stroke affects a staggering proportion of the American population, and yet the available options for treating the disease are extremely limited. Treatments targeting the peri-infarct expansion of damage are the most logical post-ischemic intervention for limiting cell death. Due to the rapid nature of cell death in the ischemic core, this area is extremely difficult if not impossible to recover after stroke has occurred. Rehabilitation, cell replacement therapy through stem cell technologies, and enhancement of the endogenous regenerative process after stroke are other long-term treatment options; however these therapies may be insufficient to alleviate severe damage to the brain after stroke. Therefore, post-ischemic neuroprotective therapy to limit the delayed and programmed cell death in the ischemic penumbra provides a rational target for time windows and mechanisms of therapeutic interventions. These treatments may be beneficial in combination with long-term rehabilitative therapies to improve overall stroke treatment in the future.

The brain has an evolutionarily conserved adaptive response to low oxygen, however in the severe oxygen and metabolic substrate deprivation of ischemia, these mechanisms are not sufficient to prevent damage. In the absence of HIF-1α, a central transcription factor mediating the response to low oxygen, brain injury is increased after stroke, suggesting that HIF-1α does provide a necessary protective function during ischemia (Baranova, O. et al., 2007). Therapeutically targeting the brain's natural response to hypoxia/ischemia provides a method to maximize a coordinate endogenous response to hypoxic stress. The current investigation provides evidence that enhanced stimulation of the oxygen-sensing response after ischemic reduces overall ischemic damage through reduction in apoptosis, maintainence of peri-infarct cerebral blood flow and activation of

a number of genes that are involved in adaptive changes during low oxygen. The stimulation of this protective response requires the transcription factor HIF-1α. The protection mediated by PHD inhibition requires both HIF-1 and the NF-κB transcription factor family. While the role of NF-κB is less well understood, the data presented here suggest that an oxygen-sensitive NF-κB-mediated regulation of HIF-1α may contribute to the dysfunction of the PHD inhibitor protective response in the absence of NF-κB p105/p50.

By enhancing the activation of the oxygen-sensing pathways after stroke, this study highlights some of the important protective features of this endogenous adaptive response. Notably, the natural oxygen-sensing response contributes to regulation of genes involved in survival, metabolic, and vascular changes that contribute to the protective nature of this pathway after stroke. This study also highlights an important integration between inflammatory pathways (NF-κB) and traditional oxygen-sensing response (HIF-1α). The information gained in these studies contributes not only to the identification of a potential therapeutic intervention for ischemic stroke, but also to a greater understanding of the pathways governing the natural response to hypoxia/ischemia in the brain.

Chapter VIII

References

Abdel-Fattah, M., Al-Sherbiny, M., Osman, A., Charmy, R., and Tsang, V. (2011). Improving the detection limit of quantitative diagnosis of anti-S. haematobium antibodies using Falcon Assay Screening Test (FAST) ELISA by developing a new standard curve. Parasitol Res *108*, 1457-1463.

Acler, M., Fiaschi, A., and Manganotti, P. (2009a). Long-term levodopa administration in chronic stroke patients. A clinical and neurophysiologic single-blind placebo-controlled cross-over pilot study. Restor Neurol Neurosci *27*, 277-283.

Acler, M., Robol, E., Fiaschi, A., and Manganotti, P. (2009b). A double blind placebo RCT to investigate the effects of serotonergic modulation on brain excitability and motor recovery in stroke patients. J Neurol *256*, 1152-1158.

Ahmad, A., Qahar, J., Siddiq, A., Majeed, A., Rasheed, J., Jabar, F., and von Knorring, A.L. (2005). A 2-year follow-up of orphans' competence, socioemotional problems and post-traumatic stress symptoms in traditional foster care and orphanages in Iraqi Kurdistan. Child Care Health Dev *31*, 203-215.

Akita, M., Mizuno, M., Tonozuka, T., Sakano, Y., Matsui, H., Hidaka, Y., Hatada, Y., Ito, S., and Horikoshi, K. (2004). Crystallization and preliminary X-ray study of isomaltodextranase from Arthrobacter globiformis. Acta Crystallogr D Biol Crystallogr *60*, 572-573.

Almehdar, H., Abdallah, H.M., Osman, A.M., and Abdel-Sattar, E.A. (2011). In vitro cytotoxic screening of selected Saudi medicinal plants. J Nat Med.

Aminova, L.R., Chavez, J.C., Lee, J., Ryu, H., Kung, A., Lamanna, J.C., and Ratan, R.R. (2005). Prosurvival and prodeath effects of hypoxia-inducible factor-1alpha stabilization in a murine hippocampal cell line. J Biol Chem *280*, 3996-4003.

Aprelikova, O., Chandramouli, G.V., Wood, M., Vasselli, J.R., Riss, J., Maranchie, J.K., Linehan, W.M., and Barrett, J.C. (2004). Regulation of HIF prolyl hydroxylases by hypoxia-inducible factors. J Cell Biochem *92*, 491-501.

Aragones, J., Schneider, M., Van Geyte, K., Fraisl, P., Dresselaers, T., Mazzone, M., Dirkx, R., Zacchigna, S., Lemieux, H., Jeoung, N.H., *et al.* (2008). Deficiency or inhibition of oxygen sensor Phd1 induces hypoxia tolerance by reprogramming basal metabolism. Nat Genet *40*, 170-180.

Astrup, J., Symon, L., Branston, N.M., and Lassen, N.A. (1977). Cortical evoked potential and extracellular K+ and H+ at critical levels of brain ischemia. Stroke *8*, 51-57.

Baranova, O., Miranda, L.F., Pichiule, P., Dragatsis, I., Johnson, R.S., and Chavez, J.C. (2007). Neuron-specific inactivation of the hypoxia inducible factor 1 alpha

increases brain injury in a mouse model of transient focal cerebral ischemia. J Neurosci *27*, 6320-6332.

Barkett, M., and Gilmore, T. (1999). Control of apoptosis by Rel/NF-kappaB transcription factors. Oncogene *18*, 6910-6924.

Bergeron, M., Yu, A.Y., Solway, K.E., Semenza, G.L., and Sharp, F.R. (1999). Induction of hypoxia-inducible factor-1 (HIF-1) and its target genes following focal ischaemia in rat brain. Eur J Neurosci *11*, 4159-4170.

Blondeau, N., Widmann, C., Lazdunski, M., and Heurteaux, C. (2001). Activation of the nuclear factor-kappaB is a key event in brain tolerance. J Neurosci *21*, 4668-4677.

Bonventre, J.V., Huang, Z., Taheri, M.R., O'Leary, E., Li, E., Moskowitz, M.A., and Sapirstein, A. (1997). Reduced fertility and postischaemic brain injury in mice deficient in cytosolic phospholipase A2. Nature *390*, 622-625.

