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

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Abstract

Pathological oxygen deprivation inhibits prolyl hydroxylase (PHD) activity and stimulates a protective cellular oxygen-sensing response in part through the stabilization and activation of the Hypoxia Inducible Factor (HIF) 1 α transcription factor. The present investigation tested the therapeutic potential of enhanced activation of oxygen-sensing pathways by competitive pharmacologic PHD inhibition after stroke, hypothesizing that post-ischemic PHD inhibition would reduce neuronal cell death and require the activation of HIF-1 α . The PHD inhibitor dimethyloxaloylglycine (DMOG, 100 μ M) reduced cell death by oxygen glucose deprivation (OGD), an *in vitro* model of ischemia, and the protection required HIF-1 α . *In vivo*, DMOG (50mg/kg, i.p.) administered 30 or 60 min after distal occlusion of the middle cerebral artery (MCA) in mice enhanced the activation of HIF-1 α protein, enhanced transcription of the HIF-regulated genes vascular endothelial growth factor, erythropoietin, endothelial nitric oxide synthase, and pyruvate dehydrogenase kinase-1, reduced ischemic infarct volume and activation of the pro-apoptotic caspase-3 protein, reduced behavioral deficits after stroke, and reduced the loss of local blood flow in the MCA territory after stroke. Inhibition of HIF-1 α *in vivo* by Digoxin or Acriflavine abrogated the infarct sparing properties of DMOG. These data suggest that supplemental activation of oxygen-sensing pathways after stroke may provide a clinically applicable intervention for the promotion of neurovascular cell survival after ischemia.

Keywords

Focal cerebral ischemia; hypoxia inducible factor; prolyl hydroxylase; preconditioning; postconditioning; dimethyloxaloylglycine

Introduction

Ischemic stroke is the third leading cause of human death and disability in the United States (American Heart Association, 2008 Update). Development of an effective therapy for acute

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ischemic attack is an urgent issue in basic and preclinical research. Ischemic stroke is characterized by a vascular occlusion in the brain that reduces tissue perfusion and starves neurons of nutrients and oxygen supply causing rapid depolarization of neurons, and initiating a cascade of events that lead to both acute necrotic, delayed apoptotic and excitotoxic cell death (Lipton, 1999).

The evolutionarily conserved cellular pathway that detects and reacts to varying tissue oxygen availability is a potent endogenous protective mechanism against hypoxic/ischemic injury. Key elements in this homeostatic pathway are the prolyl hydroxylase (PHD) oxygen-sensing enzymes and the hypoxia inducible factor (HIF) transcription factor (Jiang et al., 1996; Wang and Semenza, 1993c, 1995). Under normal oxygen availability, PHDs constitutively hydroxylate HIF-1 α on two conserved proline residues (Epstein et al., 2001), mediating the interaction of HIF-1 α with the E-3 ubiquitin ligase von Hippel Lindau (Ivan et al., 2001; Jaakkola et al., 2001). Therefore, under normal oxygen tension, hydroxylated HIF-1 α is poly-ubiquitinated and targeted for proteasomal degradation (Sutter et al., 2000). When HIF-1 α is unhydroxylated, the stable protein accumulates, and translocates to the nucleus. HIF-1 α and heterodimeric partner HIF-1 β , activate gene transcription at hypoxia-responsive elements including genes involved in promotion of cell survival (Zaman et al., 1999), angiogenesis (Forsythe et al., 1996; Palmer et al., 1998), and anaerobic metabolism (Semenza, 1994). HIF-1-regulated genes include the vascular endothelial growth factor (VEGF) (Forsythe et al., 1996), endothelial nitric oxide synthase (NOS) (Coulet et al., 2003), erythropoietin (EPO) (Wang and Semenza, 1993b), and pyruvate dehydrogenase kinases 1 and 4 (Aragones et al., 2008; Kim et al., 2006; Papandreou et al., 2006).

