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

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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) 1α 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-1α. The PHD inhibitor dimethyloxaloylglycine (DMOG, 100 μM) reduced cell death by oxygen glucose deprivation (OGD), an *in vitro* model of ischemia, and the protection required HIF-1α. *In vivo*, DMOG (50 mg/kg, i.p.) administered 30 or 60 min after distal occlusion of the middle cerebral artery (MCA) in mice enhanced the activation of HIF-1α 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-1α *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.

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 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 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-1α on two conserved proline residues (Epstein et al., 2001), mediating the interaction of HIF-1α with the E-3 ubiquitin ligase von Hippel Lindau (Ivan et al., 2001; Jaakkola et al., 2001). Therefore, under normal oxygen tension, hydroxylated HIF-1α is poly-ubiquitinated and targeted for proteasomal degradation (Sutter et al., 2000). When HIF-1α is unhydroxylated, the stable protein accumulates, and translocates to the nucleus. HIF-1α and heterodimeric partner HIF-1ß, 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 (eNOS) (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-1α 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-1α 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

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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. The present study investigated 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-1α.

Materials and methods

Primary neurons

Neuronal 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 μM) 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 24 h (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 (120 mM NaCl, 25 mM Tris–HCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, pH to 7.4 with NaOH) cells were then incubated in a calibrated hypoxia chamber perfused with 5% CO_2 and balanced nitrogen for a final ambient oxygen level of 0.2% for 2 h. Oxygen level was established, maintained and monitored by the ProOx 360 sensor (Biospherix, NY). After 2 h, 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 24 h, 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-1α shRNA forward: 5′-CCGGGCTGGAGACACAATCATATATCTCGAGATATATGATTGTGTCTCC-AGTTTTTG-3′; Mouse Hif-1α shRNA Reverse: 5′-AATTCAAAAAGCTGGA-GACACAATCATATATCTCGAGATATATGATTGTGTCTCCAGC-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. Cortical neurons were treated with lentivirus containing media on *in vitro* culture day 10, 48 h prior to OGD allowing for expression of the virus. Knockdown was verified by western blotting for HIF-1α.

Western blot analysis

Proteins were isolated, electrophoresed, and immunoblotted as previously described (Liu et al., 2009). Primary antibodies: anti-HIF-1α (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 or peri-infarct cortex tissue with Trizol (Invitrogen). Reverse transcription was preformed with 1 μg total RNA with a High capacity cDNA kit (Applied Biosystems). SYBR green qRT-PCR was used to assess relative levels of target genes using Applied Biosystems StepOnePlus machine. Primer sequences are found in Table 1. Fold change was calculated by the Δ (ΔCt) 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 et al., 2007) with some modifications. Briefly, adult male B6129PF2/J mice (Jackson Labs) weighing 20–25 g 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 reperfused.

PHD inhibitor administration

Dimethyloxaloylglycine (DMOG) (Frontier Scientific) was dissolved in DMSO at a concentration of 1 M. For animal administration, DMOG solution was diluted further in sterile saline and injected at a final concentration of 50 mg/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-1α, DIG or ACF were administered at 2 mg/kg i.p. 24 h 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 72 h following stroke. The brains were sliced into 1-mm coronal sections using a mouse brain matrix (Harvard Bioscience, South Natick, MA), and incubated in 2%

Table 1

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