Bouchelouche, P., Belhage, B., Frandsen, A., Drejer, J., and Schousboe, A. (1989). Glutamate receptor activation in cultured cerebellar granule cells increases cytosolic free Ca2+ by mobilization of cellular Ca2+ and activation of Ca2+ influx. Exp Brain Res *76*, 281-291.

Bouet, V., Boulouard, M., Toutain, J., Divoux, D., Bernaudin, M., Schumann-Bard, P., and Freret, T. (2009). The adhesive removal test: a sensitive method to assess sensorimotor deficits in mice. Nat Protoc *4*, 1560-1564.

Bouet, V., Freret, T., Toutain, J., Divoux, D., Boulouard, M., and Schumann-Bard, P. (2007). Sensorimotor and cognitive deficits after transient middle cerebral artery occlusion in the mouse. Exp Neurol *203*, 555-567.

Broughton, B.R., Reutens, D.C., and Sobey, C.G. (2009). Apoptotic mechanisms after cerebral ischemia. Stroke; a journal of cerebral circulation *40*, e331-339.

Bruick, R.K., and McKnight, S.L. (2001). A conserved family of prolyl-4-hydroxylases that modify HIF. Science *294*, 1337-1340.

Cao, Z., Dai, J., Fan, K., Wang, H., Ji, G., Li, B., Zhang, D., Hou, S., Qian, W., Zhao, J., *et al.* (2008). A novel functional motif of osteopontin for human lymphocyte migration and survival. Mol Immunol *45*, 3683-3692.

Carmichael, S.T. (2005). Rodent Models of Focal Stroke: Size, Mechanism, and Purpose. NeuroRX *2*, 396-409.

Chan, D.A., Sutphin, P.D., Yen, S.E., and Giaccia, A.J. (2005). Coordinate regulation of the oxygen-dependent degradation domains of hypoxia-inducible factor 1 alpha. Mol Cell Biol *25*, 6415-6426.

Chen, F.E., and Ghosh, G. (1999). Regulation of DNA binding by Rel/NF-kappaB transcription factors: structural views. Oncogene *18*, 6845-6852.

Chen, J., Nagayama, T., Jin, K., Stetler, R.A., Zhu, R.L., Graham, S.H., and Simon, R.P. (1998). Induction of caspase-3-like protease may mediate delayed neuronal death in the hippocampus after transient cerebral ischemia. J Neurosci *18*, 4914-4928.

Chen, L., Einbinder, E., Zhang, Q., Hasday, J., Balke, C.W., and Scharf, S.M. (2005). Oxidative stress and left ventricular function with chronic intermittent hypoxia in rats. Am J Respir Crit Care Med *172*, 915-920.

Chen, P., Zhai, W., Zhang, Y., Zhang, J., and Gu, Y. (2000). [Effects of hypoxia on expression and activity of matrix metalloproteinase-2 in hepatic stellate cell]. Zhonghua Gan Zang Bing Za Zhi *8*, 276-278.

Chen, P., Zhai, W., Zhang, Y., Zhou, X., Zhang, J., Ling, Y., and Gu, Y. (2002). [Effects of hypoxia and hyperoxia on the regulation of the expression and activity of matrix metalloproteinase-2 in hepatic stellate cell]. Zhonghua Bing Li Xue Za Zhi *31*, 337-341.

Chen, W., Jadhav, V., Tang, J., and Zhang, J.H. (2008). HIF-1 alpha inhibition ameliorates neonatal brain damage after hypoxic-ischemic injury. Acta Neurochir Suppl *102*, 395-399.

Chen, Y.F., Feng, J.A., Li, P., Xing, D., Zhang, Y., Serra, R., Ambalavanan, N., Majid-Hassan, E., and Oparil, S. (2006). Dominant negative mutation of the TGF-beta receptor blocks hypoxia-induced pulmonary vascular remodeling. J Appl Physiol *100*, 564-571.

Choi, D.W. (1988a). Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. Trends Neurosci *11*, 465-469.

Choi, D.W. (1988b). Glutamate neurotoxicity and diseases of the nervous system. Neuron *1*, 623-634.

Choi, D.W. (1996). Ischemia-induced neuronal apoptosis. Curr Opin Neurobiol *6*, 667-672.

Choi, D.W., Koh, J.Y., and Peters, S. (1988). Pharmacology of glutamate neurotoxicity in cortical cell culture: attenuation by NMDA antagonists. The Journal of neuroscience : the official journal of the Society for Neuroscience *8*, 185-196.

Coleman, M.L., Sahai, E.A., Yeo, M., Bosch, M., Dewar, A., and Olson, M.F. (2001). Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. Nat Cell Biol *3*, 339-345.

Coulet, F., Nadaud, S., Agrapart, M., and Soubrier, F. (2003). Identification of hypoxia-response element in the human endothelial nitric-oxide synthase gene promoter. J Biol Chem *278*, 46230-46240.

Cowan, W.M., Fawcett, J.W., O'Leary, D.D., and Stanfield, B.B. (1984). Regressive events in neurogenesis. Science *225*, 1258-1265.

Culver, C., Sundqvist, A., Mudie, S., Melvin, A., Xirodimas, D., and Rocha, S. (2010). Mechanism of hypoxia-induced NF-kappaB. Mol Cell Biol *30*, 4901-4921.

Cummins, E.P., Berra, E., Comerford, K.M., Ginouves, A., Fitzgerald, K.T., Seeballuck, F., Godson, C., Nielsen, J.E., Moynagh, P., Pouyssegur, J., *et al.* (2006). Prolyl hydroxylase-1 negatively regulates IkappaB kinase-beta, giving insight into hypoxia-induced NFkappaB activity. Proceedings of the National Academy of Sciences of the United States of America *103*, 18154-18159.

Davis, N., Ghosh, S., Simmons, D.L., Tempst, P., Liou, H.C., Baltimore, D., and Bose, H.R., Jr. (1991). Rel-associated pp40: an inhibitor of the rel family of transcription factors. Science *253*, 1268-1271.

Dawson, D.A., Furuya, K., Gotoh, J., Nakao, Y., and Hallenbeck, J.M. (1999). Cerebrovascular hemodynamics and ischemic tolerance: lipopolysaccharide-induced resistance to focal cerebral ischemia is not due to changes in severity of the initial ischemic insult, but is associated with preservation of microvascular perfusion. J Cereb Blood Flow Metab *19*, 616-623.

Dawson, D.A., Ruetzler, C.A., and Hallenbeck, J.M. (1997). Temporal impairment of microcirculatory perfusion following focal cerebral ischemia in the spontaneously hypertensive rat. Brain Res *749*, 200-208.