PHDs require oxygen, iron, 2-oxoglutarate, and ascorbate for the hydroxylation reaction and therefore may be inhibited by depletion or competition of these factors leading to HIF-1 α stabilization (Bruick and McKnight, 2001; Siddiq et al., 2005; Wang and Semenza, 1993b). Previous studies have indicated that preconditioning of neuronal cultures with PHD inhibitors induces HIF-1 α and a corresponding protective response that renders cells resistant to subsequent oxidative cell stress (Siddiq et al., 2005) or nerve growth factor withdrawal (Lomb 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 et al., 1994; Kitagawa et al., 1990; Liu et al., 1992; Siddiq et al., 2005). Manipulation of this robust endogenous protective pathway has been widely discussed in the literature as a potential therapeutic intervention for neuroprotection in stroke. Unfortunately, in a stroke therapeutic context, pre-treatment is not a clinically relevant paradigm since ischemic events are hardly predictable. In the present study, we sought to investigate the therapeutic potential of enhancing the oxygen-sensing pathways by delayed pharmacologic inhibition of PHD enzymes after ischemic stroke and to determine whether the mechanisms of PHD inhibitor mediated protection require HIF-1 α .

Materials and methods

Primary neurons

Cell cultures were isolated from Swiss Webster fetal mice (E14–16) by dissection of the cerebral cortex as previously described (Choi et al., 1987). Cells were maintained in Neurobasal media with B-27 serum-free culture supplement and L-glutamine (Invitrogen) until time of experiments. Apoptotic cell death model by B-27 supplement withdrawal was performed on cells after 7 days *in vitro*; cytosine arabinoside (ARA-C, 5 μ M) was added on day three of culture to halt proliferation of glial cells for a nearly pure neuronal population. B-27 contains a mix of necessary anti-oxidant and trophic support components for *in vitro* neuronal cell survival. To induce apoptosis, cells were depleted of B-27 supplement and

maintained in basal media for 24h (Farinelli et al., 1998; Zhang et al., 2003). For OGD ischemic cell death model, 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 CaCl₂, pH to 7.4 with NaOH) and then cells were incubated in a calibrated hypoxia chamber perfused with 5% CO₂ 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% CO₂ 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.

Lentiviral shRNA knockdown

The lentiviral plasmid pLKO.1 (Addgene) was used as the backbone to construct short hairpin RNA (shRNA)-expressing vectors. Forward and reverse oligonucleotide shRNA sequences with flanking EcoRI and AgeI restriction sites (IDT DNA) were annealed and cloned into the pLKO.1 vector. (Oligonucleotide Primers: Mouse Hif-1 α shRNA forward: 5'-CCGGGCTGGAGACACAATCATATATCTCGAGATATATGATTGTGTCTCCAGTT TTTG-3'; Mouse Hif-1 α shRNA Reverse: 5'-AATTCAAAAAGCTGGAGACACAATCATATATCTCGAGATATATGATTGTGTCTCCAGC-3'). The vector was virally packaged by HEK 293 co-transfection with the packaging vector (pMD2.G) and the envelope vector (pAX2) to generate lentivirus-containing medium 48 and 72h later. Cortical neurons were treated with lentivirus containing media on *in vitro* culture day 10, 48h prior to OGD to allow for expression of the virus. Knockdown was verified by western blotting for HIF-1 α .

Western blot analysis

Proteins were isolated, electrophoresed, and immunoblotted as previously described (Liu et al., 2009). Primary antibodies: anti-HIF-1 α (NB100-479, Novus Biologicals), anti-cleaved caspase-3 (AB3623, Chemicon/Millipore), and anti- β -Actin (Sigma).

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)–

Total RNA was isolated from cortical neurons at 24h after DMOG treatment with Trizol (Invitrogen). Reverse transcription was performed with 1 μ g total RNA with High capacity cDNA kit (Applied Biosystems). SYBR green qRT-PCR was used to assess relative levels of the HIF-1 target gene VEGF after DMOG treatment using Applied Biosystems StepOnePlus machine. Primer sequences are found in Table 1. Fold change was calculated by the Δ (Δ Ct) method, using 18S ribosomal RNA amplification as internal control.

