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Mechanisms of Erythropoietin-induced Neuroprotection in: in-vivo and in-vitro Models of hypoxia Ischema

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LOMA LINDA UNIVERSITY
School of Medicine
in conjunction with the
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Mechanisms of Erythropoietin-induced Neuroprotection in: *in-vivo* and
in-vitro Models of Hypoxia Ischemia

by

Rhonda Andrea Souvenir

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A Dissertation submitted in partial satisfaction of
the requirements for the degree of
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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ABBREVIATIONS

EPO	Erythropoietin
EPOR	Erythropoietin Receptor
rHuEPO	Recombinant Human Erythropoietin
ROS	Reactive Oxygen Species
HIF	Hypoxia Inducible Factor
PHD	Prolyl Hydroxylase
MMP	Matrix Metalloproteinase
TIMP	Tissue Inhibitor of Matrix Metalloproteinase
JAK	Janus Kinases
STAT	Signal Transducers and Activators of Transcription
VHL	Von Hippel–Lindau tumor suppressor factor
HI	Hypoxia Ischemia
OGD	Oxygen and Glucose Deprivation
PC-12 cells	Rat pheochromocytoma from the adrenal medulla
NGF	Neuronal Growth Factor
P-7	Postnatal Day 7
SAH	Sub-arachnoid hemorrhage
ICH	Intracerebral hemorrhage

ABSTRACT OF THE DISSERTATION

Mechanisms of Erythropoietin-induced Neuroprotection in: *in-vivo* and

in-vitro Models of Hypoxia Ischemia

by

Rhonda Andrea Souvenir

Doctor of Philosophy, Graduate Program in Microbiology and Molecular Genetics
Loma Linda University, December 2011
Dr. Jiping Tang, Chairperson

Hypoxic ischemic brain injury (HIBI) is a common cause of neonatal mortality and morbidity. Approximately 60 % of preterm babies and 2% of full term infants suffer from asphyxia. Asphyxia related death accounts for approximately 23% of neonatal mortality annually. Many therapeutic interventions show promise in the laboratory but fail in clinics. A thorough understanding of mechanisms by which promising therapeutic intervention confers its neuroprotection is necessary to promote smoother transitions from bench to the bedside. Erythropoietin (EPO), a hematopoietic growth factor that increases oxygen availability during hypoxia/ischemia is associated with cell survival and neuroprotection in: in vivo and in vitro models of hypoxia ischemia. However these studies hold limited clinical translations because the underlying mechanism remains unclear and the key molecules involved in EPO-induced neuroprotection are still to be determined. **Thus the central aim of this proposal is: to determine the key mediators of EPO-induced neuroprotection and the mechanisms by which this occurs in: *in vitro* and *in vivo* models of hypoxia ischemia.**

Two alternate pathways of EPO-induced neuroprotection were elucidated in vitro using oxygen and glucose deprivation to mimic hypoxia ischemia. Our findings showed that EPO treatment resulted in an upregulation of tissues inhibitor of matrix metalloproteinase (TIMP)-1 and inhibition of hypoxia inducible factor (HIF-1α) both of

which subsequently decreased in matrix metalloproteinase (MMP)-9 and promoted

neuroprotection. MMP-9 inhibition was associated with neuroprotection in both

pathways thus, inferring that inhibition of MMP-9 is one of the primary mechanisms of

EPO-induced neuroprotection. We also observed that EPO-induced neuroprotection was

reversed by inhibition of TIMP-1 or prolyl hydroxylase (PHD)-2. This lends to the

conclusion that TIMP-1 and PHD-2 are the key mediators of EPO-induced

neuroprotection in: in vivo and in vitro models of hypoxia ischemia.

CHAPTER ONE

CRITICAL NON-HEMATOPOIETIC FUNCTIONS OF ERYTHROPOIETIN IN

CEREBRAL HYPOXIA ISCHEMIA

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Note: Most of this chapter is taken from a review paper currently in the submission

process

Abstract

Stroke continues to be one of the leading causes of mortality and morbidity nationwide with only few therapeutic options. Recombinant human erythropoietin (rhEPO) showed promising results in animal studies, and in human neonates. However rhEPO treatment proved to be ineffective and sometimes detrimental against subarachnoid or ischemic stroke in adults. The purpose of this review was to elucidate the critical non-hematopoietic function of EPO-induced neuroprotection in the different stroke models. Major biomedical bibliographical databases (MEDLINE, ISI, and Cochrane Library) were searched with use of major keywords such as erythropoietin, stroke, neonatal hypoxia ischemia and multiple terms denoting components of EPO signaling pathways. EPO activates common signaling pathways for both hematopoietic and non hematopoietic functions. However, there are important distinctions in the molecular mechanisms of EPO-induced neuroprotection depending upon the level of EPOR expression, injury type and developmental stage of affected individuals. Both high dose EPO treatment and long term therapy tends to initiate notable erythropoiesis, coagulation and thrombosis. Thus in order to harness the beneficial non-hematopoietic functions of EPO in stroke therapy, the dose and duration of EPO treatment needs to be carefully optimized and potential side effects closely monitored.

Introduction

Stroke is the altering of brain, blood supply either by the rupturing or obstruction of a vessel (WHO). The manner in which the blood supply is interrupted determines if the stroke is hemorrhagic or ischemic. Hemorrhagic strokes are less common but more detrimental than ischemic strokes. Alternately, a hemorrhagic stroke could lead to an ischemic stroke (ASA; WHO). Thus, we'll focus primarily on cerebral ischemia. There are two main categories of stroke: ischemia and hemorrhage can be further categorized according to the region of the brain affected. Subarachnoid hemorrhage (SAH), intracerebral hemorrhage (ICH), global ischemia, focal ischemia, neonatal hypoxia ischemia are all modeled in laboratory animals and was shown to be attenuated by erythropoietin treatment. Systemic administration of single or multiple doses of recombinant Erythropoietin (EPO) pre- or post- insult was shown to confer neuroprotection in subarachnoid hemorrhage (Alafaci et al., 2000; Grasso, 2001; Grasso et al., 2002a; Grasso, 2004), intracerebral hemorrhage (Lee et al., 2006d; Grasso et al., 2009b), global ischemia ((Sakanaka et al., 1998; Calapai et al., 2000; Catania et al., 2002), focal ischemia (Bernaudin et al., 1999; Brines et al., 2000; Zhang et al., 2010a), neonatal hypoxia-ischemia (Kumral et al., 2004; Kumral et al., 2003), tramautic brain injury (Lu et al., 2005; Chen et al., 2007) and even spinal cord injury (Gorio et al., 2002) , Parkinson's (Kanaan et al., 2006) and multiple sclerosis (Sattler et al., 2004). Thus, EPO was hailed as the panacea in stroke therapy and rapidly advanced to clinical trials.

Clinical trials for the use of EPO in SAH treatment yielded inconclusive results due to a small population size; but showed promising results in Phase I for the treatment of neonatal HI and adult cerebral ischemia (Meyer et al., 2003). These findings served to bolster confidence in EPO's potential for stroke therapy and accelerated its advance into Phase II clinical trial (McPherson & Juul, 2010b) Hence, it was a resounding blow to EPO researchers and the stroke community at large when Ehrenreich and colleagues

reported that EPO not only failed to improve injury but also promoted tumor formation and reduced quality of life for stroke patients in a multicenter clinical trial (Ehrenreich et al., 2009). The failure of EPO in Phase II/III clinical trials raised several questions: Was the route of administration too traumatic? Was the potential seen in animal models exaggerated? Were the proposed mechanisms of action not clear? Or is EPO, like its primary regulator HIF-1α a biphasic molecule, that is beneficial or detrimental depending on the dose and time of administration? EPO has been administered intravenously for treatment of anemia over the past ten years with no major complication. Additionally Taylor and colleagues showed that there were no difference in the prevalence of thrombotic and hypertensive side between patients administered EPO intravenously compared to subcutaneously (Taylor et al., 1994). Thus if the route of administration affected the outcome it would have played a minor role in EPO's failure in Phase II/III clinical trial. A comprehensive review of EPO treatment in animal models of stroke with Meta analysis still predicts that EPO should show positive result in stroke therapy (Jerndal et al., 2010; Minnerup et al., 2009). Since two of the four critical questions were previously answered we'll focus on the critical non hematopoietic function of EPO and the mechanisms by which EPO accomplishes these functions.

Hypoxia Ischemia

Cerebral ischemia is associated with impaired cerebral blood flow (CBF), skewed pH and electrolyte balance and increased free radical formation, intracranial pressure (ICP) and inflammation (Dearden, 1985). Reduced CBF limits energy availability and forces the cell into anaerobic respiration, which creates an acidic environment and shifting in the sodium, potassium, calcium equilibrium (Siesjo et al., 1985a; Siesjo et al., 1985b). Hypoxia is the physiological point at which the demand for oxygen supersedes the available oxygen supply thus forces the body/cells to drastically alter its O_2

expenditure. Hypoxia triggers anaerobic respiration which results in an increase in acidity. Low oxygen or acidic environment results in an accumulation of HIF-1α in the cytoplasm (Jelkmann, 2005; Semenza, 2000b; Semenza, 2000a).

Erythropoietin Regulation

Cerebral hypoxia ischemia promotes an accumulation of hypoxia inducible factor 1 (HIF)-1α in neurons. HIF-1α has over 70 targets genes most of which are up-regulated in response to hypoxia (Zagorska & Dulak, 2004).HIF-1α downstream gene EPO is upregulated during ischemia in the brain of most mammals (Prass et al., 2003a). The expression of EPO and EPO-receptor (EPOR) is regulated by HIF. HIFs are heterodimeric transcription factors consisting of alpha and beta subunits (Chandel et al., 2000). HIF-1α and-2α shares 40% homology.HIF-2α has been shown to bind to the beta subunit of HIF1α during hypoxia (Krieg et al., 2000). An accumulation of HIF-1α or HIF-2α during hypoxia is tissue dependant. The specific HIF in neuronal tissue is still highly debate. HIF-1α regulates both pro and anti-apoptotic genes. Thus it's considered to be biphasic (Halterman & Federoff, 1999). Previous studies have suggest that the an accumulation of HIF-1α is beneficial in the up-regulation of vascular endothelial growth factor (VEGF) (Mu & Chang, 2003), EPO (Prass et al., 2003b), glucose transporter-1 (GLUT-1), glycolytic enzymes (Jones & Bergeron, 2001). However the early increase in HIF-1α also lead to up-regulation of detrimental genes BNIP3(Bruick, 2003), Nix (Sowers et al., 2001), p53 and the caspases (Li et al., 2005b). Our unpublished findings show that exogenous administration of EPO inhibits HIF-1α in a dose dependent manner. The mechanism by which this occurs warrants further studies.

Erythropoietin

EPO is a hematopoietic growth factor that stimulates red blood cell (RBC) production and is released in response to low oxygen (Haroon et al., 2003; Siren et al., 2001b; Siren et al., 2001a). EPO secretion switches from the liver in the fetus to the kidney in adults and is the primary regulator of erythropoiesis in mammals (Dame et al., 2004). EPO is a part of the hypoxic response element and a known downstream gene of HIF-1α. Under physiological conditions serum EPO levels ranges from 4-27 mU/mL (Caro & Erslev, 1988). However during hypoxia ischemia or asphyxia associated condition there is more than a hundredth folds increase in serum EPO; this in turn increases circulating reticulocytes, oxygen carrying capacity of the blood and overall tissue oxygenation (Siren et al. 2001b; Siren et al. 2001a). In addition to hematopoietic tissue, EPOR expression was observed on ovarian (Masuda et al., 2000), testicular (Magnanti et al., 2001; Kobayashi et al., 2002), intestinal, pancreatic, cardiovascular (Wu et al., 1999c; Wu et al., 1999b; Wu et al., 1999a) and neuronal tissues (Liu et al., 1997; Tsai et al., 2006). In addition to its hematopoietic functions EPO is also associated with cellular differentiation, proliferation and inhibition of apoptosis (Brines et al. 2000; Sakanaka et al. 1998; Sun et al., 2005). It prevents damage against mechanical trauma, excitotoxins, and neuro-inflammation (Brines et al. 2000; Sakanaka et al. 1998; Sun et al. 2005).Whether or not the non-hematopoietic functions of EPO are transmitted via the same receptor and signaling pathway as the hematopoietic functions is heavily debated. Casals-Pascual speculated that the erythroid functions are carried out via the homodimer form of the EPOR whereas cellular proliferation, survival and differentiation in non hematopoietic cells are carried out by the β-receptor subunit (βcR) which is a heterodimer (Casals-Pascual et al., 2009). A clear understanding of EPO signaling in hematopoietic and non-hematopoietic events is pertinent to the clinical future of EPO in stroke therapy. Should two different isoforms of EPOR be identified, the signaling

pathway still presents a challenge because the current dogma states that EPO signals via the Janus Kinase (JAK) -2 pathway and its downstream signaling pathway in both events.

Signaling Pathways of Erythropoietin

It is believed that binding of EPO to EPOR promotes the phosphorylation of receptor associated tyrosine kinase, JAK-2 (Lacombe & Mayeux, 1999; Constantinescu et al., 1999). Phosphorylated JAK-2, phosphorylates cytosolic transcription factor, signal transducers and activators of transcription (STAT) allowing for the transcription of proteins such as: cytokines, growth factors, cell survival and differentiation factors (Ihle, 1995a; Ihle, 1995c; Shuai et al., 1992a; Kaplan & Di, 1996b).

Janus Kinases are a family of receptor associated tyrosine kinases which phosphorylates the receptor it's allied with (Ihle 1995a; Ihle 1995b).The specific receptor recruits different signaling molecules such as STAT proteins 1-6 for phosphorylation (Ihle, 1995b; Ihle, 1995d; Shuai et al., 1992b; Kaplan & Di, 1996a). The phosphorylated protein is translocated to the nucleus where it binds to the promoter of the specific gene and initiates transcription of that gene (Ihle 1995a; Ihle 1995b). Previous studies have shown that EPO and other hematopoietic growth factors are associated with the JAK-2 tryosine kinase (Witthuhn et al., 1993). However, the specific STAT that is phosphorylated by each growth factor differs. STAT-3 is associated with EPOR activation (Kretz et al., 2005) whereas STAT-6 and STAT-4 are responds to interleukin (IL)-4 (Kaplan 1996a) and IL-12 (Kaplan 1996b) respectively. STAT-5 one of the most widely studies transcription factors is also associated with EPOR binding. Studies have shown that EPO activated STAT-5 promotes the transcription of anti apoptotic gene BCL_{-XL} and activation of Ras mitogen activated protein kinase (MAPK), ERK-1/-2, and PI3K/Akt pathways (Kilic et al., 2005). STAT-3 is associated with propagation of growth

in mature neurons and cell survival (Cheng et al., 2011). Studies have shown that STAT-3 binds to the promoter of sequences of TIMP-1, which is associated with cell survival, growth and differentiation in the presence of EPO (Kadri et al., 2000; Bugno et al., 1995)

TIMPs are endogenous inhibitors of MMPs (Jiang et al., 2002; Baker et al., 2002). They are a family of four secreted proteins (TIMPs 1-4) that function in maintaining extracellular matrix homeostasis (Gomez et al., 1997; Stetler-Stevenson et al., 1990). TIMP-1 and TIMP-2 are believed to promote cell proliferation and enhanced erythroid activity (Kadri et al., 2000). Subsequently TIMP-1 a 28.5-kDa glycoprotein was found to be regulated by EPO. EPO triggers an increase in both mRNA and protein levels of TIMP-1 (Kadri et al., 2000). TIMP-1 forms a 1:1 complex with MMP-9 and inhibits its proteinase activities. MMPs are a family of protein-degrading zinc dependant endopeptidases. MMPs cleave most of the extracellular matrix (ECM) and are involved in the breakdown and remodeling of many tissues and organs (Isaksen & Fagerhol, 2001; Szabova et al., 2005). Studies have shown an inverse correlation with MMP-9 expression and activity and cell survival (Kadri et al., 2000). The same pathways utilized in hematopoietic cells via EPO to promote differentiation, maturation, and survivals of erythroid cells are utilized during EPO-induced neuroprotection. Thus EPO-induced cell survival is more dependent on EPOR expression that cell type.

Erythropoietin and the Brain

Endogenous EPO signaling has been studied in the development of the central nervous system and in neuroprotection. Increased neuronal apoptosis was demonstrated in EPO-null and EPOR-null mice (Yu et al., 2001; Yu et al., 2002). EPO has been regarded as an important regulator of the differentiation of neural progenitor cells (NPCs). The importance of EPOR expression and activation to the neuroprotective effects of EPO has been demonstrated by Wang and colleagues (Wang et al., 2004;

Wang et al., 2006). EPOR expression on NPCs seems necessary for NPCs differentiation into mature neurons but less important in mature neurons. EPOR-null mice exhibited significant lower levels of NPCs in the subventricular zone compared to the wild-type counterparts. Transgenic mice (EPOR$^{-/-}$) were less resistant to hypoxia ischemia and exhibit defect in NPCs maturation and migration to the ischemic penumbra (Tsai et al., 2006). In addition to its role in NPCs differentiation, maturation and migration EPO and EPOR also play significant role in neuronal survival in the different stroke models (Studer et al., 2000; Shingo et al., 2001).