Demchuk, A.M., Morgenstern, L.B., Krieger, D.W., Linda Chi, T., Hu, W., Wein, T.H., Hardy, R.J., Grotta, J.C., and Buchan, A.M. (1999). Serum glucose level and diabetes predict tissue plasminogen activator-related intracerebral hemorrhage in acute ischemic stroke. Stroke *30*, 34-39.

Demetrius, L. (2005). Of mice and men. When it comes to studying ageing and the means to slow it down, mice are not just small humans. EMBO Rep *6 Spec No*, S39-44.

Driessler, F., Venstrom, K., Sabat, R., Asadullah, K., and Schottelius, A.J. (2004). Molecular mechanisms of interleukin-10-mediated inhibition of NF-kappaB activity: a role for p50. Clin Exp Immunol *135*, 64-73.

Elvidge, G.P., Glenny, L., Appelhoff, R.J., Ratcliffe, P.J., Ragoussis, J., and Gleadle, J.M. (2006). Concordant regulation of gene expression by hypoxia and 2-oxoglutarate-

dependent dioxygenase inhibition: the role of HIF-1alpha, HIF-2alpha, and other pathways. J Biol Chem *281*, 15215-15226.

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. (1998). A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature *391*, 43-50.

Epstein, A.C., Gleadle, J.M., McNeill, L.A., Hewitson, K.S., O'Rourke, J., Mole, D.R., Mukherji, M., Metzen, E., Wilson, M.I., Dhanda, A., *et al.* (2001). C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell *107*, 43-54.

Erecinska, M., Nelson, D., Yudkoff, M., and Silver, I.A. (1994). Energetics of the nerve terminal in relation to central nervous system function. Biochemical Society transactions *22*, 959-965.

Erecinska, M., and Silver, I.A. (1994). Ions and energy in mammalian brain. Progress in neurobiology *43*, 37-71.

Farinelli, S.E., Greene, L.A., and Friedman, W.J. (1998). Neuroprotective actions of dipyridamole on cultured CNS neurons. J Neurosci *18*, 5112-5123.

Ferrer, I., Friguls, B., Dalfo, E., Justicia, C., and Planas, A.M. (2003). Caspase-dependent and caspase-independent signalling of apoptosis in the penumbra following middle cerebral artery occlusion in the adult rat. Neuropathol Appl Neurobiol *29*, 472-481.

Fink, K., Zhu, J., Namura, S., Shimizu-Sasamata, M., Endres, M., Ma, J., Dalkara, T., Yuan, J., and Moskowitz, M.A. (1998). Prolonged therapeutic window for ischemic brain damage caused by delayed caspase activation. J Cereb Blood Flow Metab *18*, 1071-1076.

Folbergrova, J., Zhao, Q., Katsura, K., and Siesjo, B.K. (1995). N-tert-butyl-alpha-phenylnitrone improves recovery of brain energy state in rats following transient focal ischemia. Proceedings of the National Academy of Sciences of the United States of America *92*, 5057-5061.

Forsythe, J.A., Jiang, B.H., Iyer, N.V., Agani, F., Leung, S.W., Koos, R.D., and Semenza, G.L. (1996). Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol *16*, 4604-4613.

Francis, K., Wallace, G.C., Zhou, X., Yu, S.P., and Wei, L. (2009). Enhanced Cell Survival and Functional Benefits for Neural Stem Cell Therapy after Ischemic Stroke. Journal of Neurochemistry *108*, 156-156.

Freret, T., Bouet, V., Leconte, C., Roussel, S., Chazalviel, L., Divoux, D., Schumann-Bard, P., and Boulouard, M. (2009). Behavioral deficits after distal focal cerebral ischemia in mice: Usefulness of adhesive removal test. Behav Neurosci *123*, 224-230.

Fuchs, Y., and Steller, H. (2011). Programmed cell death in animal development and disease. Cell *147*, 742-758.

Gerondakis, S., Morrice, N., Richardson, I.B., Wettenhall, R., Fecondo, J., and Grumont, R.J. (1993). The activity of a 70 kilodalton I kappa B molecule identical to the carboxyl terminus of the p105 NF-kappa B precursor is modulated by protein kinase A. Cell Growth Differ *4*, 617-627.

Ghosh, S., and Karin, M. (2002). Missing pieces in the NF-kappaB puzzle. Cell *109 Suppl*, S81-96.

Gidday, J.M., Fitzgibbons, J.C., Shah, A.R., and Park, T.S. (1994). Neuroprotection from ischemic brain injury by hypoxic preconditioning in the neonatal rat. Neuroscience Letters *168*, 221-224.

Goss, G.G., Woodside, M., Wakabayashi, S., Pouyssegur, J., Waddell, T., Downey, G.P., and Grinstein, S. (1994). ATP dependence of NHE-1, the ubiquitous isoform of the Na+/H+ antiporter. Analysis of phosphorylation and subcellular localization. The Journal of biological chemistry *269*, 8741-8748.

Gout, O., Bonnaud, I., Weill, A., Moulignier, A., Quenet, J.J., Moret, J., and Bakouche, P. (1999). Facial diplegia complicating a bilateral internal carotid artery dissection. Stroke *30*, 681-686.

Greijer, A.E., van der Groep, P., Kemming, D., Shvarts, A., Semenza, G.L., Meijer, G.A., van de Wiel, M.A., Belien, J.A., van Diest, P.J., and van der Wall, E. (2005). Up-regulation of gene expression by hypoxia is mediated predominantly by hypoxia-inducible factor 1 (HIF-1). J Pathol *206*, 291-304.

Grunwald, I.Q., Wakhloo, A.K., Walter, S., Molyneux, A.J., Byrne, J.V., Nagel, S., Kuhn, A.L., Papadakis, M., Fassbender, K., Balami, J.S., *et al.* (2011). Endovascular stroke treatment today. AJNR Am J Neuroradiol *32*, 238-243.

Guan, H., Hou, S., and Ricciardi, R.P. (2005). DNA binding of repressor nuclear factor-kappaB p50/p50 depends on phosphorylation of Ser337 by the protein kinase A catalytic subunit. J Biol Chem *280*, 9957-9962.

Gwag, B.J., Koh, J.Y., DeMaro, J.A., Ying, H.S., Jacquin, M., and Choi, D.W. (1997). Slowly triggered excitotoxicity occurs by necrosis in cortical cultures. Neuroscience *77*, 393-401.

Harada, H., Itasaka, S., Kizaka-Kondoh, S., Shibuya, K., Morinibu, A., Shinomiya, K., and Hiraoka, M. (2009). The Akt/mTOR pathway assures the synthesis of HIF-1alpha protein in a glucose- and reoxygenation-dependent manner in irradiated tumors. J Biol Chem *284*, 5332-5342.