Focal cerebral ischemia

All *in vivo* experimental procedures were approved by the Institutional Animal Care and Use Committee. Middle cerebral artery occlusion (MCAO) was conducted as previously described (Li et al., 2007b, Whitaker, 2007 #557) with some modifications. Briefly, adult male B6129PF2/J mice (Jackson Labs) weighing 20–25g were anesthetized with 4% chloral hydrate. The right middle cerebral artery (MCA) supplying the barrel cortex was permanently ligated by 10-0 suture and the common carotid arteries (CCA) were occluded for 7 min and then reperused.

PHD inhibitor administration

Dimethylloxaloylglycine (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 1×10^{-5} mL/kg). Animals were i.p. injected with DMOG or Saline/DMSO vehicle 30 or 60 min after reperfusion of the CCAs.

HIF inhibitor administration

Digoxin (DIG) and Acriflavine Hydrochloride (ACF) (Sigma) were dissolved in sterile DMSO or phosphate buffered saline (PBS), respectively, and then diluted in PBS. In order to inhibit HIF-1 α , DIG or ACF were administered at 2mg/kg i.p. 24h prior to surgical stroke and daily thereafter until sacrifice (Lee et al., 2009; Yoshida et al., 2010; Zhang et al., 2008).

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°C for 5 min. Brain sections were scanned and the unstained vs. stained TTC area was determined using NIH ImageJ on the ventral side of six brain slices per animal. The infarct volume (mm³) 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.

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 (Li et al., 2007a). 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. 5D. A 3×3mm 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, Stockholm 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 (Fabricius and Lauritzen, 1996). 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.

Immunohistochemistry

Brains were frozen in optimal cutting temperature media (Sakura Finetek) at −80°C. Frozen sections were cut (10 μ m) on a cryostat microtome. For active caspase-3 staining, sections were fixed 2 min in 10% buffered formalin, washed in PBS three times, then incubated 10 min in −20°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°C. Slides were washed and incubated with donkey anti-rabbit 488 conjugated secondary antibody (Invitrogen) and donkey anti-mouse Cy3 (Jackson Immunological).

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 200 μ m apart) as previously described (Whitaker et al., 2007). 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.

Behavioral Testing

Forelimb sensorimotor deficits after stroke were analyzed by a modified adhesive removal test (Bouet et al., 2009; Freret et al., 2009). Briefly, the time to remove sticker was recorded one day prior to stroke, 1–3, 7, 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 fore-paw. Equal pressure was applied to the opposite, non-tested paw for each trial. 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.

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).

RESULTS

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

We first investigated the concentration at which PHD inhibitor DMOG induced oxygen-sensing signaling in cultured cortical neurons using the HIF-1 α protein as a marker. HIF-1 α protein is highly stabilized in low oxygen (Wang and Semenza, 1993a) or by PHD inhibition (Wang and Semenza, 1993b). As expected, protein expression of HIF-1 α increased in normoxic cortical neurons treated with 50–500 μ M of the 2-oxoglutarate analog DMOG for 24h (Fig. 1A). HIF-1 α protein blots frequently show multiple bands around the expected molecular weight of 115 kDa representing multiple post-translational modifications of the HIF-1 α protein. We observed a non-hypoxia inducible band in all samples around 150 kDa which is suspected to be a non-specific band. Expression of HIF-1 α target gene VEGF mRNA also increased after 24h exposure to DMOG (Fig. 1B) demonstrating that the 2-oxoglutarate analog induces hypoxia-sensitive gene transcription under normal oxygen conditions in cortical neuron cultures.

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

Previous work has demonstrated that preconditioning with sub-lethal hypoxia or ischemia attenuates cell death when administered prior to an otherwise lethal ischemic challenge (Gidday et al., 1994; Kitagawa et al., 1990). PHD inhibition prior to *in vitro* ischemia is untested. To determine whether PHD inhibition is sufficient to reduce neuronal ischemic cell death we employed an *in vitro* ischemia-reperfusion model of combined oxygen and glucose deprivation (OGD) followed by simulated reperfusion by return to normal oxygen and media. After 2h of OGD and 24h reperfusion, $29.72 \pm 2.88\%$ of neuronal cells die. Preconditioning with DMOG (100 μ M) for 24h prior to OGD induces protein stabilization of HIF-1 α (Fig. 1A) and significantly reduces OGD-induced cell death to $9.05 \pm 2.75\%$ (Fig. 2A). Post-treatment with the PHD inhibitor 2h *after* initiation of OGD at the start of the reperfusion phase significantly attenuated cell death to $13.69 \pm 1.77\%$ (Fig. 2A). Post-treatment protection did not statistically differ from DMOG pre-treatment. Western blotting demonstrates that HIF-1 α protein is mildly enhanced 3h after OGD in the DMOG post-treatment group compared to OGD-control (Fig. 2B). 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.