Erythropoietin-induced Neuroprotection in-vitro

In vitro studies have shown that EPO is neuroprotective in cultured hippocampal and cortical neurons against glutamate toxicity (Morishita et al., 1997), oxygen and glucose deprivation (OGD) (Sinor & Greenberg, 2000) and chemically induced hypoxia (Siren et al., 2001b). Primary neuronal cultures exposed to OGD were protected via EPO administration in a dose and time dependent manner. EPO induced neuroprotection in vitro was associated with suppression of Bad and upregulation of JAK-2, STAT-5, PI3K, AKT and Bcl$_2$ (Ruscher et al., 2002). In addition to the anti-apoptotic properties of EPO antioxidant properties were also detected in cultures. Studies have shown that EPO stabilizes mitochondrial membrane potential and decrease ROS in Abeta (25-35)-induced neuronal toxicity in PC-12 cells, up-regulated anti-apoptotic and down regulate pro-apoptotic proteins (Li et al., 2005a). The protective effects of EPO were not limited to in vitro findings.

Erythropoietin-induced neuroprotection in Subarachanoid Hemorrhage

Despite the difference in pathophysiology, EPO was shown to be neuroprotective in more than one animal models of hemorrhagic stroke. EPO was shown to reduce

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neuronal damage and S-100 expression in cerebrospinal fluid of SAH rabbits 24, 48 and

72 hours after insult. Decrease S-100 was correlated with improved neurological

outcome (Grasso et al., 2002). In a rat intracisternal blood injection SAH model, a single

low dose of EPO (400IU/Kg) was shown to improve mean arterial blood pressure and

cerebral blood flow autoregulation (Springborg et al., 2003) . Increase in CSF, EPO

expression, reduced neurological deficits, vasoconstriction and attenuation of

vasospasm was observed in EPO treated SAH white rabbits (Grasso et al., 2002b).

Previous studies have shown that EPO gene administration prompts an increase in

phospho endothelial nitric oxide synthase (eNOS) and phospho-AKT with a

corresponding decrease in eNOS. This finding infers that EPO confers neuroprotection

and reduce vasospasm by modulating phosphorylated AKT and eNOS in SAH

(Santhanam et al., 2005). Alternately it appears that the neuroprotective mechanism of

EPO is conserved between models.

Erythropoietin-induced neuroprotection in Intracerebral Hemorrhage

Neuroprotection from collagenase induced ICH in rats by EPO was associated

with reduced hematoma formation, edema, apoptosis and inflammation and improved

neurological outcomes (Lee et al., 2006; Grasso et al., 2009). The dose and form of

EPO did not appear to hamper its neuroprotective properties. Intraperitoneal

administration of 500 to 5000IU/kg of recombinant human (rHu) EPO or 1000IU/kg

Darbepoetin alfa conferred neuroprotection after ICH (Lee et al., 2006b; Grasso et al.,

2009a). The neuroprotection observed in ICH was correlated decrease caspase, 3, 8

and 9 activity, TNFα and Fas ligand expression (Lee et al., 2006a; Grasso et al., 2009c).

There was also notable increase in STAT-3, pAkt , pERK and eNOS activity observed in

EPO treated ICH rats. This observation alludes to an overlap in the mechanisms of EPO

induced neuroprotection with the other animal models of stroke such as neonatal HI, global and focal ischemia and SAH.

Erythropoietin-induced neuroprotection in adult cerebral ischemia

EPO-induced neuroprotection in cerebral ischemia is associated with neurogenesis, angiogenesis, oligodendrogenesis, and improved cerebral blood flow and blood brain barrier integrity (Li et al., 2007; Chi et al., 2008; Zhang et al., 2010b). Sakanaka et al. demonstrated that intraventricular infusion of EPO in a gerbil model of forebrain ischemia decrease synapse degeneration and neuronal loss. The neuroprotective effects of EPO infusion was rapidly reversed by the addition of a soluble EPOR which enhance the ischemic core and total neuronal loss in the animals (Sakanaka et al, 1998). EPO-induced neuroprotection in both transient and permanent MCAO appeared to be associated with increased pSTAT-5, Bcl-xL, (X-linked inhibitor of apoptosis protein) XIAP and decreased caspase 3 and 9 in neuronal tissue (Zhang et al., 2006; Fan et al., 2006). The upregulation of STAT-5 and the pan inhibition of caspase-3 and -9 appear to be conserved across the different stroke models.

Erythropoietin-induced neuroprotection in Neonates

Single or multiple doses of EPO administered pre- or post- HI was shown to promote neuroprotection in a rat model of neonatal hypoxia ischemia (Sun et al., 2004); (Kim et al., 2008). EPO-induced neuroprotection in neonatal rats is associated with reduced inflammation, apoptosis, brain atrophy, caspase 3 and 9 and increase expression of heat shock protein (HSP) 70 expression, JAK/STAT and TIMP-1 activity . Previous studies on the anti-inflammatory properties of EPO-induced neuroprotection in neonates showed that EPO inhibits/reduces cerebral expression of CD4+ and CD68+ cells interlukin (IL)-1β and tumor necrosis factor (TNF) α expression (Sun et al. 2005). In

addition to the anti-apoptotic and anti-inflammatory properties, EPO was also shown to stimulate progenitor cell proliferation in the dentate gyrus and subventricular zone of hypoxic ischemic (HI) neonates (Fan et al., 2011). These finding along with positive results in phase I/II clinical trial for EPO use in the treatment of neonatal hypoxic ischemic brain injury offer a glimmer of hope for EPO in stroke therapy (McPherson & Juul, 2010a)

Clinical Use of Erythropoietin in Stroke Therapy

Clinical trials in three different categories of stroke yielded divergent results. Single center study in China revealed that intravenous administration of 250 IU/kg of EPO thrice weekly for four weeks, significantly improved motor function in preterm infants at six months after birth (He et al., 2008). Subsequent studies were then conducted to determine the safety and efficacy of EPO used in neonates for the treatment of hypoxic ischemic brain injury (HIBI). Two independent single center studies revealed that high dose EPO improved neurodevelopment outcomes in very preterm infants and was safe and effective in doses ranging from 1000-2500IU/Kg (Fauchere et al., 2008; Juul et al., 2008). The dose was extrapolated to adults and yielded inconclusive or negative results (Tseng et al., 2010); Ehrenreich et al. 2009).

Clinical analysis of the neuroprotective effects of EPO in SAH patients' based on age, sepsis and concomitant Statin use revealed that the presence of sepsis attenuate EPO's protection. Considering that sepsis is an attack on the hematopoietic system it is not surprising that it is difficult to harness its non-hematopoietic function of EPO in the presence of infection (Tseng et al., 2010). Harnessing the non-hematopoietic benefits of EPO in stroke therapy is a delicate balancing act.

The past decade has shown a staggering number of failed clinical trials in stroke research. Many therapeutic interventions show promise in the laboratory but failed in the

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clinics. Thus, a thorough understanding of mechanisms by which promising therapeutic intervention confers its neuroprotection is necessary to promote smoother transitions from bench to the bedside for EPO in stroke therapy

       Thus the central aim of this proposal is: to determine the key mediators of EPO-induced neuroprotection and the mechanisms by which this occurs in: in-vitro and in-vivo models of hypoxia ischemia. In support of this aim we formed the following hypothesis: **exogenous administration of erythropoietin confers neuroprotection is dependent on its up-regulation of tissue inhibitor of matrix metalloproteinasee (TIMP)-1 and inhibition of hypoxia inducible factor (HIF)-1α**. This hypothesis is based on the following observations **Firstly**: Previous studies have confirmed that EPO triggers an increase in both mRNA and protein levels of TIMP-1 (Kadri et al. 2000e). TIMP-1 forms a 1:1 complex with MMP-9 and inhibits its proteinase activities. Increased MMP-9 mediated apoptosis thus, its inhibition is associated with improved cell survival in both in vivo and in vitro model of hypoxia ischemia (Chen et al. 2009?; Jiang et al. 2002;Baker et al. 2002). **Secondly**: HIF-1α target genes such as matrix metalloproteinases (MMPs), vascular endothelial growth factor (VEGF), cyclooxygenase (COX), nitric oxide synthase (NOS) and p53 plays a role in vascular permeability, angiogenesis, inflammation and cell death. Previous studies have shown that inhibition of HIF-1α is neuroprotective (Chen 2008). Additionally preliminary findings in our lab indicate that EPO administration during hypoxia ischemia inhibits HIF-1α protein expression in a dose dependent manner. Based on these observations, two aims are designed to test this hypothesis:

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Specific Aims

Specific Aim One

Determine if tissue inhibitor of matrix metalloproteinase (TIMP)-1 mediates EPO-induced neuroprotection in vivo and in vitro. <u>Sub-aim 1A:</u> is descriptive, it will demonstrate that EPO is neuroprotective in both models. The correlation between neuroprotection and increases in JAK-2/ STAT-3 and TIMP-1 activities and the subsequent down-regulation of MMP-9 will be shown. The optimum dose of EPO will be determined by cell death and infarction in vitro and in vivo respectively. JAK2 and STAT-3 will be assessed by western blotting for the phosphorylated forms of the protein. MMP-9 and TIMP-1 activity will be assessed by zymography and reverse zymography respectively. <u>Sub-Aim 1B:</u> is mechanistic: we will investigate if pharmacological inhibition of JAK-2 or TIMP-1 negates EPO induced neuroprotection during hypoxia ischemia *in vitro* and *in vivo*. Neuroprotection will be assessed by cell death and infarct volume *in vitro* and *in vivo* respectively.

Specific Aim Two

Determine the mechanism by which EPO regulates HIF -1α expression. <u>Sub-aim: 2A</u> is descriptive: We will profile the expression of HIF-1α and HIF -2α in response to EPO treatment during OGD. HIF-1α and HIF-2α has 40% homology. HIF-2α has been shown to bind to the beta subunit of HIF1α during hypoxia (Krieg et al., 2000b). Thus it is necessary to profile both isoforms to determine if EPO is inhibiting both. Cell survival during oxygen and glucose deprivation will be used to determine the optimum dose of EPO. HIF-1α and HIF-2α protein expression and activity will be determined by western blotting and reverse transcriptase polymerase chain reaction (RT-PCR) respectively. It is hypothesized that both HIF-1 and not HIF-2α will decrease with EPO treatment.

Sub-aim 2B: is mechanistic and will determine the profile of HIF-1 alpha regulators ROS, PHD-2 and VHL in EPO treatment of OGD cells. Two techniques dichlorofluorescein and myeloperoxidase assay will be used to assess ROS production. Both PHD-2 and VHL expression will be assessed by western blotting and RT-PCR. It is expected that there will be an increase in PHD and VHL expression and a decrease in the generation ROS in the EPO treated cells. Sub-aim 3C: Will verify the mechanism of EPO's inhibition of HIF-1α using pharmacological manipulations of EPOR and PHD2. EPOR receptor antagonist EMP9 will be used to block the binding of EPO during OGD in EPO treated cells. PHD2 inhibitor FG4095 will be used to inhibit PHD 2 expression in OGD cells. HIF-1 α and PHD-2 expression and activity and the generation of ROS in all groups will be compared to control. It is expected that both EPOR antagonist and PHD-2 will negate the neuroprotective effects of EPO as well as its inhibition of HIF-1α.
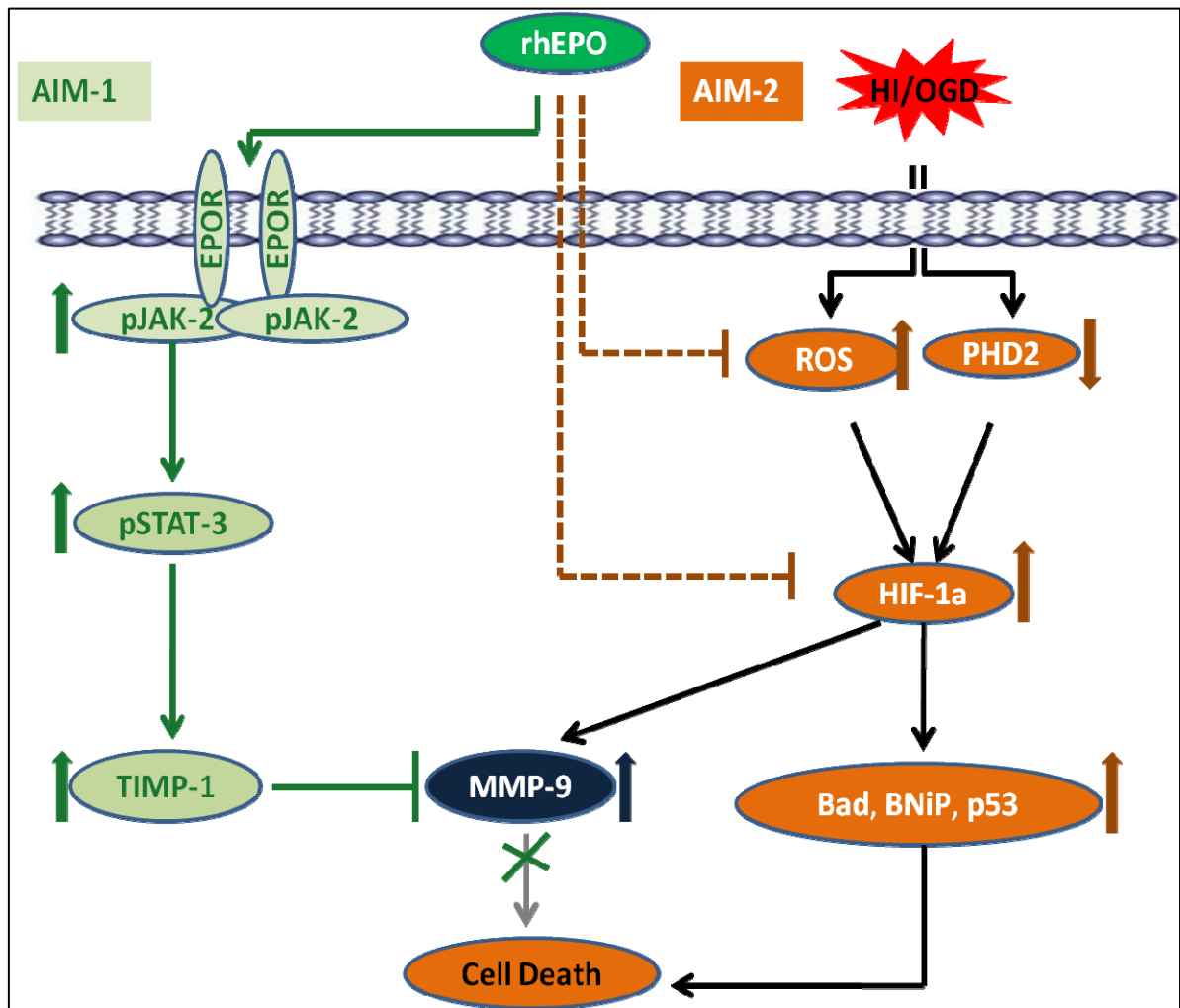
Figure 1.1. Schematic representation of the overall research proposal: the central hypothesis and aims being studied

Aim 1 illustrated in green proposes that binding of exogenous rhEPO to its receptor stimulates the phosphorylation of Janice Kinase 2 which in turn phosphorylates STAT-3. The phosphorylated STAT-3 is then translocated to the nucleus where it initiates TIMP-1 transcription. The increase in TIMP-1 inhibits MMP-9 thereby promoting cell survival.

Aim 2 illustrated in orange shows the pathophysiological response to hypoxia ischemia or oxygen and glucose deprivation. This aim proposes that treatment with exogenous rhEPO inhibits HIF-1a. The possibility of direct or indirect inhibition via ROS or PHD-2 will be examined.

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CHAPTER TWO

TISSUE INHIBITOR OF MATRIX METALLOPROTEINASE-1 MEDIATES

ERYTHROPOIETIN-INDUCED NEUROPROTECTION IN HYPOXIA ISCHEMIA

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Abstract

Previous studies have shown that erythropoietin (EPO) is neuroprotective in both in vivo and in vitro models of hypoxia ischemia. However theses studies hold limited clinical translations because the underlying mechanism remains unclear and the key molecules involved in EPO-induced neuroprotection are still to be determined. This study investigated if tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) and if its upstream regulator signaling molecule Janus kinase-2 (JAK-2) are critical in EPO-induced neuroprotection. Hypoxia Ischemia (HI) was modeled in-vitro by oxygen and glucose deprivation (OGD) and in-vivo by a modified version of Rice-Vannucci model of HI in 10-day-old rat pups.

EPO treated cells were exposed to AG490, an inhibitor of JAK-2 or TIMP-1 neutralizing antibody for 2 hours with OGD. Cell death, phosphorylation of JAK-2 and signal transducers and activators of transcription protein (STAT)-3, TIMP-1 expression, and matrix metalloproteinase-9 (MMP-9) activity were measured and compared with normoxic group. Hypoxic ischemic animals were treated one hour following HI and evaluated 48 hours after. Our data showed that EPO significantly increased cell survival, associated with increased TIMP-1 activity, phosphorylation of JAK-2, STAT-3, and decreased MMP-9 activity in vivo and in vitro. EPO's protective effects were reversed by inhibition of JAK-2 or TIMP-1 in both models. We concluded that JAK-2, STAT-3 and TIMP-1 are key mediators of EPO-induced neuroprotection during hypoxia ischemia injury.

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Abbreviations

EPO: Erythropoietin

HI: Hypoxia Ischemia

JAK: Janus Kinase

MMP: Matrix Metalloproteinase

NGF: Neuronal Growth Factor

OGD: Oxygen and Glucose Deprivation

STAT: Signal Transducer and Activator of Transcription

TIMP: Tissue Inhibitor of matrix Metalloproteinase

Introduction

Asphyxia is the disruption of placental/ pulmonary gas exchange. It is associated

with hypoxemia, respiratory and systemic acidosis, which are linked to cerebral hypoxia

ischemia in neonates (Vannucci 2000). Erythropoietin (EPO), a hematopoietic growth

factor, with non-hematopoietic functions, is upregulated during hypoxia ischemia in the

brain of most mammals (Prass et al. 2003). Although the upregulation of endogenous

EPO is not enough to confer neuroprotection during hypoxia, it is able to turn on some of

the necessary pro-survival pathways (Shein et al. 2008). Previous studies have shown

that exogenous administration of EPO confers neuroprotection in both in vitro and in vivo

models of cerebral ischemia (Brines et al. 2000; Sakanaka et al. 1998; Sun et al. 2005).