Hart, M.L., Grenz, A., Gorzolla, I.C., Schittenhelm, J., Dalton, J.H., and Eltzschig, H.K. (2011). Hypoxia-inducible factor-1alpha-dependent protection from intestinal ischemia/reperfusion injury involves ecto-5'-nucleotidase (CD73) and the A2B adenosine receptor. J Immunol *186*, 4367-4374.

Heidenreich, P.A., Trogdon, J.G., Khavjou, O.A., Butler, J., Dracup, K., Ezekowitz, M.D., Finkelstein, E.A., Hong, Y., Johnston, S.C., Khera, A., *et al.* (2011). Forecasting the future of cardiovascular disease in the United States: a policy statement from the American Heart Association. Circulation *123*, 933-944.

Helton, R., Cui, J., Scheel, J.R., Ellison, J.A., Ames, C., Gibson, C., Blouw, B., Ouyang, L., Dragatsis, I., Zeitlin, S., *et al.* (2005). Brain-specific knock-out of hypoxia-inducible factor-1alpha reduces rather than increases hypoxic-ischemic damage. J Neurosci *25*, 4099-4107.

Hengartner, M.O. (2000). The biochemistry of apoptosis. Nature *407*, 770-776.

Hindryckx, P., De Vos, M., Jacques, P., Ferdinande, L., Peeters, H., Olievier, K., Bogaert, S., Brinkman, B., Vandenabeele, P., Elewaut, D., *et al.* (2010). Hydroxylase inhibition abrogates TNF-alpha-induced intestinal epithelial damage by hypoxia-inducible factor-1-dependent repression of FADD. J Immunol *185*, 6306-6316.

Hirsila, M., Koivunen, P., Gunzler, V., Kivirikko, K.I., and Myllyharju, J. (2003). Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. J Biol Chem *278*, 30772-30780.

Hoffmann, A., and Baltimore, D. (2006). Circuitry of nuclear factor kappaB signaling. Immunol Rev *210*, 171-186.

Hou, S., Guan, H., and Ricciardi, R.P. (2003). Phosphorylation of serine 337 of NF-kappaB p50 is critical for DNA binding. J Biol Chem *278*, 45994-45998.

Hoyte, L.C., Brooks, K.J., Nagel, S., Akhtar, A., Chen, R., Mardiguian, S., McAteer, M.A., Anthony, D.C., Choudhury, R.P., Buchan, A.M., *et al.* (2010). Molecular magnetic resonance imaging of acute vascular cell adhesion molecule-1 expression in a mouse model of cerebral ischemia. J Cereb Blood Flow Metab *30*, 1178-1187.

Huang, Z., Huang, P.L., Ma, J., Meng, W., Ayata, C., Fishman, M.C., and Moskowitz, M.A. (1996). Enlarged infarcts in endothelial nitric oxide synthase knockout mice are attenuated by nitro-L-arginine. J Cereb Blood Flow Metab *16*, 981-987.

Iadecola, C., and Alexander, M. (2001). Cerebral ischemia and inflammation. Curr Opin Neurol *14*, 89-94.

Inoue, J., Kerr, L.D., Kakizuka, A., and Verma, I.M. (1992). I kappa B gamma, a 70 kd protein identical to the C-terminal half of p110 NF-kappa B: a new member of the I kappa B family. Cell *68*, 1109-1120.

Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J.M., Lane, W.S., and Kaelin, W.G., Jr. (2001). HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science *292*, 464-468.

Iyer, N.V., Kotch, L.E., Agani, F., Leung, S.W., Laughner, E., Wenger, R.H., Gassmann, M., Gearhart, J.D., Lawler, A.M., Yu, A.Y., *et al.* (1998). Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev *12*, 149-162.

Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J., *et al.* (2001). Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science *292*, 468-472.

Jayakumar, A.R., and Norenberg, M.D. (2010). The Na-K-Cl Co-transporter in astrocyte swelling. Metab Brain Dis *25*, 31-38.

Jean, J.C., Rich, C.B., and Joyce-Brady, M. (2006). Hypoxia results in an HIF-1-dependent induction of brain-specific aldolase C in lung epithelial cells. Am J Physiol Lung Cell Mol Physiol *291*, L950-956.

Jiang, B.H., Semenza, G.L., Bauer, C., and Marti, H.H. (1996). Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension. Am J Physiol *271*, C1172-1180.

Jiang, H., Guo, R., and Powell-Coffman, J.A. (2001). The Caenorhabditis elegans hif-1 gene encodes a bHLH-PAS protein that is required for adaptation to hypoxia. Proc Natl Acad Sci U S A *98*, 7916-7921.

Jin, K.L., Mao, X.O., and Greenberg, D.A. (2000). Vascular endothelial growth factor: direct neuroprotective effect in in vitro ischemia. Proc Natl Acad Sci U S A *97*, 10242-10247.

Jung, Y.J., Isaacs, J.S., Lee, S., Trepel, J., and Neckers, L. (2003). IL-1beta-mediated up-regulation of HIF-1alpha via an NFkappaB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. FASEB J *17*, 2115-2117.

Kaelin, W.G., Jr., and Ratcliffe, P.J. (2008). Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. Mol Cell *30*, 393-402.

Karin, M. (1999). How NF-kappaB is activated: the role of the IkappaB kinase (IKK) complex. Oncogene *18*, 6867-6874.

Kasiganesan, H., Sridharan, V., and Wright, G. (2007). Prolyl hydroxylase inhibitor treatment confers whole-animal hypoxia tolerance. Acta Physiologica *190*, 163-169.

Keogh, C.L., Yu, S.P., and Wei, L. (2007). The effect of recombinant human erythropoietin on neurovasculature repair after focal ischemic stroke in neonatal rats. J Pharmacol Exp Ther *322*, 521-528.

Kilic, E., Kilic, U., Soliz, J., Bassetti, C.L., Gassmann, M., and Hermann, D.M. (2005). Brain-derived erythropoietin protects from focal cerebral ischemia by dual activation of ERK-1/-2 and Akt pathways. FASEB J *19*, 2026-2028.

Kim, J.W., Tchernyshyov, I., Semenza, G.L., and Dang, C.V. (2006). HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metab *3*, 177-185.

Kitagawa, K., Matsumoto, M., Tagaya, M., Hata, R., Ueda, H., Niinobe, M., Handa, N., Fukunaga, R., Kimura, K., Mikoshiba, K., *et al.* (1990). 'Ischemic tolerance' phenomenon found in the brain. Brain Res *528*, 21-24.

Kitchen, C.M., Leung, S.W., Corbett, A.H., and Murphy, T.J. (2009). The mating response cascade does not modulate changes in the steady-state level of target mRNAs through control of mRNA stability. Yeast *26*, 261-272.