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

To determine whether HIF-1 α signaling plays a role in the PHD inhibitor protection, we knocked-down HIF-1 α expression in primary cortical neurons with lentiviral HIF-1 α -targeted shRNA. Neurons were treated with the shRNA virus for 48 h prior to OGD with or without PHD inhibitor DMOG. Success of the HIF-1 α knockdown was assessed by western blotting after 24 h treatment with 250 μ M DMOG to stabilize HIF-1 α . DMOG treated control cultures have enhanced HIF-1 α protein expression, while HIF-shRNA pretreated cultures have markedly reduced expression of HIF-1 α (Fig. 2C, inset). Cortical cultures with HIF-1 α knockdown displayed no protection against OGD with DMOG pre- or post- treatment (Fig. 2C) suggesting that HIF-1 α is necessary for the PHD inhibitor mediated protection from ischemic cell death. The HIF-1 α -shRNA treated cells displayed higher basal levels of cell death, and a trend of more cell death in OGD, however the difference in OGD did not reach statistical significance.

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, 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 et al., 1998; Zhang et al., 2003) in the presence of the PHD inhibitor DMOG (250 μ M) or equal volume of DMSO vehicle for 24h. In the absence of B-27 supplement, $35.3 \pm 2.2\%$ of the cortical neurons died in 24h as assessed by trypan blue nuclear staining (Fig. 3A). B-27 withdrawal induced a time-dependent increase in activated caspase-3, a key step in the apoptotic process (Fig. 3C). When PHD inhibitor DMOG was co-applied to the cells during B-27 withdrawal there was significantly less cell death ($21.3 \pm 2.9\%$) and markedly reduced activation of caspase-3 compared to vehicle treated controls (Fig. 3A, C). HIF-1 α was stabilized in the DMOG/B-27 withdrawal group, but not in control or withdrawal conditions (Fig. 3B). These data suggest that stimulation of oxygen-sensing signaling pathways during stress reduces apoptotic neuronal cell death.

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

HIF-1 α protein expression is stabilized in normal oxygen tension by DMOG (Fig. 1A, 3B). In order to test whether DMOG reaches the brain cortex after i.p. injection, HIF-1 α protein

in the cortex was assessed as a reporter of PHD inhibition. According to previous reports, injection of 100mg/kg i.p. DMOG enhances the expression of HIF-1 α in the liver and mediates a whole-body hypoxia tolerance (Kasiganesan et al., 2007). We initially tested two doses of DMOG (50 and 500mg/kg) in a time course experiment to determine whether HIF-1 α 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-1 α in the mouse brain cortex (Fig. 4A, B). Similar results were obtained with 500mg/kg (data not shown) however we chose to focus on the lower dose for the post-conditioning trials in order to minimize any possible off-target effects of the higher dose. HIF-1 α 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-1 α protein declined to nearly basal levels between 72 and 96h after a single injection (Fig. 4).

PHD inhibitor post-ischemic treatment reduced ischemic infarct formation

To determine whether delayed PHD inhibition after stroke could impact the gross ischemic infarct volume, we treated animals with DMOG (50 mg/kg, i.p.) 30 or 60 min after ischemic stroke. Small focal ischemic stroke was surgically implemented by occlusion of right MCA and combined 7 min ligation of the common carotid arteries (CCA) in adult mice as previously described (Whitaker et al., 2007). 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. 5E). Ischemic infarct volume was assessed 72h after stroke surgery since the bulk of cell death in the ischemic region occurs within the first 72h (Lipton, 1999). The ischemia was primarily cortical and located within the vascular bed supplied by the right MCA with no detectable damage to the contra-lateral hemisphere. Control stroke animals had an infarct volume of $12.44 \pm 1.25 \text{ mm}^3$ (n=12). A single DMOG administration 30 or 60 min after ischemia significantly attenuated infarct volume to 7.04 ± 1.07 or $7.15 \pm 1.22 \text{ mm}^3$ respectively (n = 10 each) indicating that delayed DMOG administration after stroke reduces infarct volume (Fig. 5A–C).