Our understanding of the mechanism by which this occurs is still unfolding.

Physiologically, EPO binds to the EPO receptor (EPOR) and promotes cellular

differentiation, proliferation and inhibition of apoptosis (Brines et al. 2000; Sakanaka et

al. 1998; Sun et al. 2005). It also prevents damage against mechanical trauma,

excitotoxins, and neuro-inflammation (Brines et al. 2000; Sakanaka et al. 1998; Sun et

al. 2005).

Previous studies have shown that EPO and other hematopoietic growth factors

are associated with the Janus Kinase (JAK)-2 tyrosine kinases (Harpur et al. 1992;

Witthuhn et al. 1993). Janus Kinases are a family of receptor tyrosine kinases

associated with membrane receptors and are phosphorylated when specific ligands bind

to its receptor. JAK-2 kinases phosphorylate cytosolic transcription factors, signal

transducers and activators of transcription (STAT), allowing for the transcription of

proteins such as: cytokines, growth factors, cell survival and differentiation factors

depending on the ligand (Ihle 1995b; Ihle 1995a; Shuai et al. 1992; Kaplan et al. 1996).

STAT-3 and STAT-5 are both associated with the EPOR and are both activated by the

binding of EPO to its receptor (Ruscher et al. 2002; Toth et al. 2008). STAT-3, the more

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primordial STAT is highly associated with embryonic development. It is the less studied

of the EPOR activated STATs, and is shown to bind to the promoter sequence of the

tissue inhibitor of matrix metalloproteinase (TIMP) -1 gene (Bugno et al. 1995,

Dominguez et al. 2008). TIMP-1 is associated with erythroid cell survival, growth and

differentiation in the presence of EPO (Kadri et al. 2000; Bugno et al. 1995).

TIMPs are a family of four secreted proteins (TIMPs 1-4) that function in

maintaining extracellular matrix homeostasis (Gomez et al. 1997; Carmichael et al.

1986; Stetler-Stevenson 1990; Stetler-Stevenson et al. 1990). TIMPs are endogenous

inhibitors of matrix metalloproteinases (MMP), a family of protein-degrading zinc

dependant endopeptidases (Jiang et al. 2002; Baker et al. 2002). The delicate balance

between TIMPs and MMPs regulates extracellular matrix, receptor shedding and growth

factor bioavailability (Sternlicht & Werb, 2001). MMPs are important in embryonic

development, angiogenesis, wound healing, inflammation, cancer, and tissue

destruction. MMPs cleave most of the extracellular matrix (ECM) and are involved in the

breakdown and remodeling of many tissues and organs (Birkedal-Hansen et al., 1993;

Isaksen and Fagerhol, 2001). Because of its ability to degrade ECM, changes in the

MMP-9 to TIMP-1 ratio leads to excessive tissue degradation and cell death. The

delicate balance is usually altered during hypoxia leading to a significant increase in

MMPs secretion that is not proportional to that of TIMPs (Reynolds, 1996; Ejeil et al.,

2003; Andrian et al., 2007). Previous studies have confirmed that EPO triggers an

increase in both mRNA and protein levels of TIMP-1 (Kadri et al. 2000). TIMP-1 forms a

1:1 complex with MMP-9 and inhibits its proteinase activities. Increased MMP-9

mediated apoptosis thus, its inhibition is associated with improved cell survival in both in

vivo and in vitro model of hypoxia ischemia (Chen et al. 2008; Jiang et al. 2002; Baker et

al. 2002). Additionally, an inverse correlation with MMP-9 expression and activity and

erythroid cell survival has been shown (Kadri et al. 2000). However, this correlation was

not shown in non-hematopoietic cells such as neurons and the relevance of TIMP-1 in EPO induced neuroprotection in vivo and in vitro is still to be elucidated.

In this study, we sought to determine whether TIMP-1 expression and anti-gelatinase activity are important in EPO induced neuroprotection during hypoxia ischemia. Cerebral hypoxia ischemia was modeled in vitro by oxygen and glucose deprivation (OGD) in PC12 cells and in vivo by permanent ligation of the right common carotid artery followed by two hours of hypoxia in 10-day-old (P-10) rat pups (Zagorska and Dulak 2004; Vannucci et al. 1999). We examined the expression of TIMP-1, phosphorylated JAK-2 and STAT-3, as well as the activities of TIMP-1 and MMP-9 with EPO treatment during OGD. We then investigated if pharmacological inhibition of JAK-2 or TIMP-1 negated EPO induced neuroprotection during hypoxia ischemia in vitro and in vivo. The endpoints used to determine neuroprotection were cell death and infarct volume in vitro and in vivo respectively.

Materials and Methods

Materials

Rat pheochromocytoma (PC-12) cells, fetal bovine serum (FBS) and horse serum (HS) were obtained from ATCC (Manassas, VA). Procrit ©, human recombinant erythropoietin was from Loma Linda University, Hospital Pharmacy (Loma Linda, CA.). Neuronal growth factor (NGF) was from Alomone Laboratories Ltd. (Jerusalem, Israel). Reverse zymography kit was from University of East Anglia, School of Biological Sciences (Norwich UK). TIMP-1 neutralizing antibody was from AbD Serotec (Raleigh, NC). AG490 was from Calibiochem (San Diego, CA). Rabbit phospho-STAT-3 (Tyr705) (D3A7) monoclonal antibody was from Cell Signaling (Danvers MA). Rabbit phospho-JAK-2 (Tyr1007/1008) polyclonal antibody, rabbit anti- JAK-2 and mouse TIMP-1, clone 7-6C1 monoclonal antibody was obtained from Millipore Biosciences (Temecula, CA).

Rabbit anti STAT-3, β−actin and all secondary antibodies were from Santa Cruz Biotechnology. Cell death ELISA was from Roche Diagnostics (Indianapolis, IN). All other reagents were obtained from Fisher Scientific (Tustin, CA).

Methods

Culturing and Differentiation of Cells

PC-12 cells passage 6 through 9 were used. Cells were grown on 100 mm2 poly-D-lysine plates in Dulbeco's modified Eagle's medium (DMEM) supplemented with: 5% FBS, 10% HS, 1% Penicillin/streptomycin , 25 mM glucose, 50 ng/ml NGF and incubated at 37°C in 5% CO2 for 7 days as previously described (Greene 1976;Dickson et al. 1986). .

Treatment of Cells

Normal media was replaced with glucose-free, supplemented DMEM with either 0,3,10 or 30 U/ml EPO. Cells were placed in a hypoxic chamber with less than 1% oxygen for 2 hours (Agani et al. 2002). Treatment media was discarded and cells were allowed to recover for 18 hours under normal conditions and collected for zymography, reverse zymography, western blotting, trypan blue exclusion and cell death ELISA. Cells were treated with 25 mM of AG490 during OGD for the inhibition of JAK/STAT and 3 μg/mL of TIMP-1 neutralizing antibody for inhibition of TIMP-1.

Protein extraction from PC-12 cells

Protein was extracted as previously described (Andrews and Faller 1991). Briefly, cells were detached by scraping, rinsed with cold PBS, centrifuged and supernatant discarded. Two hundred μL of radio-immuno-precipitation assay (RIPA) lysis buffer [20 mM Tris, pH 7.5, 150 mM NaCl, 1% NP40, 0.5% Na deoxycholate, 1 mM

EDTA, and 0.1% sodium dodecyl sulfate (SDS)] supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO) were added and the suspension shaken for 15 minutes at 4°C followed by centrifugation. Protein concentrations were estimated by Bradford Assay (BioRad, Hercules, CA). Samples not used immediately were aliquoted stored at -80°C for later use.

Cell Death Assay

Cell death was assessed by trypan blue exclusion and cell death ELISA according to manufacturer's protocol (Roche Diagnostics, Indianapolis, IN.) Briefly, cells were collected by scraping. Sixteen μl of cell suspension mixed with 4 μl of trypan blue and allowed to sit for 5 minutes. Cells were re-suspended and counted on a hemocytometer thrice by two independent investigators. The formula used to calculate percent cell death was as follows: (Trypan blue positive cells)]/ total cell counted) x 100. The average of all six counts was used. There was an n of six per group.

Western Blotting

Western blotting was done as previously described (Dominguez et al. 2008). A 30μg sample of total protein per well with Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue and 5% β-mercaptoethanol) was denatured at 90°C for 3-5 minutes prior to loading on a 4-12% Bis Tris gel (Biorad, Hercules, CA). Gel was electrophoresed and protein transferred to nitrocellulose membrane. The membrane was blocked with nonfat blocking grade milk (Biorad, Hercules, CA) and probed with the appropriate dilutions of primary and secondary antibodies to either JAK-2, pJAK-2, STAT-3, pSTAT-3, TIMP-1 or β−actin. Membranes were washed and protein visualized using ECL Plus, Chemiluminescence (GE Healthcare and Life Sciences, Piscataway, NJ). The optical densities of the bands were

calculated with Image J, version 1.0 and normalized to β-actin which was used as a loading control for all proteins studied.

Gelatin Zymography

Gelatin zymography was performed as previously described (Tang et al. 2004; Wang et al. 2000; Wu et al. 2010). Briefly, 60 μg samples of protein extract were prepared and separated by electrophoresis in 10% Tris-glycine gel with 0.1% gelatin as substrate (Bio-Rad, Hercules, CA). The gel was re-natured for one hour and then incubated with development buffer at 37°C for 48 hours. After development, the gel was stained with 0.5% coomassie brilliant blue R-250 for one hour and then destained appropriately. MMP activity was quantified. Optical densities were calculated with Image J (version 1.0) software.

Reverse Zymography

Reverse zymography was performed according to manufacturer's protocol. Briefly, 60μg samples cell lysate (total protein) was mixed with (1:2 ratio) zymography sample buffer [62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 4% SDS, 0.01% Bromophenol Blue] (Bio-Rad, Hercules, CA), and loaded on a 15% gelatinase gel. Electrophoresis was performed at 140V, and then gels were rinsed and incubated at 37oC for 48hours. Stained withTIMP-1 and TIMP-2 were observed as dark bands as indicated by TIMPs standard (East Anglia University, Norwich UK). TIMP-1 activity was quantified, and optical densities were calculated with Image J, version 1.0.

Neonatal Hypoxic Ischemic Model

All animal research was conducted in accordance with protocols approved by Loma Linda University Institutional Animal Care and Use Committee (IACUC). Time

pregnant female Sprague-Dawley rats (n=5) (Harlan Laboratories, Indianapolis, IN) with their dams were housed in light and temperature controlled environment with food and water ad libitum for the duration of the study. Hypoxia ischemia (HI) was performed as previously described (Calvert *et al.* 2002). Briefly, the right common carotid artery of anesthetized (2.5% isoflourane in 30% O_2 and 70% medical air) post-natal day 10 pups (n=56) were permanently ligated, followed by one hour of rest and 2.5 hours of hypoxia (8% O_2 balance N_2). The pups were returned to their respective dams and maintained at ambient temperature for 24 hours. Animals were sacrificed under deep anesthesia at 48 hours post hypoxia and samples collected for 2, 3, 5-triphenyltetrazolium chloride monohydrate (TTC) staining, imunohistochemistry, western blotting, zymography or reverse zymography.

Intracerebral Ventricular Injection

Intracerebral ventricular injection was performed 1 hour prior to HI, as previously described by Pang et al. (Pang et al. 2003). All drugs were concentrated so that animals received no more than 0.5 µl of fluid in the ventricle. Dose response curves for both TIMP-1 neutralizing antibody and AG490 were performed to determine the optimum doses. The doses presented in this study were 300 ng per pup of TIMP-1 neutralizing antibody and 15 µM of AG490. The vehicles used were IgG and 10% DMSO respectively. Sham operated animals were given a needle stick using the same coordinates as vehicle and treated animals to minimize/eliminated difference due to surgical techniques.

Infarct Volume

Infarct volume was assessed by TTC staining as previously described (Yin et al. 2003). Briefly, pups were euthanized by isoflurane inhalation. Brain was immediately

removed and sectioned into 2 µm slices. The slices were submerged in 2% TTC solution

for 5 minutes at 37°C and rinsed with cold phosphate buffered saline (PBS). Sections

were then fixed with 10% formaldehyde. The slices were photographed and analyzed

using Image J, version 1.0 software by different researchers both of whom were blinded

to the groups in this study. The following formula was used to calculated percent infarct

[(total area of contralateral hemisphere X 2) – (area of uninfarcted area of ipsilateral

hemisphere)]/ (total area of contralateral hemisphere X 2) for each slice.

Immunohistochemistry

Transcardial perfusion was performed as previously described (Hu et al. 1999;

Leonardo et al. 2010). Briefly, following anesthesia with 3.0% isoflurane for 3 to 5

minutes, pups were thoracotomized. A catheter was placed in the apex of the left

ventricle and an incision was made on the right atrium. The pups were perfused with 40

ml of PBS followed by 40 ml of 10% formalin. Collected brain tissues were first fixed in

10% formalin and then cryopreserved in 30% sucrose solution. The tissues were frozen

in optimal temperature cutting (OTC) solution (Fisher Scientific, Tustin CA) and

sectioned into 10 µm coronal slices by cryostat (CM3050S, Leica Microsystems,

Bannockburn, IL). The 10 µm sections were incubated with primary mouse anti- TIMP-1,

rabbit anti-MMP-9 (Millipore) or goat anti-MAP-2 (Santa Cruz Inc.) overnight at 4°C.

Fluorescence dye-conjugated secondary antibodies (Jackson Immuno Research, West

Grove, PA) fluorescein isothiocyanate (FITC; green fluorescence) donkey anti-mouse,

Texas red-conjugated donkey anti-rabbit IgG and AMCA-conjugated affinity pure donkey

anti-goat (Blue) were used. Sections were then observed under an Olympus B 51

microscope with a digital camera and Image-Pro plus software.

Statistical Analysis

Data are presented as mean ± standard deviation. All data were validated using one way ANOVA followed by Tukey or Dunn's test. Statistical evaluation was performed using Sigma Stat software version 3.5 (DUNDAS software LTD. Germany).

Results

Determining the Optimum Dose of Erythropoietin *in vitro*

To investigate the most effective dose of EPO during OGD, cells were treated with 0,3,10 or 30 U/ml of EPO. After 18 hours of recovery, cell death was determined quantitatively by trypan blue exclusion (figure 2.1.A, n=6) and qualitatively by ELISA (figure 2.1.B, n=5). As compared with untreated, medium dose EPO 10 U/ml conferred the most protection. Both low (3 U/ml) and high (30 U/ml) doses were ineffective in minimizing cell death during OGD. Medium dose (10 U/mL) conferred significant neuroprotection ($p < 0.05$) compared to the untreated group making this the most effective dose (Figure 2.1.).
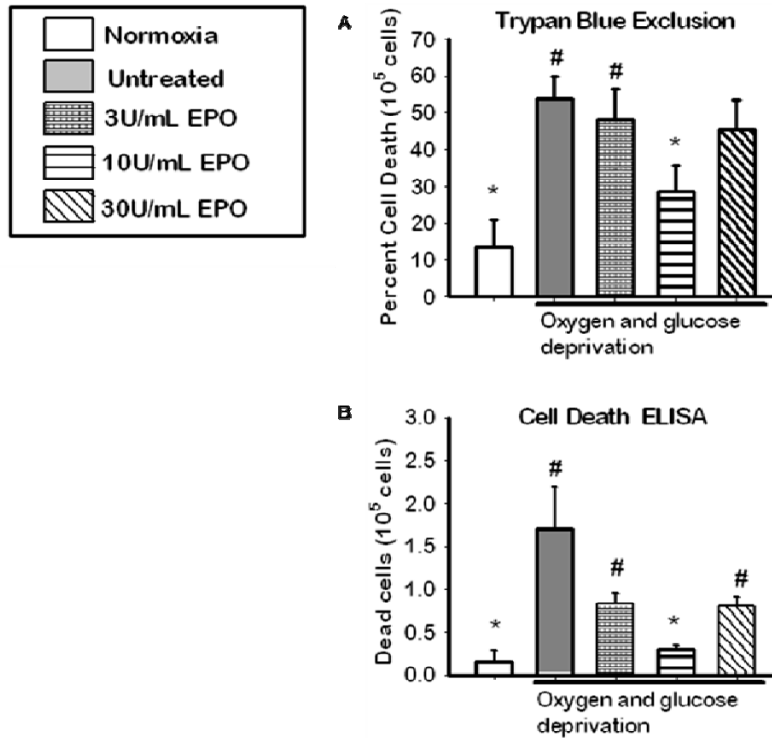
Figure 2.1. The Optimum dose of Erythropoietin for neuroprotection during Oxygen and Glucose Deprivation in vitro. (A) Cell viability as assessed by trypan blue exclusion following HI and 18hrs of recovery. Data represent the mean (±SD): n = 6 in all groups. (B) Cell viability as determined by cell death ELISA. Data represent the mean (±SD): n = 5 in all groups. *P < 0.05 compared to untreated. # P< 0.05 compared to 10U/ml EPO group. 10U/mL conferred the most neuroprotection.

