Molecular mechanisms of neuroprotection in the anoxia tolerant freshwater turtle

By

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MOLECULAR MECHANISMS OF NEUROPROTECTION IN THE ANOXIA TOLERANT FRESHWATER TURTLE

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ABSTRACT

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Cardiac ischemia, stroke and some neurodegenerative disorders are all characterized by cell damage and death due to low oxygen levels. Comparative studies show that anoxia tolerant model systems present a unique opportunity to study “survival" instead of death in the complete absence of oxygen. The freshwater turtle (Trachemys scripta elegans) is unique in its ability to survive total oxygen deprivation for hours to days, as well as reoxygenation insult after anoxia. The broad objective of this study is to understand the modulation of key molecular mechanisms involving stress proteins and VEGF that offer neuroprotection and enhance cell survival in the freshwater turtle through anoxia and reoxygenation.

In vivo analyses have shown that anoxia induced stress proteins (Hsp72, Hsp60, Grp94, Hsp60, Hsp27, HO-1); modest changes in the Bcl2/Bax ratio and no change in cleaved caspase-3 expression suggesting resistance to neuronal damage. These results

were corroborated with immunohistochemical evidence indicating no damage in turtle brain when subjected to the stress of anoxia and A/R. To understand the functional role of Hsp72, siRNA against Hsp72 was utilized to knockdown Hsp72 in vitro (neuronally enriched primary cell cultures established from the turtle). Knockdown cultures were characterized by increased cell death associated with elevated ROS levels.

Silencing of Hsp72 knocks down the expression of Bcl2 and increases the expression of Bax, thereby decreasing the Bcl2/Bax ratio. However, there was no increase in cytosolic Cytochrome c or the expression levels of cleaved Caspase-3. Significant increase in AIF was observed in the knockdown cultures that increase through anoxia and reoxygenation, suggesting a caspase independent pathway of cell death.

Expression of the master regulator of hypoxia, HIF1 alpha and its target gene, VEGF, were analyzed at the mRNA and protein levels. The results showed no significant increase in HIF-1 alpha levels but anoxia induced VEGF. The levels of stress proteins and VEGF returned to control levels during reoxygenation suggesting robust ROS protection mechanisms through reoxygenation.

The present study thereby emphasizes *Trachemys scripta* as an advantageous model to examine anoxia and reoxygenation survival without major damage to the brain due to it's modulation of molecular mechanisms.

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CHAPTER 1: INTRODUCTION

1.1. Anoxia and Failure of Brain

Cardiac ischemia, stroke and some neurodegenerative disorders are all characterized by cell damage and death due to low oxygen levels. Apparent functional impairment of the central nervous system within 5 sec of complete anoxia and total loss of consciousness in 8-12 sec are clinically observed in human brains (Oehmichen and Meissner, 2006); 10 sec of global cerebral ischemia leads to unconsciousness and death occurs within 10 min (Nedergaard and Dirnagl, 2005) demonstrating the sensitivity of neurons to inadequate oxygen supply. Despite occupying only 2 % of total body weight, the brain consumes a substantial portion of the energy budget of the total body (20% of total body oxygen) (Raichle and Gusnard, 2002; Lutz et al., 2003) and thereby becomes the most susceptible organ to energy failure when there is a decrease in oxygen supply. Interruption in oxygen supply to the brain results in a cascade of destructive physiological and molecular events in the neurons, posing a major clinical challenge in the event of stroke or other ischemic events. Hallmark events of anoxic brain failure events include loss of ATP and lethal physiological changes due to inadequate supply of ATP, increased anaerobic glycolysis, acidosis, increased calcium flux, increased excitatory neurotransmitters and collapse of mitochondrial functions and apoptosis/necrosis triggered in part by changes in the molecular balance of pro- and anti-apoptotic factors (Macdonald and Stoodley, 1998; Lipton, 1999, Milton and Prentice,

2007). Upon reoxygenation or reperfusion, additional damage occurs due to an increase in the production of reactive oxygen species (ROS) that overwhelms innate defenses.

ATP depletion and depolarization: Mammalian brains, unable to adjust energy demand and supply during anoxia, rapidly lose ATP due to aberrant changes in mitochondrial oxidative phosphorylation (Kreisman et a., 1981; LaManna et al., 1984; Nilsson and Lutz, 2004). The fall in ATP levels result in failure of Na^+/K^+ ion pumps resulting in decreased ion transport across the membrane (Allen et al., 2005). Within a few minutes of anoxia, there is a loss of ionic gradients with decreases in intracellular K+ and increases in Na^+ leading to depolarization; eventually the neurons cease to function (Hansen, 1985). The rapid increase in extracellular K^+ leads to another catastrophic event known as "anoxic depolarization" (Hansen., 1985; Lutz et al., 2003). The anoxic depolarization inundates the extracellular space with large amounts of neurotransmitters (Glutamate, dopamine, aspartate, GABA (γ-amino butyric acid), glycine and taurine) (Globus et al., 1988; Globus et al., 1989; Perez-Pinzon et al., 1993). High extracellular concentrations of neurotransmitters is not only due to the massive release but also to a chain reaction of neurotransmitters triggering near by cells to release more neurotransmitters, coupled to failed uptake mechanisms (Perez-Pinzon et al., 1993, Milton et a., 2002). High concentrations of neurotransmitters are toxic to the neurons due to over stimulation of their receptors, resulting in fatal consequences. These neurotransmitters include excitatory neurotransmitters dopamine and glutamate. A few minutes into anoxic depolarization and energy depletion, cells die rapidly due to proteolysis, lipolysis, and microtubule disintegration (Hansen, 1985).

Anaerobic glycolysis and lactate acidosis

The negative effects of anoxia/ischemia are exacerbated by the effects of increased anaerobic metabolism. Glucose is the only energy source for the brain metabolism. When oxygen is available, breakdown of glucose (glycolysis) followed by mitochondrial oxidative phosphorylation produces 36 ATP. However, rapid dissipation of oxygen during anoxia switches on anaerobic glycolysis for ATP production which yields only $1/18^{th}$ the energy of oxidative phosphorylation. Conventionally, aerobic glycolysis ends in pyruvate and anaerobic glycolysis ends in lactate (Chih and Roberts, 2003). Focal ischemia increases lactate levels tremendously up to 20 fold higher than the normoxic lactate content of the brain (Ginsberg et al., 1990; Hata et al., 2000).

Formation of lactate during anaerobic glycolysis serves an important role since it regenerates NAD+ from NADH and pyruvate; and NADH is required for oxidation of glucose by astrocytes (Hertz, 2008). However, the production of lactate results in the release of hydrogen ions [H+], and thereby decreasing the pH and making the intracellular environment acidic. The depletion in glucose and ATP levels have also been attributed to decrease in pH levels in the brains of gerbils and rats at the end of ischemia (Munekata and Hossmann, 1987; Paschen et al., 1987). Severe acidosis damages brain function by suppressing glutamate transport (Swanson et al., 1995), triggering lipid peroxidation (Siesjo et al., 1985) and suppressing antioxidant capacity (Ying et al., 1999), although mild acidosis helps in attenuating focal ischemic damage (Simon et al., 1993). Acidosis also induces glycolytic flux (hyperglycemia) that further exacerbates ischemic injury (Myers and Yamaguchi, 1977; Siesjo et al., 1988) and ischemia studies

indicated that the deleterious effects of hyperglycemia in ischemia are due to acidosis (Li and Siesjo, 1997).

Dopamine

Energy loss also results in the release of excitotoxic levels of neurotransmitters such as glutamate and dopamine. Dopaminergic neurons are mostly localized in the substantia nigral region of the brain (Cooper et al., 1996); they send their axons to various parts of the brain controlling movement, behavior, emotions and learning (Bozzi and Borrellli, 2006). Dopamine mediates its effects through dopamine receptors, mainly D1 and D2 receptors that are GPCRs (G protein coupled receptors). Dopamine receptors have been found involved in both neurotoxic and neuroprotective pathways. The neurotoxic pathways act through dopamine receptors activating Erk and c-jun pathways (Chen et al., 2004; Charvin et al., 2005) but antagonists to dopamine receptors induce apoptosis in rat striatum by inhibiting the action of Akt, suggesting a potential neuroprotective role for dopamine (Ukai et al., 2004). While the loss of dopaminergic neurons results in several neurodegenerative disorders such as Parkinson's disease, ischemia, Huntington's disease and epilepsy (O'Neill et al., 1998; Jakel and Maragos, 2000; Bozzi and Borelli, 2006), high dopamine levels are also toxic to neurons (Schmidt et al., 1985; Kita et al., 2003).

Studies of global ischemic animal models have shown huge increases in extracellular dopamine levels from 100- 500 fold during ischemia (Globus et al., 1989; Kondoh et al., 1995) and anoxia stimulated enhanced release of dopamine in rat striatal slices (Buykuysal and Mete, 1999). Improper functioning of dopaminergic systems and inhibition of dopamine uptake mechanisms are primarily involved in triggering the

dopamine surge during ischemia (Akiyama et al., 1991; Bozzi and Borrellli, 2006). High levels of extracellular dopamine result in neuronal injury through modulation of cerebral blood flow and glucose availability, increased ROS (Globus et al., 1988; Remblier et al., 1999), increased release of glutamate and suppression of mitochondrial respiratory chain (Hoyt et al., 1997; Ben-Shachar et al., 2004). Neuronal death was found to be triggered via apoptosis both in vivo and in vitro at increased concentrations of dopamine

(Ziv et al., 1994; Hattori et al., 1998). Recent studies have shown that the mechanisms underlying dopamine induced neuronal apoptosis might be due to increased levels of caspase 3 (Hou et al., 2001) or by inhibition of neuronal apoptotic inhibitory protein (NAIP)(Okada et al., 2005), or through decreased expression of mitochondrial VDAC (voltage dependant anion channel) (Premkumar and Simantov, 2002) .

Glutamate

Glutamate is the primary excitatory neurotransmitters and the excitotoxic effects of glutamate have been studied in greatest detail. Ischemia results in a surge of glutamate ten times higher than the basal level (Guyot et al., 2001); exogenous addition of glutamate to rat cortical neuronal cultures kills the neurons within minutes (Choi et al., 1987) and cell death due to anoxia has been largely attributed to the rise in glutamate levels (Huang et al., 1997). Glutamate exerts its effect by interacting with either the metabotropic receptors (mGluR) or through ionotropic receptors (NMDA(N-Methyl D-aspartate), AMPA (amino-3-hydroxy-5-methylisoxazole-4-propionic acid), and KA (kainic acid)) Evidence from cell culture studies shows that NMDA agonists aggravate neuronal injury and NMDA antagonists protect from ischemic, anoxic and OGD (oxygen-glucose deprived) injuries (Park et al., 1981; Goldberg et al., 1987; Choi et

al., 1987; Monyer et al., 1989; Takizawa et al., 1991), while other studies also implicated AMPA and KA in ischemic injury models (Buchan et al., 1991; Smith and Meldrum 1992). Glutamate stimulation of NMDA receptors allows excess Ca^{2+} into the cell. The role of calcium in excitotoxic neuronal injury has broadly divided the field of excitotoxicty research into NMDA and non-NMDA agonists and antagonists. AMPA/KA stimulates conductance for Na+ while NMDA allows both Na^{+} and Ca^{2}+ flux. Increased glutamate levels upon ischemia or anoxic depolarization thereby overstimulate NMDA receptors and increase cellular calcium permeability, resulting in massive build up of intracellular calcium. Entry of Na+ through AMPA/KA receptor channels and acidosis further triggers the entry of calcium (Dirnagl et al., 1999). The increased release of glutamate (Haberg and Sonnewald, 2006), failed uptake mechanisms of glutamate by astrocytes (Philis et al., 2001), and changes in the ion flux in the sodium, potassium and calcium ion channels (Barbour et al., 1988; Jiang et al., 1992; Taylor et al., 1995; Kimelberg and Mongin., 1998) all contribute to the accumulation of glutamate in the extracellular space in ischemia.

Calcium

Homeostasis of calcium ions (Ca^{2+}) is one of the fundamental cellular processes in the brain since $Ca^{2+}$is involved in many vital metabolic processes such as cell growth and differentiation, neurotransmitter release and synaptic plasticity (Pringle 2004). However, any neuronal insult such as hypoxia, ischemia or stroke alters the balance of influx and efflux of $Ca^{2+}$and very high levels of calcium become toxic to neuronal cells (Mattson et al., 2000). In normoxic conditions, calcium is more concentrated extracelllularly and the influx of calcium is tightly regulated by voltage dependant

calcium channels (VDCC) and the NMDA receptor channels (Chad et al., 1984; Nowak et al., 1984; Pringle, 2004). The efflux of calcium is regulated by calcium ATPase channels and Na^+ / Ca^{2+}ion exchange channel that rely upon Na^+ K^+ ATPase activity. Internal calcium concentration is also maintained by calcium sequestration either by (i) mitochondria or endoplasmic reticulum (ER) or by (ii) binding of calcium to calmodulin and calbindin proteins (Pringle, 2004).

During ischemia with the ATP levels plummeting, a chain of cellular processes takes place that increase intracellular calcium levels. Calcium influx and efflux are altered by the hyperactivation of NMDA receptor (excitotoxicity) (Choi., 1987) and reversal of Na^+ K^+ ATPase , all resulting in a massive build up of intracellular calcium and ultimately to neuronal death (Manev et al., 1989; Arundine and Tymianski., 2003).

High intracellular calcium activates proteases, lipases and nucleases leading to the degradation of macromolecules and due to depletion of ATP during ischemia, resynthesizing these compounds become a challenge (Kristian, 2004). Excessive calcium, in general has been attributed to neuronal death (Choi., 1985; Arundine and Tymianski., 2003) through activation of proteolytic enzymes and disintegration of cytoskeketal proteins including actin and laminin (Furukawa et al., 1997; Chen and Strickland, 1997). Enhanced levels of calcium are observed in the focal ischemic model, where neurodegeneration occurs (Kristian et al., 1998); using antagonists against VDCC and NMDA receptors (O'Neill et al., 1997 ; Pringle et al., 1997; Colbourne et al., 1999); blockade of Na^+ Ca^{+2} ion exchange channels (Breder et al., 2000) and intracellular calcium release (Rao et al., 2000), or calcium chelators (BAPTA) to buffer intracellular calcium are all found to offer neuroprotection (Tymianski et al., 1993).

Blocking calcium entry into mitochondria also prevented neuronal damage during hypoxia/reoxygenation in rodents suggesting a critical role of mitochondria in calcium regulation (Schild et al., 2003).

Functional failure of mitochondria, ROS, cell death

A rise of intracellular calcium above the threshold level makes mitochondria sequester calcium (Zaiden and Sims, 1994), however an overload of calcium into the mitochondria depolarizes mitochondrial membrane potential, opens the mitochondrial permeability transition pore (MPTP), alters the dynamics of the mitochondria and activates apoptotic pathways (Sims and Pulsinelli., 1987; Starkov et al., 2004; Pringle., 2004). Excessive calcium results in uncoupling oxidative phosphorylation in the mitichondria, produces arachidonic acid on activation of Phospholipase A2, and increases reactive oxygen species (ROS) production(Ouyang and Giffard, 2004). Blockade of the mitochondrial transition pore with cyclosporin greatly attenuated calcium and ROS levels (Sullivan et al., 1999) and also prevented neuronal damage in transient forebrain ischemia and hypoglycemia (Uchino et al., 1995; Friberg et al., 1999).

Reoxygenation and ROS

Reoxygenation/reperfusion causes further ROS release, adding to the ischemic damage. Reactive oxygen species are produced endogenously from the mitochondrial electron transport chain or can be induced by external stimuli like cytokines and carcinogens (Adler et al., 1999). Reduction of molecular oxygen results in several forms of ROS that include oxygen free radicals (superoxide anion, singlet oxygen, and the hydroxyl radical) and hydrogen peroxide. When produced at lower concentrations, ROS act as signaling molecules (Sauer and Wartenburg, 2005) but at higher concentrations

ROS damage proteins, lipids and DNA (Valko et al., 2006, Calabrese et al., 2007). Free radicals attack the poly unsaturated fatty acids (PUFA) and produce different lipid peroxidation products that further cause oxidative damage to DNA and proteins (Marnett, 2002). Due to the basal production of ROS, all organisms are endowed with defense mechanisms against ROS overload in the form of antioxidant enzymes, vitamins and amino acids (Droge, 2002), but when the system gets overwhelmed, oxidative stress, cell degeneration and death occurs.

Mitochondria, interestingly, generate ROS and are also susceptible to ROS damage by triggering the apoptotic pathway. Mitochondrial DNA (Mt DNA) alteration along with the increases in ROS generation and apoptosis has been identified as the prime factors for aging and neurological disorders in humans (Copeland, 2003; Lee and Wei, 2007).

Further, opening of MPTP because of high levels of ROS, triggers an avalanche of events: loss of mitochondrial membrane potential, free movement of ions across the membrane, swelling and rupturing of the outer mitochondrial membrane whiich releases Cyt c. Cyt c release triggers the apoptotic cascade by recruiting procaspase-9 to the apoptosome, and ultimately activating Caspase-3 (Dirnagl et al., 2008).

1.2. Ischemic Preconditioning: Endogenous Neuroprotective Mechanisms

However, the mammalian brain does exhibit some defensive mechanisms against ischemic damage. Ischemia triggers gene expression that shifts cells towards a protected phenotype. Endogenous neuroprotective mechanisms came to light after the discovery of the phenomenon of “ischemic tolerance/ischemic preconditionng” (IP/IT). IP/IT demonstrates that small doses of harmful agents/events can prepare the organism to better

face future lethal challenges by shifting gene expression towards a protected, pro-survival phenotype (Webster et al., 1995; Dirnagl et al., 2003). Murry et al. (1986) first reported the phenomenon of myocardial protection utilizing several short cycles of ischemia and intermittent periods of reperfusion, known as Ischemic preconditioning (IP) and delayed effects of IP were observed after 12-72 h (Second window of protection, Marber et al., 1993). Both cardiomyocytes and neurons are highly sensitive to oxygen deprivation (Steiger and Hanggi, 2007), and IP was therefore suggested as a strategy for protection against brain ischemic injury (Matsuhima and Hakim, 1995, Sharp et al., 2004, Blanco et al., 2006). Exposure to hypoxia for 3 h protected neonatal and adult rat brains from the injury of hypoxia combined with ischemia 24 h later showing that "hypoxic preconditioning" is protective in ischemia (Ran et al., 2005). Like myocardial preconditioning, IP in brain involves two distinct phases of protection: the first phase initiates the protective mechanisms immediately with in minutes while a second window of protection develops over hours to days (Dirnagl et al., 2003). IP is therefore a phenomenon that has the extraordinary potential to separate pathological events from endogenously increased protective mechanisms (Dirnagl et al, 2003). Dirnagl et al. (2003) have classified endogenous neuroprotectants involved in IP/IT into three broad categories. (i) "Sensors" that detect the presence of low oxygen and trigger the initial response (ii) "transducers" that transmit the initial trigger response to the effectors and (iii) the "effectors" that mediate the defensive response of the ensuing neuronal protection.

"Sensors" or "Triggers"

The onset of hypoxia/anoxia activates a series of signaling cascades that aids in initiating the defense mechanisms. A number of candidate proteins that may sense the drop in molecular oxygen at cellular level have been suggested, including HIF (Hypoxia inducuble factor) (Semenza, 2007) Cyt b/ NAD(P)H complex (Zhu et al., 1999), K_{ATP} channels (Rodrigo and Standen, 2005), AMP kinase (Tokunaga et al., 2004), Adenosine (Dirnagl et al., 2003) and mTOR (Mammalian target of rapamycin) (Swiech et al., 2007).

HIF-1 is the most important regulator of oxygen sensing mechanisms at the cellular level. HIF-1 consists of two sub units; HIf-1- alpha and HIF-1 beta. HIF-1 beta is constitutively expressed, whereas HIF-1 alpha is oxygen regulated. Under normoxic conditions HIF-1 alpha gets hydroxylated by Prolyl hydroxylase (PH) that facilitates the binding of Von-Hippel Landau factor and directs the complex to degradation through a ubiquitin-proteasome pathway. Under hypoxic conditions, PH is inactivated and HIF-1 alpha dimerizes with HIF-1 beta in the nucleus where the complex regulates the transcription of a range of over seventy genes including glycolytic enzymes and VEGF (Semenza, 2004, 2007). These responses trigger myriad signaling cascades that either initiate the hypoxic defense mechanisms or prepare for reoxygenation stress (Hochachka and Lutz, 2001).

Hypoxia defense mechanisms are also linked to the purine adenosine, which increases in hypoxia due to ATP break down (Wardas, 2002). The role of adenosine and ROS in preconditioning is not fixed as they were reported as triggers (Baxter, 2002; Rodrigo and Standen, 2005; Cohen and Downey, 2008) or mediators (Otani, 2004; Cohen

and Downey, 2008). In both the categories, adenosine was found offering endogenous neuroprotection through increasing cerebral blood flow, suppressing synaptic activity and inhibiting the release of excitatory neurotransmitters (Rudolph et al., 1992). The protective effect of adenosine is mediated through adenosine receptor A1R. Agonists to A1R receptors conferred neuroprotection by suppressing Ca2+ influx and glutamate release (Rudolphi et al., 1992; Wardas, 2002). Opposite effects are mediated through A2A (subtype of A2) receptors that enhances excitability and synaptic transmission. Recent studies implicate A2A antagonists in the treatment of Parkinsons disease where blocking of A2A receptors inhibits defective dopamiergic transmissions (Chen et al., 2003).

"Transducers"or "Mediators"

Transducers typically involve protein kinases that phosphorylate the final effector proteins that aid in suppressing pro-death pathways (Dirnagl et al, 2008). The multiple cellular cascades that mediate the danger signal include: mitochondrial ATP sensitive potassium channels (K_{ATP} channels) (functions both as initiator and transducer) (Oldenburg et al., 2002), Mitogen activated protein kinases (MAP kinases) (Nishimura et al., 2003) , Phosphokinase C or PKC (Raval et al., 2003) and PI3K(Phosphoinositide-3 Kinase) / Akt (Hillion et al., 2006) signaling that can modulate the action of effector proteins.

Mito K_{ATP} channels

Noma (1983) first reported that K+ channels are regulated by the presence of ATP levels and has shown that increased levels of ATP suppressed K^+ channel activity in the mammalian heart. Decreased ATP levels open the K_{ATP} channels, increase the

conductance of K^+ and thereby prevent membrane excitability in the heart during ischemia (Smallwood et al., 1990; Nichols et al., 2004). K_{ATP} channel openers enhanced ischemic preconditioning and mostly the protection was offered by the mito K_{ATP} channels rather than the plasma membrane K_{ATP} channels (Liu et al., 1998; Sanada et al., 2001). Bajgar et al (2001) have first reported the presence of mitochondrial K_{ATP} channels in the rat brain. Mito K_{ATP} channels were found protective in the heart and the brain against ischemia (Grover et al, 2003; Raval et al., 2007). Opening of mito K_{ATP} channel prevents from ischemic injury by suppressing ROS formation (Mayanagi et al., 2007), blocking Bax translocation and release of cytochrome c (Liu et al., 2002), reducing calcium levels, and preventing the formation of the mitochondrial transition pore (Wu et al., 2006).

MAP kinases

MAP kinases are serine-threonine kinases and signal transducing enzymes that are involved in a wide variety of cellular functions including cell growth and differentiation and cell death (Milton et al., 2008). MAP kinases are part of the three tier signaling cascade where an external stimulus such as a growth factor stimulates the MAP kinase kinase kinase that in turn activates MAP kinase kinase and ends in phosphorylation of MAP kinases. Activation or phosphorylation of MAP kinases regulates transcription factors that eventually modulate gene expression and alter the fate of the cell (Craig et al., 2008). Mammalian MAP kinases are broadly classified based on the regulatory functions: ERK (Extracellular signal regulated kinase) activates a cell survival mechanism through activation of pro-survival proteins such as cAMP response element binding protein (CREB), Bcl2, and suppression of apoptotic proteins such as

Bad, Bim and caspases (Hetman and Gozdz, 2004). Though ERK was mostly considered protective, the action of ERK as a pro- or anti- survival was found to be determined by the factors that activate ERK, for example, when activated by ROS or cytokines ERK is detrimental (Zhuang and Schnellmann, 2006; Sawe et al., 2008). JNK (c-jun Kinase) and p38 are stress activated kinases that are apoptotic inducers (Irving et al., 2000), though the role of JNK as pro/anti apoptosis is still a topic of debate in preconditioning (Guan et al., 2006).