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

To analyze the change in perfusion of the MCA territory before, during and after surgical ischemia, we used Laser Doppler perfusion imaging. The mean perfusion was analyzed in six measurements of a $3 \times 3 \text{ mm}$ area overlying the medial border of the occluded branch of the right MCA (Fig. 5D–F) before, during, and 12, 24, 48 or 72h after stroke in each animal. 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. 5F). Upon release of the bi-lateral CCAs, the area surrounding the occluded MCA was gradually reperfused to surviving tissue. 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 5F). 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. 5E, F).

PHD inhibitor treatment reduced activation of caspase-3 in the ischemic cortex

We hypothesized that DMOG treatment may reduce 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. Activated caspase-3 co-stained with the neuronal marker NeuN within and adjacent to the ischemic core. Positive cells had apoptotic morphology with shrunken/condensed nuclei and reduced cell volume.

DMOG post-treatment significantly reduced the number of caspase-3-positive cortical neurons 24h after stroke (Fig. 6).

PHD inhibitor post-ischemic treatment reduces behavioral deficits after stroke

Our distal MCA focal stroke model targets the barrel somatosensory field that controls whisker and fore-limb function (Murphy and Corbett, 2009; Sigler et al., 2009; Wei et al., 1995). The adhesive removal task has been previously described as a sensitive measure of sensorimotor function in mouse focal ischemic stroke (Bouet et al., 2009; Bouet et al., 2007; Freret 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. The control stroke animals displayed significantly impaired function of the forelimb contra-lateral to the ischemic injury as measured by the time required to remove the adhesive sticker from the forepaw (Fig. 7). Performance was compared to baseline assessment prior to stroke, and between treatment groups by 2-way ANOVA. DMOG treated animals were not significantly impaired 7 or 14 days after stroke (Fig. 7). The DMOG animals performed the adhesive removal task significantly faster than vehicle-treated animals at both 7 and maintained the same trend at 14 days (Fig. 7) and were not statistically different from their baseline performance before stroke. These data correlate the smaller stroke volume in the DMOG group 72h after stroke with less severe functional deficits. Right forepaw function was not affected.

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

HIF-1 α protein expression was increased in the ischemic cortex 3h after stroke compared to sham-operated animals. Correlated with the reduced infarct volume and improved sensorimotor function, HIF-1 α protein was significantly enhanced 3h after stroke by DMOG post-treatment compared to stroke with vehicle post-treatment (Fig. 8A). While HIF-1 α is only slightly elevated 3h after DMOG injection alone (Fig. 4) or 3h after stroke, the combination of stroke + DMOG injection contributes to a more robust stabilization of HIF-1 α protein.

Peri-infarct mRNA expression of HIF-1 α -regulated genes, HIF-1 α , VEGF, EPO, eNOS, and PDK1, is enhanced 12h after stroke in animals that received DMOG injection versus vehicle control (Fig. 8B).

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

To determine whether HIF-1 α is responsible for the beneficial outcome after DMOG post-ischemic treatment, we tested the effect of DMOG post-treatment in combination with two different HIF-1 α inhibitors DIG and ACF. To evaluate the effectiveness of HIF inhibition by DIG and ACF in the brain, adult male mice were injected with DIG, ACF (2mg/kg i.p.), or vehicle. After 6h, DMOG (50mg/kg) or vehicle was injected to induce HIF-1 α stabilization in the brain. After 24h, a time sufficient to induce HIF-1 α and HIF-1 downstream gene transcription, cortical brain tissue was harvested for protein and mRNA analysis. DMOG stabilized HIF-1 α protein expression compared to vehicle control. As expected, DIG pre-treatment tissue did not undergo an increase in HIF-1 α with DMOG treatment, since DIG inhibits HIF-1 α transcription. ACF pre-treated animals did have a small increase in HIF-1 α upon DMOG treatment compared to ACF alone; however it was not as large an increase as control animals and was not statistically significant (Fig. 9A). Since ACF inhibits HIF-1 activity, but not protein expression (Lee et al., 2009), we next analyzed the HIF-1 α target gene VEGF expression after DMOG treatment with or without the HIF inhibitors. We found that 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-1 α inhibitors DIG or ACF (Fig. 9B). These results suggest that DIG and ACF block the DMOG-induced activity HIF-1 α .