Phosphorylation of JAK-2 and STAT-3 *in vitro*

To examine if phosphorylation of JAK-2 and STAT-3 are involved in EPO induced neuroprotection we profile the expression of the phospho-proteins in the presence and absence of their inhibitors and EPO during OGD in NGF differentiated PC-12 cells. Figure 2:2 B shows a significant increase in JAK-2 phosphorylation ($p < 0.05$) in EPO treated group compared to normoxic, untreated and JAK inhibitor group (25 µM AG490). The expression of phosphorylated STAT-3 was also significantly increased in the 10U/mL EPO treated group ($p < 0.05$) compared to that of all other groups Figure 2.2.C. This indicates EPO has an effect on both JAK-2 and STAT-3 phosphorylation when used to treat NGF differentiated PC-12 cell during OGD.
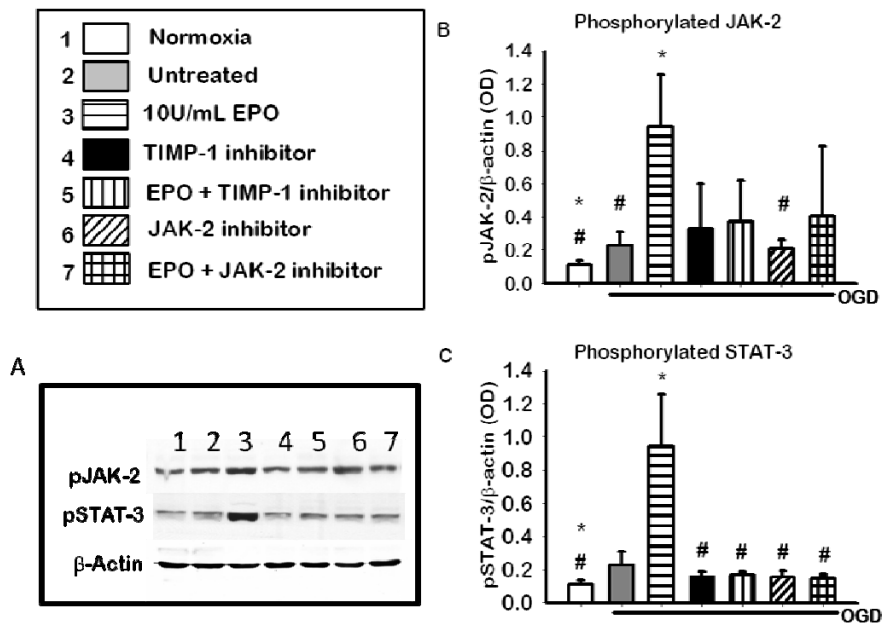
Figure 2.2. The profile of pJAK-2 and pSTAT-3 after the inhibition JAK-2 and TIMP-1 during 2 hours of OGD. (A) Shows representative blots of pJAK-2, pSTAT-3 and the b-actin used as the loading control. (B) JAK-2 phosphorylation in the presence of JAK-2 and TIMP-1 inhibitors.(c) STAT-3 phosphorylation in the presence of JAK-2 and TIMP-1 inhibitors. Data represent the mean (±SD): n = 5 in all groups. .* P< 0.05 compared to untreated # P< 0.05 compared to the 10U/ml EPO treated group. Phosphorylation of both JAK-2 and STAT-3 was increased with EPO treatment and reversed by inhibitors.

TIMP-1 Expression and TIMP-1 and MMP-9 activity Following OGD in
Cultures

To determine the importance of TIMP-1 expression in EPO induced

neuroprotection, we profiled the expression and activity of TIMP-1 and its substrate

MMP-9 in the presence and absence of TIMP-1 inhibition and EPO during OGD. A

neutralizing antibody was used to inhibit TIMP-1 (3 µg/ml). Thus, it was not surprising

that although there was a visible increase in TIMP-1 expression figure 2.3.A row 1

representative blot and figure 2.3(b) densiometric quantification. It was expected that

only the biological activity and not the expression of TIMP-1 would be altered by the use

of a neutralizing antibody, which was shown in figure 2.3.A row 3 representative gel and

figure 2.3.C densiometric quantification .TIMP-1 activity was significantly increased in

EPO treated group ($p<0.05$) compared to the other groups. There was an inverse

correlation between TIMP-1 and MMP-9 activity figure 2.3.A representative gel and

figure 2.3.D densiometric quantification. MMP-9 activity was significantly decreased

($p<0.05$) in the presence of EPO treatment compared to untreated.
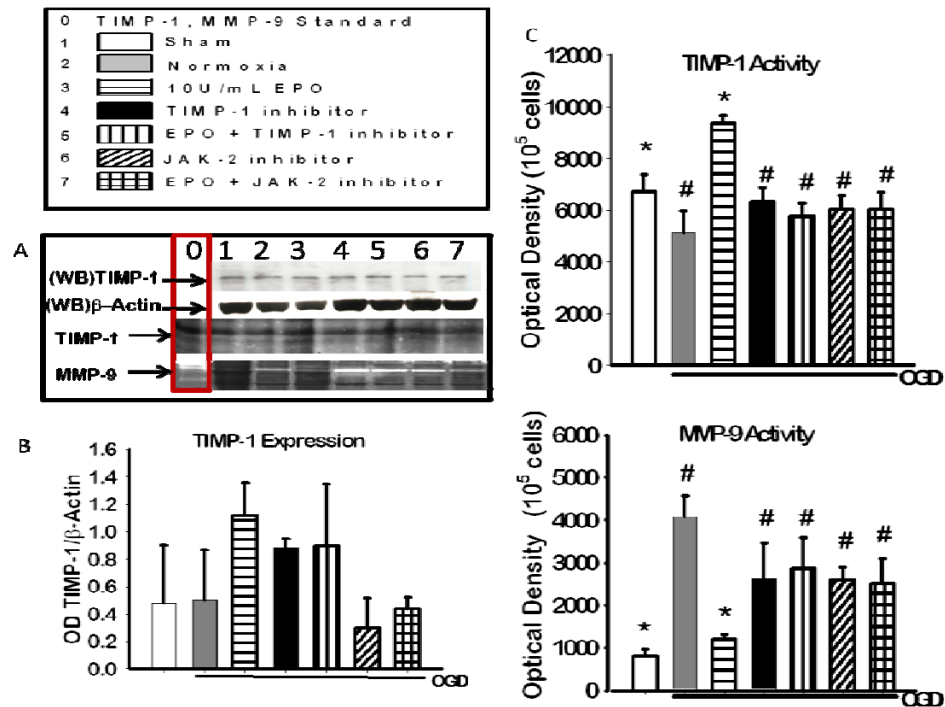
Figure 2.3. The Expression of TIMP-1 and Activity of TIMP-1 and MMP-9 in the presence of JAK-2 and TIMP-1 inhibitors 18 hours after OGD. (A) Representative blot (WB) of TIMP-1 and b-actin expression and coomassie blue stain of TIMP-1 activity (dark bands) and MMP – activity (light bands), column 0 shows positive controls for TIMP-1 and MMP-9. (B) Densitometric quantification of western blot for TIMP-1 expression. (C) Densiometric calculation of reverse zymography of TIMP-1 activity. (D) Densiometric quantification of zymography of MMP-9 activity. Data represent the mean (±SD): n = 6 in all groups represented in B,C and D. * $P < 0.05$ in all group compared to untreated. # $P < 0.05$ compared to 10U/mL EPO treated group. EPO treatment increased TIMP-1 activity and expression and reduced MMP-9 activity.

Reversing EPO's Neuroprotection

TIMP-1 or JAK-2 inhibitors were added to OGD cells in the presence or absence

of EPO to determine if these molecules were necessary for EPO-induced

neuroprotection. Neuroprotection was assessed by cell death assays. Inhibition of either

JAK-2 or TIMP-1 negated the neuroprotective effects of EPO Figure 2.4. Both trypan

blue exclusion Figure 2.4.A and cell death ELISA Figure 2.4.B showed a significant

increase in cell survival in the presence of EPO treatment compared to untreated and

inhibitor groups. EPO induced cell survival was attenuated in the presence of both

inhibitors.
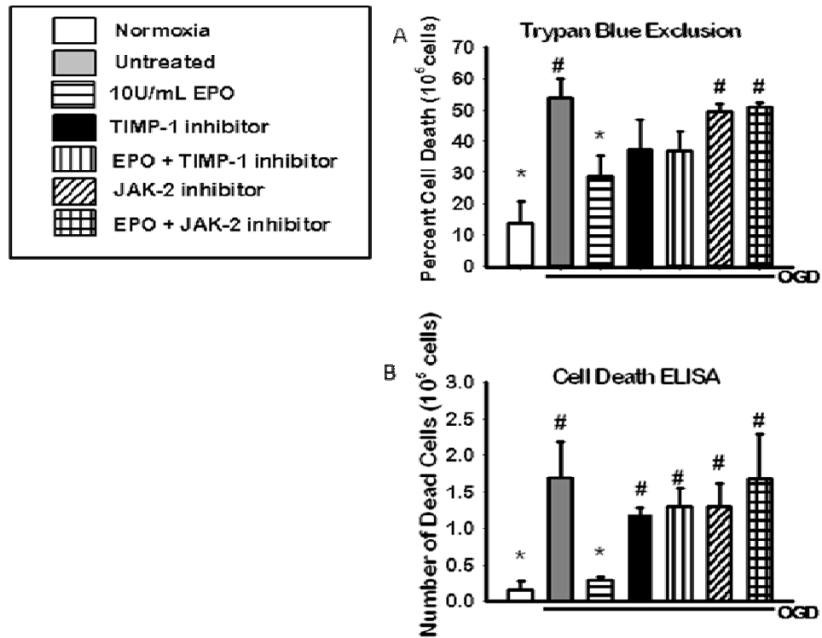
Figure 2.4. The effect of 2 hours of inhibition of JAK-2 and TIMP-1 during OGD on cell death. Cell death assays showing the neuroprotective effects of EPO is reduced in the presence of JAK-2 and STAT-3 inhibitors during OGD. Cell viability as assessed by: (A) trypan blue exclusion (n=6) and (b) cell death ELISA (n=5) following HI and 18hrs of recovery. Data represent the mean (±SD). * P< 0.05 compared to untreated. #P < 0.05 in all group compared to 10U/EPO treated group.

Phosphorylated JAK-2, Phosphorylated STAT-3 is Necessary for
Protection *in vivo*

To determine if the role of JAK-2 and STAT-3 in EPO-induced neuroprotection is similar in animal to that observed in cultures, we profile the phosphorylation of JAK-2 and STAT-3 in the presence and absence of their inhibitors and EPO in the ipsilateral cortex of neonatal hypoxic ischemic pups. Figure 2.5.C shows a significant increase in JAK-2 phosphorylation ($p < 0.05$) in sham, IgG +EPO and DMSO +EPO compared to IgG vehicle and DMSO vehicle as well as TIMP-1 inhibitor with and without EPO. The expression of phosphorylated STAT-3 was also significantly increased in the IgG+ EPO and DMSO + EPO groups ($p < 0.05$) compared to TIMP-1 inhibitor groups with and without EPO and JAK-2 inhibitor groups with and without EPO, Figure 2.5.C. This suggests a role for JAK-2 and STAT-3 in EPO-induced neuroprotection in vivo.
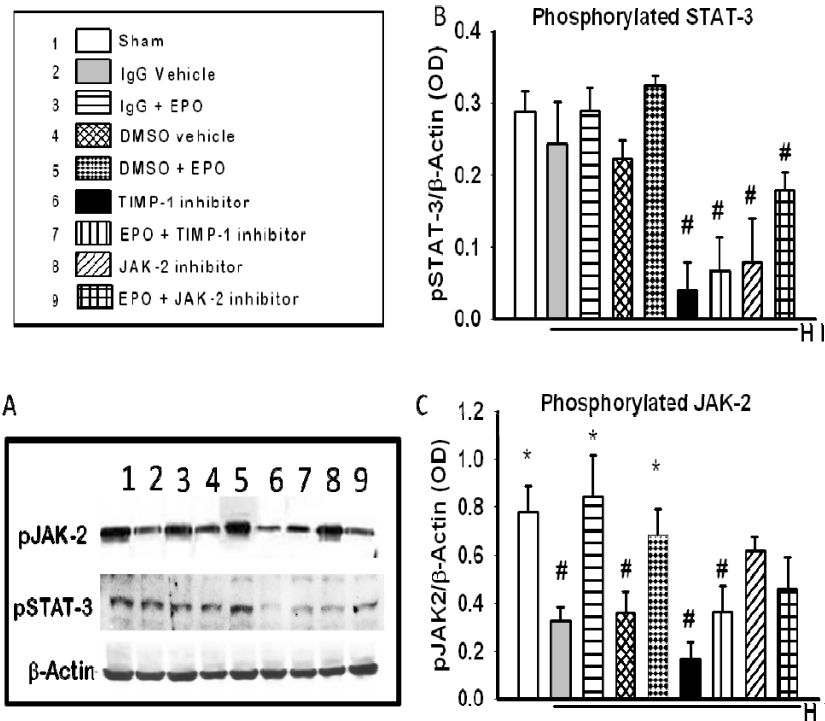
Figure 2.5. The profile of phosphorylated JAK-2 and phosphorylated STAT-3 in the ipsilateral cortex of the brain of HI rats (48 hrs) after inhibition of JAK2 or TIMP-1 (A) Shows a representative blots of pJAK-2, pSTAT-3 and the b-actin used as the loading control. (B) JAK-2 phosphorylation in the presence of JAK-2 and TIMP-1 inhibitors. (C) STAT-3 phosphorylation in the presence of JAK-2 and TIMP-1 inhibitors. Data represent the mean (±SD): n = 6 in all groups. .* P< 0.05 compared to untreated. # P< 0.05 compared to the 10U/ml EPO treated group. EPO treatment increase the phosphorylation of JAK-2 and STAT-3 and the inhibitors decreased both.

TIMP-1 Expression and TIMP-1 and MMP-9 Activity *in vivo* 48 hours
after Hypoxia Ischemia

To determine the importance of TIMP-1 expression in EPO induced

neuroprotection, we profiled the expression and activity of TIMP-1, and its substrate,

MMP-9 in the presence and absence of TIMP-1 inhibition and EPO during HI. A

neutralizing antibody was used to inhibit TIMP-1 (300ng/pup). Neutralizing antibodies

binds to the active site of the protein thereby physically inhibiting the interaction of that

protein with its substrate. Thus, it was not surprising that although there was a visible

increase in TIMP-1 expression figure 2.6.A row 1 representative blot and figure 2.6.B

densiometric quantification there was no significant difference between the TIMP-1

inhibitors group and the IgG + EPO groups. . It was expected that only the biological

activity and not the expression of TIMP-1 would be altered by the use of a neutralizing

antibody, which was shown in figure 2.6.A row 3 representative gel and figure 2.6.A

densiometric quantification .TIMP-1 activity was significantly increased in the IgG +EPO

group ($p < 0.05$) compared to the other groups. There was an inverse correlation between

TIMP-1 and MMP-9 activity figure 2.6.A row 4 representative gels and figure 2.6.D

densiometric quantification. MMP-9 activity was significantly decreased in the IgG +EPO

group ($p < 0.05$) compared to IgG vehicle, EPO+ TIMP-1 inhibitor and EPO + JAK-2

inhibitor. Suggesting that both JAK-2 and TIMP-1 are involved in EPO induce

neuroprotection in vivo.
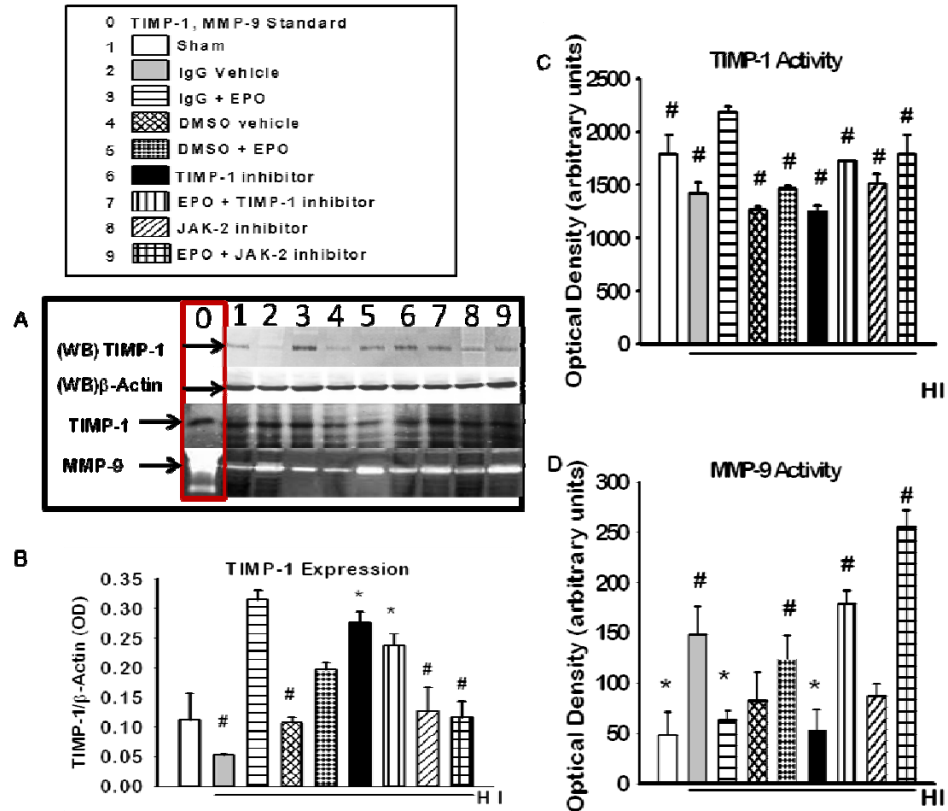
Figure 2.6. The Expression of TIMP-1 and Activity of TIMP-1 and MMP-9 in the presence of JAK-2 and TIMP-1 inhibitors 48 hours after HI in the brain. (A) Representative blot (WB) of TIMP-1 and b-actin expression and coomassie blue stain of TIMP-1 activity (dark bands) and MMP –9 activity (light bands), column 0 shows positive controls for TIMP-1 and MMP-9. (B) Densitometric quantification of western blot for TIMP-1 expression. (C) Densiometric calculation of reverse zymography of TIMP-1 activity. (D) Densiometric quantification of zymography of MMP-9 activity. Data represent the mean (±SD): n = 6 in all groups represented in B, C and D. * P < 0.05 in all group compared to IgG vehicle # P < 0.05 compared to IgG + EPO group. EPO treatment increased TIMP-1 activity and expression and reduced MMP-9 activity.