Akt and PKC

Akt/PKB (phosphokinase B) is a pro-survival, serine-threonine kinase that is triggered by growth factors and acts through phosphorylation of PI3K (Phosphoinositide-3 kinase) (Zhao et al., 2005). Downey et al (2008) have shown that PI3K activates ERK that in turn activates mito K_{ATP} channels that result in increase in K+ and ROS. Increases in ROS activate PKC (Protein kinase C). PKC activated in the presence of Ca+2 and DAG (Diacyl glycerol) is involved in several cellular processes including cell differentiation and changes in ion channel conductance. PKC epsilon, an isoform of PKC family, is a potential therapeutic target for neuroprotection (Van Kolen et al., 2008). Ischemic preconditioning in rat brains resulted in increased activity of ERK, Akt and PKC epsilon while no significant changes were observed with JNK and p38 (Raval et al., 2003; Jones and Bergeron, 2004; Gao et al., 2008). Recent studies have shown pro-survival mechanisms ERK and AKT are active even during reperfusion/postconditioning in myocardial preconditioning and were termed as Reperfusion Injury Salvage Kinases (RISK) (Hausenloy and Yellon, 2007).

EFFECTORS

Effectors constitute the final executioners of endogenous neuroprotective mechanisms. Ischemic Preconditioning consists of two phases: an early or rapid phase that is transient and remains effective for only a few hours, and a second phase that offers protection 24- 48 h later. The second phase is called late preconditioning or the second window of protection (SWOP) where there is an opportunity to synthesize proteins that delay cell death (Dirnagl et al., 2003; Yellon and Downey, 2003). It has been suggested that triggering the end-effectors in "late preconditioning" (Yellon and Downey, 2003) that mediate protection against cell death could provide a route for future therapeutic agents. Such effectors include heat shock proteins, Bcl2, Vegf, ROS and GABA.

Heat shock proteins

Heat shock proteins (Hsp's) are molecular chaperones that aid in the maturation and repair of proteins. The presence of heat shock proteins were first discovered in *Drosophila* subjected to heat stress (Rittosa, 1962). More than fifty years of research have shown numerous functions associated with the heat shock proteins that are evolutionarily conserved from prokaryotes to eukaryotes. Heat shock proteins are induced by are stressors other than heat such as toxins, metals, alcohol and hypoxia/ischemia and thereby popularly known as stress proteins. The mammalian heat shock proteins are categorized based on their molecular weight as high molecular weight Hsp's (Hsp110- 60 kD ; Hsp90, Hsp70, Hsp60) and low molecular weight or small Hsp's (Hsp11- 30kD ; Hsp25/Hsp27) (Garrido et al, 2001). The Hsp70 family of proteins is the most studied heat shock protein group and has several isoforms ; the constitutively expressing Hsc73/Hsp73, inducible Hsp70/Hsp72, Grp 78 (the

Endoplasmic reticulum isoform) and Hsp75/Mortalin (the mitochondrial isoform) (Giffard et al., 2008). Hsp72/73 are heavily involved in myocardial protection as end-effectors in anti-apoptotic mechanisms (Corneleusen et al., 2003, Guisasola et al., 2006) and also in neuroprotection during ischemia and stroke (Hoehn et al., 2001, Yenari et al., 2005, Badin et al., 2006). Hsp72, apart from its chaperoning abilities, has the ability to inhibit apoptosis by its interaction with pro-apoptotic proteins and also by enhancing the levels of anti-apoptotic proteins (Giffard and Yenari, 2004). Hsp72 acts in the apoptotic cascade (Yenari et al., 2005) at various points including elevating the levels of Bcl2 (Kelly et al., 2002), binding with apoptosome activating factor (Apaf-1) to prevent the formation of apoptosome (Beere et al., 2000) and blocking the action of the pro-apoptotic JNK pathway (Stankiewicz et al., 2005).

VEGF

Another effector mechanism involves vascular endothelia growth factor (VEGF), one of the major HIF targeted genes that regulates vascular permeability, angiogenesis, and neurogenesis and also acts as a neurotrophic factor (Storkebaum et al., 2004). In neonatal rats ischemia increased the levels of HIF-1 alpha and VEGF simultaneously (Ran et al., 2005); blocking of VEGF or blocking the mediator pathways such as Akt in neuronal cell cultures attenuated the protective effect (Wick et al., 2002); while exogenous addition of VEGF to oxygen glucose deprived cells (OGD simulating ischemia) rescued the neuronal cells from cell death (Jin et al., 2000).

Bcl2

The Bcl2 family of proteins are major players in the mitochondrial apoptotic cell death pathway. The Bcl2 family consists of a number of members with opposing actions,

there are both pro- and anti- apoptotic proteins classified based on the presence of BH (Bcl2-homology) domains (Adams and Cory, 2001). BH3 only domain proteins (Bax, Bik, Bim) are killer proteins that either sequester the anti-apoptotic Bcl2 proteins (Bcl2, Bcl_{XL}) or bind to another protective protein, and render them inactive (Adams and Cory, 2001). The localization of these Bcl2 family members to the mitochondria initiates the apoptotic cascade: the binding of Bax keeps VDAC open and thereby inducing apoptosis with the release of cytochrome c that forms the apoptosome with caspase 9 and Apaf1 and binding of Bcl2 to VDAC inhibits apoptosis (Shimizu et al., 1999). So, the balance of Bcl2 family members decides the fate of the cell either by triggering the sequestering or formation of the apoptosome (Admas and Cory, 2001). IP in rat hearts reduces apoptosis by enhanced levels of Bcl2 (Maulik et al., 1999) and overexpression of Bcl2 in a focal ischemia model has significantly attenuated neuronal death (Zhao et al., 2003) promising a huge potential for Bcl2 as a target for therapeutic purposes.

ROS

Along with increased cell death when produced in excess, ROS are considered a trigger, mediator and effector for ischemic preconditioning (Otani, 2004). Increased extracellular glutamate, dopamine and calcium during ischemia and reperfusion leads to mitochondrial dysfunction and a surge in ROS production. Increased production of ROS works in early phase myocardial preconditioning through the activation of tyrosine kinases, src-kinases (Otani, 2004) that further activate PKC epsilon, ERK and the PI3k/Akt survival pathways (Liu et al., 2008). During reperfusion ERK and Akt act on target proteins that aid in pro-survival pathways while PKC epsilon targets Mito K_{ATP} channels that mediate IP; ROS, mito K_{ATP} , and PKC epsilon thus act in concert to

provide tolerance during ischemia/reperfusion (de Ruijter et al., 2003; Penna et al., 2006). IP/hypoxic preconditioning also reduced ROS levels and increased antioxidant enzymes suggesting IP maintains the redox equilibrium (Nakatsuka et al., 2000; Perez-Pinzon, 2007).

GABA

GABA (Gamma amino butyric acid) plays a central role in the brain due to its effects opposing the actions of glutamate (Schwartz-Bloom and Sah, 2001). GABA is the main inhibitory neurotransmitter in mammals (Siesjo, 1978); GABA is synthesized from glutamate and its effects are mediated through $GABA_A$ and $GABA_B$ receptors, conducting Cl^- and HCO_3^-, respectively (Bowery, 1989; Burt and Kamatchi., 1991). Activated GABA receptors increase the conductance of Cl^-, HCO_3^-, and K^+, increasing the membrane potential and hyperpolarizing the cell.

During ischemia, levels of GABA were elevated in the cortical region, striatum and hippocampus of the mammalian brain (Globus et al., 1991; Haberg et al., 2006). Several mechanisms are thought to affect the accumulation of GABA during ischemia: synthesis of GABA from glutamate is carried out without oxygen and mitochondrial function (Mrsulja et al., 1978), while GABA breakdown is oxygen dependant; reversal of GABA transporters or conductance across ion channels, and exocytotic release of GABA are due to increased Ca^{+2}(Saransaari and Oja, 2008). Increased levels of GABA shifted the balance away from the excitatory effects of the neurotransmitter glutamate in rat ischemic model during IP, and thus offered neuroprotection (Dave et al., 2005).

Dirnagl et al. (2003) suggested that IP in the mammals has two distinct advantages. One is that it shows enhanced endogenous protective mechanisms during

ischemia and second is that these protective mechanisms elicit "memory" of rescue mechanisms to defend against future ischemic insults. The amazing ability of survival strategies used by some vertebrates during hibernation, diving and anoxia follow a similar pattern of elevated innate defense mechanisms.

1.3 Anoxia and Brain that Survives

Freshwater turtles of the genera *Trachemys* and *Chrysemys* constitute the best studied species that survive extended periods of anoxia for days to months depending upon the temperature (Jackson, 2000). The amazing ability to tolerate oxygen deprivation is not something common to all reptiles or lower vertebrates , as most of the reptiles can survive only for short period of time(30- 90 min) without oxygen (Belkin, 1963; Storey, 2006) (Table I). There are only a few species other than the turtle, such as the crucian carp, that can sustain life without oxygen (Nilsson, 1990). This shows that anoxia tolerance is a result of complex physiological and molecular adaptations in these unique organisms that can survive in oxygen depleted environments, and the mechanisms of anoxia-tolerance demonstrate how turtles or other anoxia tolerant vertebrates have evolved to survive without oxygen. The physiology behind anoxia tolerance in the freshwater turtles (*Trachemys scripta elegans),* has been studied for the past two decades with a shift over the last few years of turtle research towards understanding the molecular mechanisms. The focus of the present section is to understand the critical pathways of survival in the turtle brain and the similarities/dissimilarities between endogenous neuroprotective mechanisms during IP in the mammals to the anoxia induced processes in the turtle (Fig 1). Anoxia tolerance in the turtle can be broadly divided into three distinct phases: early phase or the initial transition to anoxia (first few hours)

resisting the stress of oxygen deprivation (1), late phase or maintenance phase of anoxia (h-days) (2) exhibiting drastic downregulation of metabolic activities (comatose) but still maintaining neuronal function and to recover and resist oxidative stress of ROS in reoxygenation phase (3) in the face of oxygen availability (Milton and Prentice, 2007) . Each phase has unique physiological and molecular adaptations.

Initial transition phase

The first few minutes to first few hours of anoxia can be considered an initial transition phase. Severe reduction in metabolic activities (hypometabolism) is the immediate response to survive in the initial few hours of anoxia, so that the energy demand is reduced and the energy produced by anaerobic glycolysis is sufficient to carry out basal metabolic activities. Unlike mammals where ATP levels drop in few seconds of anoxia, ATP levels are maintained at a steady state in anoxia in the turtles (Lutz et al., 1984). The core strategy of the turtle to survive anoxia therefore depends on balancing the equation of energy supply and demand.

Physiological adaptations

A drop in ATP levels was noticed in the initial transition phase i.e in the first 1h of anoxia (Lutz et al., 1984) because of the decrease in energy and oxygen supply, but this is soon matched with downregulation of the metabolic activities that demand energy (Nilsson and Lutz, 2004). Metabolic levels drop by 40% from the control normoxic conditions in the turtle brain (Kelley and Storey, 1988). Although the ATP levels are maintained through out anoxia, the initial phase experiences decreased ATP levels that correspond with increases in the levels of adenosine (Nilsson and Lutz, 1992). As in the mammal, elevated levels of adenosine were observed to have multiple protective effects

in the turtle such as increased glycolysis, increased cerebral blood flow, and decreased release of excitatory neurotransmitters, and thus aids in avoiding anoxic depolarization (Nilsson and Lutz, 1992; Perez-Pinzon et al., 1993; Hylland et al., 1994; Milton et al., 2002; Milton and Lutz, 2005) and acts as an inhibitory neuromodulator that aids in initial metabolic suppression (Nilsson and Lutz, 2004).

The turtle avoids anoxic depolarization by shutting down its ion channel conductance ("channel arrest") because ion flux is one of the most energy expense process in the brains utilizing almost 50% of total energy consumed (Lutz et al., 2003). The conductance of K^+ ions decreases by 50% at the end of 1 h of anoxia and reduces further to 70% basal by 2 h anoxia (Pek and Lutz, 1998). Opening of K_{ATP} channels and activation of adenosine receptors (A1AR) reduces the efflux of K^+ ions, avoiding depolarization (Pek and Lutz, 1997; Pek and Lutz, 1998). Na^+ channels were also found inactivated by 42% of the basal resulting in attenuating the threshold levels of the action potential ("spike arrest") (Perez-Pinzon et al., 1992, Sick et al., 1993). Calcium influx also reduces as the animal progresses into anoxia indicating inhibition of Ca^{2+} channels (Bickler and Gallego, 1993). Reduction in Ca^{2+} influx was found to be mediated by suppressed activity of NMDA receptors that decrease by 50-60% of basal with in the first 10 min of anoxia (Bickler et al., 2000).

Pamenter and Buck (2008) reported that regulation of GABA receptors, K^+ channels and antioxidant enzymes, in concert, protected from "mild anoxic depolarization" (MAD) during the first hour of anoxia in the turtle cortex. They have also observed that MAD, unlike mammalian neurons, does not lead to release of excitotoxins and the membrane potential returns to resting membrane potential on reoxygenation.

One other strategy of survival during anoxia is maintenance of low levels of excitatory amino acids (Milton and Lutz, 1998). Extracellular glutamate does not increase due to decreased release and sustained reuptake mechanisms (Milton et al., 2002). Like glutamate, dopamine levels are also kept low during anoxia due to suppressed release and continued reuptake of dopamine (Milton and Lutz, 1998; Milton and Lutz, 2005). Interestingly, the decreased release of glutamate and dopamine were mediated by activation of adenosine A1R receptors and the K_{ATP} channels. Blocking either of these resulted in increases in glutamate and dopamine. This also fits the energetics of turtle anoxia as activation of K_{ATP} channels and adenosine lower the release of neurotransmitters and thereby reduce energy expensive reuptake mechanisms (Milton and Lutz, 2002, Milton and Lutz, 2005, Milton and Prentice, 2007).

Molecular adaptations

There have been recent reports of adaptations for anoxic survival at the molecular level as well. High constitutive levels of heat shock proteins Hsp72 and Hsc73 were observed in normoxic conditions. Exposure to 4 h of anoxia increased the expression levels of both Hsp72 and Hsc73 (Prentice et al., 2004). Unlike mammals, the induction of cognate Hsc73 in the turtle brain suggests "constitutive preconditioning" that may prepare the turtle to anoxia and reoxygenation stress.

Milton et al (2008) have shown upregulation of pro-survival pathways Akt/PI3k and ERK during the first hour of anoxia. Also seen was the inhibition of p38 expression during the first hour of anoxia. Blocking adenosine receptor through aminophylline resulted in suppression of ERK and Akt and increased levels of p38, indicating the role of adenosine in regulating the MAP kinases and Akt pathways (Milton et al, 2008).

Increased adenosine levels in the first few hours and increases in phospho ERK and Akt suggest a combined defense mechanism against anoxia.

Increased levels of adenosine, decreased levels of glutamate and opening of K_{ATP} channels are the mechanisms of neuroprotection found common in anoxic turtles and in hypoxia/ischemic preconditioned rats (Schultz et al., 1994; Perez-Pinzon et al., 1998; Johns et al., 2000; Perez-Pinzon, 2007). Increases in heat shock proteins are also similar to the mammalian ischemic response and show that the turtle is under stress during anoxia. MAP kinases are activated at different time points in the mammals following cerebral ischemia: JNK or nuclear JNK was found activated at the end of 1 h after reperfusion in the dying neurons or the ischemic core (Ferrer et al., 2003) but Repici et al. (2007) have shown the expression of pJNK in the penumbra by the end of 6 h after ischemia and blocking of JNK reduced the infarct size. Anoxia did not alter the expression of pJNK in the turtle brains. Focal ischemia in rats resulted in increased expression of phosphorylated cytoplasmic ERK, p38 and JNK, 4 h after reperfusion in the penumbral region of the infarct (Ferrer et al., 2003) whereas the upregulation of pro-survival MAP kinases and shut down of ion channels with in the first few hours of anoxia, are therefore unique to the turtle, enabling it to counteract the deleterious effects of anoxia.

Maintenance phase

After surviving the initial hour or few hours without oxygen and combating the potential destructive events associated with energy loss, the freshwater turtle now faces the challenge of maintaining the functionality of the brain during prolonged anoxia into

long hours to days and weeks (Milton and Prentice, 2007). Again, we see adaptations at both the physiological and molecular level.

Physiological adaptations

Subsequent to the initial drop, the ATP levels return to basal when the animal progresses into long term anoxia (h –days), (Lutz and Milton, 2004). Continued anaerobic glycolysis replenishes the ATP levels to carry out the basal metabolic activities such as electrical activity, ion channel flux and neurotransmitter release that aid the animal to maintain functionality.

However, long term anaerobic glycolysis results in the accumulation of high levels of lactic acid. Turtles have evolved a mechanism to avoid acidosis; utilizing the buffering capacity of the turtle shell enables the removal of lactic acid by fluxing carbonate from the shell to the cells to buffer lactic acid and lactic acid moves from the cells to the shell to get buffered (Jackson, 2000).

Extracellular concentrations of glutamate and dopamine are stabilized due to continued release and reuptake mechanism during extended anoxic periods (Milton et al., 2002; Milton et al., 2005). The cycle of release and reuptake of neurotransmitters is maintained at a scaled down level in the turtle in long term anoxia since some level of neurotransmitters is apparently essential to maintain functional integrity of the neuronal network (Biegon et al., 2004). As the ATP levels becomes steady in anoxia , the K_{ATP} channels close and the reduction in glutamate release relies on adenosine and GABA receptors (Thompson et al., 2007).

Opposite to the levels of excitatory neurotransmitters, the extracellular and tissue concentrations of inhibitory GABA increase by 45-60% at the end of 2-4 h of anoxia and

by 13 h of anoxia, GABA increases by 127% (Nilsson and Lutz, 1990). With such a high concentration of GABA, the effect might mimic the anesthetic effect that is protective in mammalian ischemia (Nilsson and Lutz, 2004). The levels of GABA start to rise at around the same time that levels of adenosine start falling, suggesting modulations of physiology during long term anoxia. Along with GABA, the levels of GABA receptors are also increased during prolonged anoxia (Nilsson et al., 1990). In mammalian ischemic studies, IP results in elevated levels of GABA, glutamate decarboxylase(GAD) and $GABA_B$ receptors suggesting GABA as one of the key factors offering neuroprotection, mediated through $GABA_B$ receptors (Dave et al., 2005). Preliminary results in our laboratory have indicated the presence of GAD during normoxia and anoxia. Jin et al (2003) have suggested a model where a complex is formed between glutamate, GABA and Hsc73 that facilitates the packaging of GABA. The presence of considerable levels of GAD and Hsc 73 during anoxia supports such a mechanism for GABA release for long tem anoxic survival.

Molecular adaptations

Heat shock Proteins

Long term anoxia also leads to changes in the expression of heat shock proteins. In long term anoxia, increased expression of the constitutive heat shock protein Hsc73 was reported by Prentice et al. (2004). Continued increase in the expression of Hsc73 might be due to its role in GABA synthesis during long term anoxia (Jin et al., 2003). Increased levels of Hsc73 also might play a role in maintaining synaptic integrity during anoxia as observed in mouse brain under hyperthermia. Recent reports have shown induction of constitutive Hsc73 and relocation to synapse rich areas during hyperthermia

in mouse brain where it aids in refolding the proteins suggesting a neuroprotective role for Hsc73 in maintaining synaptic function during stress (Brown, 2007; Chen and Brown, 2007).

High basal expression of inducible Hsp72 that was further induced in short term anoxia but was found to decline to normoxic levels by the end of 12 h of anoxia (Prentice et al., 2004). Upregulation of Hsp72 suggests the plausible protective role during transition to anoxia and might also prepare the animal for the stress of reoxygenation.

Kv channels

One of the important physiological adaptations against anoxia in the turtle brain is reduction of ionic flux or channel arrest. mRNA levels of Kv channels that regulate the flux of potassium were downregulated to 18.5 % to the normoxic control during 4 h of anoxia and returned to basal levels after reoxygenation (Prentice et al., 2003). Reduced gene expression of Kv channels would decrease excitability and thereby conserve energy utilization during anoxic energy crisis (Lutz and Milton, 2004). Regulation of Kv channels is a significant observation since the expression levels are modulated by the presence of oxygen in the turtle brain. These results underscore the role of Kv channels as oxygen sensors similar to the mammalian Kv channels in smooth muscle cells that are hypoxia regulated (Osipenko et al., 2000; Sweeney and Yuan, 2000; Patel et al., 2001).

HIF1- alpha (Hypoxia inducible factor1-alpha)

Out of the many putative oxygen sensors one major oxygen sensor is a transcription factor, HIF1- alpha. Hypoxia in mammalian fetal brain or adult brain stabilized and induced HIF1-alpha protein levels while expression of mRNA was found either stabilized or induced based on the experimental model (Jin et al., 2000; Sharp et

al., 2001; Trollman et al., 2003). Anoxia did not induce HIF1- alpha mRNA levels in the turtle brain, suggesting post translational modification of HIF1 alpha (Prentice et al., 2003).

NF- kappa B

Another transcript factor of key importance is NF-kappa B. NF-kappa B is a transcription factor that is activated in ischemia in the brain and also regulates the inflammatory responses and apoptosis (Haddad et al, 2002; Martindale and Holbrook, 2002). Lutz and Prentice (2002) have shown maximal binding of NF- kappa B to the DNA at the end of 6 h of anoxia suggesting a role in the maintenance phase.

Bcl2 family

Haddad (2007) has shown the differential regulation of the apoptotic regulators of Bcl2 family, Bcl2 and Bax in *Chrysemys picta* during submerged hypoxia. Increased levels of Bax were found in long term hypoxia: at the end of 3 days of hypoxia and also at the end of 1 week of hypoxia. Bcl2 was also elevated but the increase was 3-4 folds lower than the Bax protein levels, suggesting prolonged hypoxia in turtles increases Bax/Bcl2 ratio and thereby inducing pro-apoptotic mechanisms.

MAP kinases and Akt

The serine –threonine kinases ERK and Akt phosphorylate multiple proteins involved in cell survival, including the apoptotic regulators. Submerged anoxia in turtles did not alter the levels of MAP kinases (ERK, JNK andp38) in *Chrysemys* (Greenway and Storey, 2000) while exposure to anoxia (Nitrogen) resulted in differential expression of MAP kinases in *Trachemys* (Milton et al., 2008). ERK and Akt that were upregulated after exposure to 1 h anoxia in the freshwater turtle returned to basal levels at the end of 4

h of anoxia; suppressed levels of p38 also returned to base levels and JNK was not affected by 4 h of anoxia (Milton et al., 2007). The initial surge in adenosine declines, (Nilsson and Lutz, 1991) ATP levels return to normoxic levels and protein synthesis decrease (Fraser et al., 2001) by 4 h of anoxia when the animal enters into a comatose mode and survives on anaerobic glycolysis (Lutz et al., 2003). Therefore, long term anoxic phase depends on maintenance of a basal mode of survival and might not need greater modulation of proteins than the initial vulnerable transition phase where MAP kinases and Akt were differentially regulated.

Although brain ischemia halts general protein synthesis, increased synthesis of critical proteins such as stress proteins has been previously noted in preconditioned mammals (Frasier et al., 2001; Furuta et al., 1993; Kokubo et al., 2003). Like mammals, the turtles upregulate selected genes/proteins (Storey, 2007) such as, induced Hsp72, cognate Hsc73, Bcl2, MAP kinases and Neuroglobin during anoxia (Milton and Prentice, 2007). Prolonged anoxia matches up with the late preconditioning phase of the mammals where enhanced levels of effectors proteins were observed that attempt to rescue the ischemic penumbra.