Koong, A.C., Chen, E.Y., and Giaccia, A.J. (1994). Hypoxia causes the activation of nuclear factor kappa B through the phosphorylation of I kappa B alpha on tyrosine residues. Cancer Res *54*, 1425-1430.

Krishnamachary, B., Berg-Dixon, S., Kelly, B., Agani, F., Feldser, D., Ferreira, G., Iyer, N., LaRusch, J., Pak, B., Taghavi, P., *et al.* (2003). Regulation of colon carcinoma cell invasion by hypoxia-inducible factor 1. Cancer Res *63*, 1138-1143.

Kunsch, C., Ruben, S.M., and Rosen, C.A. (1992). Selection of optimal kappa B/Rel DNA-binding motifs: interaction of both subunits of NF-kappa B with DNA is required for transcriptional activation. Mol Cell Biol *12*, 4412-4421.

Lang, V., Janzen, J., Fischer, G.Z., Soneji, Y., Beinke, S., Salmeron, A., Allen, H., Hay, R.T., Ben-Neriah, Y., and Ley, S.C. (2003). betaTrCP-mediated proteolysis of NF-kappaB1 p105 requires phosphorylation of p105 serines 927 and 932. Mol Cell Biol *23*, 402-413.

Lavista-Llanos, S., Centanin, L., Irisarri, M., Russo, D.M., Gleadle, J.M., Bocca, S.N., Muzzopappa, M., Ratcliffe, P.J., and Wappner, P. (2002). Control of the

hypoxic response in Drosophila melanogaster by the basic helix-loop-helix PAS protein similar. Mol Cell Biol *22*, 6842-6853.

Lee, D.W., Rajagopalan, S., Siddiq, A., Gwiazda, R., Yang, L., Beal, M.F., Ratan, R.R., and Andersen, J.K. (2009). Inhibition of prolyl hydroxylase protects against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity: model for the potential involvement of the hypoxia-inducible factor pathway in Parkinson disease. J Biol Chem *284*, 29065-29076.

Lee, J.-M., Grabb, M.C., Zipfel, G.J., and Choi, D.W. (2000). Brain tissue responses to ischemia. The Journal of Clinical Investigation *106*, 723-731.

Lee, K., Qian, D.Z., Rey, S., Wei, H., Liu, J.O., and Semenza, G.L. (2009a). Anthracycline chemotherapy inhibits HIF-1 transcriptional activity and tumor-induced mobilization of circulating angiogenic cells. Proc Natl Acad Sci U S A *106*, 2353-2358.

Lee, K., Zhang, H., Qian, D.Z., Rey, S., Liu, J.O., and Semenza, G.L. (2009b). Acriflavine inhibits HIF-1 dimerization, tumor growth, and vascularization. Proc Natl Acad Sci U S A *106*, 17910-17915.

Lee, S., Nakamura, E., Yang, H., Wei, W., Linggi, M.S., Sajan, M.P., Farese, R.V., Freeman, R.S., Carter, B.D., Kaelin, W.G., Jr.*, et al.* (2005). Neuronal apoptosis linked to EglN3 prolyl hydroxylase and familial pheochromocytoma genes: developmental culling and cancer. Cancer Cell *8*, 155-167.

Li, J., Lu, Z., Li, W.L., Yu, S.P., and Wei, L. (2008). Cell death and proliferation in NF-kappaB p50 knockout mouse after cerebral ischemia. Brain Res *1230*, 281-289.

Li, X., and Stark, G.R. (2002). NFkappaB-dependent signaling pathways. Exp Hematol *30*, 285-296.

Li, Y., Lu, Z., Keogh, C.L., Yu, S.P., and Wei, L. (2007). Erythropoietin-induced neurovascular protection, angiogenesis, and cerebral blood flow restoration after focal ischemia in mice. J Cereb Blood Flow Metab *27*, 1043-1054.

Lin, L., and Ghosh, S. (1996). A glycine-rich region in NF-kappaB p105 functions as a processing signal for the generation of the p50 subunit. Mol Cell Biol *16*, 2248-2254.

Linnik, M.D., Zobrist, R.H., and Hatfield, M.D. (1993). Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. Stroke *24*, 2002-2008; discussion 2008-2009.

Liou, H.C., Nolan, G.P., Ghosh, S., Fujita, T., and Baltimore, D. (1992). The NF-kappa B p50 precursor, p105, contains an internal I kappa B-like inhibitor that preferentially inhibits p50. EMBO J *11*, 3003-3009.

Lipton, P. (1999). Ischemic cell death in brain neurons. Physiol Rev *79*, 1431-1568.

Liu, Y., Kato, H., Nakata, N., and Kogure, K. (1992). Protection of rat hippocampus against ischemic neuronal damage by pretreatment with sublethal ischemia. Brain Research *586*, 121-124.

Loenarz, C., Coleman, M.L., Boleininger, A., Schierwater, B., Holland, P.W., Ratcliffe, P.J., and Schofield, C.J. (2011). The hypoxia-inducible transcription factor pathway regulates oxygen sensing in the simplest animal, Trichoplax adhaerens. EMBO Rep *12*, 63-70.

Lomb, D.J., Straub, J.A., and Freeman, R.S. (2007). Prolyl hydroxylase inhibitors delay neuronal cell death caused by trophic factor deprivation. J Neurochem *103*, 1897-1906.

Majid, A., He, Y.Y., Gidday, J.M., Kaplan, S.S., Gonzales, E.R., Park, T.S., Fenstermacher, J.D., Wei, L., Choi, D.W., and Hsu, C.Y. (2000). Differences in vulnerability to permanent focal cerebral ischemia among 3 common mouse strains. Stroke *31*, 2707-2714.

Markgraf, C.G., Velayo, N.L., Johnson, M.P., McCarty, D.R., Medhi, S., Koehl, J.R., Chmielewski, P.A., and Linnik, M.D. (1998). Six-hour window of opportunity for calpain inhibition in focal cerebral ischemia in rats. Stroke *29*, 152-158.

Martin, E., Rosenthal, R.E., and Fiskum, G. (2005). Pyruvate dehydrogenase complex: metabolic link to ischemic brain injury and target of oxidative stress. J Neurosci Res *79*, 240-247.

Mayer, M.L., and Westbrook, G.L. (1987). Permeation and block of N-methyl-D-aspartic acid receptor channels by divalent cations in mouse cultured central neurones. The Journal of physiology *394*, 501-527.

McDonough, M.A., Li, V., Flashman, E., Chowdhury, R., Mohr, C., Lienard, B.M., Zondlo, J., Oldham, N.J., Clifton, I.J., Lewis, J., *et al.* (2006). Cellular oxygen sensing: Crystal structure of hypoxia-inducible factor prolyl hydroxylase (PHD2). Proc Natl Acad Sci U S A *103*, 9814-9819.