Co-localization of TIMP-1 and MMP-9, 48 hours after hypoxia
ischemia *in vivo*

To determine that the increase in TIMP-1 expression seen with EPO treatment in vitro also occurred in vivo, we performed immunohistochemistry staining for TIMP-1 and MMP-9 in HI animals with EPO using JAK-2 or TIMP-1 inhibitor (Figure 2.7). There was an increase in TIMP-1 expression and a corresponding decrease in MMP-9 expression in the cortex and hippocampus of animals and visible co-localization between TIMP-1 and MMP-9 in neurons indicating that there is interaction between the two. The optimum dose of both JAK-2 inhibitor (AG490) and TIMP-1 neutralizing antibody was determined by dose response curves (data not shown). The dose that reduced JAK-2 expression and TIMP-1 activity without exacerbating injury was used. The optimum dose of AG490 used in vivo was 15 µM/pup. The dose of TIMP-1 neutralizing antibody used was 300 ng/pup. There was no visible difference between the inhibitor only groups and the inhibitor with EPO groups so only the inhibitors plus EPO group were shown in the figure 2.7.
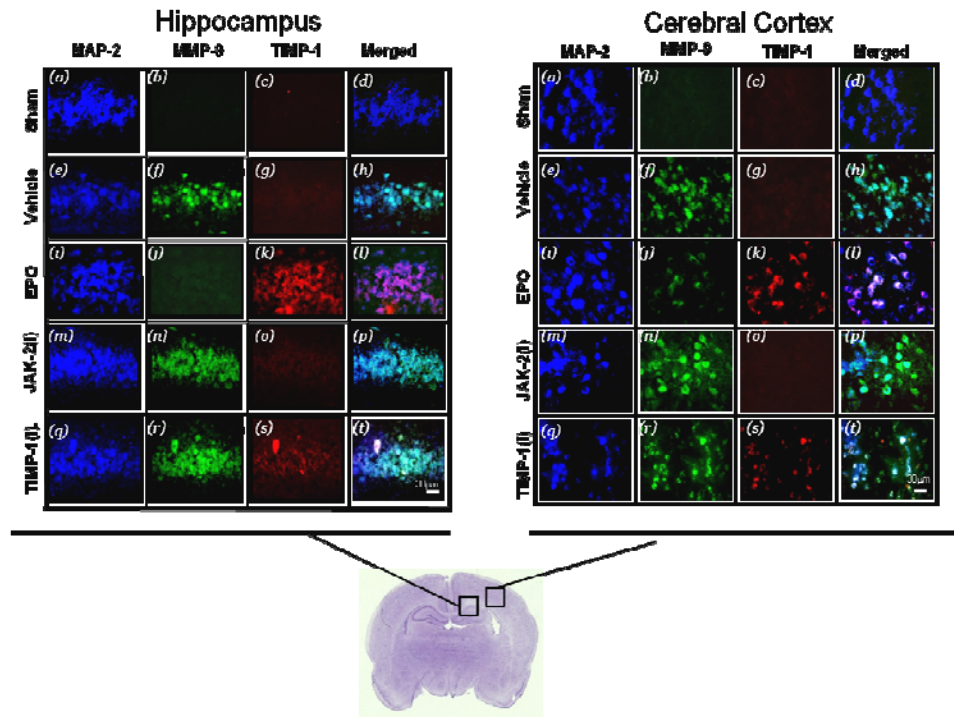
Figure 2.7. TIMP-1 and MMP-9 expression in the brain of neonatal hypoxic ischemic rats after inhibition of JAK-2 and TIMP-1. The expression of TIMP-1 (Texas Red) and MMP-9 (green, FITC) in neurons (blue, AMCA) in the hippocampus (A, left panel) and cortex (B, right panel) of neonatal HI animals treated with (5U/g) EPO. Merge of TIMP-1 and MMP-9 with and without EPO are shown in the right panes. The co-localization of TIMP-1 and MMP-9 in neurons 48 hours after hypoxia ischemia in EPO treated neonatal rats. Both inhibitors suppressed the upregulation of TIMP-1 in the presence of EPO treatment.

Inhibition of JAK-2 or TIMP-1 reverses EPO's neuroprotection *in vivo*

EPO was effective in conferring neuroprotection in 10 day old neonatal hypoxic ischemic pups. Similar to the in vitro results of EPO's neuroprotective effects were reversed by inhibition of either JAK-2 or TIMP-1 in vivo resulting in increased tissue infarction figure 2.8.A representative brain slices, figure (2.8.A quantification of infarction. Tissue infarction/cell death was significantly decreased in EPO treated animals without inhibitor compared to both vehicle and inhibitor treated animals.
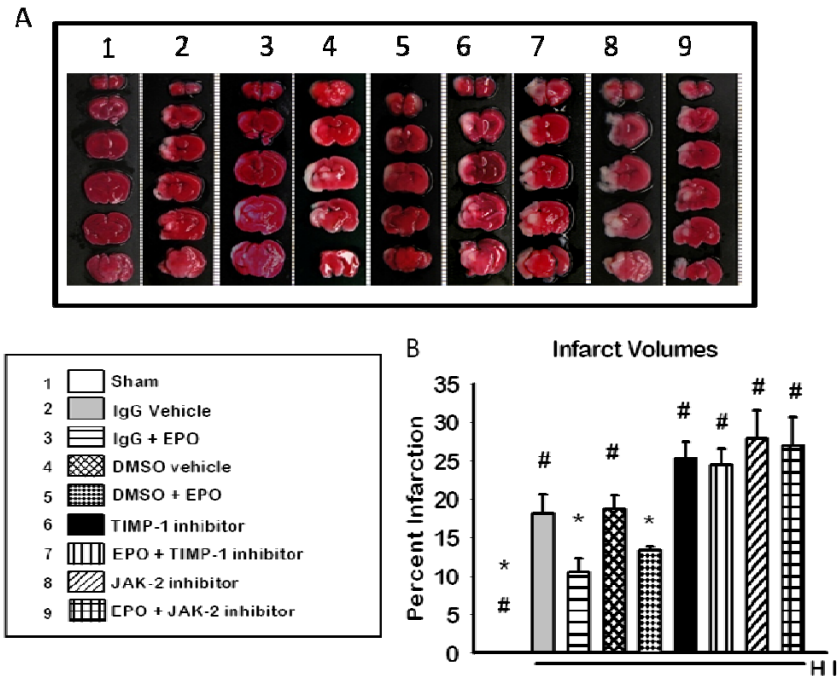
Figure 2.8. Triphenyltetrazolium chloride (TTC) staining of brain tissue of neonatal hypoxic ischemic pups after inhibition of JAK-2 and TIMP-1 (a) Representative cross-sections from the brain of ischemic rat pups are depicted from anterior top) to posterior (bottom). The white areas show the area of infarction for that pup at that particular cross-sectional level. The mean (±SD) percent area of infarction is represented on the graph in (b). (n=6 for sham) (n= 8 for all other groups). * P < 0.05 in all group compared to IgG vehicle: #P < 0.05 in all group compared to 10U/EPO treated group. Inhibition of JAK or TIMP-1 reverses EPO's protection in vivo.

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Discussion

Our findings showed that the upregulation of TIMP-1 by exogenous EPO is necessary for EPO-induced neuroprotection following neonatal hypoxia ischemia or oxygen and glucose deprivation. We also found that EPO-induced neuroprotection was associated with increased phosphorylation of the JAK-2 receptor, downstream signal transducer STAT-3 and its transcription product TIMP-1 and significantly lower MMP-9 activity.

Erythropoietin is up-regulated during ischemia in the brain of most mammals (Prass et al., 2003). EPO released in response to low oxygen promotes cellular differentiation, proliferation and inhibition of apoptosis (Brines et al. 2000; Sakanaka et al. 1998; Yoshimura 1998). Previous studies have shown that exogenous administration of EPO is associated with neuroprotection in vivo and in vitro which was corroborate by our findings (Sun et al. 2005; Ruscher et al 2002). The optimum dose of EPO needed to produce these effects varies from cell type to cell type (Ruscher et al. 2002; Kadri et al. 2000; Renzi et al. 2002). Chan et al. showed that the optimum dose for JAK-2 activation and MMP-9 inhibition and subsequent protection of cultured ischemic hearts was 5 U/mL (Chan et al 2007). Interestingly EPO was shown to confer neuroprotection at a significantly lower dose of 0.1 U/mL (Rusher et al. 2002). Our results showed that 10 U/mL of EPO were the optimum dose for neuroprotection in NGF differentiated PC-12 cells. We demonstrated the mechanism by which this occurred and the importance of TIMP-1 activity in this protection. Several studies have shown that EPO is associated with the family of JAK receptor tyrosine kinases. It is believed that binding of EPO to its receptor EPOR promotes the phosphorylation of JAK-2 (Lacombe and Mayeux 1999a; Lacombe and Mayeux 1999b; Constantinescu et al. 1999). The phosphorylated receptor recruits different signaling molecules such as STAT proteins 1-6 for phosphorylation, depending on the specific ligand bound to the receptor (Ihle 1995b; Ihle 1995a; Shuai et

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al. 1992; Kaplan et al. 1996b). The phosphorylated STAT is translocated to the nucleus where it binds to the promoter of specific genes and initiates transcription of that gene (Ihle 1995b; Ihle 1995a; Shuai et al. 1992; Kaplan et al. 1996b). The specific STAT that is associated with EPO is still controversial. According to Chen et al STAT-5, one of the most widely studies transcription factors, is associated with EPOR binding. STAT-5 promotes the transcription of anti-apoptotic gene BCL-XL and activation of Ras mitogen activated protein kinase (MAPK), ERK-1/-2, and PI3K/Akt pathways (Chen et al. 2003; Kilic et al. 2005). However, contrary to previous suggestion that STAT-3 is not involved in EPO's signaling; our results show that phosphorylated STAT-3 is involved in EPO induced neuroprotection (Rusher et al 2002). This contradiction could possibly be explained by the passage of cells and the age of the animals studied. In our studies we used neonatal models of hypoxia ischemia. Neonatal animals are more likely to express higher levels of STAT-3 than their adult counterparts because STAT-3 is a primordial STAT associated with embryonic development (Murphya et al 2005.). Additionally, STAT-3 is shown to be induced in response to injury in peripheral nerves. It is also shown to be involved in axonal regeneration (Schwaiger et al. 2000; Sheu et al. 2000; Qiu et al. 2005). Studies have shown that STAT-3 binds to the promoter sequences of TIMP-1, which is associated with erythroid cell survival, growth and differentiation in the presence of EPO (Kadri et al. 2000b; Bugno et al. 1995). Our study demonstrates that in non-erythroid cells, EPO up-regulates TIMP-1 by phosphorylation of its transcription factor STAT-3. Concordantly, there was a significant decrease in cell viability of EPO treated cells if the JAK/STAT pathway was inhibited or there was a lower expression of phosphorylated STAT-3. We did not study STAT-5 as the role of STAT-5 in EPO induced neuroprotection was previously shown. We did not detect an induction of MMP-2 during OGD in vitro and we observed that MMP-2 was constituently expressed in vivo 48 hours after HI thus we only focused on MMP-9.

In this present study EPO treatment was accompanied by increased phosphorylation of JAK-2, STAT-3 and TIMP-1 expression and activity. Kadri et al. showed EPO induced increase in TIMP-1 expression in erythroid cells (Kadri et al. 2000b; Bugno et al. 1995). However, since EPO is shown to have different effects in non hematopoietic cells, we needed to verify if TIMP-1 is important for EPO induced neuroprotection. Our findings showed that TIMP-1 neutralizing antibody (figure 2.3.A and 2.3.B) did not significantly alter TIMP-1 expression which was not surprising. Neutralizing antibody alters/inhibits the biological function/activities of cells/receptors/proteins but does not change the protein levels. We observed an inverse correlation between TIMP-1 and MMP-9 activity in the presence of EPO. This finding is consistent with studies done by earlier authors which show similar correlations in erythroid cells (Kadri et al. 2000b; Bugno et al. 1995). Our in vivo studies also reflect a similar correlation between TIMP-1 and MMP-9 expression. There was a higher expression of MMP-9 as shown by imunohistochemistry in the vehicle group compared to EPO treated or sham operated animals. This finding seems to contradict previous studies which showed that EPO increased MMP2/9 secretion into the media of endothelial cells (Wang L. et al). This apparent disparity could be explained by the difference in media and whole cell lysate. Additionally, endothelial cells make up a small proportion of brain tissue and thus would not impact total MMP-9 activity. In vivo inhibitions of either JAK-2 or TIMP-1 show a notable decrease in TIMP-1 expression (immunohistochemistry) even with EPO treatment which we also saw with our TIMP-1 activity but not expression. Immunohistochemistry preserves protein structure and integrity thus allowing for continued interaction between the active site of TIMP-1 and the neutralizing antibody. However, during western blotting, the protein is run under denaturing conditions thus allowing for separation of the neutralizing antibody from the epitope. Thus it not surprising that visible TIMP-1 suppression was observed via

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immunohistochemistry in the TIMP-1 neutralizing antibody group that was not seen in the western blot results. The interplay between EPO, TIMP and MMP appeared to be occurring primarily in neuron, whether this interaction is autocrine or paracrine is still to be elucidated.

The mechanism by which EPO confers its neuroprotection is still unfolding. In 2002, Ruscher et al observed that inhibition of the JAK/STAT pathway with pharmaceutical inhibitor AG490 abolished EPO's neuroprotection in an in-vitro model of hypoxia ischemia (Ruscher et al. 2002). This was validated by our findings both in vitro and in vivo. However the reversal of EPO's neuroprotection by inhibition of TIMP-1 that we observed was not previously shown in neuronal cell in vitro or in vivo. Thus, based on this observation we concluded that in addition to the activity of the JAK/STAT pathway TIMP-1 also plays a crucial role in EPO-induced neuroprotection in in-vivo and in vitro models of hypoxia ischemia. This finding provides important information for expanding our understanding of EPO as a potential treatment for neonatal hypoxic-ischemic brain injury.

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CHAPTER THREE

ERYTHROPOIETIN INHIBITS HIF-1α EXPRESSION VIA UPREGULATION OF PHD-2 TRANSCRIPTION AND TRANSLATION IN AN INVITRO MODEL OF HYPOXIA ISCHEMIA

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Abstract

Erythropoietin (EPO), a hematopoietic growth factor, increases oxygen availability during hypoxia/ischemia and is associated with neuroprotection in: in vivo and in vitro models of hypoxia ischemia. Hypoxia inducible factor (HIF)-1α is the central transcriptional factor for regulation of oxygen associated genes in response to hypoxia. EPO a downstream gene of hypoxia inducible factor was shown to inhibit HIF-1α in a dose dependant manner in an in-vitro model of hypoxia ischemia. This study elucidates the key mediator of EPO-induced HIF-1α inhibition and subsequent neuroprotection. Oxygen and glucose deprivation was used to model hypoxia ischemia in vitro. Our findings showed that EPO treatment resulted in an increase in prolyl hydroxylase (PHD)-2 transcription and translation, inhibition of HIF-1α expression, ROS formation and MMP-9 activity which resulted in neuroprotection. We also observed that EPO-induced neuroprotection was reversed by siRNA silencing of PHD -2. This led to the conclusion that PHD-2 is a key mediator of EPO-induced HIF-1α inhibition and subsequent neuroprotection in an *in vitro* model of hypoxia ischemia.

Abbreviations

EPO: Erythropoietin

HIF: Hypoxia Inducible Factor

MMP: Matrix Metalloproteinase

NGF: Neuronal Growth Factor

OGD: Oxygen and Glucose Deprivation

PHD: Prolyl Hydroxylase

ROS: Reactive Oxygen Species

VHL: Von Hippel Lindau

Introduction

Stroke continues to be among the leading cause of mortality and morbidity; with ischemic strokes accounting for more than 75% of all incidents (ASA, AHA). Despite extensive stroke research there are few effective treatments available. Clinically, tissue plasminogen activator (tPA) is commonly used. However the therapeutic window for tPA is only three hours (Su et al., 2008). Several animal studies have shown promising results for the use of stroke therapy however, recent clinical trials proved otherwise (Sun et al., 2005); (Ehrenreich et al., 2009). Thus it's imperative that we determine the key mechanisms of EPO- induced neuroprotection. This will allow us to harness the beneficial effects EPO treatment while minimizing the detrimental effects EPO therapy.

Cerebral hypoxia ischemia promotes an accumulation of hypoxia inducible factor (HIF)-1α in neurons. HIF-1-α is innate modulator of oxygen homeostasis in most aerobes (Zagorska & Dulak, 2004). During conditions of hypoxia it initiates transcription of VEGF (Mu & Chang, 2003), erythropoietin (EPO)(Prass et al., 2003), glucose transporter-1 (GLU-1), glycolytic enzymes (Jones & Bergeron, 2001a), and several prosurvival genes. However HIF-1α also prompts the transcription of proapoptotic genes such as, MMP-9 (DeNiro et al., 2010), BNIP3(Bruick, 2003), Nix (Sowers et al., 2001), p53 and the caspases (Li et al., 2005a).