Reoxygenation or recovery phase

Physiological and Molecular adaptations

The mammalian brain upon ischemia-reperfusion is prone to experience massive burst of ROS. The brain is highly susceptible to ROS damage due to its high consumption of oxygen, lipid content and low antioxidant capacities (Leutner et al., 2001). “Redox homeostasis” is necessary for regular functioning of brain, and the exaggerated

production of ROS, acts as a critical signaling molecule in immune responses, the cell death cascade, MAPK cascade, aging, and disease (detailed review by Droge W, 2002).

Much less data is available regarding the mechanisms involved in the recovery phase in the turtle. In the turtle, tissues are reoxygenated within ten minutes of oxygen supply restoration (Fernandes et al., 1997) with minimal side effects. The defense strategy to prevent ROS damage in turtles is presumed to be high antioxidant levels with increased levels of SOD in anoxia and overall higher levels of SOD, catalase and GPOX in comparison to other ectothermic vertebrates (Willmore et al., 1997). The turtle brain has 2-3 times higher concentration of ascorbic acid than the mammals (Rice et al., 1995) with no lipid peroxidation damage in anoxia and reoxygenation (Rice at al., 1995). Elevated levels of protective genes like Hsp72/73, c-fos, and c-jun were found in turtle which are protective during hypoxia in mammals (Lutz and Prentice, 2002). Milton et al (2007) for the first time measured physical levels of ROS both *in vivo* and *in vitro* of the turtle. Release of ROS in turtles in reoxygenation did not increase in relation to the normoxic levels of ROS; levels of ROS decreased during anoxia and returned only to the normoxic levels on reoxygenation in both the tissues and the cell culture samples. Blocking adenosine receptors resulted in increased ROS production while adenosine receptors increased survival rate and decreased the production of ROS (Milton et al., 2007). We have also found that turtle neurons are vulnerable to exposure of hydrogen peroxide, showing that the turtle survives the stress of repeated anoxic-reoxygenation events not by being immune to high levels of ROS but by activating mechanisms that eventually lead to suppression of ROS (Milton et al., 2007).

Inhibition of ROS production might be a result of complex physiological and molecular adaptations that rescued the turtle from anoxia and later maintained the neuronal integrity in long-term anoxia, such as the time course of adenosine release, K_{ATP} channels opening and activation of ERK and Akt and also increased expression of heat shock proteins and antioxidant enzymes.

Oxidative stress restrains the ability of neurons to resist apoptosis and excitotoxicity (Mattson et al., 1999). An increase in extracellular glutamate levels due to activation glutamate/NMDA receptors leads to massive influx of intracellular calcium and results in neuronal damage in cerebral ischemia (Yin et al., 2005) and Huntington's disease (Fan and Raymond, 2007). Glutamate excitotoxicity in neuronal damage or death is not only related to the rise in calcium but also due to the breakdown of mitochondrial membrane potential and the release of ROS (Kannurpatti et al., 2004, Kahlert et al., 2005). Cell death of nigral dopaminergic neurons in Parkinson's disease is primarily because of oxidative stress (Yoo et al., 2003). Subjecting the dopaminergic neurons in cell culture to 6-hydroxy dopamine results in cell death, confirming the susceptibility of these neurons to oxidative stress (Collier et al., 2003).

During anoxia, the turtle avoids the major catastrophe of excitotoxicty by silencing the NMDA receptors, decreasing the release of calcium, increasing adenosine levels, opening of K_{ATP} channels and ultimately lowering glutamate and dopamine levels. Blocking of adenosine resulted in increased ROS, glutamate and dopamine release, emphasizing that there are interlocking pro-survival mechanisms that might be efficiently operating from anoxia through reoxygenation. Haddad (2007) has shown differential regulation of MAP kinases, Bcl2, Bax and Procaspases on reoxygenation; p38

was decreased, Bcl2 did not alter to a large extent but the levels were lower than Bax; Bax was enhanced 4 times the basal and procaspases were elevated without cleavage after 1 day reox/3 d anoxia suggesting that pro-survival and the apoptotic pathways are in constant modulation in these anoxia tolerant species to ensure cell survival.

1.4. Goals of the Study

The overall objective of the present study is to understand the modulation of key molecular mechanisms that offer neuroprotection and enhances cell survival in the freshwater turtle through anoxia and reoxygenation.

The goals of this study are to analyze (1) the stress protein expression in anoxia and reoxygenation, specifically Hsp72 and its role in regulation of cell syrvival and also (2) to determine the expression levels of VEGF and HIF1 alpha in the anoxic tolerant brain of the freshwater turtle.

It is known that high levels of inducible Hsp72 were found in normoxia and anoxia induced cognate Hsc73 and Hsp72, suggesting the importance of constitutive preconditioning and the role of heat shock proteins during anoxia (Prentice et al., 2004). The objective of the stress proteins study in chapter 2 is to examine the modulation of heat shock proteins and apoptotic regulators during anoxia and reoxygenation. Here we report immunohistochemical studies of neuronal preservation in the face of anoxia and reoxygenation in the turtle brain. This study is based on the hypothesis that freshwater turtle prevents neuronal damage during anoxia and reoxygenation by upregulating stress proteins and stabilizing the apoptotic proteins.

In furthering our understanding of stress proteins, we hypothesized that upregulation of Hsp72 prevents the activation of pro-apoptotic pathways and thereby

increases cell survival. In chapter 3 the effect of knocking down the expression of Hsp72 through siRNA and its downstream effects on apoptosis are studied. In order to understand the functionality of Hsp72, the major step will be to develop neuronal cell cultures. As the isolation of neuronal cell cultures will be the first time, crucial goal of the project is also to validate the cell cultures with neuronal markers and then perform the gene knockdown experiment to understand the downstream pathways.

Stabilization of mRNA levels of HIF-1 alpha during anoxia has been observed in the turtle brain (Prentice et al., 2002). There were no studies on the expression of one of the key targets of HIF-1 alpha, VEGF, a neuroprotectant, in anoxia and reoxygenation in the freshwater turtle. The hypothesis was anoxia triggers HIF1 alpha and VEGF protein levels and attenuates neuronal damage during anoxia and reoxygenation. The major objectives in chapter 4 are to (a) analyze the mRNA and protein levels of VEGF (b) analyze the protein levels of HIF-1 alpha and (3) determine the DNA binding of HIF-1 alpha during normoxia and anoxia.

Tables

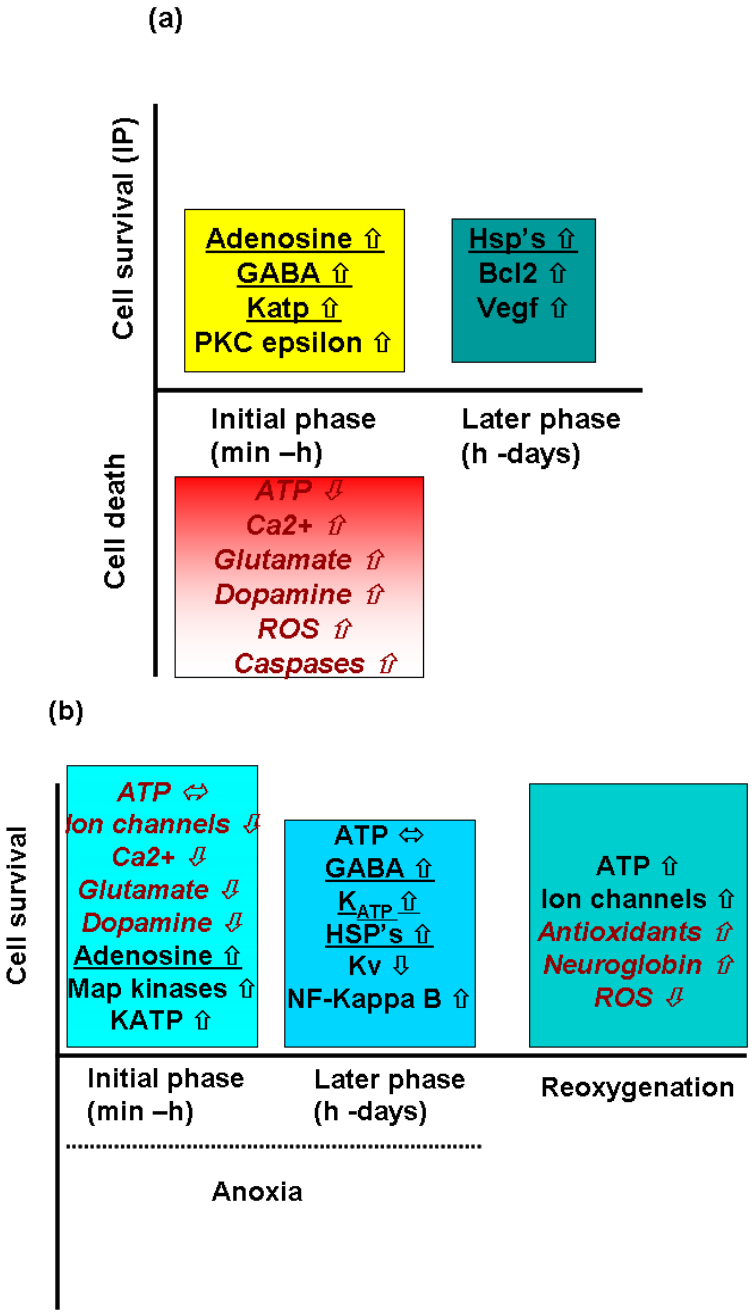
Table I: Anoxia and freeze tolerance in reptiles (Adapted from Belkin, 1963 and Storey, 2006)

Family/ Species	Anoxia Tolerance at 22±3 ^{0}C	Freeze Tolerance below -4 ^{0}C
Testudinae(Turtles: Chrysemys, Trachemys)	945 min	Chrysemys- 3-7 d Trachemys- 3 d
Gekkonidae (Lizards)	31 min	40 min – 2 h
Viperidae (Snakes)	95 min	2-3 h

Figures

Figure 1: Regulation of cellular mechanisms during cerebral ischemia and anoxia (Adapted from Dirnagl et al., 2003; Lutz and Milton, 2004). (a) Mammalian neurons are vulnerable to global ischemia, leading to a cascade of destructive events resulting in cell death as shown in the bottom. Endogenous protective mechanisms (Ischemic preconditioning, Section 1.2) shown in the top part that defend against further ischemic attacks resulting in cell survival. (b) Turtle neurons survive anoxia with no apparent cell damage by complex physiological and molecular adaptations. The figure compares mammalian ischemia with turtle anoxia; similar mechanisms of protection are underlined and different mechanisms are in red font.

Figure 1


(a)
Cell survival (IP)
Adenosine ⇧
GABA ⇧
Katp ⇧
PKC epsilon ⇧
Hsp's ⇧
Bcl2 ⇧
Vegf ⇧
Initial phase
(min –h)
Later phase
(h -days)
Cell death
ATP ⇩
Ca2+ ⇧
Glutamate ⇧
Dopamine ⇧
ROS ⇧
Caspases ⇧
(b)
Cell survival
ATP ⇔
Ion channels ⇩
Ca2+ ⇩
Glutamate ⇩
Dopamine ⇩
Adenosine ⇧
Map kinases ⇧
KATP ⇧
ATP ⇔
GABA ⇧
K_ATP ⇧
HSP's ⇧
Kv ⇩
NF-Kappa B ⇧
ATP ⇧
Ion channels ⇧
Antioxidants ⇧
Neuroglobin ⇧
ROS ⇩
Initial phase
(min –h)
Later phase
(h -days)
Reoxygenation
Anoxia

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CHAPTER 2: MODULATION OF STRESS PROTEINS AND APOPTOTIC REGULATORS

2.1. Introduction

In mammals, neuronal death due to extended anoxia is inevitable, but some organisms possess the extraordinary ability to survive hours to days of anoxia without apparent damage to the brain. One such anoxia-tolerant species is the freshwater turtle Trachemys scripta elegans. Earlier studies have shown that anoxia tolerance results from the modulation of complex cellular processes including the controlled release and reuptake of neurotransmitters (Milton and Prentice, 2007), arrest of ion channel function (Perez-Pinzon et al., 1992; Bickler et al., 2000), and regulation of MAP kinases (Milton et al., 2008). Studies on preconditioning in rodent models have identified multiple genes/proteins that confer increased tolerance to ischemia (Chen et al., 1996). We have proposed that the constitutive expression and subsequent modulation of such genes/proteins could also play a role in the high tolerance of the turtle to anoxia (Milton and Prentice, 2007). High basal levels and a further upregulation of Hsp72 and Hsp60 (Chang et al., 2000; Ramaglia et al., 200; Prentice et al., 2004) have been reported in the anoxic turtle brain, while anoxia also increases levels of phosphorylated extracellular regulated kinase (ERK 1/2) and Akt in the turtle brain (Haddad, 2007; Milton et al., 2008). Molecular pathways thought to incur cell death, on the other hand, are suppressed,

including activation of the c-jun kinase (JNK) pathway and p38MAPK (Milton et al., 2008).

Induction of cytoprotective heat shock proteins (HSPs) in response to physiological stressors (e.g. heat shock, osmotic stress, hypoxia/anoxia) has been well-studied in several model systems which together suggest they shift cellular equilibrium towards survival (Obrenovitch, 2008; Lanneau et al, 2008). Increased expression of Hsp70 is associated with increased ischemic tolerance in preconditioning (Nishi et al., 1993) and permanent cerebral ischemia (van der Weerd et al., 2005). Other HSPs upregulated after ischemia that may play a role in preconditioning include Hsp60, HO-1 (Heme oxygenase 1, also named Hsp32), Hsp27, and the glucose-regulated proteins Grp94 and Grp78 (Kato et al., 1994; Geddes et al., 1996; Chen et al, 1996; Massa et al., 1996; Kirino, 2002; Valentim et al., 2001; Badin et al., 2006; Hwang et al., 2007).

In models of rodent ischemia, however, intrinsic cellular protective responses to ischemia-hypoxia cannot be easily separated from secondary reactions to neuronal death. The turtle brain thus presents a unique model to understand the regulation of stress protein expression to promote cell survival in the face of complete anoxia. Histological examination of the turtle brain to determine damage following anoxia or reoxygenation has not been previously performed, on the assumption that turtle survival was accompanied by a lack of cellular damage. In the present study, we determined histologically that the turtle brain remains overtly intact after 24 h of anoxia and examined several stress-related proteins likely to be important in anoxic survival, including Hsp70, Hsp60, Hsp27, HO-1, Grp94 and Grp78, as well as the downstream modulators of apoptosis: Bcl-2, Bax, apoptosis inducing factor (AIF) and caspase-3. The

involvement of apoptotic cell death pathways in cerebral ischemia, hypoxia, and neurodegenerative disorders is well studied in rodent models (Cao et al., 2001; Cao et al., 2002; Saito et al., 2005, Mehta et al., 2007), though little work has been done in turtles (Haddad, 2007), who presumably are able to avoid triggering apoptotic pathways.

This study revealed simultaneous regulation of Bcl-2 and Bax, and suggests that apoptotic pathways may be initiated but not executed. We hypothesize that increases in HSPs in the anoxic turtle brain may prevent the activation of key pro-apoptotic regulators such as Bax and caspase-3 (Garrido et al., 1999; Stankiewicz et al., 2005; Matsumori et al., 2006). The present study is the first report to analyze the freshwater turtle's resistance to anoxia with histological methods and to characterize alterations in the expression of multiple major stress inducible heat shock proteins and apoptotic regulators during different time points in anoxia and in anoxia-reoxygenation.

2.2. Materials and Methods

Induction of anoxia

Experiments conducted in this study were approved by the Florida Atlantic University Institutional Animal Care and Use Committee. Freshwater turtles (*Trachemys scripta,* female*)* were obtained from commercial suppliers (Clive Longdon, Tallahassee, FL). Animals were kept in freshwater aquaria in an in-lab facility on a 12h light/dark cycle and fed three times weekly with commercial turtle food.

To induce anoxia, turtles were placed individually in a tightly sealed container at room temperature (22-23°C) with positive pressure flow-through nitrogen gas (99.99% N_2, Air Gas, Miami, FL) for the experimental time period. Animals subjected to reoxygenation were allowed to recover in room air. Control animals (N=5) were utilized

directly from the aquaria. For the study of stress proteins, turtles were subjected to anoxia for 1 h, 4 h, or 24 h anoxia in groups of n=4-6. A separate group was exposed to 4 h anoxia followed by 4 h of reoxygenation. The animals were decapitated, and the brains removed and snap frozen in liquid nitrogen in less than 2 min. For histological studies, groups of n=3 turtles were subjected to 24 h anoxia followed by 1 or 3 d of reoxygenation and were then perfusion fixed (as described below). N=5 control animals were fixed in parallel.

Protein extraction

Proteins were extracted from the frozen brains in cell lysis buffer (0.15 M Nacl, 5 mM EDTA pH 8, 1% Triton X100, 10 mM Tris-Cl pH 7.4; with added 5 M DTT , 100 mM PMSF, 5 M mercaptoethanol diluted 1:1000) using a glass homogenizer at 4°C . The homogenate was centrifuged at 13,000 rpm at 4°C for 10 min and the supernatant collected for further analysis. Protein concentrations were determined using a standard BCA assay following the manufacturer's protocol (Pierce Biotechnology, Inc., Rockville, IL).

Western blotting

The antibodies used for this study are as follows: rabbit polyclonal anti Hsp70 (SPA-812,), rabbit polyclonal anti Caspase-3 (AAP-113), rabbit polyclonal anti Hsp60 (SPA-805), mouse monoclonal anti HO-1 (OSA-111), rabbit polyclonal anti Hsp25 (SPA-801) and rabbit polyclonal anti Grp78(SPA-826) were obtained from Stressgen (Victoria, BC, Canada). Rabbit polyclonal anti-AIF (SC-5586) and mouse monoclonal anti-Bax (SC-7480) were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Polyclonal rabbit anti-Bcl2 (AB1720) was obtained from Chemicon (Temecula, CA).

Polyclonal rabbit anti-Grp94 (RB-10642-P1) was obtained from Neomarkers (Union City, CA). All the antibodies except for Hsp70 (1:4000) were diluted at 1:1000 in 2% non-fat milk. All the secondary antibodies (goat anti rabbit and rabbit anti mouse) were obtained from Southern Biotech (Birmingham, AL).

Equal amounts of protein (50 µg /lane) were separated on a 15% SDS-Polyacrylamide gel for 1.5 h at 150 V. The proteins were transferred onto a Hybond nitrocellulose membrane (Amersham Biosciences, Pittsburgh,PA) for 1.5 h at 0.3 A. The membrane was blocked in 5 % non-fat milk in TBS (25 mM Tris-Cl, pH 7.5, at 24ºC, 150 mM NaCl) for 2 h at room temperature followed by incubation with the respective primary antibody overnight at 4°C. The membranes were washed 3X 10 min in TBS and probed with appropriate HRP conjugated secondary antibody for 2 h at room temperature. Immunoreactive protein bands were then visualized with ECL (Amersham Biosciences). Relative density of the bands was determined using NIH Image J software. Results were normalized to percent of β-actin (utilized as a loading control, no change in anoxia, Prentice et al., 2004; Milton et al., 2008) and relative changes expressed as percent of control (normoxia).

Perfusion fixation and cryostat sectioning

Perfusion fixation was carried out to achieve optimal fixation and to avoid the manifestation of neuronal artifacts that impair the evaluation of cell damage. A window was prepared in the ventral shell (plastron) and the heart exposed. A blunt steel cannula was inserted through the tip of the heart, advanced towards the left aorta, i.e. the left-most vessel in the ventral position giving rise to the brachiocephalic artery (Cameron, 1989), and clamped in place. The right atrium was incised to allow for the escape of perfusate.

About 200-300 ml of 0.9% saline were slowly injected, followed by 300-400 ml of 4% paraformaldehyde. Brains were postfixed for one day in 4% paraformaldehyde and moved to PBS for storage at 4° C. Following cryoprotection in 20% sucrose for 1- 3 d, brains were immersed into a plastic template filled with Tissue Tek (Sakura) and frozen in 2-methylbutane (Sigma) chilled to – 30-40° C over dry ice. Sections were prepared at 40 µm thickness in the frontal plane (levels A4.8 to P0.8 according to the atlas of Powers and Reiner, 1993) on the cryostat (Microm). Serial sections were taken from the knife and placed into 24-well plates (Costar ® 3526, Corning Inc., Corning, NY) with 2 ml PBS per well. PBS was prepared with 0.5 ml/l antibacterial- antimycotic liquid (10,000 unit/ml penicillin G sodium; 10,000 µg/ml streptomycin sulfate; and 25 µg/ml amphotericin B; (GIBCO, Invitrogen). The immunohistochemical analysis included regions of the turtle brain between levels A4.8 and A 3.2 as defined by Powers and Reiner (1993), e.g. the cortex (cortex medialis, dorsalis and pyriforme), pallial thickening, primordium hippocampi, dorsal ventricular ridge (DVR) of the telencephalon including the core nucleus, paleostriatum augmentatum, globus pallidus, and ventral paleostriatum Other sections were floated onto poly-L-lysine coated slides (Poly-PrepTM slides, Sigma P0425-72EA) and air-dried for cresyl violet staining.

Immunohistochemistry

Immunohistochemical labeling was performed using a free-floating method in which relatively thick sections were reproducibly exposed to fixed volumes of antibody solutions (Schmidt-Kastner et al., 1991; Schmidt-Kastner et al., 2005). Reactions were carried out using six sections from 3-4 animals, including a control, in one batch. The peroxidase-antiperoxidase (PAP) method was used due to its high reproducibility and

successful prior use on sections of the turtle brain. Sections were moved to PBS containing 0.3% Triton X-100 (PBS-Tr) and 2% normal rabbit or swine serum (matching to the species of the second antibody) for 1 h, and then rinsed in PBS-Tr for 1 h. Reactions in primary antibodies were carried out overnight at +4° C. We used mouse monoclonal antibodies to NeuN (1:250; kind gift of Dr. Mullen, Univ. of Utah) and rabbit antibodies to GFAP (1:500; DAKO, Carpintera, CA). Following a rinse in PBS-Tr for 1 h, sections were reacted with the matched secondary antibodies, i.e. swine anti-rabbit IgG (DAKO) or rabbit anti-mouse IgG (DAKO), diluted 1:100 in PBS-Tr for 1 h. Subsequently, sections were rinsed for 1 h in PBS-Tr and then placed into rabbit-PAP complex (DAKO) or mouse monoclonal PAP-complex (DAKO) diluted 1:200 in PBS-Tr for 2 h. Following a rinse in PBS for 1 h, peroxidase activity was visualized using 0.05% diaminobenzidine (DAB, Sigma) and 0.01% H_2O_2 for 5 to 20 min. Following a prolonged rinse in PBS, sections were floated onto poly-L-lysine coated glass slides. Dried sections were moved through distilled H_2O, 70%, 90% and 100% ethanol, two changes of xylene, and finally coverslipped with DePeX (Sigma).

Cresyl violet (CV) staining

Cresyl Violet acetate (Sigma C1791-1G) was prepared with 1 g/ l in distilled H_2O (0.1%). For delipidation, sections were moved through 70%, 90%, and 100% ethanol, 2 x xylene, 100%, 90%, and 70% ethanol, and then washed in distilled water. Sections were immersed in CV solution for 5 min and quickly moved through distilled H_2O, 70%, 90% and 100% ethanol, two changes of xylene, and finally coverslipped with DePeX (Sigma).