Minet, E., Ernest, I., Michel, G., Roland, I., Remacle, J., Raes, M., and Michiels, C. (1999). HIF1A gene transcription is dependent on a core promoter sequence encompassing activating and inhibiting sequences located upstream from the transcription initiation site and cis elements located within the 5'UTR. Biochem Biophys Res Commun *261*, 534-540.

Minet, E., Michel, G., Remacle, J., and Michiels, C. (2000). Role of HIF-1 as a transcription factor involved in embryonic development, cancer progression and apoptosis (review). Int J Mol Med *5*, 253-259.

Mitani, A., Andou, Y., Matsuda, S., Arai, T., Sakanaka, M., and Kataoka, K. (1994). Origin of ischemia-induced glutamate efflux in the CA1 field of the gerbil hippocampus: an in vivo brain microdialysis study. Journal of neurochemistry *63*, 2152-2164.

Moorthy, A.K., and Ghosh, G. (2003). p105.Ikappa Bgamma and prototypical Ikappa Bs use a similar mechanism to bind but a different mechanism to regulate the subcellular localization of NF-kappa B. J Biol Chem *278*, 556-566.

Moorthy, A.K., Savinova, O.V., Ho, J.Q., Wang, V.Y., Vu, D., and Ghosh, G. (2006). The 20S proteasome processes NF-kappaB1 p105 into p50 in a translation-independent manner. EMBO J *25*, 1945-1956.

Mukae, N., Enari, M., Sakahira, H., Fukuda, Y., Inazawa, J., Toh, H., and Nagata, S. (1998). Molecular cloning and characterization of human caspase-activated DNase. Proceedings of the National Academy of Sciences of the United States of America *95*, 9123-9128.

Murphy, T.H., and Corbett, D. (2009). Plasticity during stroke recovery: from synapse to behaviour. Nat Rev Neurosci *10*, 861-872.

Murry, C.E., Jennings, R.B., and Reimer, K.A. (1986). Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation *74*, 1124-1136.

Nambu, J.R., Chen, W., Hu, S., and Crews, S.T. (1996). The Drosophila melanogaster similar bHLH-PAS gene encodes a protein related to human hypoxia-inducible factor 1 alpha and Drosophila single-minded. Gene *172*, 249-254.

Namura, S., Zhu, J., Fink, K., Endres, M., Srinivasan, A., Tomaselli, K.J., Yuan, J., and Moskowitz, M.A. (1998). Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. J Neurosci *18*, 3659-3668.

Niquet, J., Baldwin, R.A., Allen, S.G., Fujikawa, D.G., and Wasterlain, C.G. (2003). Hypoxic neuronal necrosis: protein synthesis-independent activation of a cell death program. Proc Natl Acad Sci U S A *100*, 2825-2830.

Nunn, P.B., Davis, A.J., and O'Brien, P. (1991). Carbamate formation and the neurotoxicity of L-alpha amino acids. Science *251*, 1619-1620.

O'Neill, L.A., and Kaltschmidt, C. (1997). NF-kappa B: a crucial transcription factor for glial and neuronal cell function. Trends Neurosci *20*, 252-258.

Ogle, M.E., Gu, X., Espinera, A.R., and Wei, L. (2012). Inhibition of prolyl hydroxylases by dimethyloxaloylglycine after stroke reduces ischemic brain injury and requires hypoxia inducible factor-1alpha. Neurobiol Dis *45*, 733-742.

Olney, J.W. (1986). Inciting excitotoxic cytocide among central neurons. Adv Exp Med Biol *203*, 631-645.

Olney, J.W., Price, M.T., Samson, L., and Labruyere, J. (1986). The role of specific ions in glutamate neurotoxicity. Neurosci Lett *65*, 65-71.

Owen, J.B., Opii, W.O., Ramassamy, C., Pierce, W.M., and Butterfield, D.A. (2008). Proteomic analysis of brain protein expression levels in NF-kappabeta p50 -/- homozygous knockout mice. Brain Res *1240*, 22-30.

Palmer, L.A., Semenza, G.L., Stoler, M.H., and Johns, R.A. (1998). Hypoxia induces type II NOS gene expression in pulmonary artery endothelial cells via HIF-1. Am J Physiol *274*, L212-219.

Papandreou, I., Cairns, R.A., Fontana, L., Lim, A.L., and Denko, N.C. (2006). HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metab *3*, 187-197.

Pappalardi, M.B., McNulty, D.E., Martin, J.D., Fisher, K.E., Jiang, Y., Burns, M.C., Zhao, H., Ho, T., Sweitzer, S., Schwartz, B., *et al.* (2011). Biochemical characterization of human HIF hydroxylases using HIF protein substrates that contain all three hydroxylation sites. Biochem J *436*, 363-369.

Patel, T.H., Kimura, H., Weiss, C.R., Semenza, G.L., and Hofmann, L.V. (2005). Constitutively active HIF-1alpha improves perfusion and arterial remodeling in an endovascular model of limb ischemia. Cardiovasc Res *68*, 144-154.

Perkins, N.D., Schmid, R.M., Duckett, C.S., Leung, K., Rice, N.R., and Nabel, G.J. (1992). Distinct combinations of NF-kappa B subunits determine the specificity of transcriptional activation. Proc Natl Acad Sci U S A *89*, 1529-1533.

Pignataro, G., Meller, R., Inoue, K., Ordonez, A.N., Ashley, M.D., Xiong, Z., Gala, R., and Simon, R.P. (2008). In vivo and in vitro characterization of a novel neuroprotective strategy for stroke: ischemic postconditioning. J Cereb Blood Flow Metab *28*, 232-241.

Prass, K., Ruscher, K., Karsch, M., Isaev, N., Megow, D., Priller, J., Scharff, A., Dirnagl, U., and Meisel, A. (2002). Desferrioxamine induces delayed tolerance against cerebral ischemia in vivo and in vitro. J Cereb Blood Flow Metab *22*, 520-525.

Pulsinelli, W.A., and Brierley, J.B. (1979). A new model of bilateral hemispheric ischemia in the unanesthetized rat. Stroke; a journal of cerebral circulation *10*, 267-272.

Rajendrasozhan, S., Chung, S., Sundar, I.K., Yao, H., and Rahman, I. (2010). Targeted disruption of NF-{kappa}B1 (p50) augments cigarette smoke-induced lung

inflammation and emphysema in mice: a critical role of p50 in chromatin remodeling. Am J Physiol Lung Cell Mol Physiol *298*, L197-209.

Rehni, A.K., Bhateja, P., and Singh, N. (2009). Diethyl dithiocarbamic acid, a possible nuclear factor kappa B inhibitor, attenuates ischemic postconditioning-induced attenuation of cerebral ischemia-reperfusion injury in mice. Can J Physiol Pharmacol *87*, 63-68.