Under normoxic condition HIF-1α is continuously synthesized and degraded within 5 minutes by the ubiquitin–proteasome pathway (Huang et al., 1998; Kallio et al., 1999; Salceda & Caro, 1997). HIF-1α first undergoes hydroxylation by prolyl hydroxylase (PHD)-2 which allows it to interact with the product of the von Hippel-Lindau (VHL) tumor suppressor gene (Ivan & Scaiano, 2003a; Jaakkola et al., 2001b; Lee et al., 2003). This interaction allows for the poly ubiquitination and proteasome-dependent degradation of HIF-1α.

PHDs have a strikingly low O2 affinity which is ideal for oxygen sensing (Epstein et al., 2001d; Hirsila et al., 2003). Reduced oxygen or increased ROS allows for the stabilization and accumulation of HIF-1α (Cash et al., 2007; Evans et al., 2005; Kamura et al., 1999; Lisztwan et al., 1999; Stebbins et al., 1999) .The absence of oxygen or the presence of ROS promotes the destabilization of PHD-2 (Cash et al., 2007a; Epstein et al., 2001; Evans et al., 2005; Jones & Bergeron, 2001). HIF degradation pathways require either hydroxylation or acetylation which is PHD-2 and oxygen dependant.

HIF-α target gene, EPO, has been shown to modulate other target genes of HIF-1α such as matrix metalloproteinases (MMPs), vascular endothelial growth factor (VEGF), cyclooxygenase (COX), nitric oxide synthase (NOS) and p53 (Wang et al., 2008). However the effect of EPO on HIF-α or its primary regulator PHD-2 warrants further investigation. EPO was shown to stabilize mitochondrial membrane potential and decrease ROS in Abeta(25-35)-induced neuronal toxicity in PC12 cells, up-regulated anti-apoptotic and down regulate pro-apoptotic proteins (Li et al., 2005b). Alternately ROS promotes HIF-α accumulation. Therefore we hypothesized that EPO-induced neuroprotection will reduce HIF-1α accumulation during hypoxia ischemia in vitro by increasing PHD-2 and inhibiting ROS formation. We explored this hypothesis using oxygen and glucose deprivation (OGD) of NGF differentiated PC-12 cells to model cerebral hypoxia ischemia in vitro. The expression of HIF-1α, HIF-2α, PHD-2 and VHL, as well as the accumulation of ROS in the presence and absence of EPO treatment in during OGD were assessed. We then investigated if pharmacological inhibition of EPOR or siRNA silencing of PHD-2 increases ROS accumulation and negates EPO-induced neuroprotection during hypoxia ischemia *in vitro*. Neuroprotection was assessed by cell death.

Materials and Methods

Materials

The cell line (rat pheochromocytoma cells, PC-12 cells) and serum supplements were obtained from ATCC (Manassas, VA.). Human recombinant erythropoietin (Procrit ©) was from Loma Linda University, Hospital Pharmacy (Loma Linda, CA.). Neuronal growth factor (NGF) was from Alomone Laboratories Ltd. (Jerusalem, Israel). Rat primary antibodies toward HIF-1α and HIF-2α were from ABD Serortec (Raleigh, NC). HIF-PHD-2 antibody, β−actin and all secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Gelatin gel, TRIZOL reagent, and primers were from Invitrogen (Carlsbad, CA). Cell death ELISA was from Roche Diagnostics (Indianapolis, IN). All other reagents were obtained from Fisher Scientific (Tustin, CA).

Methods

Culturing and Differentiation of Cells

Rat pheochromocytoma cells (PC-12 cells) (ATCC Manassas,VA) passage 6 through 9 were be use. Cells were grown in Dulbecco's modified Eagle's medium (DMEM)(Sigma Aldrich ST.Louis MO) with 5% Fetal bovine serum (FBS) and 10% horse serum (HS)(ATCC Manassas,VA), 1% Penicillin/streptomycin and 25mM glucose as previously described (Greene, 1976).Cells were incubated in 5% carbon dioxide incubators at 37°C. At 75-80% confluence cells were sub-cultured on poly-L- lysine coated dishes (BD San Diego CA) and allowed to attach for 24 hours. Neuronal growth factor (NGF)(Alamones lab Israel) 50ng/mL was then added and cells allowed to differentiate. . Approximately 75% of media was replaced every 2-3   days until 60-70% of the cells were differentiated (Dickson et al., 1986).

Treatment of Cells

Normal media was replaced with glucose-free, supplemented DMEM with either 0 or 10 U/ml EPO. Cells were then placed in a hypoxic chamber with less than 1% oxygen for 2 hours (Agani et al., 2002). The media was then replaced with normal media and cell allowed to recover for 18 hours. Cells were then collected for cell death assay, western blotting, gelatin zymography or reverse transcriptase polymer chain reaction (RT-PCR).

Protein extraction from PC-12 cells

Protein was extracted as previously described (Andrews and Faller 1991). Briefly, cells were detached by scraping, rinsed with cold PBS, centrifuged and supernatant discarded. Two hundred µL of radio-immuno-precipitation assay (RIPA) lysis buffer [20 mM Tris, pH 7.5, 150 mM NaCl, 1% NP40, 0.5% Na deoxycholate, 1 mM EDTA, and 0.1% sodium dodecyl sulfate (SDS)] supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO) were added and the suspension shaken for 15 minutes at 4°C followed by centrifugation. Protein concentrations were estimated by Bradford Assay (BioRad, Hercules, CA). Samples not used immediately were aliquoted stored at -80°C for later use.

Transfection

Cells were transfected Silencer® selected pre-designed siRNA to HIF-PHD-2 using Lipofectamine ^{TM} reagent according as previously described (Lee et al., 2008). Briefly 24 hours prior to transfection cells were trypsinized and plated at a density of 2 x10^5 cells in polly D-lysine coated 6 well plates (BD biosciences) with Opti-MEM® reduced serum media. Plus reagent and siRNA were allowed to incubate for 15 minutes in a 1:1 ratio. Lipofectamine ^{TM} LTX reagent was then added and mixture incubated for

25 minutes at room temperature. Reduced serum media was removed from cells and replaced with media containing 5nMconcentration of siRNA complexed with Lipofectamine ^{TM}. Plates were incubated for 24 hours and transfection efficiency assessed and cells used for experiments.

Western Blotting

SDS-PAGE electrophoresis was done as previously described (Dominguez et al. 2008). A 40ug sample of total protein per well with a 1:1 ratio of sample buffer was denatured at 95°C for 3-5 min prior to loading 4-12% Bis Tris gel (Bio Rad). Gels were electrophoresed and protein transferred to nitrocellulose membrane. The membranes were blocked with nonfat blocking grade milk (Biorad) and probed with the appropriate dilutions of primary to HIF-1α (Abcam), HIF-2α (ABD Serotec), PHD-2 (Santa Cruz Biotechnology, Santa Cruz, CA.), VHL (cell signaling Technologies) or loading control -actin (Santa Cruz Biotechnology). Membranes were washed and protein visualized using ECL Plus, Chemiluminescence (GE Healthcare and Life Sciences, Piscataway, NJ). The optical densities of the bands were calculated with Image J, version 1.0 and normalized to β-actin which was used as a loading control for all proteins studied.

Gelatin Zymography

Gelatin zymography was performed as previously described (Tang *et al.* 2004; Wang *et al.* 2000; Wu et al. 2010). Briefly, 60 μg samples of protein extract were prepared and separated by electrophoresis in 10% Tris-glycine gel with 0.1% gelatin as substrate (Bio-Rad, Hercules, CA). The gel was re-natured for one hour and then incubated with development buffer at 37°C for 48 hours. After development, the gel was stained with 0.5% coomassie brilliant blue R-250 for one hour and then destained for 18

to 24 hours. MMP activity was visualized and photographed with BioRad Versa Doc imager. Optical densities were calculated with Image J (version 1.0) software.

Assessment of Reactive Oxygen Species Formation

ROS was assessed immediately after OGD using OxiSelect™ ROS Assay Kit (Cell Biolabs Inc. San Diego CA). The assay used 2, 7-Dichlorodihydrofluorescin diacetate (DCFH-DA) and was performed according to manufacturer's protocol. Briefly cell were washed with Dulbecco's phosphate buffer saline (DPBS) and incubated 1mM of DCFH-DA in serum free media for 1 hour. Media was discarded and cells washed with DPBS to remove unabsorbed DCFH-DA. DCF was then evaluated by spectrophotometry at wavelength 405 nm and reference wavelength in a 96 well plate reader (BioRad, Hercules CA).

Reverse Transcriptase Polymerase Chain Reaction

RNA was extracted using TRIZOL reagent (Invitrogen). DNA was synthesized using SuperScript® First-Strand Synthesis System for RT-PCR (Invitrogen). PCR for HIF-1, PHD-2, VHL and internal standard GAPDH was performed with using the following primers (forward 5'GGCTTTGTTATGTGCTAAC3' and reverse 5'ACTTGATGTTCATCGTCCTC3' for HIF-1α), (forward 5'TAAACGGCCGAACGAAAGC3' and reverse 5'GGGTTATCAACGTGACGGACA3' for PHD-2), (forward 5'ACAGGATGTGCAAGGACAAGTG3' and reverse 5'TAGTCTGCAGCATTCCGTGAGT3' for VHL) and (forward 5'ACCACAGTCCATGCCATCAC3' and reverse 5'TCCACCACCCTGTTGCTGTA3' for GAPDH). Samples were cycled using a 2720 thermal cycler (Applied Biosciences).The samples were denatured at 95°C for 5 minutes followed by 30 samples of amplification for HIF-1α, PHD-2 and VHL and 25 cycles for GAPDH. The amplication cycle consisted

of 30 seconds denaturation at 94°C 30 seconds annealing at 56°C, 1 minute extension at 72°C and a final extension of 5 minutes at 72°C. Samples were electrophoresed on 2% agarose gel with 0.5ug/mL etidium bromide. Gels were visualized under UV light and photographed, and optical densities of the bands were analyzed with Quantity One software (Bio-Rad).

Cell Death Assessment

Cell death was also determined using an Cell Death ELISA kit from Roche Diagnostics (Indianapolis, IN) according to manufacturer's instruction. Briefly the supernatant of lysed cells was conllected and the concentration of mono- and oligonucleosomes present in whole cell lysate using an ELISA kit. The concentration was determined by spectrophotometry at wavelength 405nm with a reference wavelength of 495nm using a 96 well plate reader (BioRad, Hercules CA).

Statistical Analysis

Data are presented as mean ± standard deviation. All data were validated using one way ANOVA followed by Tukey or Dunn's test. Statistical evaluation was performed using Sigma Stat software version 3.5 (DUNDAS software LTD. Germany).

Results

Determining if both HIF-1α and HIF-2α are Inhibited by EPO
Treatment

To determine if the reduction of HIF-1α expression we observed was a result of an upregulation of HIF-2α, we evaluated the expression of both HIF-1α and HIF-2α following OGD in NGF-differentiated PC-12 cells. HIF-1α expression was significantly lower ($p<0.5$) in EPO treated cells compared to untreated cell figure 3.1.B. However HIF-2α expression remained relatively unchanged via EPO treatment figure 3.1.C. Thus, suggesting that HIF-1α is the more prevalent HIF during OGD in PC-12 cells. Consequently a significant increase ($p< 0.5$) in PHD-2 expression was also observed in the EPO treated group compared to normoxic and untreated group.
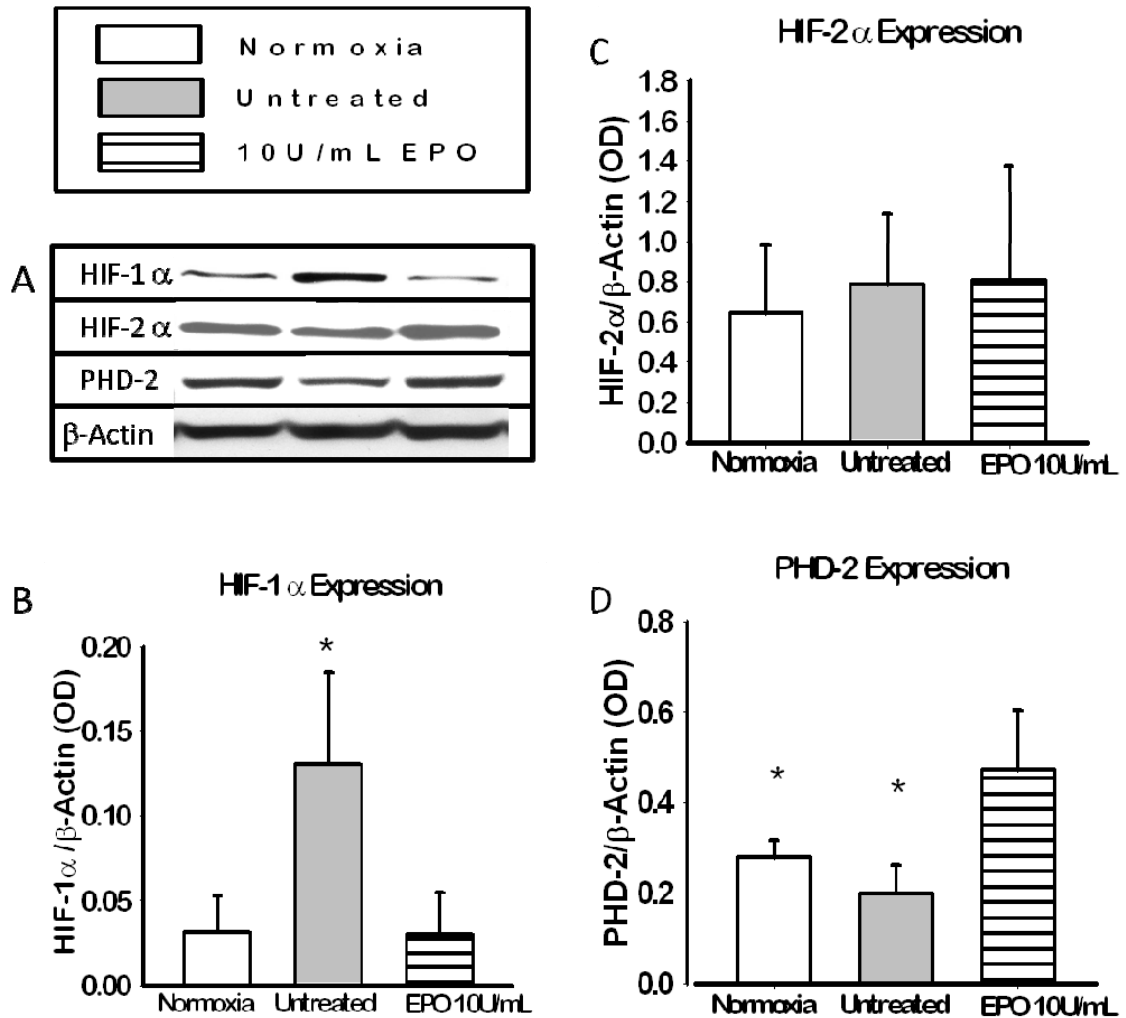
Figure 3.1. The profile of HIF-1α, HIF-2α, and PHD-2 following 2 hours of OGD. (A) A representative image of a western blot of HIF-1α, HIF-2α, PHD-2 and the β-actin as the loading control (B) Densiometric quantification of HIF-1α expression. (C) HIF-2α expression following EPO treatment. (D) PHD-2 expression following OGD. Data represent mean (± SD) n=6 in all groups. *P<0.05 compared to 10U/ml EPO treated group. HIF-1α but not HIF-2α expression was altered in the presence of EPO treatment.

The Profile of the Extracellular (media) pH and Electrolytes Following
OGD

To determine if the change in HIF-a expression was mediated by a change in pH and electrolytes we evaluated the pH, calcium and potassium of the media.We hypothesized that intracellular changes in pH and electrolytes would be reflected in the media since it is an artificial representation of the extracellular fluid. The pH was unaffected by either OGD or EPO treatment figure 3.2.A. OGD prompted an increase in extracellular calcium which was unaltered by EPO treatment figure 3.2.B. Hypoxia also led to a modest increase in extracellular potassium in all groups except 10U/mL EPO and control siRNA. Thus 10U/mL EPO treated group was significantly ($p<0.05$) higher than normoxia but significantly lower ($p<0.05$) that all other groups except control siRNA group figure 3.2.C.
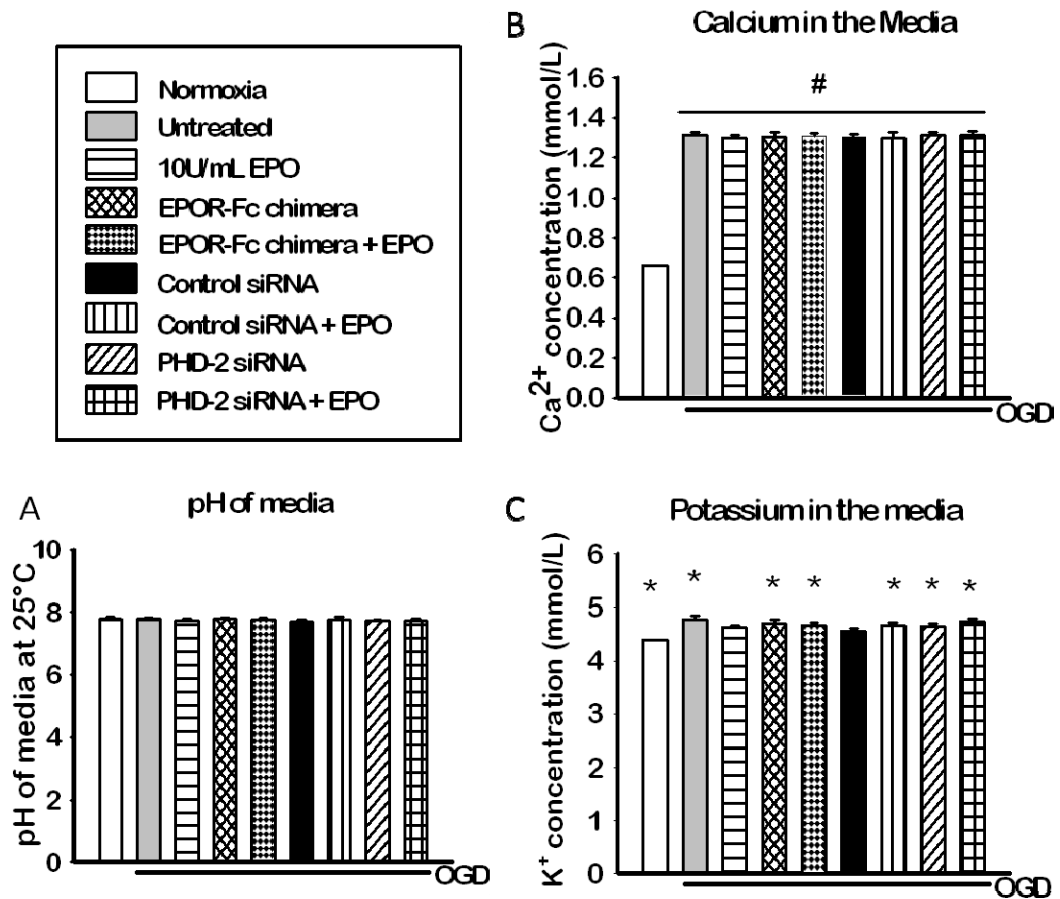
Figure 3.2. The pH, calcium and potassium content in the media following OGD.
(A) The pH of the media following 2 hours OGD with and without EPO treatment. (B) The
calcium present in the media following 2 hours OGD with and without EPO treatment.
(C) The calcium present in the media following 2 hours OGD with and without EPO
treatment. Data represent the mean and (± SD) n=6 in all groups. *P<0.05 compared to
10U/ml EPO treated group. #P<0.05 compared to normoxia. EPO treatment had no
effect on media pH or calcium.