Evaluation of pathology

The analysis was based on CV staining and immunohistochemical labeling for neurons (NeuN) and astrocytes (GFAP), with the presumption that three days is sufficient for the manifestation of serious neuronal damage or infarction in the turtle brain. As a note of caution, protein synthesis and degradation are known to be reduced during anoxia in the turtle brain (Fraser et al., 2001; Storey, 2007), and it is unclear how such changes would influence the manifestation of tissue damage during reoxygenation. A search for selective neuronal necrosis and infarction was first carried out in CV-stained sections. Major structures such as cortex, DVR, core nucleus of the DVR, paleostriatum augmentatum, globus pallidus, septum and ventral areas were examined. The cortex was inspected for interruptions of the pyramidal cell layer, cell loss and gross swelling. Only clear cut signs of ischemic neuronal necrosis such as severe shrinkage and clumping of the nucleus were considered; darkly stained and compressed neurons were not considered as irreversibly damaged. Areas of swelling with decreased background labeling and vacuolization were cautiously considered. NeuN antibodies detect a marker protein for neurons ("NeuN" for neuronal nuclei) (Mullen et al., 1992; Wolf et al., 1995). The antibodies visualize neurons in multiple species (Mullen et al., 1992). Loss of neurons manifests as reduction of the NeuN signal. The basic anatomy of GFAP labeling in the turtle brain has been described (Kalman et al., 1994), whereby labeled glial fibers but not stellate astrocytes were found. GFAP labeled sections were searched for increased signals in reactive astrocytes after 1 and 3 d of reoxygenation (i.e. indicating a reaction to damage). GFAP labeled sections were also inspected for subtle changes of glial processes and for changes in perivascular glia.

Statistical analysis

Statistical significance of the data was tested using Minitab 15 (Minitab Inc, State college, PA, USA). One way analysis of variance (ANOVA) was used to compare multiple experimental groups followed by Tukey's post hoc test. For the reoxygenation experiments after anoxia, data was compared using Student two tailed t-test. All the data are represented as Mean ± SE, and $p < 0.05$ was considered as significant.

2.3. Results

Absence of overt damage to neurons during anoxia and reoxygenation

The results of the histological studies on 24 h anoxia and anoxia/recovery animals confirms that anoxia tolerance at which the whole animal survives is indeed accompanied by a lack of overt damage to neurons. Representative images are shown in Fig 1.

Controls

NeuN labeling showed strong nuclear and weak cytoplasmic staining (Fig 1d). In the cortex, neurons were densely packed into one layer whereas the broad dendritic layer showed few neuronal cell bodies. Different brain nuclei and subregions showed regionally specific densities of neurons, e.g. strong cluster formation was seen in the dorsal ventricular ridge of the telencephalon. GFAP labeling occurred in fine glial processes but not in the cytoplasm of astrocytes (Fig 1g). Radial glial fibers spanned the cortex. Enhanced density of glial fibers was seen in the subpial and subependymal regions. Several large vessels were also outlined by glial processes. CV staining showed neurons and glial cells organized into different layers, nuclei and subregions as described by Powers and Reiner (1993). Densely packed neurons formed a broad layer in the cortex (Fig 1a). Diverse types of neurons were seen in various brain nuclei. A particular feature of the turtle brain is the high density of neurons in many brain areas and the formation of

very tight clusters. The cellular features of the turtle brain differ substantially from those of the rat brain and make it difficult to evaluate cell damage. Triangulated and darkly stained neurons were seen in several sections which were considered to be mild fixation artifacts. There were sometimes vacuolated regions around the cortical neurons in CV staining which appeared to be due to mechanical stress on the thin cortical sheath during tissue processing. Subsequently, we surveyed the sections immunostained for NeuN for overt loss of neurons, the GFAP-labeled sections for increases of signals indicative of reactive astrocyte formation, and the CV stained sections for neuronal deficits and gliosis. This approach enabled us to detect infarction and selective neuronal necrosis in circumscribed regions.

Anoxia for 24 h

NeuN labeling did not show areas of neuronal loss (Fig 1e). Most neurons had normal outlines. GFAP labeling showed swelling of the subpial region in two animals leading to a distortion of the subpial fibers (Fig 1h). The (open) lumen of the vasculature appeared to be more prominent than in controls. CV staining showed an overall intact architecture with no evidence for infarction or major neuronal loss (Fig 1b). A line of light labeling separated the neuronal layer from the ependyma in the cortex of two animals. Most neurons showed normal morphology and no signs of widespread shrinkage or nuclear disintegration were found. In areas of light background labeling, neuronal outlines were fuzzy suggesting the presence of edema. Perivascular halos of light staining were noted in basal areas.

Reoxygenation for 1d

NeuN labeling did not reveal neuronal deficits. Most neurons had normal outlines and distinct nuclear labeling. In some regions, neurons appeared to be compressed by small vacuoles. GFAP labeling did not indicate any areas with formation of reactive glial cells, i.e. star-shaped astrocytes were not seen. The overall intensity of labeling of fine glial fibers was variable among brain regions. CV staining showed vacuolization between the ependyma and cortical neuronal layer. Perineuronal vacuolization was also seen in the cortex which lead to some dark staining, but overt ischemic necrosis was not seen. Some fiber rich areas in the basal regions showed vacuolization.

Reoxygenation for 3d.

No areas of neuronal loss or infarction were seen in NeuN-labeled sections (Fig 1f). The neuronal layer of the cortex was swollen in some but not all animals. GFAP staining showed a regular pattern of glial processes with no evidence for the formation of reactive astrocytes (Fig 1i). CV staining showed minor swelling of the cortex (Fig 1c). No infarction was evident.

Induction of Stress proteins under anoxia

It has been previously suggested that physiological stress in the anoxia tolerant turtle is greatest during the initial transition phase (1-2 h) to the hypometabolic state, associated with a drop in ATP and the specific upregulation of protective MAPK pathways (Lutz and Milton 2004; Milton et al., 2008). The results of this study show increased expression of several heat shock proteins during the initial transition phase which remain elevated throughout the extended maintenance phase of long-term anoxia (Fig 2).

Hsp72

As previously reported by Prentice et al. (2004), Hsp72 was expressed at high basal concentrations in the normoxic turtle brain, in direct contrast to the rodent brain where Hsp72 is typically not detectable in the normal brain unless induced by stress. Hsp72 increased significantly to 207.0 ± 12.2% of basal during the initial hour of anoxia and remained elevated at 4h and 24 h anoxia (Fig 2a).

Hsp60

Exposure to 1 h and 24 h of anoxia resulted in a moderate increases to 111.3 ±10.6% and 119.3±7.2% of basal, respectively (Fig 2b). Hsp60 levels at 4 h anoxia were also elevated, but the difference from control was not statistically significant.

HO-1

HO-1 levels were relatively low, though detectable, in normoxic controls. Anoxia-stimulated increases in HO-1, however, were similar to other stress proteins. HO-1 protein levels increased by 1h anoxia (163.0±10.6%) and remained elevated through 24 h anoxia, increasing to 227.9±28.1 of basal (Fig 2c).

Hsp27

Relatively low levels of Hsp27 were detected under normoxic conditions; expression of Hsp27 was 1.8-fold to 2-fold over normoxic controls at 1 h and 24 h anoxia (Fig 2d).

Grp78 and Grp94

To further understand the resistance of freshwater turtles to cell damage in anoxia, we investigated the expression of two ER-specific stress-induced chaperones, Grp78 and Grp94 (Fig 2e - 2f), which are thought to protect cells against ER-related

stress in ischemia (Kudo et al., 2008; Paschen, 2001). Interestingly, the levels of Grp78 were not altered on exposure to anoxia (Fig 2e). By contrast, Grp94 was strongly and progressively induced through anoxia. Anoxia increased the levels of Grp94 2.5-fold by 1 hr anoxia and four-fold by the end of 24 h of anoxia (Fig 2f).

Bcl2 and Bax

Based on rodent studies, where increased levels of the pro-apoptotic protein Bax and decreased levels of the protective Bcl-2 are associated with apoptosis, we hypothesized that the anoxia resistant turtle brain would instead increase Bcl-2 protein levels and possibly decrease Bax (Zhang et al., 2001) but more complex changes in both Bcl-2 and Bax were encountered. Levels of both proteins varied over the course of 24 h of anoxia, but surprisingly the changes in Bcl-2 and Bax at each time point were in the same direction. The expression levels of both proteins initially decreased significantly by the end of 1h anoxia, with the decrease in Bcl-2 levels slightly greater (to 32% of basal) than decreases in Bax (to 58% of basal expression). The expression of Bcl2 then increased through 4 h anoxia but remained below basal levels, at 67.0± 7.9% of control, while Bax did not change significantly from levels detected at 1 h anoxia (60.5±12% of normoxic control). By 24 h anoxic exposure, the expression of Bcl2 had increased to 109.6±23.8% and Bax to 129.8±10.2% of normoxia These results suggest that exposure to anoxia and reoxygenation do not alter the overall ratio of Bcl2 to Bax, then, except for an initial decrease in the first hour of anoxic exposure (Fig 3a -3c).

Caspase 3

Levels of cleaved/active caspase-3, a key player in the apoptotic cascade, were unchanged by anoxia. While levels of the inactive zymogen procaspase-3 increased

continuously up to 2.3 fold by the end of 24 h anoxia, levels of cleaved (active) caspase-3 did not change significantly through anoxia and reoxygenation (Fig 3e).

Apoptosis Inducing Factor

Neuronal death in ischemia also occurs through non-caspase mediated cell death pathways (Cregan et al., 2002; Cao et al., 2003) by way of alternate mediators like AIF whose nuclear translocation directs DNA fragmentation (Plesnila et al., 2004). Exposure to 4 h anoxia increased the expression of AIF significantly to 180.7±28.9% of the basal level. Protein levels were not significantly different from the control at other timepoints in anoxia (Fig 3d).

Protein levels during reoxygenation.

Damage to mammalian systems is also significant upon reoxygenation, due in large part to resulting increases in oxygen free radical production that overwhelm antioxidant protections and trigger further cell death. However, cell death in vitro upon reoxygenation in turtles is low (Milton et al., 2007) presumably due both to high antioxidant levels (Rice et al., 1995; Storey, 2006) and the suppression of ROS formation upon reoxygenation (Milton et al., 2007). As HSPs are also protective upon reperfusion in mammals, it was thus of interest to determine if stress proteins would be further stimulated by reoxygenation in turtles (4 h anoxia/ 4 h recovery).

While Hsp72, Hsp60, Hsp27 and Grp94 levels were elevated above controls at 4 h of anoxia, no further upregulation of these proteins was evident following 4 h of reoxygenation (Fig 4a). In fact, the expression of Hsp72, Hsp60 and Hsp27 tended to return towards basal level after reoxygenation from anoxic increases, though the difference was not significant from 4h anoxia. By contrast, the expression of HO-1

increased further during reoxygenation to 134.45±3.4% above 4h anoxic levels, or 2.5 fold above the normoxic basal levels.

The apoptotic regulators also changed, with significant increases in levels of Bcl2, Bax and Procaspase-3 by the end of 4 h of reoxygenation when compared to 4 h of anoxia (Fig 4 b). The increases in Bcl2 and Bax returned them to basal levels upon reoxygenation, resulting again in a constant Bcl2 to Bax ratio. Procaspase-3 also increased during reoxygenation, though the cleaved form of Caspase-3 remained unchanged.

2.4. Discussion

We investigated the expression of stress proteins and apoptotic regulators in the anoxia-tolerant turtle during anoxia and reoxygenation: in the initial transition phase to anoxic hypometabolism (1 h), during long term anoxia (4 and 24 h), and in the reoxygenation phase. The results of our study show a general pattern of high constitutive levels and further rapid induction of stress proteins within the first hour of anoxia; elevated levels continue through 24 h of anoxia, suggesting activated endogenous protective mechanisms. Previous studies in our laboratory reported the presence of high basal levels and further upregulation of inducible Hsp72 during anoxia, suggesting a degree of constitutive preconditioning in the turtle brain (Prentice et al., 2004). Our present study reconfirms the presence and further anoxia-induced upregulation of Hsp72 as well as reports increases in other stress proteins including Hsp27, Hsp60, HO-1 and Grp94 during anoxia. Unlike the mammalian brain, where Hsp27 is not detectable prior to ischemic stress and requires as long as 24 h to increase significantly (Higashi et al., 1994; Wagstaff et al., 1996), Hsp27 was also detectable under basal conditions in the

turtle and increased significantly during the initial hour of anoxia. HSP increases are associated with neuronal protection, and indeed, our histological and immunohistochemical studies indicate that a period of 24 h of anoxia does not lead to overt neuronal loss or infarction despite variable vacuolization in the cortex, nor does 1 to 3 d recovery following anoxia. This is the first study to examine the brain directly for indications of anoxia/reoxygenation damage, confirming histologically the anoxia resistance of the turtle brain. Thus, the changes in stress proteins found here with Western blot analysis occurred in tissue that is going to survive overtly intact for at least three days.

Elevated heat shock proteins may protect against apoptosis. Apoptosis is induced by two distinct but interconnected pathways: the extrinsic pathway activated in response to stimulation of death receptors, and the intrinsic pathway initiated by cellular stresses that activate pro-apoptotic members of the Bcl-2 family, which in turn target the mitochondria. In intrinsic apoptosis, pro-apoptotic proteins cause permeabilization of the mitochondria, resulting in the release of apoptogenic factors that activate downstream effector caspases, including caspase-3 (Cao et al., 2001; Cao et al., 2002; Clemons et al., 2005). Caspase-independent apoptosis may also occur, as through AIF (Cao et al., 2003) or the calpains. In contrast to the significant increases in most HSPs examined, changes indicative of apoptosis were not observed in the anoxic or reoxygenation; neither the decrease in the Bcl-2:Bax ratio which occurred in the initial transition to anoxia nor AIF increases were apparently significant enough to trigger further apoptosis in the face of elevated intracellular protection, due not only to the HSPs but most likely also to

increased adenosine (Nilsson and Lutz, 1992) that in turn affects activated ERK1/2 and Akt (Milton et al., 2008).

Much of this conferred neuroprotection is thought to occur through interactions that block key steps of the intrinsic apoptotic pathways and shift cellular machinery towards cell survival (Abe and Nowak, 2004; Halaby et al., 2004; Zhu et al., 2007), including through the inhibition of the c-jun kinase (JNK) pathway (Gabai et al., 2000; Volloch et al., 2000; Gabai et al., 2002), blocking the translocation of the pro-apoptotic protein Bax to the mitochondria (Wang et al., 1999), and preventing Cyt-c release, caspase-3 activation and the translocation of AIF (Li et al., 2000; Li et al., 2002; Wang et al., 2002; Ruchalski et al., 2006). Such mechanisms allow Hsp72 to be protective even during times of ATP depletion (Wang and Borkan, 1996), which in mammalian cells is also when Bcl-2 levels decrease and Bax increases (Feldenberg et al., 1999). Interestingly, the greatest decrease in Bcl2 and change in the Bcl-2: Bax ratio in the turtle brain occurred in the initial hour of anoxia, when it has been shown that ATP levels temporarily decrease (Lutz et al., 1984). Changes in phosphorylation of both Bcl-2 and Bax by MAP kinases may have lead to the rapid decrease of proteins detectable with the present antibodies (Ashraf et al., 2001). The parallel decline of Bcl-2 and Bax early after the onset of anoxia is not paralleled by changes in the ischemic rodent brain, and it appears to be a specific feature of the turtle brain.

Also unlike mammalian models, where increases in many HSPs are delayed, increases in Hsp72, Hsp27, and HO-1 all were apparent in the turtle within the initial hour of anoxia, which makes it likely they could offer neuroprotection concurrent with ATP depletion. It is also during this initial hour, when physiological stress is presumably

the greatest, that phospho-Akt levels temporarily increase to over 300% of basal (along with a 6-fold increase in phosphorylated ERK) (Milton et al., 2008); Hsp27's protective role is thought to occur in part through PI3K activation and Akt increases that inhibit Bax activation (Havasi et al., 2008). Akt and ERK activation have also been associated with HO-1 protection against H_2O_2-induced apoptosis (Kim et al., 2008).

Ischemic tolerance conferred by Hsp60 might be also be due to its anti-apoptotic interactions with Bax and Caspase-3 (Kirchoff et al., 2002, Chandra et al., 2007). While found primarily in the mitochondria, some Hsp60 is also located in the cytosol (Lin et al., 2007) where it complexes with Bax; thus decreases in Hsp60 permit the translocation of Bax to the mitochondria. In the turtle, Hsp60 was one of the stress proteins largely unchanged during anoxia, though as in mammalian cells high constitutive levels were found. Lin et al. (2007), however, reported recently that while Hsp60 levels may remain unchanged overall, cytosolic proteins may shift to the plasma membrane, allowing apoptosis to be triggered. While our study did not examine the subcellular localization of any HSP, it is apparent that any subcellular changes were not sufficient to trigger apoptosis.

Another heat shock protein that has been found to offer neuroprotection is HO-1. Neuroprotective effects of HO-1 (also known as Hsp32) have been mainly attributed to its cleavage of the pro-oxidant heme into antioxidants (bilirubin and biliverdin) that may ameliorate the oxidative stress of ischemia (Nimura et al., 1996; Bergeron et al., 1997; Chen et al., 2000), while decreases in heme levels protect against lipid peroxidation and oxygen radical formation (Panahian et al., 1999). Overexpression of HO-1 resulted in reduced infarct size in the rat focal ischemic model (Panahian et al., 1999) and neurons

overexpressing HO-1 are more resistant to glutamate excitotoxicity (Chen et al., 2000). HO-1 in mammals is mainly induced in astroglial or microglial cells (Geddes et al., 1996), and its synaptic localization led Bechtold and Brown (2000) to suggest that HO-1 may thus be involved in the protection and repair of synapses. The large increases we observed in anoxia and upon reoxygenation, then, are of interest as it has been suggested that the turtle maintains synaptic function during long-term anoxia, with the continued low-level release and reuptake of neurotransmitters by both glia and neurons (Milton et al., 2002; Thompson et al., 2007) and periodic bursts of electrical activity (Fernandes et al., 1997). Turtle neurons are also known to be highly resistant to glutamate toxicity (Wilson and Kriegstein, 1991). It would thus be of future interest to ascertain the cellular and subcellular localizations of anoxia-induced HSPs.

Hsp27 is also primarily expressed in astroglia post-ischemia (Kato et al., 1995, Currie et al., 2000), and Hsp27 gene transfer offers protection against ischemia (Badin et al., 2006). The protective effect of Hsp27 might be due to its multiple anti-apoptotic interactions, e.g. preventing formation of the apoptosome (Garrido et al., 1999) and activation of Bax (Havasi et al., 2008), sequestration of cytochrome c (Bruey et al., 2000), and interactions with Akt (Lannaeu et al, 2008) and Bid (Conconnaon et al., 2001). In whole turtle brain, levels of activated Akt increase in the initial hour of anoxia (Milton et al., 2008), while here we report decreases in Bax. While changes in Akt levels are related to adenosine stimulation, the link between adenosine, Akt, and cellular protection is not necessarily direct, and it is likely that the turtle has multiple and redundant interacting pathways for neuroprotection. The observation that anoxia-induced increases in Hsp27 were among the largest changes detected again suggests that

the protection and function of glial cells may be a critical, and previously overlooked, aspect of brain survival in the anoxic turtle.

Involvement of ER stress chaperones in ischemic protection is relatively well established, though reports of Grp78 and Grp94 elevations after ischemia in rodent models are variable (Chen et al., 1996; Hayashi et al., 2003; Kim et al., 2003). Grp94 is again thought to be primarily a glial response (Kim et al., 2003; Bando et al., 2003; Jeon et al., 2004), while Grp78 responses are neuronal (Kudo et al., 2008). In the anoxic turtle brain, Grp94 was upregulated whereas Grp78/BiP did not change, which could be due to regulation by different transcription factors controlling the ER stress response (Schroeder and Kaufman, 2005) or again reflect differences in neuronal and glial responses to anoxia. Further studies on the ER stress response in the turtle brain would be of interest, particularly as the ER is a significant intracellular calcium store.

In contrast to large increases at 1h anoxia with further elevation by the end of 24 h of anoxia in the primarily glial (in mammals) Hsp27, Grp94 and HO-1, Hsp72 increases were not observed after 4 h. Preliminary studies with immunohistochemistry indicate neuronal expression of Hsp72 in the normal and anoxic turtle brain (Kesaraju and Schmidt-Kastner, unpublished observations) which is in line with neuronal expression in rodents after ischemia (Vass et al., 1988). Together, then, the data suggest that expression in astrocytes and/or microglial cells in mammals is a common denominator for stress genes which show a greater increase of protein levels during anoxia in the turtle brain. This leads to the intriguing question of whether astrocytes are under increasing metabolic stress in the turtle brain, because their working load for maintaining basal neuron functions must be augmented during anoxia. In mammals, astrocytes provide nutritional

support to neurons, and Pellerin and Magistretti (1994) have shown that the uptake of glutamate stimulates astrocytes to shuttle lactate to neurons. The turtle brain is known to undergo swelling during anoxia (Cserr et al., 1988) and much of this swelling can be predicted to reflect intracellular edema in astrocytes (Sykova et al., 1998). The present immunolabeling for GFAP may also be taken to indicate swelling of the cortical astrocytes during anoxia. In view of the lively debate regarding astrocyte-to-neuron transfer of energy-rich metabolites (Meeks and Mennerick, 2003), it is tempting to speculate that astrocytes may continue to support neurons throughout anoxia whereas neurons themselves shut down much of their function.

Pro-caspase-3 levels also increased significantly in the anoxic turtle brain, as has been seen at the mRNA and protein levels in the post-ischemic rodent brain, both in vulnerable neurons and in neurons protected by preconditioning (Cao et al., 2002; Tanaka et al., 2004). Importantly, however, the active cleaved caspase-3 was not elevated in the anoxic turtle brain, a condition also reported in the preconditioned mammalian model (Tanaka et al., 2004). One explanation is that HSPs blocked upstream caspases or calpain during anoxia (Garrido et al, 2001). These initial studies show that the turtle brain expresses regulators of apoptosis under basal conditions, but that protective changes induced by anoxia prevent drastic alterations in Bax:Bcl2 ratios and caspase-3 activation. Increases in Procaspase3 have also been found in mammalian neurons that are not dying, suggesting other potential, non-apoptotic roles related perhaps to other functions like long term synaptic plasticity (Bravarenko et al., 2006).

Reoxygenation for 4 h following 4 h anoxia did not further stimulate expression of Hsp70, Hsp60 or Hsp27, nor were there changes in the Bcl2/Bax ratio, indicative again

of the stabilization of apoptotic regulators. Additional increases in HO-1 in reoxygenation beyond the already significant increases observed in anoxia suggest involvement in protection against potential oxidative stress. The turtle is endowed with superior protective mechanisms to cope with reoxygenation stress including enhanced antioxidant systems (Rice et al., 1995; Wilmore et al., 1997; Storey, 2006) and suppression of ROS (reactive oxygen species) formation (Milton et al., 2007; Pamenter et al., 2007) and high levels of HO-1 would add further protection. So unlike mammalian systems, where reoxygenation often triggers massive increases in ROS release and cell death, the turtle appears to experience little reoxygenation oxidative stress, and thus the unaltered expression of most stress proteins after reoxygenation would be due to an absence of triggers for stress induction. Alternatively, of course, high "banked" levels of stress proteins may be getting used up as the turtle emerges from its hypometabolic state and returns to basal metabolism, with the requisite upregulation of transcription (Prentice et al., 2003) and protein synthesis (Fraser et al., 2001). The return of elevated stress protein levels exactly to basal, however, argues that this is a controlled return to normoxic status rather than a response to increases in protein synthesis and/or damage, and in turtles protein synthesis likewise returns rapidly to pre-anoxic levels upon recovery (Fraser et al., 2001). By contrast, hypoxia/ ischemia studies in rodent models show induction of major stress proteins within 1 h -7 d after insult in the salvageable tissue (Chen et al., 1996, Kokubo et al., 2003, Nishino and Nowak, 2004) despite an overall reduction in protein synthesis extended till 72 h after the insult (Kokubo et al., 2003).