Having established that DIG and ACF inhibit HIF-1 α in our system, we next tested the effect of HIF-1 α inhibitors on the post-ischemic DMOG-mediated protection. 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 α , one group of animals was sacrificed 12h after stroke and peri-infarct cortical tissue was harvested. HIF-1 α 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-1 α protein than DMOG treated controls (Fig 9C). mRNA expression of VEGF and EPO were increased 12h after stroke by DMOG; HIF-1 α inhibitors DIG and ACF abrogated this increase (Fig 9D).

Infarct volume analysis by TTC demonstrated that both HIF-1 α inhibitors, DIG and ACF, block the infarct-sparing effects of DMOG post-ischemic treatment (Fig. 9E, F). The infarct of the animals treated with HIF-1 α inhibitors trended towards larger than control, but did not reach statistical significance. These data suggest that HIF-1 α is an integral part of the mechanism of PHD inhibitor post-ischemic neuroprotection.

DISCUSSION

Modulation of PHDs, a major preconditioning target, provides a method of triggering endogenous protective mechanisms without applying hypoxic or invasive ischemic treatments (Siddiq et al., 2005). The present investigation explored whether globally enhancing oxygen-sensor signaling after ischemia with PHD inhibitor DMOG could provide neuroprotection and improve stroke outcome. Administration of DMOG after focal ischemia reduces neuronal damage and behavioral deficits correlating with the enhanced expression of HIF-1 α protein and HIF-1-regulated gene expression in peri-infarct cortical tissue. Multiple lines of evidence suggest that the enhanced activation of HIF-1 α in part mediates the reduction of cortical cell death.

In a focal ischemic stroke, a small core of ischemic tissue is acutely affected by restricted oxygen and nutrients; however, over hours and days the infarct expands due excitatory amino acid release, loss of ion homeostasis, decreased pH, inflammation, and edema causing additional damage and apoptosis in the surrounding areas of the brain parenchyma (Broughton et al., 2009; Paciaroni et al., 2009). The strategy for post-ischemic neuroprotective therapies is to target the peri-infarct or 'penumbra' region to prevent or rescue the spreading damage of the initial infarct. HIF-1 is pathologically stimulated in the ischemic region as part of the endogenous protective response to low oxygen; however, DMOG injection (i.p.) inhibits PHDs throughout the brain and whole body (Kasiganesan et al., 2007). Post-stroke DMOG treatment enhanced HIF-1 α expression in the peri-infarct cortex after stroke and significantly reduced cortical neuron apoptotic cell death in vitro and in vivo. DMOG post-treated animals had a significantly smaller infarct at 72h, after the majority of ischemic cell death occurs (Lipton, 1999). The protective effect of DMOG treatment was associated with an improvement of blood flow in the peri-infarct region 24 – 72h after stroke. The extent of restoration of cerebral blood flow after stroke generally correlates to the size of the ultimate ischemic infarct region (Del Zoppo et al., 2011). The measurement of more flow in DMOG-treated animals suggests a smaller infarct size and a greater region of healthy tissue. Consistent with less brain injury, DMOG-treated animals displayed fewer deficits in sensorimotor function after stroke.