The Effect of EPO and PHD-2 Inhibition on ROS Formation and HIF-
1α and PHD-2 Expression

The primary mediators of HIF-α expression are ROS and hypoxia. Therefore we

determined the consequence of EPO treatment on ROS formation in the presence of an

EPOR-Fc chimera which competitively binds to EPO thereby preventing it interaction

with the naïve EPOR and a PHD-2 siRNA. Transfection increases the porusness of the

cell membrane and is of itself an form of injury thus its not surprising that the basal levels

in the siRNA control group is higher that that of the untreated groups. The formation of

ROS was enhanced during OGD in all groups except the 10U/mL group and the control

siRNA +EPO group figure 3.3.B. Accordingly HIF-1α expression was positively

correlated with ROS formation. Elevated in all of the groups exhibiting high ROS

($p<0.05$) and decreased in EPO treated groups figure 3.3.C. Conversely PHD-2

expression was inversely correlated with ROS formation and HIF-1α expression. PHD-2

expression was notably increased in 10U/mL group ($p<0.05$) and compared to

untreated, EPOR-Fc chimera, control siRNA, PHD-2 siRNA and PHD-2 siRNA + EPO

figure 3.3.D. This suggests that EPO either directly or indirectly alters ROS formation,

HIF-1α and PHD-2 expression when used with NGF-differentiated PC-12 cells.
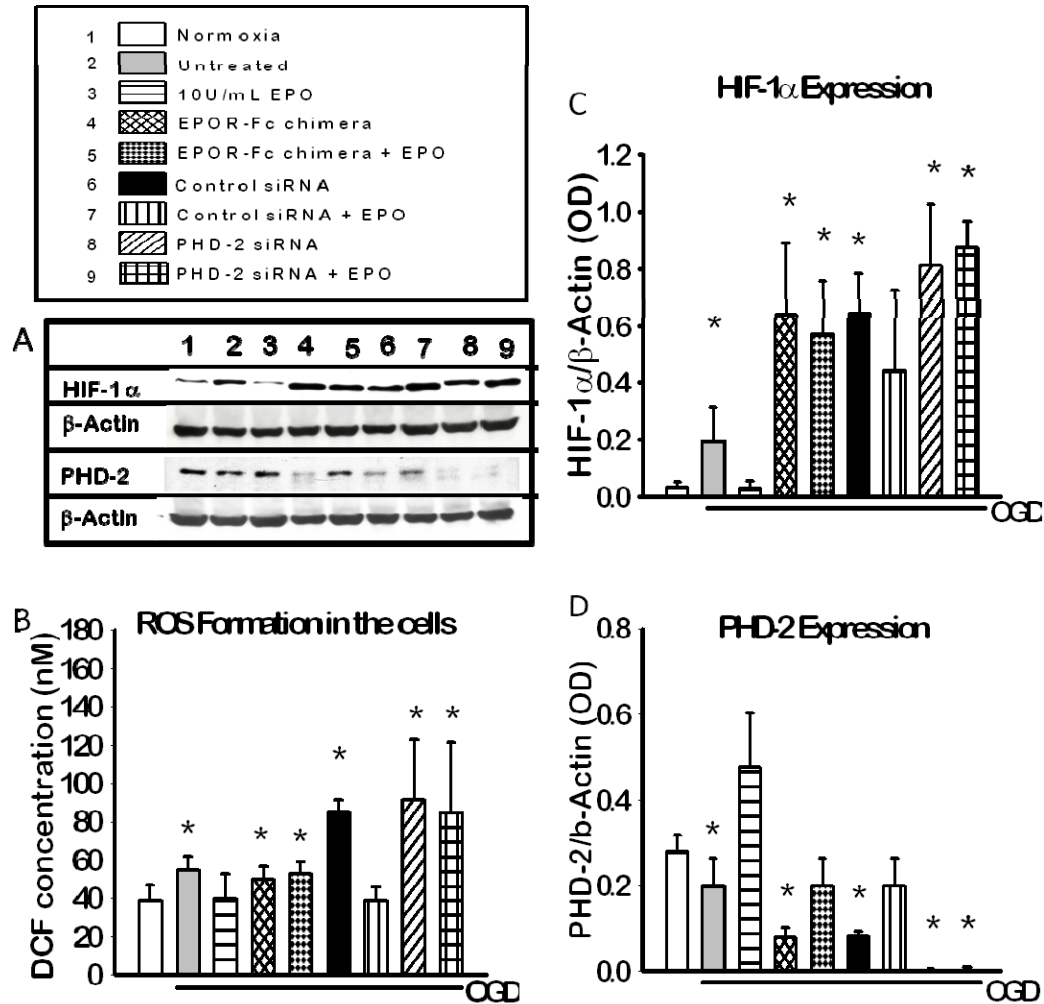
Figure 3.3. ROS formation, HIF-1α and PHD-2 expression in the presence of EPO and PHD-2 inhibition. (A) Shows a representative blot of HIF-1α, PHD-2 and the loading control β-actin. (B) ROS formation during OGD in the presence of inhibitors. (C) HIF-1α expression after OGD in the presence of inhibitors. (D) PHD-2 expression in after OGD in the presence of inhibitors. Data represent the mean and ($\pm$ SD) n=6 in all groups. *P<0.05 compared to 10U/ml EPO treated group. Inhibition of EPO or PHD-2 enhanced HIF-1α expression and ROS formation

Determining the Effects of EPO Treatment on mRNA Levels of HIF-1α, PHD-2 and VHL

Changes in protein expression are not always indicative of transcriptional changes. Thus to determine if the changes we observed in HIF-1α and PHD-2 expression with EPO treatment and inhibitor were at the transcription or the translational levels we did RT-PCR for these protein figure 3.4. Silencing of PHD-2 via siRNA triggered a significant increase ($p < 0.05$) in HIF-1α transcription compared to 10U/mL EPO group. On the contrary competitive inhibition of EPO did not affect HIF-1α transcription with and without EPO treatment figure 3.4.B. Alternately either inhibition of EPOR or silencing of PHD-2 significantly lowered mRNA levels of PHD-2 figure 3.4.C. Downstream effecter of HIF-1α degradation VHL appeared unaffected by EPO treatment as well as inhibition of EPO or PHD-2, figure 3.4.E. This observation alluded to PHD-2 as one of the primary effecter of HIF-1α inhibition and subsequent neuroprotection in NGF-differentiated PC-12 cells.
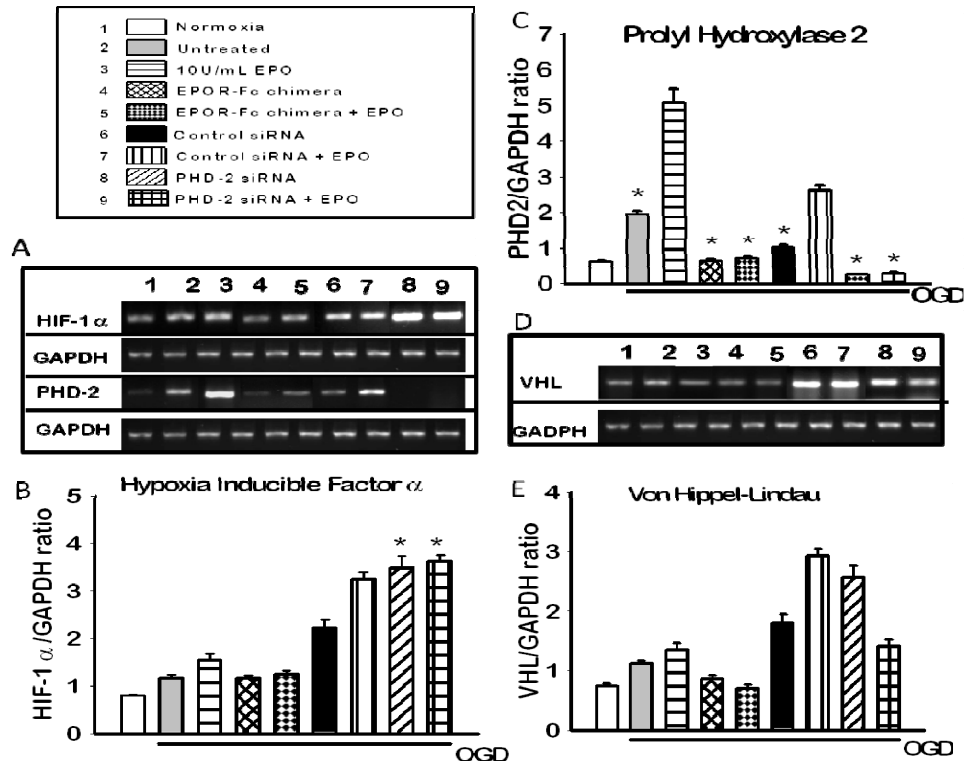
Figure 3.4. mRNA levels of HIF-1α, PHD-2 and VHL in the presence of EPO and PHD-2 inhibition. (A) Shows representative gel of HIF-1α, PHD-2 and the housekeeping gene gapdh. (B) HIF-1α mRNA expression after OGD in the presence of inhibitors. (C) PHD-2 mRNA expression in after OGD in the presence of inhibitors. (D) Shows representative gel of VHL and the housekeeping gene gapdh. (E) VHL mRNA expression after OGD in the presence of inhibitors. Data represent the mean and (± SD) n=6 in all groups. *P<0.05 compared to 10U/ml EPO treated group. Inhibition of PHD-2 enhanced HIF-1α transcription in the presence and absence of EPO.

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Inhibition of EPO or PHD-2 alters MMP-9 Activity and Reversed
EPO's Neuroprotection

Downstream molecule of HIF-1α, MMP-9 is associated with exacerbated injury and cells death in the acute stages of hypoxia. Thus to determine if EPO-induced HIF-1 inhibition affects downstream genes expression and subsequent cell death we assessed MMP-9 activity and cell death. According to expectations EPO treatment was associated with lower MMP-9 activity. However inhibition of PHD-2 rapidly reversed this resulting in significantly higher level of MMP-9 activity in PHD-2 siRNA + EPO group compared to 10U/mL EPO and control siRNA + EPO figure 3.5.B. Alternately EPOR-FC chimera did not appear to negatively affect MMP-9 activity in the presence of EPO. Consequently cell death was positively correlated with MMP-9 activity. Inhibition of EPO or PHD-2 in the presence of or absence of EPO treatment visibly reversed EPO-induced neuroprotection compared to 10U/mL ($p<0.05$) EPO figure3.5.C.
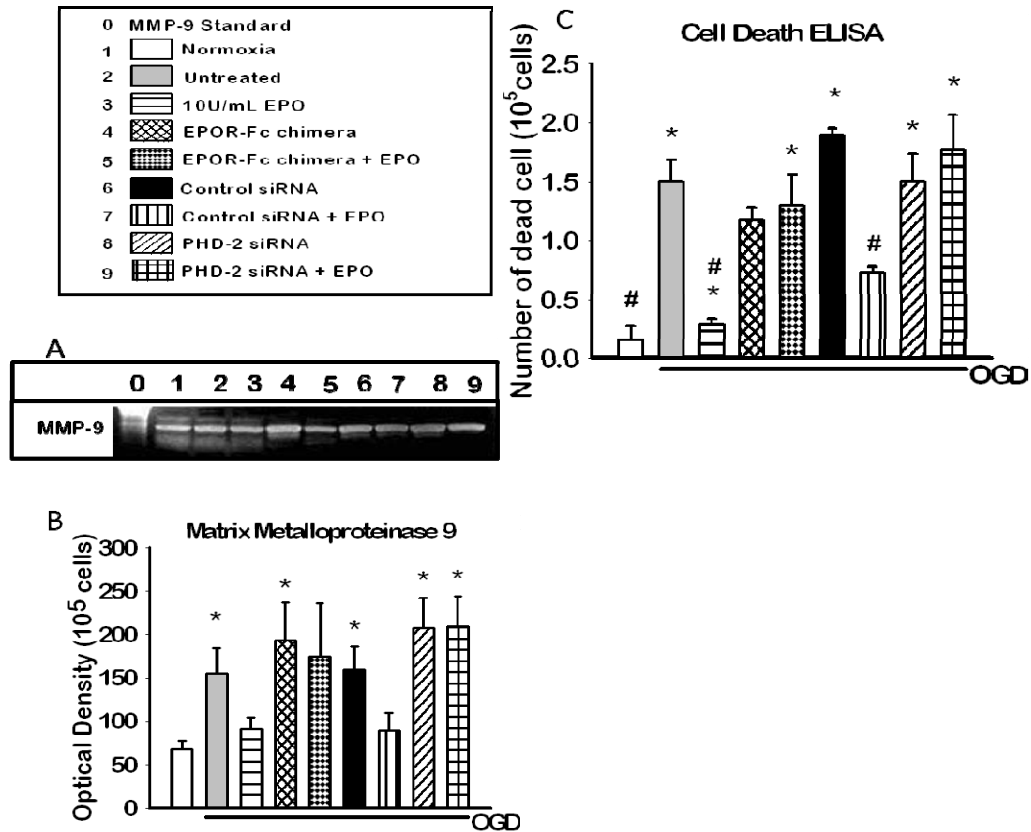
Figure 3.5. The effects of EPO treatment on MMP-9 activity and subsequent cell death after OGD. (A) Shows representative gel of MMP-9 activity (light bands). (B) Densiometric quantification of MMP-9 activity as detected via gelatin zymography. (C) Shows cells death assessment via ELISA. Data represent the mean and (± SD) n=6 in all groups. *P<0.05 compared to 10U/ml EPO treated group. #P<0.05 compared to untreated. Inhibition of PHD-2 significantly increased MMP-9 activity and cell death with and without EPO.

Discussion

Our findings showed that EPO-induced inhibition of HIF-1α which was associated with elevated transcription and translation of PHD-2. Inhibition of EPO had prompted an increase in HIF-1α protein expression but had no effect on mRNA. EPO-induced elevation of PHD-2 was coupled with reduced ROS formation, MMP-9 and cell death.

HIF-1α and-2α shares 40% homology. HIF-2α has been shown to bind to the beta subunit of HIF-1 during hypoxia (Krieg et al., 2000). An accumulation of HIF-1α or HIF-2α during hypoxia is tissue dependant. The specific HIF in neuronal tissue is still highly debate (Yeo et al., 2008). However in our study a profile of both HIFs revealed that, HIF-2α protein expression in cells was relatively unchanged during OGD with or without EPO treatment. This inferred that HIF-1α and not HIF-2α is the primary HIF associated with hypoxia in NGF differentiated PC-12 cells. On the contrary HIF-1α was notably increased by OGD and diminished with EPO treatment. Reduction of HIF-1α protein expression by the administration of exogenous EPO was not previously shown and thus warranted further investigation. EPO is a downstream gene of HIF-1α (Semenza, 1998).Therefore; we speculated that the apparent EPO-induced inhibition of HIF-1α was probably caused by a negative feedback inhibition. However a profile of the primary regulators of HIF-1α degradation, PHD-2 and ROS alluded to a much more complex mechanism.