Taken together, then, the data point to complex molecular mechanisms that result in stable apoptotic machinery and increased stress protein protection (Table I). In the early phase of anoxia, as the brain transitions to a state of deep hypometabolism, a rapid induction of stress proteins and depression of Bcl2 and Bax stabilizes the cell at a time of elevated physiological stress and decreased ATP levels. During the ensuing long term maintenance phase of anoxia, there is a steady increase in overall stress protein levels and a constant low level of pro-apoptotic proteins, tipping the balance towards survival. Although the localization of stress proteins in the turtle brain are not yet known, interestingly, the stress proteins that were most enhanced are the ones mostly localized to glial cells in mammalian studies, suggesting an increased functionality of glial cells for maintenance of neuronal integrity that has not been previously noted. Reoxygenation did not further elevate the stress protein levels, supporting earlier studies indicating that turtles possess innate mechanisms to suppress oxidative stress. A better understanding of the molecular mechanisms that promote survival in the anoxia tolerant brain, honed though millions of years of evolution, may yield new therapeutic targets for mammalian anoxic/ischemic deficits.

Tables

Table I: Overview of the expression of stress proteins and apoptotic regulators

Overview of the expression of stress proteins and apoptotic regulators in three different phases of anoxic survival in an anoxia tolerant freshwater turtle (After Lutz and Milton, 2004). Downregulation of metabolism (Hypometabolic phase) occurs in the initial transition to full anoxia (1-2 h), followed by the maintenance phase of long term anoxia (> 2h to days); the recovery phase represents a return to basal metabolism during reoxygenation and the potential for oxidative stress. Throughout both stages of hypometabolism , there is an increase in the expression of stress proteins and decrease in the expression of proteins involved in apoptosis that may help maintain the structural integrity of the brain and prevent significant damage. ⇔ represents expression at the basal level, ⇧ represents upregulation, ⇧⇧ represents continued upregulation , ⇩ represents decreased expression. * represents elevated constitutive expression of Hsp's normally undetectable in the basal mammalian brain,

Table I

Protein	Hypometabolism Transition (1-2 h anoxia)	Maintenance (h – d anoxia)	Recovery (h – d Reox)
Hsp72*	⇧	⇧	⇔
Grp78	⇔	⇔	⇔
Hsp60	⇧	⇧	⇔
HO-1	⇧	⇧⇧	⇧⇧
Hsp27*	⇧	⇧⇧	⇔
Grp94	⇧	⇧⇧	⇧
Bcl2	⇩	⇩	⇔
Bax	⇩	⇩	⇔
Procaspase 3	⇧	⇧⇧	⇧⇧
Caspase 3	⇔	⇔	⇔

Figures

Figure 1: Analysis of the cortex of the turtle for neuronal damage. a– c) Cresyl violet staining; d – f) Immunolabeling for the neuronal marker NeuN; and g – i) Immunolabeling for the glial marker GFAP. First column (a, d, g) = control animals; second column (b, e, h) = anoxia; third column (c, f, i) = anoxia followed by three days of survival. Note preservation of neuronal cell band at all time points in cresyl violet staining and NeuN-immunolabeling. GFAP signals are not increased at three days. Magnification bar shown in i) is equivalent to 200 μm for all.

Figure 1

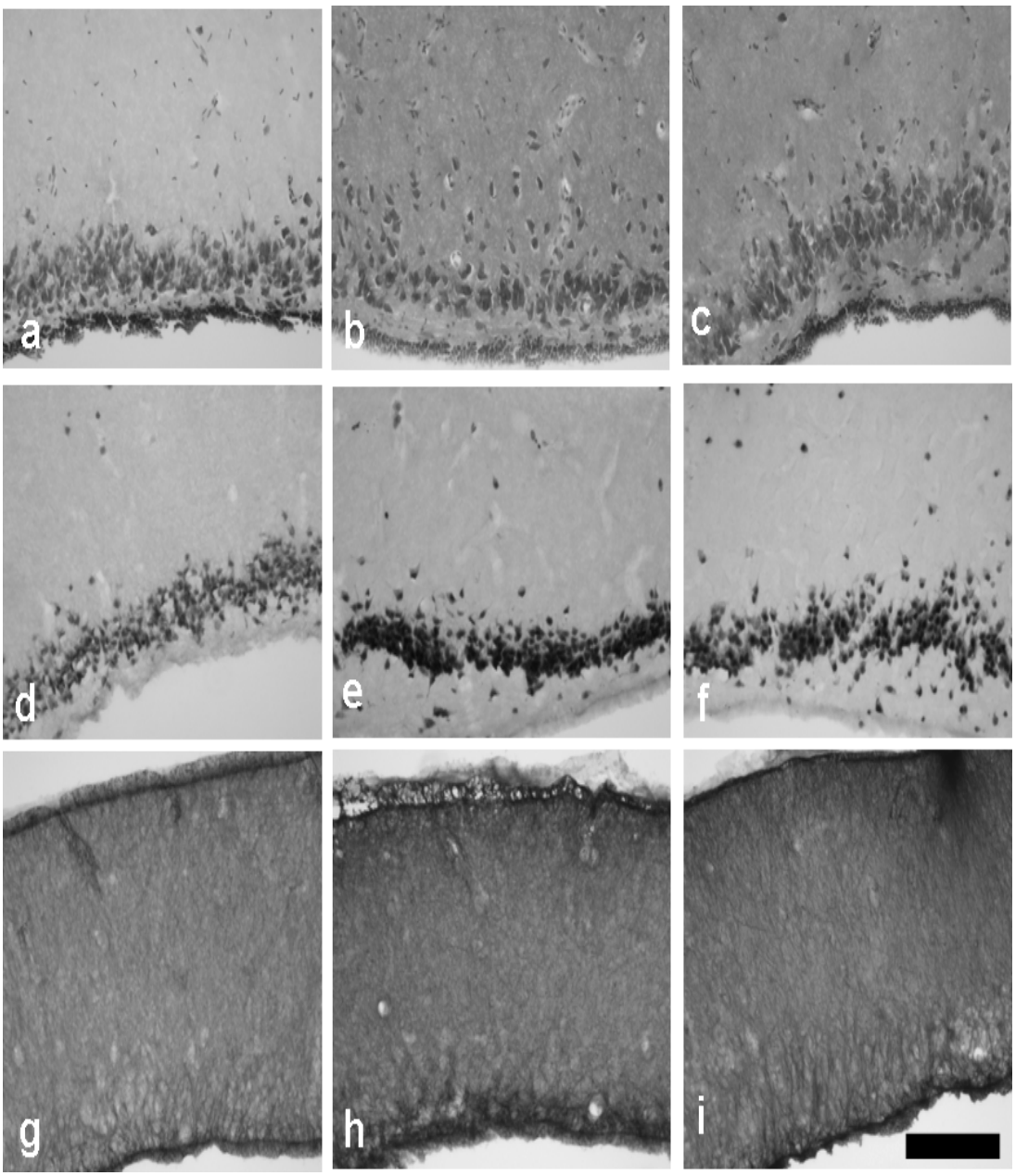


Figure 2: Multiple stress proteins are upregulated by short- and long-term anoxia in the turtle brain.

Representative western blots and densitometric analyses (expressed as % of control) of turtle brain whole cell lysates are shown. For each blot, the order of loading from left is as follows: normoxic control, 1 h anoxia, 4 h anoxia and 24 h anoxia. (a) Hsp72 (b) Hsp60, (c) HO-1 (d) Hsp27 (e) Grp78 (f) Grp94. Data are expressed as mean ± SE. *shows significance shows significant difference from normoxic control ($p<0.05$) "#" = s.d. from 1 h anoxia.

Figure 2

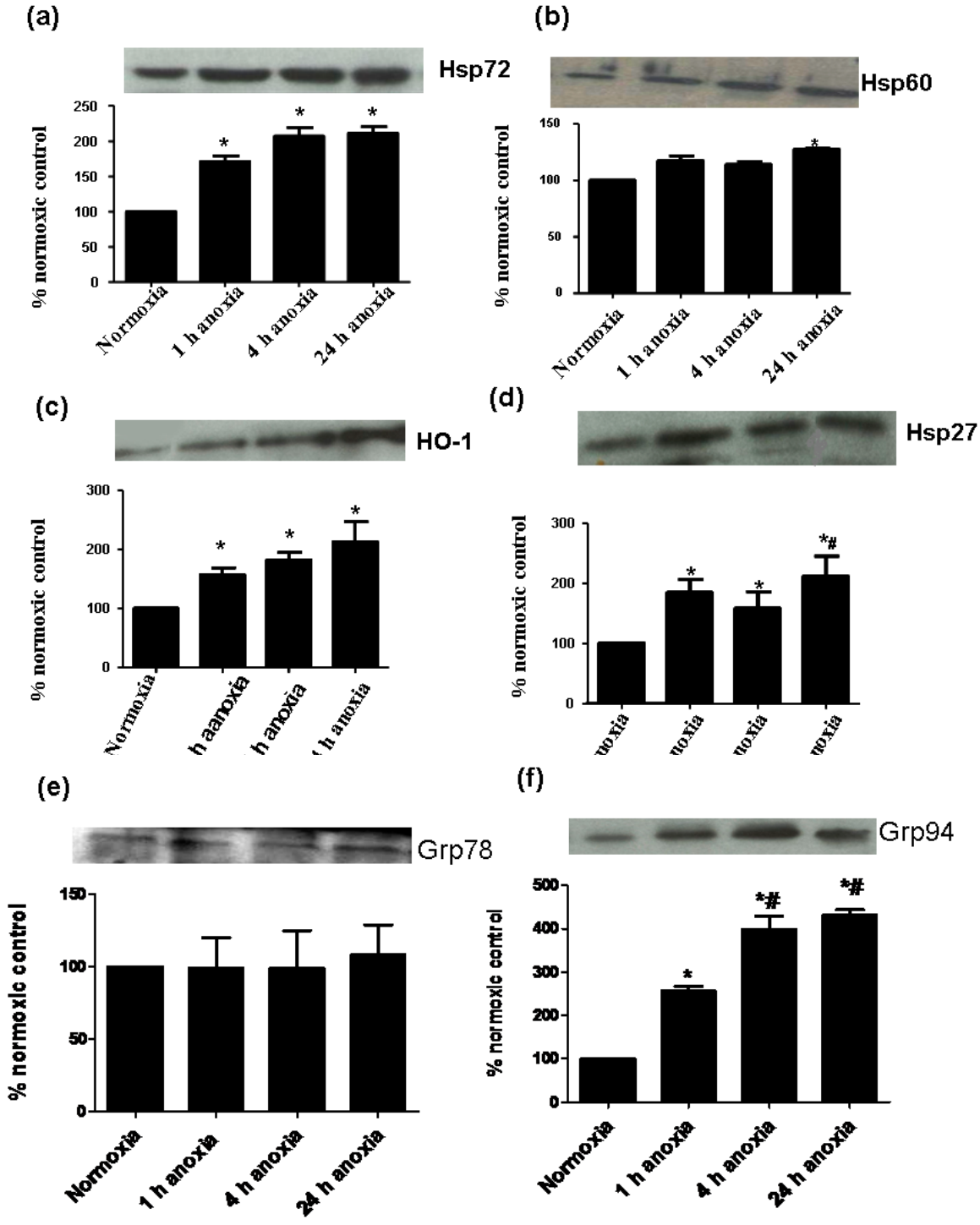

(a)
Hsp72
% normoxic control
250
200
150
100
50
0
Normoxia
1 h anoxia
4 h anoxia
24 h anoxia
(b)
Hsp60
% normoxic control
150
100
50
0
Normoxia
1 h anoxia
4 h anoxia
24 h anoxia
(c)
HO-1
% normoxic control
300
200
100
0
(d)
Hsp27
% normoxic control
300
200
100
0
(e)
Grp78
% normoxic control
150
100
50
0
Normoxia
1 h anoxia
4 h anoxia
24 h anoxia
(f)
Grp94
% normoxic control
500
400
300
200
100
0
Normoxia
1 h anoxia
4 h anoxia
24 h anoxia

Figure 3: Apoptosis regulator proteins are differentially regulated by short- and long-term anoxia in the turtle brain.

Representative western blots and densitometric analyses (expressed as % of control) of turtle brain whole cell lysates are shown. Relative changes in expression of (a) Bcl2 (b) Bax, (c) Ratio of the expression of Bcl2 to Bax obtained from the mean densities (d) AIF and (e) Procaspase 3 and Caspase 3. * shows significant difference from normoxic control ($p<0.05$) "#" = s.d. from 1 h anoxia, $ = s.d from 24 h anoxia.

Figure 3

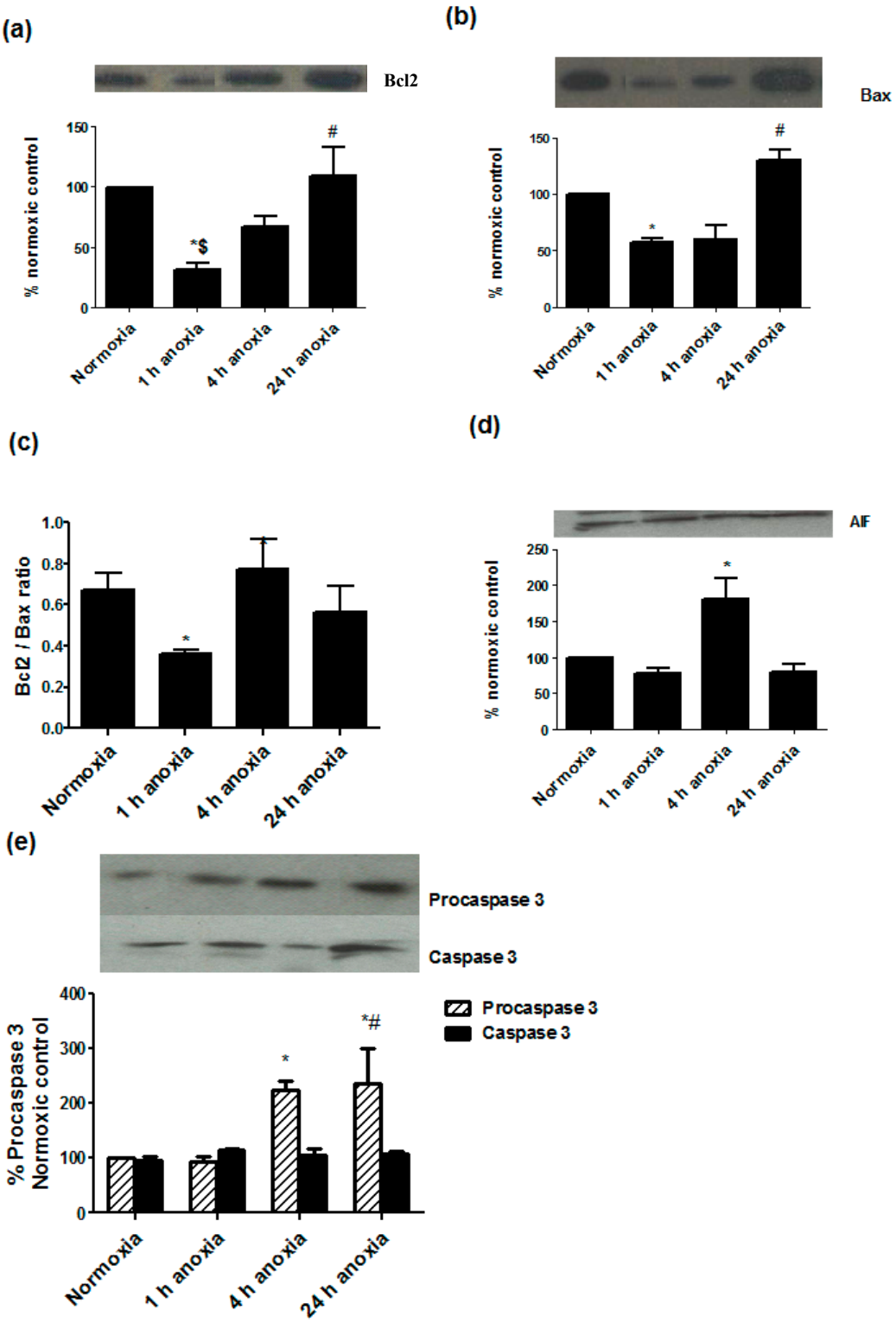
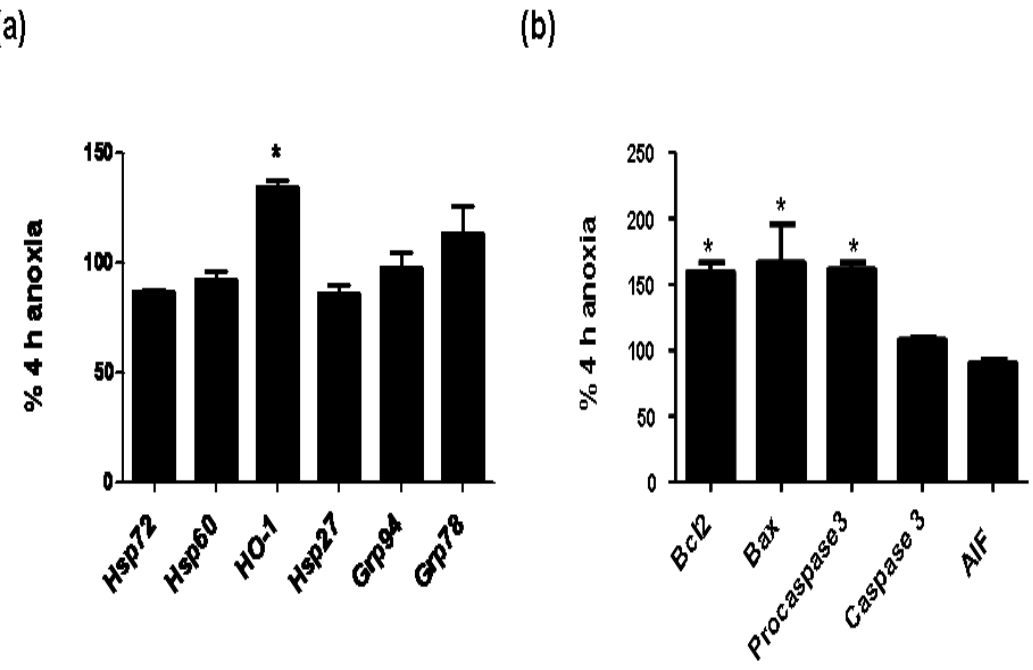

(a)
Bcl2
% normoxic control
150
100
50
0
*$
#
Normoxia
1 h anoxia
4 h anoxia
24 h anoxia
(b)
Bax
% normoxic control
150
100
50
0
*
#
Normoxia
1 h anoxia
4 h anoxia
24 h anoxia
(c)
Bcl2 / Bax ratio
1.0
0.8
0.6
0.4
0.2
0.0
*
Normoxia
1 h anoxia
4 h anoxia
24 h anoxia
(d)
AIF
% normoxic control
250
200
150
100
50
0
*
Normoxia
1 h anoxia
4 h anoxia
24 h anoxia
(e)
Procaspase 3
Caspase 3
% Procaspase 3
Normoxic control
400
300
200
100
0
*
*#
Procaspase 3
Caspase 3
Normoxia
1 h anoxia
4 h anoxia
24 h anoxia

Figure 4: Densitometric analysis of protein expression of all the stress proteins and the apoptotic regulators in response to reoxygenation after anoxia.

The data are expressed as percent of the 4 h anoxia to analyze the relative change of protein levels in reoxygenation after 4 h anoxia.. The data are analyzed by two tailed t-test. *shows significance ($p<0.05$).

Figure 4


(a)
(b)
% 4 h anoxia
150
100
50
0
*
Hsp72
Hsp60
HO-1
Hsp27
Grp94
Grp78
% 4 h anoxia
250
200
150
100
50
0
*
*
*
Bcl2
Bax
Procaspase3
Caspase 3
AIF

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CHAPTER 3: NEUROPROTECTIVE ROLE OF HSP72 AGAINST ANOXIA AND REOXYGENATION

3.1 Introduction

The freshwater turtle (*Trachemys scripta*) is one of the few anoxia tolerant species that survives without oxygen for days to months depending on the temperature (Jackson, 2000). Milton and Prentice (2007) have suggested that survival is a result of complex physiological and molecular adaptations that defend the turtle against the stress of oxygen deprivation. Turtles therefore provide an ideal alternative animal model to study anoxia/reoxygenation (A/R), as neuronal function in turtles and mammals is similar, including such protective responses to anoxia as alterations in heat shock proteins, MAPK pathways, increases in neuroprotectants like adenosine and GABA, and the opening of ATP-dependent potassium channels (Nilsson and Lutz, 1991; Nilsson and Lutz, 1992; Nilsson and Lutz, 1993; Pek-Scott and Lutz, 1997; Lutz and Milton, 2004).

Differential modulation of MAP kinases and Akt pathways were implicated in anoxic survival (Milton et al., 2008) and freshwater turtles are also capable of surviving the stress of reoxygenation by innate suppression of reactive oxygen species (ROS)(Milton et al., 2007). One molecular mechanism described in the turtles was high basal expression of both constitutive Hsc73 and inducible Hsp72 that were further elevated during anoxia (Prentice et al., 2004).

Hsp72 or the inducible Hsp70, a member of the 70- kilodalton heat shock family of proteins containing 11 different isoforms (Giffard et al., 2008) is one of the most studied proteins due to its protective effects in cerebral ischemia (Massa et al., 1996; Giffard et al., 2008). Hsp72 acts as a molecular chaperone to assist in protein refolding, trafficking, maturation and ubiquitination (Hartl, 1995; Giffard and Yenari, 2004). Sato et al. (1996) have described Hsp72 as protective in rat primary culture neurons where Hsp72 attenuated cell death due to heat shock (42^0C for 30 min) while antisense treatment of Hsp72 reduced the neuroprotective effect. Overexpression of Hsp70 ameliorated the ischemic and heat shock effects in dorsal root ganglion neuron cell culture of rodents and the amount of protection was correlated with the concentrations of Hsp70 (Amin et al., 1996). Overexpression of Hsp72 through viral vectors or through transgenic animals also reduces the infarct size in ischemia-stroke models (Plumier et al., 1997; Rajdev et al., 2000; Kelly et al., 2002; van der Weerd et al., 2005). The protective effects of Hsp70 are observed in several organs experiencing ischemia; overexpression of Hsp70 rescued ATP depleted renal epithelial cells and suppressed caspase activity (Wang et al., 2002), while overexpression of Hsp72 rescued the myocardium from ischemia/reperfusion injury (Corneleusen et al., 2003, Guisasola et al., 2006).

Beere (2001) has suggested that the fate of cell survival is decided by the equilibrium established between stress proteins and the apoptotic pathway of cell death. The cytoprotective function of Hsp72 has been ascribed to its inhibitory effects on apoptotic and necrotic cell death pathways (Giffard et al., 2008). Cell death due to apoptosis can be mediated through intrinsic pathways (intracellular signaling) or extrinsic pathways (extracellular signaling). The final executioners of both the pathways are

caspases that ultimately lead to cell death. Induction of intrinsic cell death pathways has been implicated in several cerebral ischemic models, initiated by the translocation of Bax to the mitochondria that triggers the release of Cytochrome c (Cyt c) from the mitochondria, recruits caspase 9 and Apaf 1 to form the apoptosome complex and activate the final effector caspases (Giffard et al., 2008). Hsp72 has been implicated in suppression of a number of pro-apoptotic events such as translocation of Bax from the cytosol to the mitochondria (Stankiewicz et al., 2005), release of Cyt c from the mitochondria involving loss of mitochondrial membrane potential (Tsuchiya et al., 2003), and formation of the apoptosome through interaction with Apaf 1 and also through binding with caspases (Matsumori et al., 2006). The interaction of Hsp72 with Apaf 1 is a matter of debate, though Ravagnan et al (2001) have shown that overexpression of Hsp70 rescued Apaf 1 (-/-) cells from serum withdrawal, suggesting the presence of an alternative mechanism. Further studies by the same group and others have shown that Hsp72 prevents cell death by inhibiting the release of AIF (apoptosis inducing factor) from the mitochondria and its translocation to the nucleus, where it leads to condensation of the chromatin and apoptosis independent of the action of caspases (Ravgnan et al., 2001; Matsumori et al., 2005). In either case, Hsp72 is clearly protective against ischemia-induced neuronal injury.