Rius, J., Guma, M., Schachtrup, C., Akassoglou, K., Zinkernagel, A.S., Nizet, V., Johnson, R.S., Haddad, G.G., and Karin, M. (2008). NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1alpha. Nature *453*, 807-811.

Roger, V.L., Go, A.S., Lloyd-Jones, D.M., Benjamin, E.J., Berry, J.D., Borden, W.B., Bravata, D.M., Dai, S., Ford, E.S., Fox, C.S., *et al.* (2012). Heart disease and stroke statistics--2012 update: a report from the american heart association. Circulation *125*, e2-e220.

Rothman, S. (1984). Synaptic release of excitatory amino acid neurotransmitter mediates anoxic neuronal death. The Journal of neuroscience : the official journal of the Society for Neuroscience *4*, 1884-1891.

Rytkonen, K.T., and Storz, J.F. (2011). Evolutionary origins of oxygen sensing in animals. EMBO Rep *12*, 3-4.

Sakahira, H., Enari, M., and Nagata, S. (1998). Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature *391*, 96-99.

Saleh, A., Smith, D.R., Balakrishnan, S., Dunn, L., Martens, C., Tweed, C.W., and Fernyhough, P. (2011). Tumor necrosis factor-alpha elevates neurite outgrowth through an NF-kappaB-dependent pathway in cultured adult sensory neurons: Diminished expression in diabetes may contribute to sensory neuropathy. Brain Res *1423*, 87-95.

Samdani, A.F., Dawson, T.M., and Dawson, V.L. (1997). Nitric oxide synthase in models of focal ischemia. Stroke *28*, 1283-1288.

Schofield, C.J., and Zhang, Z. (1999). Structural and mechanistic studies on 2-oxoglutarate-dependent oxygenases and related enzymes. Current Opinion in Structural Biology *9*, 722-731.

Schwaninger, M., Inta, I., and Herrmann, O. (2006). NF-kB signalling in cerebral ischaemia. Biochem Soc Trans.

Selak, M.A., Armour, S.M., MacKenzie, E.D., Boulahbel, H., Watson, D.G., Mansfield, K.D., Pan, Y., Simon, M.C., Thompson, C.B., and Gottlieb, E. (2005).

Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase. Cancer Cell *7*, 77-85.

Semenza, G. (2002). Signal transduction to hypoxia-inducible factor 1. Biochem Pharmacol *64*, 993-998.

Semenza, G.L. (1994). Transcriptional regulation of gene expression: mechanisms and pathophysiology. Hum Mutat *3*, 180-199.

Semenza, G.L. (2001). HIF-1, O(2), and the 3 PHDs: how animal cells signal hypoxia to the nucleus. Cell *107*, 1-3.

Semenza, G.L. (2002). Involvement of hypoxia-inducible factor 1 in human cancer. Intern Med *41*, 79-83.

Semenza, G.L. (2007). HIF-1 mediates the Warburg effect in clear cell renal carcinoma. J Bioenerg Biomembr *39*, 231-234.

Semenza, G.L., Roth, P.H., Fang, H.M., and Wang, G.L. (1994). Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem *269*, 23757-23763.

Sha, W.C., Liou, H.C., Tuomanen, E.I., and Baltimore, D. (1995). Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. Cell *80*, 321-330.

Sheppard, K.A., Rose, D.W., Haque, Z.K., Kurokawa, R., McInerney, E., Westin, S., Thanos, D., Rosenfeld, M.G., Glass, C.K., and Collins, T. (1999). Transcriptional activation by NF-kappaB requires multiple coactivators. Mol Cell Biol *19*, 6367-6378.

Siddiq, Aminova, Suh, Messer, Cappola, Geschwind, and Ratan (2008). Neuroprotection from oxidative stress-induced death by prolyl hydroxylase isoform inhibition occurs via mechanisms independent of HIF-1 and CREB. Paper presented at: Society for Neuroscience 2008 (Washington, DC, Neuroscience Meeting Planner).

Siddiq, A., Aminova, L.R., Troy, C.M., Suh, K., Messer, Z., Semenza, G.L., and Ratan, R.R. (2009). Selective inhibition of hypoxia-inducible factor (HIF) prolyl-hydroxylase 1 mediates neuroprotection against normoxic oxidative death via HIF- and CREB-independent pathways. J Neurosci *29*, 8828-8838.

Siddiq, A., Ayoub, I.A., Chavez, J.C., Aminova, L., Shah, S., LaManna, J.C., Patton, S.M., Connor, J.R., Cherny, R.A., Volitakis, I., *et al.* (2005). Hypoxia-inducible factor prolyl 4-hydroxylase inhibition. A target for neuroprotection in the central nervous system. J Biol Chem *280*, 41732-41743.

Sigler, A., Mohajerani, M.H., and Murphy, T.H. (2009). Imaging rapid redistribution of sensory-evoked depolarization through existing cortical pathways after targeted stroke in mice. Proc Natl Acad Sci U S A *106*, 11759-11764.

Sridharan, V., Guichard, J., Li, C.Y., Muise-Helmericks, R., Beeson, C.C., and Wright, G.L. (2008). O(2)-sensing signal cascade: clamping of O(2) respiration, reduced ATP utilization, and inducible fumarate respiration. Am J Physiol Cell Physiol *295*, C29-37.

Sun, G.Y., Zhang, J.P., Lin, T.A., Lin, T.N., He, Y.Y., and Hsu, C.Y. (1995). Inositol Trisphosphate, Polyphosphoinositide Turnover, and High-Energy Metabolites in Focal Cerebral Ischemia and Reperfusion. Stroke *26*, 1893-1900.

Sun, S.C. (2011). Non-canonical NF-kappaB signaling pathway. Cell Res *21*, 71-85.

Sun, Y., Jin, K., Xie, L., Childs, J., Mao, X.O., Logvinova, A., and Greenberg, D.A. (2003). VEGF-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia. J Clin Invest *111*, 1843-1851.

Sutter, C.H., Laughner, E., and Semenza, G.L. (2000). Hypoxia-inducible factor 1alpha protein expression is controlled by oxygen-regulated ubiquitination that is disrupted by deletions and missense mutations. Proc Natl Acad Sci U S A *97*, 4748-4753.

Talukder, M.A., Yang, F., Shimokawa, H., and Zweier, J.L. (2010). eNOS is required for acute in vivo ischemic preconditioning of the heart: effects of ischemic duration and sex. Am J Physiol Heart Circ Physiol *299*, H437-445.

Thornberry, N.A., Rano, T.A., Peterson, E.P., Rasper, D.M., Timkey, T., Garcia-Calvo, M., Houtzager, V.M., Nordstrom, P.A., Roy, S., Vaillancourt, J.P.*, et al.* (1997). A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. The Journal of biological chemistry *272*, 17907-17911.