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-1 α protein and transcription of VEGF, EPO, eNOS, and PDK-1 12h after ischemia. VEGF and EPO can both function as neuroprotective molecules after ischemia (Digicaylioglu and Lipton, 2001; Jin et al., 2000a, b; Li et al., 2007a). VEGF reduces cell death and caspase-3 activation in neuronal ischemic model systems (Jin et al., 2000a, b) and is required for hypoxic preconditioning-mediated neuroprotection against ischemia (Wick et al., 2002). Caspase-3 activation was reduced both in vitro and in vivo with DMOG in our models. eNOS plays a protective function during the early response to ischemia; loss of eNOS leads to larger stroke size (Huang et al., 1996). Enhanced expression of eNOS may also correlate with the enhanced blood flow, since previous studies have shown that eNOS knockout mice have reduced cortical reperfusion following stroke (Huang et al., 1996). The protective effect of ischemic preconditioning requires eNOS in both heart and liver models (Abu-Amara et al., 2011; Talukder et al., 2010), supporting the notion that eNOS may also play a mechanistic role in the DMOG post-conditioning model. 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 et al., 2005). Increased activation of PDKs in the PHD-1 knockout mouse, leads to an anaerobic metabolic switch (Aragones et al., 2008). Increased expression of PDK-1 in the peri-infarct region of DMOG-treated animals may support an ability to continue to generate energy even under reduced perfusion. Each of these genes enhanced by DMOG may contribute to the neuroprotective phenotype.

Oxygen-sensitive pathways activate HIF-1 transcription factor, but the role of HIF-1 in pre- and post-conditioning has remained inconclusive. HIF-1 α has both pro-survival and pro-death roles in the ischemic brain depending on the injury model, time point, or cell type assessed (Aminova et al., 2005; Baranova 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-2 α for neuroprotection (Siddiq et al., 2009), whereas our findings suggest that HIF-1 α is required for neuroprotection from OGD. In vitro knockdown of HIF-1 α in cortical neuron cultures showed that DMOG-mediated protection against ischemic challenge is abrogated in the absence of HIF-1 α 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, which is an essential component of ischemic injury; however, glutathione depletion and OGD insults are vastly different in cellular pathology. Moreover, the concentration of DMOG used against the oxidative stress model by Siddiq, *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. Although we show that HIF-1 α is necessary for PHD inhibitor-mediated protection and HIFs are the best-characterized targets for PHD inhibition, HIF may not be sufficient for the protection. Other pathways that are activated by PHD inhibition such as NF- κ B (Cummins et al., 2006) may be involved as well.

Given that knockout of HIF-1 α is embryonic lethal (Iyer et al., 1998), and tissue specific knockouts have given conflicting results on the role of HIF during ischemia (Baranova et al., 2007; Helton et al., 2005), we chose a pharmacologic approach to study the role of HIF-1 α in the DMOG-mediated post-conditioning effect in vivo. DIG and ACF were identified as HIF inhibitors in cancer therapeutic drug screens (Lee et al., 2009; Zhang 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-1 α (Zhang et al.,

2008). ACF inhibits HIF by interfering with dimerization to HIF-1 β , therefore inhibiting transcriptional activity of HIF-1 α (Lee et al., 2009). 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 did not suppress the endogenous HIF-1 response in the peri-infarct cortex, but they did suppress the DMOG-mediated HIF-1 signaling. This may explain why there is little exacerbation of infarct volume with the inhibitors *in vivo*.

Taken together, the findings reported here demonstrate that PHD inhibition after ischemia enhances the activation of the HIF-1 α , reduces ischemic damage, and improves functional recovery after MCAO. The beneficial effects of PHD inhibition after ischemia require the activity of HIF-1 α . These data suggest that supplemental activation of oxygen-sensing pathways after stroke may provide a clinically applicable therapeutic intervention for the promotion of neuronal cell survival after ischemia.

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Abbreviations

PHD	Prolyl hydroxylase
HIF	Hypoxia Inducible Factor
OGD	Oxygen glucose deprivation
DMOG	Dimethyloxaloylglycine
VEGF	Vascular Endothelial Growth Factor
EPO	Erythropoietin
DIG	Digoxin
ACF	Acriflavine Hydrochloride
MCA	Middle cerebral artery
CCA	Common carotid artery
CBF	cerebral blood flow

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Highlights

- PHD inhibition by DMOG after stroke reduces ischemic damage.
- Post-ischemic DMOG enhances peri-infarct HIF-1 α expression.
- Neuroprotection by DMOG in vivo requires HIF-1 α activity.