Ischemia/reperfusion injury is exacerbated by the increase of reactive oxygen species (ROS) production through membrane permeabilization of the mitochondria (Maier et al., 2006). The brain is highly susceptible to ROS damage due to its high consumption of oxygen, high lipid content, and low antioxidant capacities (Leutner et al, 2001). Although the mechanism of action is not clear, ROS might oxidize the

mitochondrial membrane pores and release Cyt C that triggers the apoptotic pathway (Simon et al, 2000) or might act as a signaling molecule for the execution of apoptosis (Carmody and Cotter, 2001). Hsp's reduce ROS effects when the antioxidant system fails to meet the challenge of ROS injury (Smolka et al., 2000). In addition to the function as molecular chaperones, the cell signaling mechanisms of heat shock proteins (Hsp's) play a vital role in tolerance against ROS damage in neurodegenerative disorders (Calabrese et al., 2004); Hsp's also prevent necrotic damage through inhibiting the activation ROS and MAP kinases, JNK in particular, in energy depleted myocardial cells (Yaglom et al., 2003).

MAP kinases differentially activate during ischemia/reperfusion although the nature of each pathway, whether it is pro-survival or not, depends on the type of the stressor and also on the amount of time these pathways remains activated (Martindale and Holbrook, 2002; Milton et al., 2008). Conventionally, JNK and p38 pathways drive the cell towards apoptosis (Wang et al., 1998; Gabai et al., 2000) while ERK is a pro-survival pathway that prevents ischemic injury (Yue et al., 2000; Gabai et al., 2000). Many studies have revealed that the anti-apoptotic function of Hsp72 is also due to suppression of the stress kinases, SAPK/JNK and p38, that prevents apoptosis by inhibiting phosphorylation of JNK and translocation of Bax to the mitochondria (Mosser et al., 1997; Gabai et al., 2000; Stankiewicz et al., 2005).

While it was reported that Hsp72 increases significantly in the anoxic turtle brain (Prentice et al., 2004), that the protective pathways ERK1/2 and Akt are also upregulated (Milton et al., 2008) and the pro-apoptotic pathways p38 and JNK as well as Bax are suppressed (Haddad, 2007a; 2007b; Milton et al., 2008), it is not known if there are

functional links between these molecular pathways or even if increases in Hsp72 are truly neuroprotective in the turtle brain. The present study therefore aims to delineate the functional role of high constitutive levels of Hsp72 in the freshwater turtle and its regulation of apoptosis. The functional role of Hsp72 in apoptosis during anoxia and reoxygenation and also in the balance of JNK/ERK pathways for regulating cell survival/cell death mechanisms was analyzed in neuronal enriched turtle primary cell cultures. Use of neuronal cultures allowed the investigation molecular pathways of apoptosis and survival in cells treated with siRNA against turtle Hsp72 to decrease specific protein expression.

3.2. Materials and Methods

Experiments conducted were approved by the Florida Atlantic University Institutional Animal Care and Use Committee.

Development of enriched neuronal cell culture: We were the first to successfully establish neuronal enriched cultures (Milton et al., 2007) from juvenile turtle brains by modification of Brewer's (1997) method of density gradient isolation of neurons. The cortex of the turtle brain is aseptically chopped and added to a cocktail of MEM–L-glutamine and Earl's Salts (Mediatech, Inc., Herndon, VA) with proteases (0.16U/ml Dispase (Gibco/Invitrogen, NY, USA); 12.5U/ml Collagenase (Gibco/Invitrogen); 6.25 mg/ml Hyalorudinase (Sigma-Aldrich, St. Louis, MO). The culture media is supplemented with 10% BSA (Gibco/Invitrogen). The protease digested medium is then applied on top of an Opti-prep gradient (Sigma-Aldrich) and centrifuged at 750 g for 15 min at room temperature. This result in a two clear bands and the fraction between the bands and above the pellet is collected as a neuronal fraction. The neuronal fraction is

than plated onto 6 well dishes. Cells are grown at 300C in a 5%CO2 incubator. For immunohisto-chemical analysis, the cells are rinsed with PBS and then fixed for 10 min at room temperature with 4% formaldehyde (Brewer, 1997). Subsequently, the cells are blocked with 0.5% Triton X, 3% normal goat serum in PBS for 30 min at room temperature. The cells are then rinsed and incubated with primary antibodies overnight at 40C: Alexa fluor conjugated NeuN (1:100, Chemicon, Temecula, CA), neurofilament (1:100, Chemicon) and GFAP (1:100, Dako, Carpintera, CA) to confirm the neuronal phenotype of the cells. Cells were then washed 3X with PBS, probed with fluorescent conjugated Alexa fluor- anti-mouse and Alexa fluor anti-rabbit secondary antibodies (1:500, Molecular probes, Eugene, OR), rinsed with PBS and viewed under the confocal microscope.

RNA isolation and RT-PCR

Total RNA was isolated from the turtle brain and from cell cultures using Trizol reagent following the manufacturer's protocol. The RNA sample was treated with DNase (Invitrogen, Eugene, OR) and RNA was quantified using UV spectrophotometry; 1 μg of total RNA was used for RT-PCR. Reverse transcription was performed at 50°C for 55 min using 5 units Superscript III, 125 pM forward and reverse primers (IDT DNA), 1X First strand synthesis buffer, 0.1 mM DTT , and 2 units of RNAse OUT ribonuclease inhibitor. All the reagents for RT-PCR were obtained from Invitrogen, (Eugene, OR) To detect Hsp72, PCR was performed using 100 ng of template, 200μM dNTP, 50 pM Forward and Reverse primer, 1X PCR buffer with 1.5 mM Mg and 5 U Taq polymerase. All PCR reagents were obtained from Fisher Scientific (Pittsburgh, PA). The amplification cycle consisted of 5 min initial denaturation at 94°C followed by 35 cycles

of 94°C for 1min; 58°C for 1 min and 72°C for 1min. Degenerate primers were made to detect Hsp72; Forward Primer: 5' GTGTAGAAGTCGATGCCCTCA 3' and Reverse primer: 5' AANGAGCCCAGCGCNCCC3' where "N" could be A/G/C/T with a 300 bp PCR product . For all the reactions actin was run as an internal control that does not change with change in experimental conditions (Prentice et al., 2004). All the data was normalized with actin and expressed as percent control.

siRNA design and transfection

Target siRNA against turtle Hsp72 was identified by using the siRNA design tool of Invitrogen/Molecular Probes (Eugene, OR) from the turtle Hsp72 specific sequence. Specific siRNA sequences against Hsp72 and the scrambled control are as follows: 5' UGUCCCGCUUGUGCUUGCGCUUGAA3';5'UGUCGCGUUCGUGUUCGCUU-CGGAA3'. All the controls in the siRNA experiments were performed using scrambled siRNA. Transfection was performed using Lipofectamine-2000 as per the manufacturer's protocol. The amount of siRNA was optimized for different experimental conditions. Protein /Cell lysate was harvested from the transfected cell cultures for further analysis. Transfection efficiency of lipofectamine was analyzed using GFP. Cells were transfected with 1 ug GFP plasmid following a similar protocol as experimental samples.

Cell viability assay

Propidium iodide (PI) staining was used to obtain the ratio of dead cells to total cells for each treatment, including in the cell cultures transfected with siRNA against Hsp72 and the control siRNA. The ratio of live to dead cells ratio was obtained by using 1.5 μM of PI (Molecular Probes) where the dying cells take up the red stain. Cells were

viewed under confocal microscope and at least 5 fields were counted per each experimental condition.

Hydrogen peroxide (H_2O_2) release assay

Although the turtle is able to survive repeated anoxia/reoxygenation events, turtle neurons were found susceptible to extrinsic stress of hydrogen peroxide (Milton et al., 2007). Hydrogen peroxide (H_2O_2) by itself is inactive but it can diffuse freely across the cell membrane and can transform into the highly reactive hydroxyl radical (Cui et al., 2004). The extent of H_2O_2 released into the extracellular medium of the control and knockdown cultures was measured by using Amplex Red assay kit (Molecular Probes) utilizing the manufacturer's protocol. A mix of horseradish peroxidase (HRP) and the fluorescent Amplex Red was added to 50 µl of the culture medium of each condition and the fluorescence emitted (on reduction of H_2O_2) was read using a microplate reader at 560 nm.

Western blot analysis

The primary antibodies used were Hsp72 (Stressgen, Victoria, BC, Canada); Rabbit (Chemicon, Temecula, CA); Bax (Santa Cruz Biotechnology, Santa Cruz, CA); Cyt C (Abcam Inc, Boston, MA) Caspase 3 (Cell Signaling Technology, Danvers, MA); AIF (Stressgen); pJNK(Cell Signaling Technology); and pERK (Cell Signaling Technology). All primary antibodies except for Hsp72 (1:4000) were used 1:1000 dilution.

Total cell protein was extracted using RIPA cell lysis buffer (0.15 M Nacl, 5 mM EDTA pH 8, 1% Triton X100, 10 mM Tris-Cl pH 7.4; with added 5 M DTT , 100 mM PMSF, 5 M mercaptoethanol diluted 1:1000) and 10 µg of the protein was loaded on a

12% SDS-PAGE gel. The gel was transferred onto a nitrocellulose membrane (Hybond ECL, Amersham Biosciences, Piscataway, NJ) for 1 h at 0.3 mA. The membrane was blocked in 5% milk for 1 h and then incubated with the respective primary antibody overnight at 4°C in 2% milk. The blot was washed 3X in TBS-T and incubated with respective secondary antibody (Southern Biotech #4050, Birmingham, AL) at room temperature for 2 h and after 3X rinses, the bands were visualized by ECL chemiluminescence (Amersham Biosciences). The band intensity was measured using NIH ImageJ software.

Separation of mitochondrial and cytosolic fractions

Mitochondrial and cytosolic fractions were isolated to analyze the levels of Cyt c using Cytochrome C release apoptosis assay kit (Calbiochem, Gibbstown, NJ). Cells that were grown to confluence were first washed with PBS and then the cells were scraped using a sterile cell scraper. The cells were then centrifuged at 600g for 10 min at 4°C. The pelleted cells were suspended into the cytosolic buffer and incubated for 10 min on ice. The cells were then homogenized using a glass douncer and the homogenate centrifuged at 700 g for 10 min at 4°C. The supernatant was collected as the cytosolic fraction and the pellet suspended in the mitochondrial extraction buffer.

Statistical analysis was performed using Mintab (Minitab Inc, State college, PA, USA). Results were analyzed using one way ANOVA (Analysis of variance) and two way ANOVA and the data were expressed as Mean ± SE.

3.3. Results

Isolation of neurons from juvenile turtles

The isolation of adult neurons is a challenge due to complex neuronal networks and the inability of adult neurons to divide *in vivo* (Brewer, 1997). Brewer (1997, 1999) and Brewer and Torricelli (2007) have shown that adult rat hippocampal neurons can be cultured from young and old rats by separating the neural cells in a density gradient and then providing trophic factors to stimulate growth. We isolated neural cells from juvenile freshwater turtles by optimizing Brewer's protocol. After plating the cells, cell survival and the proportion of cells positive for neuronal markers was recorded to ensure that cells were not being overgrown by fibroblasts. Cell survival at 10 and 21 DIV averaged 78.7 ± 6.0 and 76.3 ± 5.7 % of cells present at day 4, respectively. (130cells / mm^2 at 4 d and 88 cells / mm^2 by day 21 (Fig 1).

The growing cells show neuronal morphology by 14-21 DIV (Fig 2a) .Cells 10-14 DIV were positive for the neuronal marker NeuN and Neurofilament (Fig 2b-2c) indicating that these cultures are populated with neuron like cells. Cells positive for neuronal markers had several asymmetric tapered processes by 10-14 DIV, though extensions and branching were observed to be less extensive than typical mammalian neuronal morphologies (Fig. 2).GFAP positive cells in 2 weeks old culture were less than 20% of the total cell number showing that the cultures were enriched with neurons.

Upregulation of Hsp72 in neuronal enriched primary cultures

Western blot analysis with the antibody against Hsp72 was used on the proteins extracted from the cell cultures. Protein used were from 3 different anoxic time points (4 h, 8 h, and 16 h) along with the normoxic controls (n=5). The cell cultures were subjected

to anoxia by placing them in Bactron hypoxia chamber (Sheldon manufacturing, Cornelius, OR). As reported in whole brain (Prentice et al, 2004; Kesaraju et al., in prep), there was significant upregulation of Hsp72 in neuronal cultures through anoxia. *In vitro* the expression levels of Hsp72 increased by 163.8±23.4 % by 4h anoxia, followed by a decrease to basal at 8h and a further 2-3 fold increase by16 h (Fig 3 ($P<0.05$) indicating a likely role for Hsp72 both in the transition to hypometabolism and in long-term anoxic cell survival.

Analysis of gene knockdown by targeting siRNA against Hsp72

It has already been suggested that Hsp72 is neuroprotective in turtle neurons against anoxia (Chang et al., 2000; Prentice et al., 2004) due to the detection of enhanced levels on exposure to anoxia. To test this hypothesis and further characterize the role of Hsp72 in anoxia and reoxygenation, siRNA (small interference RNA) was used to specifically knockdown Hsp72. A 300 bp sequence of Hsp72 gene was sequenced from an RT-PCR product of the whole brain RNA of turtle and used to make a specific turtle Hsp72 siRNA product. Using GFP-positive cells as a marker of transfection efficiency with lipofectamine showed 70% successful transfection after 48 h. siRNA treatment produced a 50-60% decrease ($p<0.05$) in the expression of Hsp72 when compared to treatment with controls using scrambled siRNA in the normoxic conditions The knockdown effect was achieved by utilizing 100 pM of siRNA in the normoxic (Fig 4) conditions; greater concentrations of siRNA, 250 pM, were needed in anoxia (Fig 4b) and A/R (Fig 4c) to achieve the same level of reduction, due presumably to the native increase in Hsp72 expression.

Hsp72 is critical for survival of the neurons during anoxia and reoxygenation

The present study and earlier studies have shown increased levels of Hsp72 both *in vivo* and *in vitro* during anoxia. However, it was not known if this increase was indeed neuroprotective during anoxia or anoxia/reoxygenation stress. Consistent with our hypothesis, we found that siRNA silencing of Hsp72 significantly increased cell death in anoxia and A/R. In the normoxic condition, the percentage of dead cells increased from the control group to the knockdown group from 16.5%± 2.3% to 28.7±_2.2%. Anoxia alone increased cell death in the control group to 25.5± 0.8% but knockdown further increased cell death to 56.84±10.34% of total cells. The stress of reoxygenation is apparent as an increase in the proportion of dead cells even in the control group, and Hsp72 knockdown significantly increased cell death in neurons after reoxygenation by an additional 35% of the untreated controls ($p<0.05$) (Fig 5).

While it is apparent, then, that Hsp72 provides neuroprotection to turtle neurons in the case of anoxia and reoxygenation stress, the mechanisms are yet unknown. Neuroprotection may occur through an upregulation of protective mechanisms and/or via the suppression of pro-apoptotic pathways (Kelly et al., 2002; Matsumori et al., 2005; Stankiewicz et al., 2005). The effect of Hsp72 knockdown on several key survival and apoptotic effectors was thus investigated.

Interaction of Hsp72 with the apoptotic pathways prevents cell death during anoxia and reoxygenation

Hsp72 and Bcl2

Mammalian ischemic studies have shown that the interaction of Hsp72 at several points upstream and downstream of mitochondria in the apoptotic pathway aids in

preventing cell death. Kelly et al. (2002) have shown overexpression of Hsp72 resulted in increases in the expression of the anti-apoptotic protein Bcl2, showing a positive correlation between the two proteins that inhibits cell death. As Hsp72 knockdown increased cell death, it was thus expected that silencing the expression of Hsp72 in the turtles would lower the expression of Bcl2. Anoxia alone (scrambled siRNA controls) resulted in a larger but not statistically significant increase of Bcl2 compared to normoxic controls. While exposure to anoxia decreased the expression of Bcl2 in knockdown cultures slightly (101.96±7.06%) compared to control siRNA treatment (110±2.5% of normoxia), the difference was not statistically significant. Reoxygenation induced greater changes in Bcl2 expression in the control samples (130.2±9.7%) and was significantly downregulated (112.1±3.3% over baseline) in the Hsp72 knockdown samples (Fig 6a). However, as it seems unlikely that this decrease in Bcl-2 expression alone is responsible for the approximate 2-fold increase in cell death detected in knockdown cells after anoxia/reoxygenation, the effects on other apoptotic effectors was also examined.

Hsp72 and Bax

In the present study we analyzed Bax expression in response to 4 h anoxia and 4 h anoxia/4 h reoxygenation in the presence of siRNA against Hsp72 (Fig 6b). Hsp72 knockdown increased Bax expression under all conditions. The normoxic expression of Bax increased in knockdown samples to146±2.1% over basal, while in anoxia, Bax levels were higher (127±7.34%) than in control siRNA treated samples in comparison to the normoxic control (91.1±4.8%). Reoxygenation induced Bax significantly in siRNA treated cultures (145.7±7.14%) in comparison to the control (112.6±8.3%).

Bcl2: Bax and Hsp72

Stressors such as ischemia and exercise decrease Bcl2/Bax expression ratios (Kwak et al., 2006). Bax, a pro-apoptotic protein, aids in dislodging Bcl2 from the mitochondrial membrane and opening the mitochondrial pore. So, high levels of Bax inactivate Bcl2 (and vice versa). Therefore, the ratio of Bcl2 to Bax, rather than absolute values, might provide a better idea of whether the balance is tipped towards either pro-survival or pro-apoptotic pathways in the anoxia tolerant neuron. In this study, blocking the expression of Hsp72 in the neurons of the turtle resulted in lower levels of Bcl2 compared to controls and higher levels of Bax, thus decreasing the Bcl2 to Bax ratio in comparison to the control (Fig 6c).

Cell death in Hsp72 knockdown neuronal cultures does not depend on the alterations of Cyt c and caspase 3

Hsp72 and Cyt c

If a decrease in the Bcl2/Bax ratio shifts the internal balance of a cell towards apoptosis, then one would expect to see increases in the downstream effectors of cell death, including Cytochrome c and the caspases. There may also be interactions of Hsp72 with effector mechanisms downstream of the mitochondria, though these are still under debate. Many reports have suggested that Hsp72 inhibits apoptosis irrespective of Cyt c release (Li et al., 2000; Steel et al., 2004). Release of Cyt c from the mitochondria into the cytosol is the key step for formation of the apoptosome and activation of caspases in the intrinsic pathway of apoptosis. We probed the mitochondrial and cytosolic fractions of the control and siRNA treated cultures with Cyt c antibody. Though we found significant increases in Bax in the knockdown cultures, the expression of Cyt c

was unchanged. While the mitochondrial fraction of Cyt c decreased slightly in knockdown anoxic cultures, this change was not statistically significant and not accompanied by any increase in Cyt c in the cytosolic fraction (Fig 7 a).

Hsp72 and Caspase3

Mammalian ischemic models show that overexpression of Hsp70 attenuates ischemic injury by downregulating the expression of caspase 3 (Suzuki et al., 2002; Wang et al., 2002). Still the interaction of Hsp70 downstream of mitochondria is disputed. Stankiewicz et al. (2005) have suggested that the protective function of Hsp70 is limited only while the mitochondria membrane potential (MMP) is preserved, and, once the MMP is lowered and Cyt c released, Hsp70 cannot inhibit apoptosis. Preliminary observations have suggested mild mitochondrial membrane depolarization of the neuronal cells in the knockdown cultures during anoxia and reoxygenation (results not shown). The presence of cleaved caspase 3 was noted even under normoxic conditions in the control and remains unchanged over anoxia and reoxygenation (Fig 7 b). As was seen with Cyt c, then, the partial silencing effect of Hsp72 did not have an effect on the expression of cleaved caspase 3.

Hsp72 prevents cell death via inhibiting the activation of apoptosis inducing factor

Hsp72 and AIF

The lack of any effect of Hsp72 knockdown on the expression of key executioners of the intrinsic pathway were contrary our findings that knockdown leads to increased cell death. To investigate the contradictory results, the expression of AIF was analyzed in the control and knock down cells (Fig 7c). In the control samples, the expression was not altered during anoxia but reoxygenation induced the expression of AIF (138.2±6.7%)

compared to basal. By contrast, reoxygenation in the knockdown samples led to significant increases of AIF (211± 11.6%) compared to the basal, thus presenting a possible reason for increased cell death during reoxygenation in the Hsp72 silenced neuronal cultures. . Normoxic and anoxic exposure in the knockdown experiment also triggered additional AIF increases that were not significant.

Hsp72 aids in suppression of ROS formation upon reoxygenation

Even though AIF was highest during reoxygenation, Hsp72 may protect cells by other mechanisms as well. We investigated the release of ROS in the knockdown cultures to understand the interaction Hsp72 and ROS (H_2O_2) formation. In untreated controls, it has been shown that ROS levels fall by 4h anoxia, and increase upon reoxygenation only to basal, without the massive overproduction which occurs in mammalian cells following anoxia/ischemia (Milton et al., 2007). The levels of hydrogen peroxide in the medium were higher in Hsp72 knockdown cultures in normoxic and anoxic samples, though the increase was modest. In sharp contrast to control cultures, however, 4 h Anoxia/ 2 h reoxygenation in Hsp72 knockdown cultures resulted in a five-fold increase in H_2O_2 (Fig 8 a)($p<0.05$).

Hsp72 differentially regulates MAP kinases during anoxia and reoxygenation

MAP kinases are triggered not by ischemia alone but also by ischemia/reperfusion indicating ROS as a mediator in their activation (Armstrong, 2004). In energy depleted cardiac cells, overexpression of Hsp72 suppressed the activation of JNK and had no effect on ERK (Gabai et al., 2000) and thereby prevented apoptosis and necrosis. It can be thus predicted that knockdown of Hsp72 might affect JNK and ERK expression in our anoxia/reoxygenation -tolerant model system. In the whole brain, phosphorylated JNK

levels are largely unaltered by 4 h anoxia or anoxia/reoxygenation, while activated ERK levels increase significantly by 6-fold in the first hour of anoxia before returning to basal. A similar but slower pattern appears in control cultures where the expression of JNK remains unchanged while ERK progressively increases from normoxia to anoxia and peaks during reoxygenation. As seen in vivo, the ERK upregulation during anoxia was not seen in vitro as we have not measured that initial time point. Contrary to the mammalian models, suppression of Hsp72 did not alter the expression levels of pJNK (Fig 8b). But, the expression of pERK in Hsp72 silenced cultures increased over anoxia to reoxygenation significantly from the controls in anoxia and reoxygenation (Fig 8c). In addition, when neuronal cell cultures were incubated with specific pERK blocker, U0126, the expression of Hsp72 increased two fold by the end of 4 h reoxygenation after anoxia ($p<0.05$)(Fig 8d).

3.4. Discussion

Freshwater turtles are highly resistant to anoxia, and histological results show no apparent neuronal damage during anoxia or anoxia/reoxygenation (Kesaraju et al., in prep). Turtles also express high constitutive levels of Hsp72 in vivo that are induced by anoxia, concurrent with the apparent suppression of pro-apoptotic factors. In vitro analysis using neuronal cultures has likewise shown high basal levels of Hsp72 that are further elevated during anoxia. Therefore, we hypothesized that Hsp72 is neuroprotective through its interaction with pro-survival and pro-apoptotic pathways, as suggested by mammalian models. In rodent models Hsp72 is expressed at minimal to undetectable levels under normal conditions but is induced after insults such as experimental ischemia by middle cerebral artery occlusion (Chen et al., 1996; Weinstein et al., 2004). In

mammals, the expression of Hsp72 determines the delineation of the unsalvageable ischemic core and the salvageable penumbra in a cerebral infarction (Sharp et al., 2000; Weinstein et al., 2004), and is thus also a significant factor in preconditioning models (Sharp, 2000). The time course of the expression of Hsp72 after preconditioning corresponds to the window of protection, indicating a prominent role in ischemic tolerance (Chen et al., 1996; Sato and Matsuki, 2002). Overexpression of Hsp72 through viral vectors or transgenic animals also reduced the infarct size and induced protective pathways (Hoehn et al., 2001; van der Weerd et al., 2005; Badin et al., 2006). This is in direct contrast to turtles, which appear to bank high levels of stress proteins and thus may be "constiutively preconditioned".