PHDs are oxygen sensors that use oxygen as a co-substrate and 2-oxoglutarate and iron as cofactors. Because of the iron dependence iron compounds could be used for hypoxic mimicry (Cioffi et al., 2003; Lieb et al., 2002). PHDs are extremely sensitive to low O_2 concentration which is ideal for oxygen sensing (Epstein et al., 2001b; Hirsila et al., 2003b). They are dioxygenases that catalyze oxygen-dependent hydroxylation of HIF-1α prolyl residues. PHD-2 promotes the degradation of both HIF-1α and HIF-2α

(Chang et al., 2005). Thus it was not surprising when we observed an inverse correlation between HIF-1α and PHD-2 protein expression after hypoxia (figure 3.1. and figure 3.3) and a marked increase in ROS formation that mirrored HIF-1α expression. Our data corroborated published reports that, reduced oxygen or increased ROS allows for the stabilization and accumulation of HIF-1α (Cash et al., 2007b; Evans et al., 2005b; Kamura et al., 1999b; Lisztwan et al., 1999b; Stebbins et al., 1999b) . Low levels of oxygen or high levels of ROS promotes the destabilization of PHD-2 (Cash et al., 2007c; Epstein et al., 2001a; Evans et al., 2005a; Jones & Bergeron, 2001b). However contrary to our expectation there were no notable changes in extracellular pH or electrolytes: Ca^{2+} and K^+. Despite this observation we are unable to say conclusively that EPO-induced inhibition of HIF-1α is independent of electrolytes regulation, because it is possible that the change were too small to be detected in the media. Studies have shown that prolyl hydroxylation and acetylation, modulates cellular HIF-1α levels by governing the physical interaction between HIF-1α and VHL. VHL binds with elongin B, elongin C, Rbx1 and Cul2 to form the E3 ubiquitin ligase. The ubiquitin is unable to bind HIF-1α during hypoxia because HIF-1α lacks hydroxylation or acetylation at this time (Ivan & Scaiano, 2003b; Jaakkola et al., 2001a). Thus, we anticipated that EPO-induced inhibition of HIF-1α would reflect corresponding increase in regulatory proteins PHD and pVHL expression but would not alter mRNA levels. On the contrary pVHL protein expression was almost undetectable in all groups (data not shown) and is therefore inconclusive. HIF-1α protein but not mRNA levels were significantly reduced by EPO treatment. This suggests that EPO-induced inhibition of HIF-α is not a direct action at the transcriptional level. Thus changes in HIF-1α protein expression was mediated by either physical interaction of EPO with HIF-1α or by transcriptional regulation of a HIF-1α regulator. Thus, we were not surprised when we observed that EPO treatment

propagated increased in both transcriptional and translational levels of PHD-2. This alluded to modulation of PHD-2 levels as the primary mechanism by which exogenous EPO inhibits HIF-1α in NGF differentiated PC-12 cells during hypoxia ischemia which is associated with neuroprotection. Conversely Ratan and colleagues showed that inhibition of PHD and activation HIF-1α was associated with neuroprotection in vivo and in vitro (Ratan et al., 2008; Lee et al., 2009; Siddiq et al., 2009). The apparent contradiction in these reports attests to the biphasic nature of HIF-1α which can be beneficial as well as detrimental (Calvert et al., 2006; Halterman & Federoff, 1999; Leker et al., 2004). The dichotomy of HIF-1α neuroprotection is best explained by Baranova and colleagues who described an oscillation of HIF-1α levels following hypoxia ischemia. Transient increases in HIF-1α within the first 24 hours after an injury was associated with elevation of proapoptotic genes however, downstream prosurvival genes of HIF-1α were upregulated with later (>24hrs) sustained elevation of HIF-1α (Baranova et al., 2007) . Thus HIF-1α is detrimental in the acute stages of injury but beneficial in the delayed/recovery stage of injury.

Silencing of PHD-2 prompted a notable increase in both protein and mRNA levels of HIF-1α, MMP-9 activity and resultant cell death even in the presence of EPO treatment. These observations lends to the conclusion that PHD-2 is a potent mediator of EPO-induced neuroprotection in NGF differentiated PC-12 cells.

Given the direct or indirect interaction of EPO with biphasic HIF-1α and its primary regulator PHD-2 caution should be exercised to harness the beneficial neuroprotective effects of EPO, without triggering the detrimental effect of delayed inhibition of HIF-1α.

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CHAPTER FOUR

DISCUSSION AND CONCLUSION

Summary/Highlight of Findings

Our findings show that EPO-induced neuroprotection was mediated by both upregulation of TIMP-1 and inhibition HIF-1α. This finding was supported by the following observations: (1) EPO-induced neuroprotection was associated with increased phosphorylation of the JAK-2 receptor, downstream signal transducer STAT-3 and its transcription product TIMP-1 and significantly lower MMP-9 activity in: *in vivo* and *in vitro* models of hypoxia ischemia. (2) Inhibition of either JAK-2 or TIMP-1 reversed the protective effects of EPO in both models. (3) The pharmacological inhibition of JAK-2 or TIMP-1 was associated with reduced expression of STAT-3 and downstream gene, TIMP-1 activity and considerable increase in MMP-9 activity. (4) HIF-1α expression and ROS accumulation were significantly reduced following EPO treatment in OGD cells. (5) EPO- induced inhibition of HIF-1α was associated with increased PHD-2 expression and a corresponding decrease in ROS formation, MMP-9 activity and cell death.

The State of the Field Prior to this study

Currently it is believed that EPO –induced neuroprotection is mediated by three major pathways downstream of JAK-2 phosphorylation namely: STAT-5, PI3K/AKT and MAPK/RAS/ERK1/2 pathways (Ruscher et al., 2002; Socolovsky et al., 1999; Ribatti et al., 1999; Shen et al., 2010). Previous studies have shown that JAK-2 phosphorylated STAT -5 which is then translocated to nucleus where it activates the gene transcription

of several proapoptotic genes suchas Bcl-xL and the X-linked inhibitor of apoptosis

protein (XIAP) (Socolovsky et al., 1999). The newly transcribed anti apoptotic proteins

inhibits both caspase dependent and caspase independent cell death, by modulating

caspase 8,3 and 9 activation as well as cytochrome c transport from the mitochindria

(Siren & Ehrenreich, 2001; Siren et al., 2001b; Siren et al., 2001a). EPO bounded to

EPOR modulates calcium-calmodulin interation resulting in a calcuim influx into the cells

(Kakihana et al. 2005). Increased intracellular calcium activates the PI3K/AKT pathway.

Prosurvival protein XIAP, Bcl2 and BclxL downstream is upregulated while proapoptotic

proteins Bad and caspase 9are downregulated via PI3K/AKT activation, thereby

promoting neurrroprotection (Dhanasekaran et al., 2008). The third major pathway that is

know to play a role in EPO-induced neuroprotection is the RAS/MAPK/ ERK1/2 pathway.

Activated EPOR phosphorylates Ras which binds to c-Raf to phosphorylate MAP2

lending to the phosphorylation of ERK1/2 which in turn activates MAPK. MAPK

subsequent inhibits Bax translocation form the cytosol and promotes Bcl-2 transcription

leading to cell survival and proliferation (Arai et al., 2002; Morrison et al., 2002). Tables

4.1 and 4.2 summarized what was known in the field prior to our study.

Table: 4.1. A Summary of Erythropoietin-induced Neuroprotection in Animal Models of Ischemic Stroke

Stroke Model	Species	Time of Administration	Outcome Measured	Effect	Reference
Focal Ischemia	Male Long Evans Rats	6, 7 and 8 days after occlusion	Neurobehavior Lesion Volume, Tissue Loss	Improved infarct volume and neurological outcomes	Belayev et al. Brain Res. 2009 Jul 14;1280:117-23.
	Male C57BL/6 mice	30 minutes before ischemia/ daily for 3, 7, 14 and 21 days	Infarct Volume. Angiogenesis. Endothelial Cell Survival. Local Cerebral Blood Flow (LCBF). Apoptosis : Caspase activities	Decrease cell death and infarct volume 3 days after stroke. Restored CBF 14 days after stroke. neurovascular protection and neurogenesis	Li et al. J Cereb Blood Flow Metab. 2007 May;27(5):1043-54
Global Ischemia	Male Sprague-Dawley Rats	20 hr before or 20 min or 1 hr after ischemia	Histology of neurons. Cell Death: DNA Fragmentation	Protected CA1 neurons of hippocampus. Decreased neuronal death	Zhang et al. J Neurosci Res. 2006 May 15;83(7):1241-51.
	Male Mongolian Gerbils	Immediately after restoration of blood flow	Brain Edema. Hippocampal neuronal death. Brain nitric oxide. Survival	Increased neuronal survival and decreased brain edema. Reduced NO levels after ischemia	Calapai et al. Eur J Pharmacol. 2000 Aug 11;401(3):349-56.
Neonatal Hypoxia Ischemia	P-7 Sprague-Dawley Rats	24 hours after insult	Inflammatory effects. Brain Weight.	Attenuated brain injury Reduced inflammatory cytokine and leukocytes infiltration.	Sun et al. Stroke. 2005 Aug;36(8):1672-8.
	P-7 Sprague-Dawley Rats	20 minutes after insult/ Day 2,4 and 6	Infarct Volume. Revascularization. Neurogenesis. Immunohistochemistry Neurological behavior	Reduced tissue infarct volume. Enhanced revascularization, increased sub-ventricular zone cell proliferation and neuronal migration. Improved neurological outcomes	Iwai et al. Stroke. 2007 Oct;38(10):2795-803

Table: 4.2 A Summary of Erythropoietin-induced Neuroprotection in Animal Models of Hemorrhagic Strokes

Stroke Model	Species	Time of Administration	Outcome Measured	Effect	References
ICH	Male Sprague-Dawley Rats	5 minutes after surgery/ Daily for 14 days	Neurobehavior: Histopathological Computation of Tissue Loss, Immunohistochemistry for NeuN positive cells	Significantly Improved Neurobehavior 1 week after insult. Reduced injury and preservation of parenchymal cytoarchitecture.	Grasso et al. Neurosurgery. 2009 Oct; 65(4):763-9
	Male Sprague-Dawley Rats	2 hours after surgery / Daily for 1 or 3 days	Neurobehavior: Hemorrhage Volume. Hemispheric Atrophy. Brain Edema. Cell Death: TUNEL staining, Caspase Activity	Better functional recovery. Reduced brain water content. Decreased mean hemorrhage volume and hemispheric atrophy. Reduced apoptosis: lower caspase activities, TUNEL positive cells	Lee et al. J Neurochem. 2006 Mar;96(6):1728-39
SAH	Male New Zealand White Rabbits	5 minutes after SAH induction/ every 8 hrs for 72 hrs	Neurobehavior: Ischemic Lesions. Analysis of Basilar Arteries diameter	Improved neurobehavioral outcome. Decreased ischemic neuronal damage. Reduced vasoconstriction of arteries	Grasso et al. Proc Natl Acad Sci USA. 2002 Apr 16;99(8):5627-31.
	Male New Zealand White Rabbits	* Immediately after injection of autologous blood.	Cerebral Angiography. Vascular Reactivity.	Reduced cerebral vasospasm. Augmented basilar arterial relaxation to acetylcholine	Santhanam et al. Stroke. 2005 Dec;36(12):2731-7
	Male New Zealand White Rabbits	10 minutes after SAH procedure/ Day 2, 4, and 6	Computed tomography perfusion. CT angiography. Neurological Function. Histology	Improved neurological outcomes. Improved cerebral blood flow and microcirculatory flow. Improved tissue perfusion	Murphy et al. J Neurosurg. 2008 Dec;109(6):1155-64.

How does our finding advance the field?

The current knowledge in the field allowed for for the use of erythropoietin in clinical trials as neuroprotective agent in stroke therapy. However erythropoiesis, coagulation and thrombosis were observed in some of the adult patients in one of the primary large center clinical trial using high dose EPO over a three week period (Ehrenreich et al., 2009). The adverse effects observed in that aforementioned study led many veteran EPO researchers to ask is there a future for EPO in stroke therapy (Minnerup et al., 2010)? In an attempt to   answer the aforementioned question we arrived at our study. The key mediators of EPO induced neuroprotection *in vivo* and *in vitro*. Our findings showed that the modulation of TIMP-1, HIF-1α, PHD-2 and MMP-9 are the primary modalities of EPO-induced neuroprotection. This information will prove invaluable in prescribing EPO for stroke therapy. TIMP-1 is a prosurvival protein that is critical for cell survival but could promote excessive proliferation of cells if over-expressed for long periods of time as was observed in cancer. The biphasic master regulator HIF-1α has both beneficial and detrimental properties. Thus extended inhibition of HIF-1α is also detrimental. Additionally MMP-9 is a major player in angiogenesis which is a critical part of wound healing. Because all of the primary mediators of EPO-induced neuroprotection are biphasic the dose and time of administration of EPO are critical to its use in stroke therapy. Thus our response to Minnerup question is that there is a future for EPO in stroke therapy if used in moderation at modest doses for acute administration. The figure below integrated our findings into the current body of know on the mechanism of EPO-induced neuroprotection in stroke therapy.
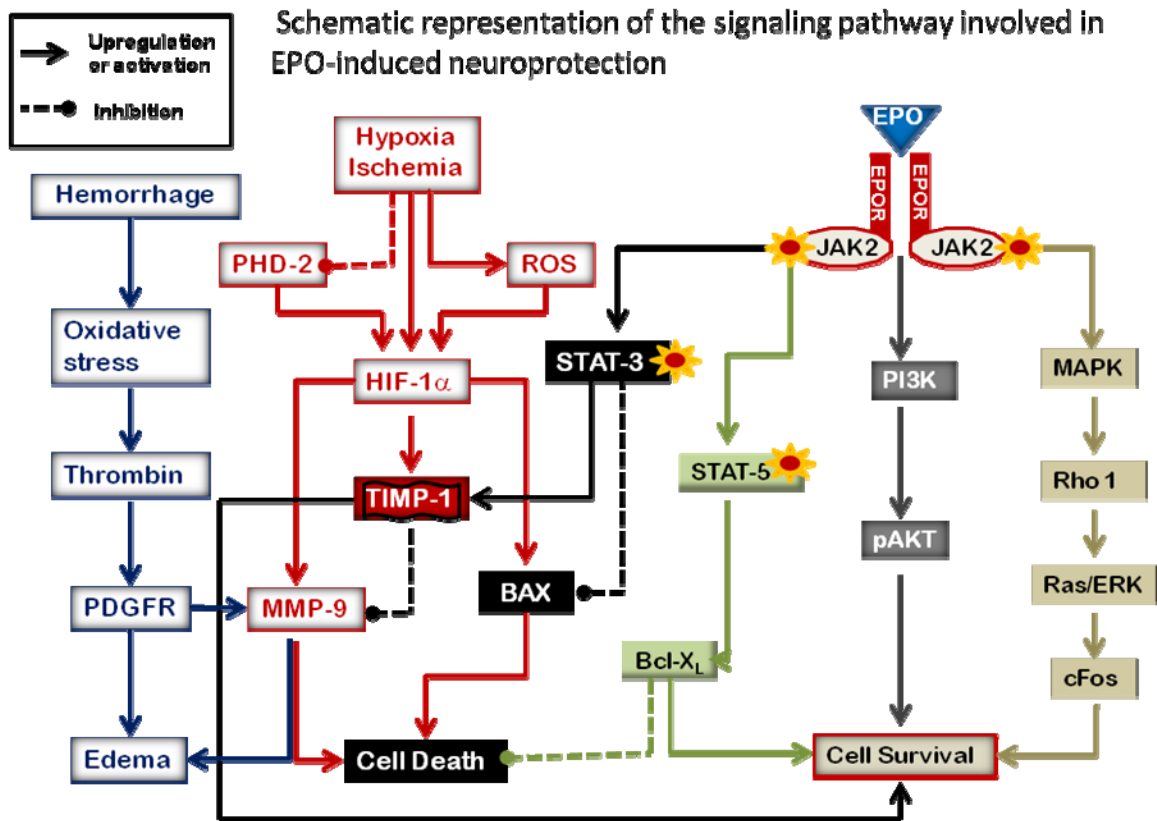
Figure 4.1. Schematic representation of how our findings fit into the current knowledge of the signaling pathways involved in EPO-induced neuroprotection. The primary mechanism of secondary injury due to hemorrhagic strokes is presented in blue. One of the primary mechanism of ischemic injury in presented in red. Matrix metalloproteinase serves as the pivotal molecule leading to pathology in both hemorrhagic and ischemic strokes. The green, grey and tan lines show the known signaling pathway activated downstream of Janus Kinase 2, prior to our study. The black lines show the signaling pathway we elucidated during this study.

Summary/Conclusion

One of the key mechanisms of EPO-induced neuroprotection in hypoxia ischemia in vivo and in vitro is the upregulation of TIMP-1 and inhibition of HIF-1α. EPO induced an upregulation of TIMP-1 via the JAK-2/STAT-3 pathway. The upregulated TIMP-1 inhibited MMP-9 thereby conferring neuroprotection. Additionally EPO also triggered an increase in PHD-2 transcription and translation which lead to inhibition of HIF-1α protein expression. The primary mediators of EPO-induced neuroprotection are STAT-3, TIMP-1 and PHD-2. These molecules appear to signal from two divergent pathways that converge at the point of MMP-9. The common denominator in both pathways, MMP-9, is a biphasic zymogen that is detrimental during acute injury but critical in wound healing. Thus EPO-induced neuroprotection could be a double edged sword beneficial or detrimental depending on the dose and time of administration. However the benefits of EPO treatment in stroke therapy are tremendous and gravely outweigh the adverse side effects of coagulation and thrombosis. Therefore, EPO should be used with cautious optimism.

Prospective

To develop an EPO analog that lacks erythropoietic function. Dissociating the non-hematopoietic function of EPO from the hematopoietic function for treatment of stroke has proven to be challenging. Thus recent efforts have been made to design pharmaceutical analogs that lack the ability to initiate erythropoiesis. Structural analogs asialoerythropoietin and carbamylated-Epo (CEPO) both confers cellular survival and proliferation with minimal erythropoiesis (Erbayraktar et al., 2003; Leist et al., 2004; Adembri et al., 2008). These analogs appear to promote neuroprotection without initiating notable erythropoiesis, coagulation and thrombosis at low doses and for short

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duration. However if the derivatives are able to withstand long treatment without the significant side effects is still to be elucidated. Should I expand on my current study I would elucidate the ability of EPO analog CEPO to confer neuroprotection at high doses for extended duration and the mechanism of the proposed neuroprotection.

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