Tong, X., Yin, L., Washington, R., Rosenberg, D.W., and Giardina, C. (2004). The p50-p50 NF-kappaB complex as a stimulus-specific repressor of gene activation. Mol Cell Biochem *265*, 171-183.

Treins, C., Giorgetti-Peraldi, S., Murdaca, J., Semenza, G.L., and Van Obberghen, E. (2002). Insulin stimulates hypoxia-inducible factor 1 through a phosphatidylinositol 3-kinase/target of rapamycin-dependent signaling pathway. J Biol Chem *277*, 27975-27981.

Umemura, A., Mabe, H., and Nagai, H. (1992). A phospholipase C inhibitor ameliorates postischemic neuronal damage in rats. Stroke *23*, 1163-1166.

Ura, S., Masuyama, N., Graves, J.D., and Gotoh, Y. (2001). Caspase cleavage of MST1 promotes nuclear translocation and chromatin condensation. Proceedings of the National Academy of Sciences of the United States of America *98*, 10148-10153.

van Uden, P., Kenneth, N.S., and Rocha, S. (2008). Regulation of hypoxia-inducible factor-1alpha by NF-kappaB. Biochem J *412*, 477-484.

Wang, G.L., and Semenza, G.L. (1993a). Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia. J Biol Chem *268*, 21513-21518.

Wang, G.L., and Semenza, G.L. (1993b). Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. Blood *82*, 3610-3615.

Wang, G.L., and Semenza, G.L. (1993c). General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. Proc Natl Acad Sci U S A *90*, 4304-4308.

Wang, G.L., and Semenza, G.L. (1995). Purification and characterization of hypoxia-inducible factor 1. J Biol Chem *270*, 1230-1237.

Ward, J.P. (2008). Oxygen sensors in context. Biochim Biophys Acta *1777*, 1-14.

Wei, E.P., and Kontos, H.A. (1999). Blockade of ATP-sensitive potassium channels in cerebral arterioles inhibits vasoconstriction from hypocapnic alkalosis in cats. Stroke *30*, 851-853; discussion 854.

Wei, L., Erinjeri, J.P., Rovainen, C.M., and Woolsey, T.A. (2001). Collateral Growth and Angiogenesis Around Cortical Stroke. Stroke *32*, 2179-2184.

Wei, L., Han, B.H., Li, Y., Keogh, C.L., Holtzman, D.M., and Yu, S.P. (2006a). Cell death mechanism and protective effect of erythropoietin after focal ischemia in the whisker-barrel cortex of neonatal rats. The Journal of pharmacology and experimental therapeutics *317*, 109-116.

Wei, L., Han, B.H., Li, Y., Keogh, C.L., Holtzman, D.M., and Yu, S.P. (2006b). Cell death mechanism and protective effect of erythropoietin after focal ischemia in the whisker-barrel cortex of neonatal rats. J Pharmacol Exp Ther *317*, 109-116.

Wei, L., Rovainen, C.M., and Woolsey, T.A. (1995). Ministrokes in rat barrel cortex. Stroke *26*, 1459-1462.

Yeager, M., Chatterjee, N., Ciampa, J., Jacobs, K.B., Gonzalez-Bosquet, J., Hayes, R.B., Kraft, P., Wacholder, S., Orr, N., Berndt, S., *et al.* (2009). Identification of a new prostate cancer susceptibility locus on chromosome 8q24. Nat Genet *41*, 1055-1057.

Yoshida, T., Zhang, H., Iwase, T., Shen, J., Semenza, G.L., and Campochiaro, P.A. (2010). Digoxin inhibits retinal ischemia-induced HIF-1alpha expression and ocular neovascularization. FASEB J *24*, 1759-1767.

Yu, F., White, S.B., Zhao, Q., and Lee, F.S. (2001a). Dynamic, site-specific interaction of hypoxia-inducible factor-1alpha with the von Hippel-Lindau tumor suppressor protein. Cancer Res *61*, 4136-4142.

Yu, F., White, S.B., Zhao, Q., and Lee, F.S. (2001b). HIF-1alpha binding to VHL is regulated by stimulus-sensitive proline hydroxylation. Proc Natl Acad Sci U S A *98*, 9630-9635.

Zaman, K., Ryu, H., Hall, D., O'Donovan, K., Lin, K.-I., Miller, M.P., Marquis, J.C., Baraban, J.M., Semenza, G.L., and Ratan, R.R. (1999a). Protection from Oxidative Stress-Induced Apoptosis in Cortical Neuronal Cultures by Iron Chelators Is Associated with Enhanced DNA Binding of Hypoxia-Inducible Factor-1 and ATF-1/CREB and Increased Expression of Glycolytic Enzymes, p21waf1/cip1, and Erythropoietin. J Neurosci *19*, 9821-9830.

Zaman, K., Ryu, H., Hall, D., O'Donovan, K., Lin, K.I., Miller, M.P., Marquis, J.C., Baraban, J.M., Semenza, G.L., and Ratan, R.R. (1999b). Protection from oxidative stress-induced apoptosis in cortical neuronal cultures by iron chelators is associated with enhanced DNA binding of hypoxia-inducible factor-1 and ATF-1/CREB and increased expression of glycolytic enzymes, p21(waf1/cip1), and erythropoietin. J Neurosci *19*, 9821-9830.

Zhang, H., Qian, D.Z., Tan, Y.S., Lee, K., Gao, P., Ren, Y.R., Rey, S., Hammers, H., Chang, D., Pili, R., *et al.* (2008). Digoxin and other cardiac glycosides inhibit HIF-1alpha synthesis and block tumor growth. Proc Natl Acad Sci U S A *105*, 19579-19586.

Zhang, S.X., Gozal, D., Sachleben, L.R., Jr., Rane, M., Klein, J.B., and Gozal, E. (2003). Hypoxia induces an autocrine-paracrine survival pathway via platelet-derived growth factor (PDGF)-B/PDGF-beta receptor/phosphatidylinositol 3-kinase/Akt signaling in RN46A neuronal cells. FASEB J *17*, 1709-1711.

Zhao, H., Sapolsky, R.M., and Steinberg, G.K. (2006). Interrupting reperfusion as a stroke therapy: ischemic postconditioning reduces infarct size after focal ischemia in rats. J Cereb Blood Flow Metab *26*, 1114-1121.

Zhong, H., Agani, F., Baccala, A.A., Laughner, E., Rioseco-Camacho, N., Isaacs, W.B., Simons, J.W., and Semenza, G.L. (1998). Increased expression of hypoxia inducible factor-1alpha in rat and human prostate cancer. Cancer Res *58*, 5280-5284.

Zhong, H., May, M.J., Jimi, E., and Ghosh, S. (2002). The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1. Mol Cell *9*, 625-636.