Knockdown studies of Hsp72 in the turtle resulted in cell death that increased progressively in anoxia and reoxygenation suggesting that, as in mammalian ischemic models, Hsp72 is critical for neuronal survival. The mechanisms behind the protection offered by Hsp72 are widely ascribed to chaperoning of misfolded proteins or preventing denaturation of the proteins under stress, but many mammalian studies in recent years have also describe Hsp72 as anti-apoptotic due to its many interactions at points in the apoptotic cascade during cerebral ischemia (Kelly et al., 2002; Giffard et al., 2008). Hsp72 has been reported to block apoptosis by interacting with Bcl2, Bax, Cyt c, Apaf1, and caspases.

Members of the Bcl2 family are key regulators of apoptosis balancing the pro and anti-apoptotic pathways (Graham et al., 2000), with Bcl2 and Bcl-Xl being the main pro-survival factors, while Bax and Bad , for example, promote apoptosis. Overexpression of Bcl2 reduced infarct size (Martinou et al., 1994), while chemical inhibition of Bax

protected neuronal cells from damage in rodent models of ischemia (Liu et al., 2008). Bcl2 and Bax are two factors thought to be influenced by HSPs: increases in Hsp72 expression increase Bcl2 (Kelly et al., 2002) during cerebral ischemia and prevents Bax translocation in heat stressed cells (Stankiewicz et al., 2005).

In mammalian models, changes in the Bcl-2:Bax ratio are better predictors of cell fate than absolute values although the exact ratio of Bcl2:Bax necessary to initiate apoptosis is not definitive. The regulation of Bcl2 and Bax is varied in different model systems; hypoxia ischemia followed by 4 h of reoxygenation in a postnatal mouse brain did not show any changes in the Bcl2: Bax ratio whereas after 24 h of reoxygenation the ratio was decreased (Zovein et al., 2004) and 3 h of hypoxia in a perinatal rat brain did not induce any changes while 6 h hypoxia decreased Bcl2 to Bax leading to increased cell death (Vert and Daval., 2006). Knockdown of Hsp72 in the turtle neuronal cultures resulted in moderate decreases in Bcl2 expression and increases in the expression of Bax, leading to 30-40% decreases in the Bcl2/Bax ratio that might have been sufficient to shift the balance towards apoptosis. In untreated neuronal cultures, the ratio of Bcl2/Bax was always greater than 1.0, while this ratio in siRNA treated cells was decreased on average to 0.7-0.8. It is difficult to conclude that the moderate decrease in Bcl2 to Bax ratio would be sufficient to trigger cell death in the turtles as we do not know the sub cellular localization of Bax, and an upregulation of Bax does not necessarily indicate a redistribution to mitochondria (Cai et al,. 2001). Ischemia/reperfusion in rodent models results in the translocation of Bax to the mitochondria and releases Cyt c that activates caspases (Lin et al., 2005). In this study, changes in Bcl2 and Bax were not accompanied

by the activation of any of their downstream effectors, indicative of the further activation of apoptotic pathways; neither cyt c nor caspase-3 levels increased in knockdown cells.

Bcl2 prevents apoptosis by inhibiting the action of Bax and also the release of Cyt c (Giffard, 2008). Release of Cyt c triggers the apoptotic cascade by activating apaptosis-protease activating factor-1 (Apaf-1) and caspase-9 (Garrido et al., 2006). Sugawara et al (1999) first showed evidence that global ischemia in rats resulted in increased expression of Cyt c in the cytosolic fractions of the hippocampus and a parallel reduction in the mitochondrial fractions. Many studies have since reported the ability of Hsp72 to inhibit the release of Cyt c during ischemia (Lee et al., 2004; Tsuchiya et al., 2003). Contradictory to these findings, knockdown of Hsp72 in turtle neuronal cultures did not have any effect on the cytosolic fractions and there was only an insignificant decrease in the mitochondrial fractions. However, Steel et al (2004) have shown that Hsp72 does not exert any influence on the binding of Apaf 1 and the release of Cyt c and activation of caspases as observed in several cell lines, suggesting the targets for Hsp72 interaction are upstream the mitochondria. It was also shown that overexpression of SOD (superoxide dismutase) inhibits the release of Cyt c; reduced levels of Cyt c in cytosolic fractions was found in SOD transgenic mice and enhanced level of SOD inhibits the activation of caspase 9 (Fujimura et al., 2000; Noshita et al., 2001) thereby preventing the formation of apoptosome and activation of effector caspases. High basal levels of anti oxidant enzymes such as SOD, glutathione peroxidase (Gpx), ascorbate and catalase are observed in freshwater turtles (Rice et al., 1995; Wilmore and Storey, 1997) suggesting that several alternate mechanisms operate to prevent cell death in addition to any direct blockade of apoptosis by Hsp72.Along with no apparent change in Cyt c, levels of active

caspase-3 were unaltered in Hsp72 knockdown cultures. Tanaka et al (2004) have shown that ischemia induced caspase 3 activation in neurons that were dying, while conversely preconditioning has shown the expression of caspase 3 in the neurons that survive, suggesting suppression of events downstream from caspase 3. Hsp expression has been shown to block caspase activation (Mosser et al., 2000; Lee et al., 2005), and overexpression of Hsp70 prevented the activation of the Caspase activated DNase (CAD), a caspase downstream regulator of apoptosis that fragments DNA, indicating functional interaction between Hsp70 and CAD (Liu et al., 2003). The caspase-3 findings presented here are in line with the Cyt c results suggesting that the targets for Hsp72 in the anoxia tolerant model system might be upstream to mitochondria or downstream of the caspases.

Although numerous studies have shown that Hsp72 also prevents the formation the apoptosome by physically binding to Apaf 1 , Ravagnan et al (2001) have shown that Hsp72 can suppress cell death even in cell systems that lack Apaf 1 (Apaf 1(-/-)) by inhibiting AIF in a caspase-independent mechanism of apoptosis. AIF is a mitochondrial protein that translocates into the cytoplasm and nucleus in response to apoptotic stimuli, leading to DNA fragmentation or chromatin condensation, and was found to be in inhibited by Hsp70 (Cande et al., 2004; Gurbuxani et al, 2003). In the turtle, expression of AIF was induced more than two-fold in the Hsp72 knock down cultures upon reoxygenation. Knockdown of Hsp72 also increased expression of AIF in normoxic and anoxic conditions by 129.2 and 161.0 % respectively, but the effect was more pronounced during reoxygenation. Because AIF release results in both apoptosis and necrosis (Daugas et al., 2000) and Hsp72 can protect the cells from both cell death

pathways (Kelly et al., 2002; Yaglom et al., 2003), cell death in anoxia and reoxygenation in the knockdown cultures may be attributed to increased AIF release . It was shown that the mechanism of release of AIF from the mitochondria was different form that of Cyt c, since inhibition of AIF rescued neurons in an Apaf (-/-) neuronal cells (Cregan et al., 2001), though AIF may still be linked to Hsp72 and Bax. Overexpression of Hsp70 in kidney cells inhibited Bax activation and AIF release, suggesting that AIF release is dependant on the activation of Bax (Ruchalski et al., 2006). As shown in our results knockdown of Hsp72 resulted in decrease of Bcl2/Bax ratio that might result in sufficient leakage of AIF from the mitochondria to trigger cell death in neurons even if the shift in Bcl2 to Bax is not large enough to trigger caspase-dependent apoptosis. However, results in vivo suggest that the brain survives with similar increases in AIF. So, other protective mechanisms such as inactivation of JNK or PARP (Poly-ADP-ribose polymerase) may counter the translocation of AIF to nucleus preventing ischemia/reperfusion injury (Song et al., 2007).

One way Hsp70 inhibits Bax is through suppressing the activation of c-jun kinase (JNK). Suppressed JNK activity inhibits Bax translocation to the mitochondria (Stankiewicz et al., 2005). However, in the turtle, levels of pJNK were found unchanged by the stress of anoxia and reoxygenation in the controls. Knockdown of Hsp72 did not change the expression of pJNK compared to untreated cells. While the results in cell culture correspond to earlier studies on MAP kinases in the turtle in vivo, that have also shown no change in the expression pJNK (Milton et al., 2008), it makes it more difficult to explain Bcl2 /Bax regulation. pJNK appears to have little role in cell survival or cell death in this model under basal conditions, nor is JNK activation affected by Hsp72. It is,

in fact, likely that other protective pathways are functioning in addition to the HSPs, and these pathways modulate cell death pathways. One such potential protective pathway is ERK1/2. ERK is thought to protect mammalian cells against apoptosis in many models because it aids in activation of Bcl2 and suppression Bax and caspases (Hetman and Gozdz, 2004).

Milton et al. (2008) have shown a temporary but significant six fold upregulation of pERK in vivo in the initial hour of transition to anoxia, suggesting a protective function on the assumption that highly upregulated functions in an anoxia tolerant model are protective. Knockdown of Hsp72 increased pERK levels during reoxygenation and blocking pERK by chemical inhibition induced 194.7± 4.0% increase in Hsp72 during reoxygenation that was not seen under control reoxygenation conditions. While these are likely to be generalized responses to cell stress, e.g. increased stress from Hsp72 knockdown increases ERK protection, while the loss of ERK protection stimulates the general HSP response, nonetheless increases in ERK to 170% of basal when Hsp induction is inhibited may be sufficient to block Bax/caspase dependent apoptotic pathways, such that cell death occurs by other mechanisms.

ERK activation is mediated through ROS in hypoxia induced neurons/neuronal cultures/astroglial cultures but the nature of ERK action as protective or destructive is a matter of debate (Hou et al., 2003 de Bernardo et al., 2004; Sun et al., 2008). ERK activation is protective in pharmacological and hypoxia/ischemia models of neuronal insult (Sun et al., 2008; Jia et al., 2008). Under the circumstance of turtle anoxia, if ERK is protective, a near two-fold induction in Hsp72 knockdown cells was not sufficient to rescue the neurons from death even if apoptotic mechanisms were inhibited. 1 h of

anoxia induced a 6 fold increase of pERK in vivo, but in vitro 4 h anoxia only increased pERK to 112.6±8.8% of basal and to 125.3±10%_ of control upon reoxygenation. While knockdown of Hsp72 induced a 1.8 fold increase in pERK upon reoxygenation, this degree of protection is apparently not sufficient enough to protect the neurons, especially not in the face of the massive release of ROS that occurs in knockdown cells.

Several studies have shown that AIF release is mediated through ROS (Kang et al., 2004; Thayyulathil et al., 2008) and the absence of Cyt c increase, increased AIF and pERK expression prompted us to study the effects of Hsp72 knockdown on ROS production. As has been shown previously (Milton et al., 2007; Pamenter et al., 2007), reoxygenation did not lead to a surge of ROS release in untreated cultures, but ROS did increase several fold in the knock down samples by the end of 2 h reoxygenation, clearly showing that Hsp72 is essential for suppression of ROS in turtles. In mammalian studies, overexpression of Hsp72 prevented the release of ROS in astrocyte cultures (Ouyang et al., 2006); increased levels of Hsp72 also increased the activity of SOD (Suzuki et al., 2002) and Hsp72 inhibits the activity of iNOS, a key enzyme in the release of NO and peroxynitrates (Feinstein et al., 1996).

There is much literature suggesting that a mitochondrial form of Hsp70 (Hsp75) is involved in ROS suppression in mammals (Liu et al., 2005; Williamson et al., 2008). This raises the question of where Hsp72 is localized in the turtle neurons. Immunohistochemical studies of Hsp72 on turtle cortical sections have shown a pattern suggesting a mitochondrial localization under high magnification. Preliminary experiments to understand the localization of Hsp72 was performed using Mitotracker Red (a mitochondrial ROS detecting probe). In normoxic controls, colocalization of a

Mitotracker Red and Hsp72 was observed (Results not shown). In mammalian models (exercise, preconditioning etc.), Hsp72 acts as an antioxidant with high levels of Hsp72 significantly reducing oxidative stress by attenuating lipid peroxidation and ROS release (Smolka et al., 2000; Moran et al., 2004; Wang et al., 2005). Therefore, in addition to high levels of anti-oxidant enzymes, high constitutive levels of Hsp72 might add to the repertoire of protective mechanisms that enable anoxia/ reoxygenaton survival.

Although all the key pathways appeared to be connected, many mechanisms act independently of each other such as the role of ROS in mitochondrial membrane permebalization and release of pro-apoptotic proteins (Cai et al., 2001) and activation of JNK and ROS pathways (Yaglom et al., 2003). The findings of our study suggest that the neuroprotective mechanisms of the turtle during anoxia occur through independent processes; Hsp72 modulates intrinsic apoptotic pathways with targets upstream of mitochondria, and cells die through caspase independent mechanisms. Hsp72 plays a critical role in establishing turtle's tolerance to anoxia and reoxygenation stress by inhibiting mitochondrial ROS and regulating the activation of pERK.

The freshwater turtle presents a unique system with high constitutive levels of Hsp72 and other HSPs, and the present study aids our understanding of the neuroprotective function of Hsp72 by examination of an anoxia tolerant model.

Figures

Figure 1: Total number of cells plated in primary neuronal culture.

Total number of cells after 4 days of in vitro growth (DIV), 10 DIV and 21 DIV. The cells were plated on 6 well plates with a grid. Cells were grown for 3 weeks and counted every week. The cells in the same field are counted at the end of 4 d, 10 d and 21 d. Mean ± SE (n=3, 4 fields per each plate).

Figure 2: Immunohistochemical analysis of neuronal cell culture. Primary neuronal enriched cell culture (14 DIV) from the turtle brain. (a). Morphology of turtle neurons under bright field microscope. Immunostaining of turtle neuronal culture under confocal microscope with (b) Anti- NeuN antibody staining nuclei (c) Anti-Neurofilament antibody staining neuronal filaments.

Figure 1

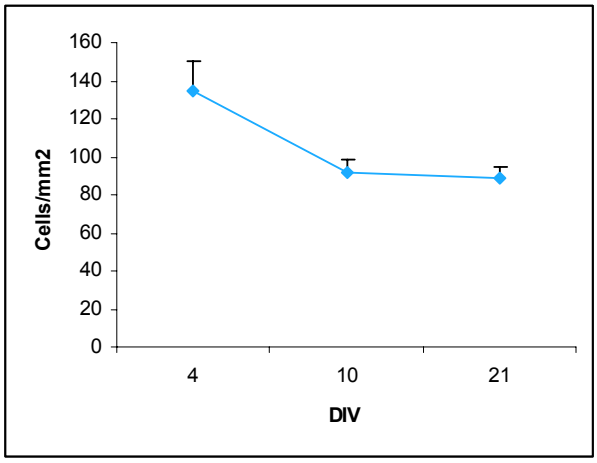


Figure 2

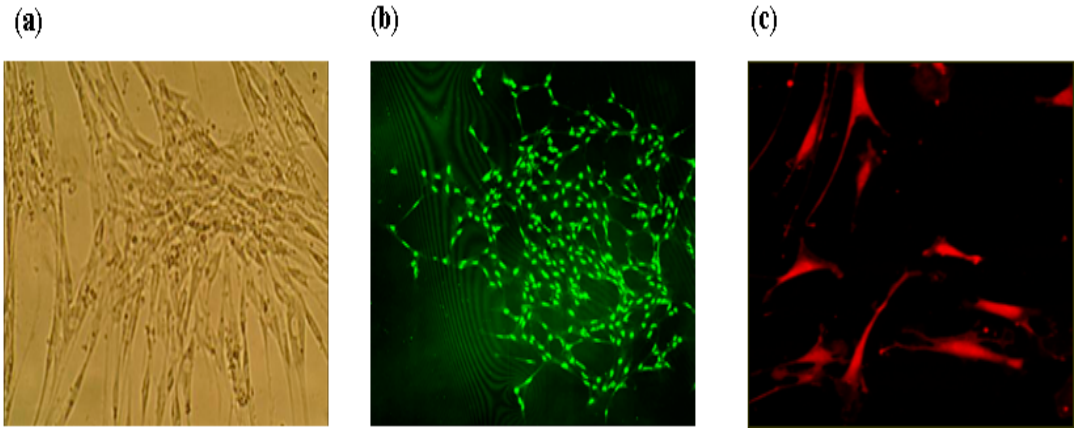


Figure 3: Anoxia induces Hsp72 expression in vitro. Protein from neuronal cell lysates (in vitro) subjected to 0 h (Normoxia / Control), 4 h, 8 h and 16 h anoxia shows marked upregulation (2-3 fold) of Hsp72, compared to the normoxic control as the animal progresses into long term anoxia. Representative western blot with densitometric analysis is shown. The samples in the western blot from left are normoxia , 4 h anoxia, 8 h anoxia and 16 h anoxia. Data is Mean ± SE (n=5), $p<0.05$. * = significantly different from normoxic control.

Figure 4: siRNA treatment against Hsp72 results in partial knockdown of Hsp72 expression in turtle neuronal cell cultures. siRNA treatment (+) partially silenced (40-60%) the expression of Hsp72 in comparison with control. Representative western blots are shown. Dose dependant knockdown of Hsp72 siRNA in

(a) Normoxia: 1. Control siRNA 2. 50 pM of target siRNA 3. 100 pM

(b) 4 h anoxia: 1. Control 2. 50 pM 3. 100 pM 4. 250 pM

(c) 4 h anoxia/ 4 h reoxygenation (4 h A/R) : 1. Control 2. 100 pM 3. 250 pM

Figure 3

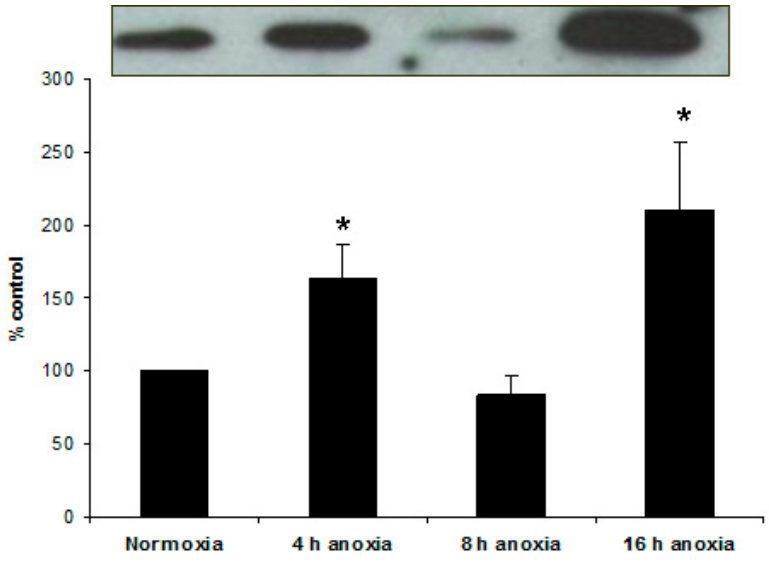


Figure 4

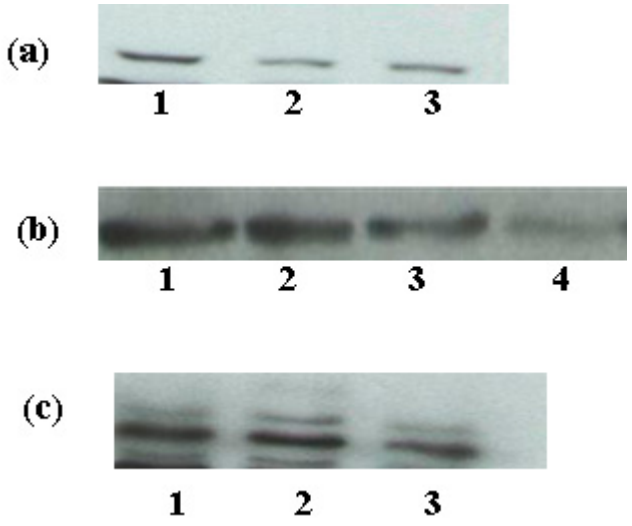


Figure 5: siRNA silencing of Hsp72 leads to increased cell death in anoxia and A/R. Cell death was assessed by counting PI+ cells to total cells in control group and the siRNA treatment group. (a) PI stained images of siRNA treated (i) Normoxia (ii) 4 h Anoxia (iii) 4 h A/R cell cultures. (b) Hsp72 knockdown significantly increased cell death in anoxic and A/R neurons by 20-35% vs. untreated controls. Data Mean ± SE, $p<0.05$. *sd normoxic controls # is sd from 4 h A/R controls.