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

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

Fig 2. PHD inhibitor attenuates OGD-induced cell death and requires 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. (* $p < 0.05$ 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 ($n = 3$). (C) Inset demonstrates successful HIF-1α knockdown. Neuronal cultures were infected with lentiviral shRNA directed against HIF-1α for 48h. Parallel control and HIF-1α-shRNA cells were treated with 250μM DMOG for 24h to induce HIF-1α protein stabilization. Control cultures but not shRNA treated cultures show HIF-1α protein expression. DMOG did not reduce cell death from OGD in cells with HIF-1α knockdown. Data are the mean $\pm$ SEM from at least three independent experiments. (*compared to parallel control cultures, $p < 0.05$, $n = 3$).

Fig 3. 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. (B) Neuronal lysate was collected after 24h and immunoblotted for HIF-1 α and β -actin loading control. (C) Western blot for the caspase-3 active fragment 3 and 6h after initiation of B-27 withdrawal. Data are the mean $\pm$ SEM from at least three independent experiments. (* p <0.05; n =3).

Fig 4. DMOG intraperitoneal injection stabilizes HIF-1 α 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-1 α or β actin. Representative western is shown. (B) Densitometry summary of western blots. (n=3–4 animals per time-point; mean $\pm$ SEM, *compared to vehicle injected animal, $p < 0.05$).

Fig 5. PHD inhibitor post-ischemic treatment reduces ischemic infarct formation and attenuates peri-infarct loss of cerebral perfusion

Representative TTC staining of ischemic infarct 72h after stroke in (A) control and (B) 60 min DMOG (50mg/kg) post-treatment. (C) Quantification of TTC infarct volume (mm³) 72h after stroke demonstrates the protective effect of PHD inhibitor post-ischemic treatment. (n=10–12 animals per group, *p<0.05). (D) Local cerebral blood flow (CBF) was examined over the territory of the right middle cerebral artery covering the ischemic border region in our focal ischemic stroke model. (E) Pseudo-colored representation of intensity of CBF before, during, and 72h after stroke in control or DMOG post-treated animals. (F) Quantification of CBF relative to initial flow. (n=8, *p<0.05).

Fig 6. PHD inhibitor treatment reduced activation of caspase-3 in the ischemic cortex
 (A) Immuno-staining 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).

Fig 7. 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). (* $p < 0.05$, $n = 10$ –12 animals per group)

Fig 8. Post-ischemic DMOG therapy enhances HIF-1α expression

(A) Expression of HIF-1α 3h after stroke with vehicle or DMOG 30 min post-treatment. (* $p < 0.05$, $n = 4$) (B) mRNA expression of HIF-1 responsive genes in control stroke animals or DMOG-treated animals 12h after stroke. (* $p < 0.05$, $n = 3-4$)

Fig 9. Inhibition of HIF-1 α abrogates the post-ischemic DMOG-mediated neuroprotection

(A) Adult male mice were pre-treated for 6h with saline or HIF-1 α inhibitors 2mg/kg DIG, or 2mg/kg ACF. Mice in each group were then treated with DMOG (50mg/kg) or vehicle. After 24h, cortical tissues were immunoblotted for HIF-1 α . Densitometry is represented as fold change to vehicle for each condition. DMOG enhances HIF-1 α expression compared to vehicle in control conditions, but not under DIG treatment. ACF shows a trend for enhanced expression of HIF-1 α with DMOG, but it is not statistically significant. (n=3 animals per group). (B) RNA was extracted from cortical tissues treated with DIG, ACF, and DMOG. Quantitative RT-PCR for the HIF-1-dependent gene VEGF 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 30m after stroke with either DMOG or vehicle. After 12h, peri-infarct cortical tissue was immunoblotted for HIF-1 α and actin loading control. DMOG enhances HIF-1 α expression compared to sham in control conditions; DIG and ACF reduce the DMOG-mediated increase in HIF-1 α . 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 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) (E) 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 protection in the HIF-1 α inhibitor groups. (F) Quantification of infarct volume. (n=8–10 in each group, * p<0.05).

Table 1

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