Figure 5

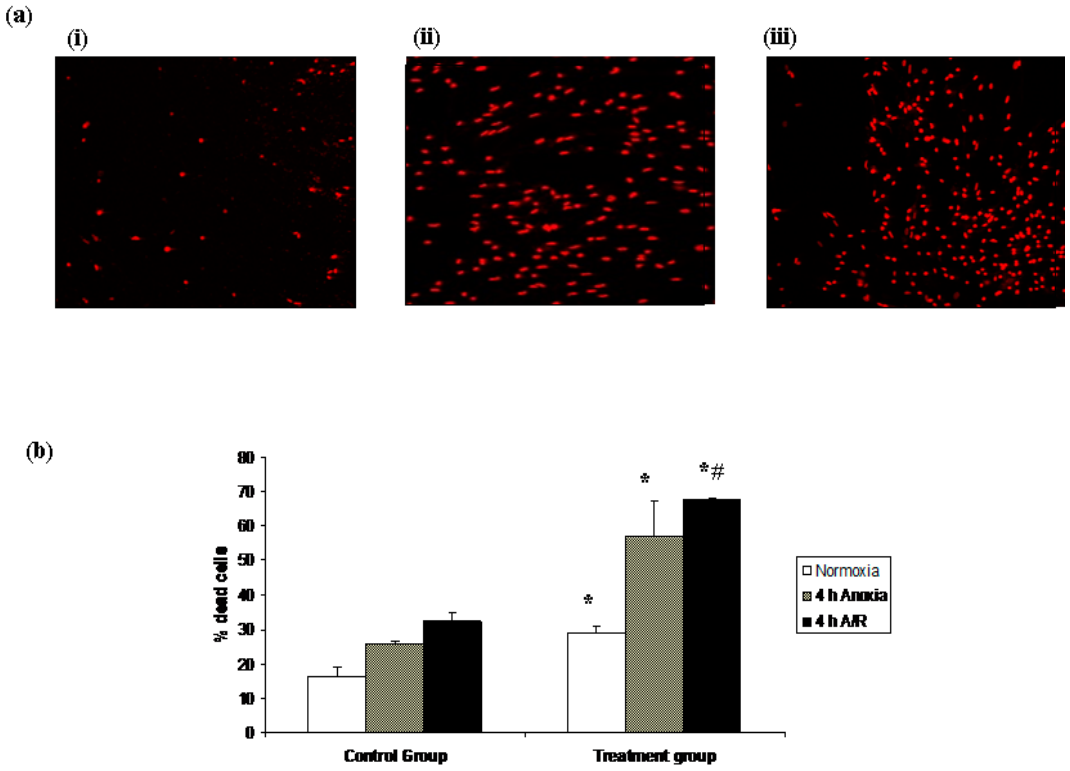

(a)
(i)
(ii)
(iii)
(b)
% dead cells
0
10
20
30
40
50
60
70
80
*
*
*#
Control Group
Treatment group
Normoxia
4 h Anoxia
4 h A/R

Figure 6. Analysis of mitochondrial proteins Bcl2 and Bax in the Hsp72 knockdown cultures. (a) Expression of Bcl2 in siRNA control and Hsp72 knockdown cultures. Inserts are representative western blots. The order of the samples in the western blot is normoxia, 4 h anoxia, 4 h reoxygenation from the left in both (i) control samples and (ii) siRNA treated samples. # sd from 4 h A/ R controls. (b) Bax levels in control and Hsp72 knock down cultures. (i) Bax protein levels in control samples (ii) Bax levels in knockdown samples. $ sd from anoxic control. (c) Ratio of Bcl2/Bax expression in the Hsp72 knockdown cultures

Figure 6

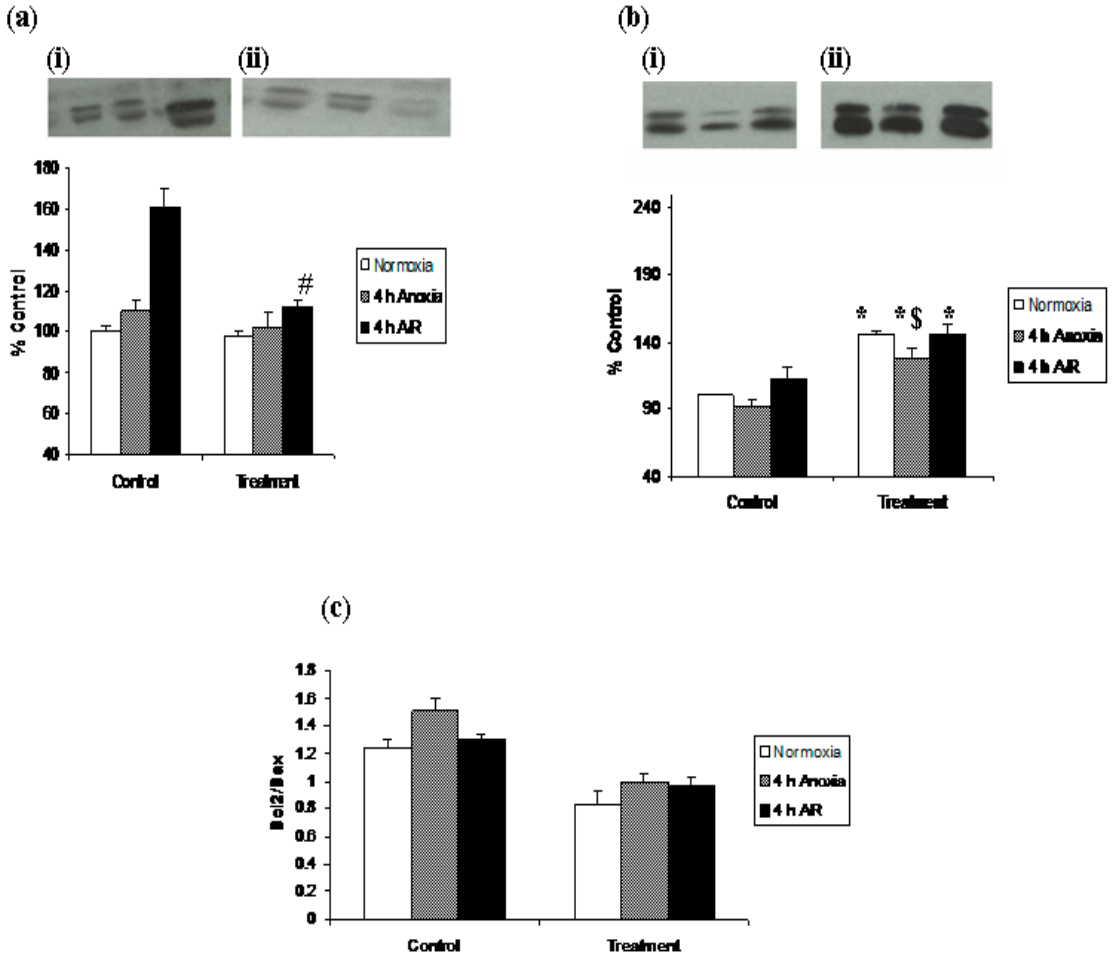

(a)
(i)
(ii)
% Control
Normoxia
4 h Anoxia
4 h AR
Control
Treatment
#
(b)
(i)
(ii)
% Control
Normoxia
4 h Anoxia
4 h AR
Control
Treatment
*
*
$
*
(c)
Bcl2/Bax
Normoxia
4 h Anoxia
4 h AR
Control
Treatment

Figure 7 Analysis of apoptotic regulators downstream of mitochondria in Hsp72 silenced cultures. (a) Expression of Cytochrome c in the mitochondrial fraction of Hsp72 Knockdown cultures (i) controls (ii) siRNA treated samples (b) Expression of activated caspase 3 in the Hsp72 knockdown cultures (i) controls (ii) siRNA treated (c) Expression of AIF in Hsp72 knockdown cultures (i) controls (ii) siRNA treated* sd from normoxic, # sd from 4 h A/ R controls.

Figure 7

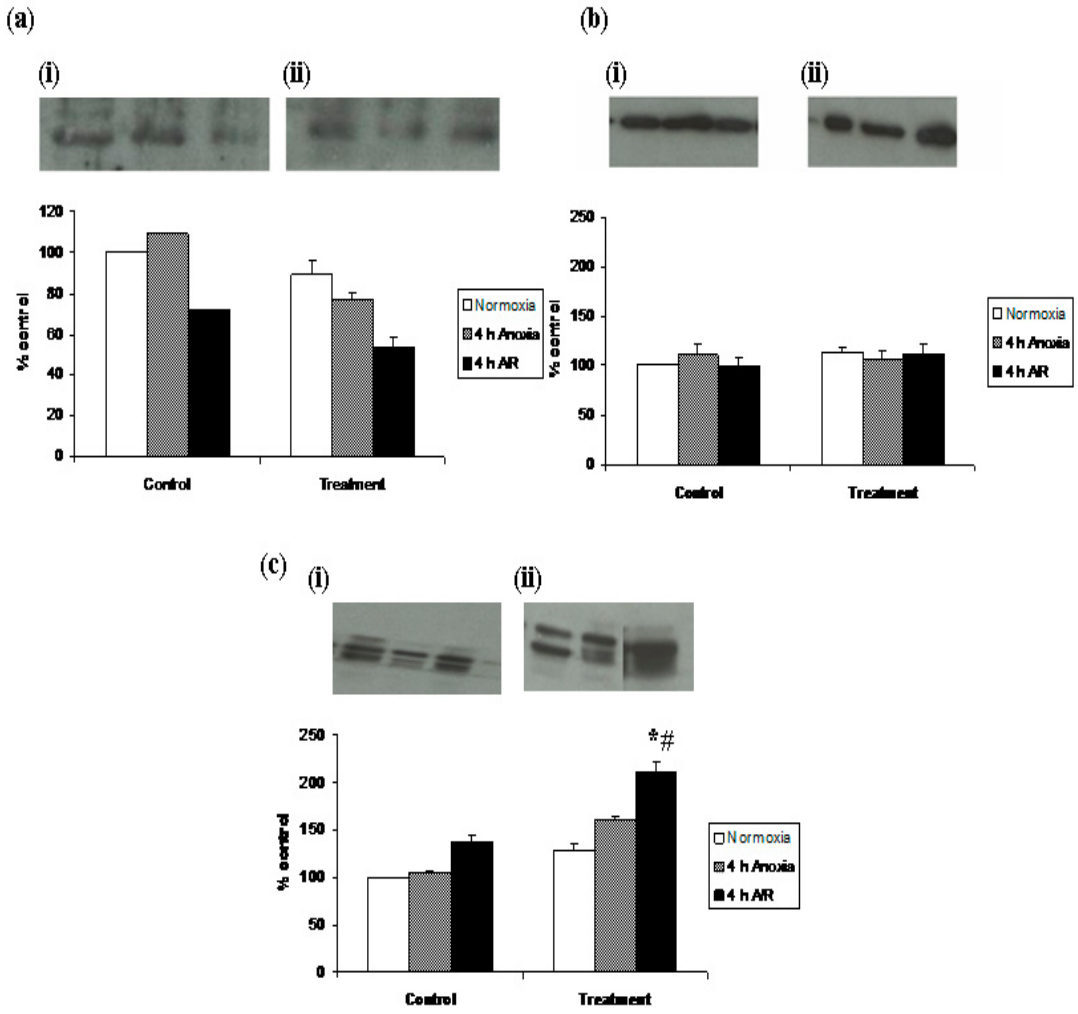
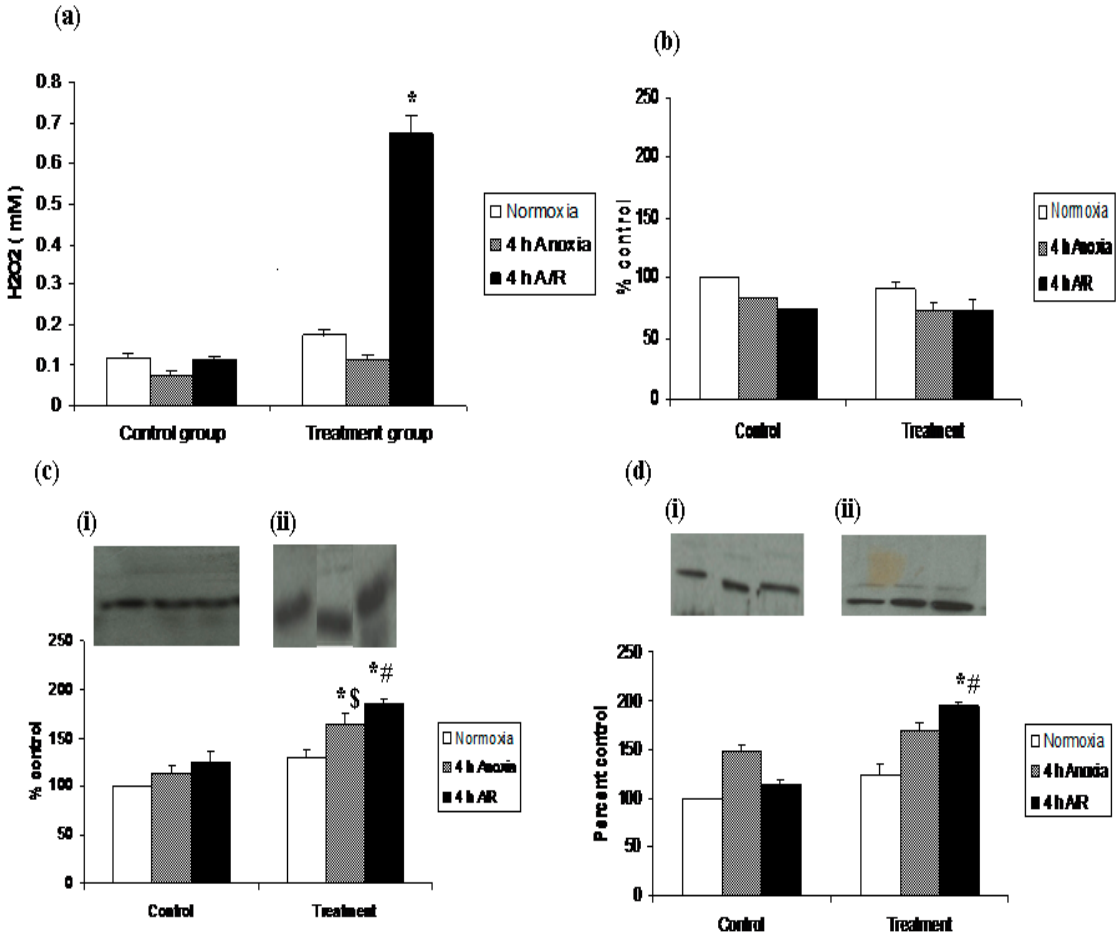

(a)
(i)
(ii)
% control
120
100
80
60
40
20
0
Control
Treatment
Normoxia
4 h Anoxia
4 h AR
(b)
(i)
(ii)
% control
250
200
150
100
50
0
Control
Treatment
Normoxia
4 h Anoxia
4 h AR
(c)
(i)
(ii)
*#
% control
250
200
150
100
50
0
Control
Treatment
Normoxia
4 h Anoxia
4 h AR

Figure 8. Analysis of Hsp72 knockdown effects on ROS and MAP kinases. (a) Knockdown of Hsp72 considerably increased the release of H_2O_2 released into the medium by 5-6 fold upon reoxygenation followed by 4 h anoxia. H_2O_2 concentration was calculated based on the standard curve. (b Densitometric analysis of expression of pJNK show no alterations in the knockdown samples compared to controls (c) Densitometric analysis with wetern blots are shown. Expression of pERK in the Hsp72 knock down cultures (i)controls (ii) siRNA treated (d)Cell cultures were treated with 10 μM of ERK blocker U0126 and analyzed for Hsp72 levels. (i) controls (ii)pERK blocked* sd from normoxic control, $sd from anoxic control and # sd from 4h A/R.

Figure 8


(a)
H2O2 (mM)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Control group
Treatment group
Normoxia
4 h Anoxia
4 h A/R
(b)
% control
250
200
150
100
50
0
Control
Treatment
Normoxia
4 h Anoxia
4 h A/R
(c)
(i)
(ii)
% control
250
200
150
100
50
0
*$
*#
Control
Treatment
Normoxia
4 h Anoxia
4 h A/R
(d)
(i)
(ii)
Percent control
250
200
150
100
50
0
*#
Control
Treatment
Normoxia
4 h Anoxia
4 h A/R

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CHAPTER 4: CHARACTERIZATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN ANOXIA AND REOXYGENATION

4.1. Introduction

Cerebral hypoxia/ischemia leading to stroke is one of the foremost reasons for morbidity and mortality in the United States (Doyle et al., 2008). Translation of successful animal neuroprotective models to human clinical trials is a formidable task as evident in multiple failed therapeutic interventions in providing neuroprotection (DeGraba and Pettigrew, 2000; Gladstone et al., 2002). One of the problems in success of clinical trials of stroke might be due to the inability to distinguish between protective vs pathological responses. However, not all organisms are equally susceptible to hypoxic/ischemic stress. An anoxia/hypoxia tolerant model system provides an opportunity to understand neuroprotective molecular and cellular events without the mixing of pathological responses and physiological adaptations.

The freshwater turtle *Trachemys scripta elegans* is one of the few anoxia-tolerant vertebrates that survive prolonged periods (up to a month at 3^{o}C) of total oxygen deprivation (Lutz and Milton, 2004, Ultsch, 2006). The turtle survives anoxia by drastic reductions in energy (ATP) demand, thereby matching demand to reduced supply (Lutz et al., 2003). The key factors that distinguish anoxia-intolerant and anoxia-resistant animals are the abilities to continue energy production and reduce demand to prevent catastrophic cell death by stabilizing pro-survival and pro-apoptotic pathways (Milton

and Prentice, 2007). Some of the physiological adaptations that enable the turtle to survive anoxia include increased anaerobic glycolysis (Hochachka and Lutz, 2001), reduced electrical activity (Sick et al., 1993; Fernandes et al., 1997), decreased ion channel conductance (Buck and Hochachka, 1993; Pek-Scott and Lutz, 1998) and modulation of neurotransmitters (decreased excitatory neurotransmitters and increased inhibitory neurotransmitters) (Nilsson and Lutz, 1991; Milton and Lutz, 1998; Thompson et al., 2007).

The mammalian brain responds to hypoxic/ischemic stress through several physiological adaptations including increased blood flow, increased glucose consumption and increased capillary density (LaManna et al., 2004). The key growth factor that stimulates the formation of these new blood vessels under hypoxia is vascular endothelial growth factor (VEGF). Like mammals, the turtle responds to hypoxic/ischemic stress through differential expression of several proteins including Hsc73, Hsp72 (Prentice et al., 2004), Kv channels (Prentice et al., 2003) and MAP kinases (Milton et al., 2008), Bcl2, Bax and Caspases (Haddad, 2007 a,b). Thus, it is of great interest to understand the neuroprotective role of vascular endothelial growth factor (VEGF) in anoxia tolerant turtle brain.

Abundant VEGF receptor transcripts were observed in the brain at early postnatal stages but these disappeared in adult mouse, suggesting a critical role for VEGF in embryonic angiogenesis in the brain (Millauer et al., 1993; Zachary, 2005). Neuronal VEGF protein levels decreased in adult rats and shifted to astroglia whereas in early developmental stages the expression was seen in cortical neurons (Ogunshola et al., 2000). The function of VEGF in adult brain is still under debate (Ogunshola et al., 2000)

although low levels of transcripts of VEGF are observed in the adult brain (Monacci et al., 1993). VEGF was initially reported as a vascular permeability factor and as an angiogenic factor (Senger et al., 1983, Leung et al., 1989) and has since been found to have a multitude of functions including neurogenesis and neuroprotection (Storkebaum et al., 2004, Greenberg and Jin, 2005). Regulation of VEGF expression has been associated with several pathological conditions such as hypoxia-ischemia, diabetes, glaucoma, stroke, Alzheimer's disease and amyotrophic lateral sclerosis (ALS) (Strokebaum et al., 2004; Ergorul et al., 2008).

Hypoxia induces VEGF in the astrocytes and endothelial cells facilitating increased permeability of the blood brain barrier (BBB) and angiogenesis (Ogunshola et al., 2000; Krum and Khaibullina, 2003; Krum et al., 2008). Hypoxia also triggers the expression of VEGF in neurons (Ogunshola et al., 2000). VEGF protected the hippocampal neurons from excitotoxic insult (Matsuzaki et al., 2001); over expression of VEGF stimulated angiogenesis (Shen et al., 2008) and neurogenesis (Wang et al., 2007) in the mouse ischemia model and exogenous addition of VEGF rescued hypoxic HN33 cells (Jin et al, 2000a) indicating a neuroprotective function of VEGF. Neuroprotective effects of VEGF were found to be mediated through signaling cascades involving PI3K/Akt (Jin et al., 2000b; Wick et al., 2002, Li et al., 2003), NFkB (Jin et al., 2000b, Sun and Guo, 2005), and Erk pathways (Matsuzaki et al, 2001). Pro-survival mechanisms of VEGF also include increased tyrosine phosphorylation of Kv channels (Qiu et al., 2003) and blockade of the apoptotic cascade (Jin et al., 2001; Raab et al., 2004).

Though the neuroprotective effects of VEGF have been demonstrated, many reports caution of adverse reactions to VEGF administration; hypoxic exposure leads to

increased expression of VEGF but also increased blood brain barrier leakage (Schoch et al, 2002). Administration of VEGF(165) 1 h after ischemia led to increased lesions and vascular leakage (Zhang et al., 2000), while angiogenic dosages after MCAO resulted in brain injury rather than recovery (Manoonkitiwongsa et al., 2004). The prime difficulty in therapeutic application of VEGF is that pathological events overwhelm beneficial physiological adaptations.

In turtles, the function of VEGF may not be angiogenesis since angiogenesis for repeated hypoxia-reoxygenation events might be pointless and potentially fatal. Although cerebral edema in anoxic turtle brain was reported (Cserr et al., 1988) but still the turtle brain is clearly protected from anoxia.

Along with VEGF, one of the primary regulators of VEGF expression is the transcription factor, Hypoxia inducible factor-1 (HIF-1), which acts as a hypoxia sensor and regulator of hypoxia tolerance in animals from *C.elegans* to humans (Semenza, 2004). Under hypoxic conditions, HIF- regulates the transcription of over seventy genes including critical enzymes for glycolysis, angiogenesis, cell growth and metabolism, and apoptosis (Wenger et al., 2005; Semenza 2004, 2007). HIF -1 alpha accumulates rapidly in hypoxic rats neurons and remains elevated for 14 days (Chavez et al., 2000); increased HIF-1 levels correspond with increased VEGF in rat hippocampal and cortical neurons after ischemia (Jin et al., 2000) while increased HIF- alpha transcripts and HIF-1 alpha induction reduce infarction in hypoxic preconditioned neonatal rats. The data thus suggest a critical role for HIF-1 alpha in sensing oxygen deprivation and thereby triggering downstream molecular events to increase survival (Sharp et al., 2001).

Therefore, the present study was designed to increase our understanding of the adaptive mechanisms against anoxia/hypoxia injury in an anoxia tolerant model system. Specifically we examined the regulation of VEGF expression during anoxia and reoxygenation and related this to the binding activity and expression of HIF-1 alpha in response to anoxia in the freshwater turtle.

4.2. Materials and Methods

Experimental Procedures: All experiments were conducted by approval of the Institutional Animal Care and Use Committee (IACUC) for Florida Atlantic University.

Whole animal induction of anoxia: Freshwater turtles (approx. 500g) were obtained from commercial suppliers (Clive Longdon, Tallahassee, FL). Animals were divided into four experimental groups: Normoxic control, 4 h anoxia, 24 h anoxia and 4h reoxygenation after 4 h anoxia (N=4-6/group). Animals were placed in sealed plastic boxes at room temperature (23-25°C) and subjected to anoxia for 4 h and 24 h by flushing the box with positive pressure 99.99% nitrogen (Air Gas, Miami, FL); control animals were taken directly from aquaria. All animals survived anoxia; for reoxygenation experiments, animals were placed in anoxia for 4 h and returned to dry tanks in air for 4 h. Immediately after the experimental conditions, animals were sacrificed by decapitation and the brain removed into liquid nitrogen in less than 2 mins. Frozen brains were then used for Western blot analysis and RT-PCR, below.

Preparation of neuronal enriched cell cultures: For in vitro work, primary neuronal enriched cultures were established from juvenile turtles (300-500g) as previously described (Milton et al, 2007). Briefly, density gradient centrifugation was utilized to separate neuronal fractions from glial fractions and debris (Brewer, 1997). The grey

matter is aseptically chopped and dissociated with a mixture of proteases: hyaluronidase (1300U/ml, Sigma-Aldrich, St. Louis, MO), collagenase (25U/ml, Gibco) and dispase (0.32U/ml, Gibco). After 4 h of incubation at room temperature the homogenate is separated by centrifugation in Opti-prep (Sigma-Aldrich, St.Louis, MO) gradient. The neuronal fraction is plated onto 6 well dishes and cells are grown at 300C in a 5% CO2 incubator in a culture media of MEM with L-glutamine and Earl's Salts (Mediatech, Inc., Herndon, VA) 10% FBS (Gibco/Invitrogen, Grand Island, NY) , 56U/ml penicillin, and 56ug/ml streptomycin (Gibco). Immunohisto-chemical analysis using NeuN, NCAM and GFAP confirmed the phenotype of the cells. Cells 14 DIV were positive for the neuronal marker NeuN and NCAM indicating that these cultures are populated >90% with neurons. For in vitro anoxic treatments, cells were placed at 30°C in a Bactron hypoxia chamber (Sheldon Manufacturing, Cornelius, OR) supplied with anaerobic gas mixture (90% N2, 5% He, 5% CO2, Air Gas, Miami, FL). Control experiments were carried out in the CO2 (5%) incubator at 30°C. For reoxygenation, cells were returned to the incubation chamber for 4 h after anoxia.

RNA isolation, RT-PCR and sequencing

Total RNA was isolated from the turtle brain and from cell cultures using Trizol reagent following the manufacturer's protocol. RNA was quantified using UV spectrophotometry and 1 μg of total RNA was used for RT-PCR. Preceding RT-PCR , the RNA sample was treated with DNase (Invitrogen, Eugene, OR). Reverse transcription was performed at 50°C for 55 min using 5 units Superscript III, 125 pM forward and reverse primers (IDT DNA), 1X First strand synthesis buffer, 0.1 mM DTT , and 2 units of RNAse OUT ribonuclease inhibitor. All the reagents for RT-PCR were

obtained from Invitrogen. To detect VEGF, PCR was performed using 100 ng of template, 200μM dNTP, 50 pM Forward and Reverse primer, 1X PCR buffer with 1.5 mM Mg and 5 U Taq polymerase. All PCR reagents were obtained from Fisher Scientific, Pittsburgh, PA. The amplification cycle consists of 5 min initial denaturation at 94°C followed by 35 cycles of 94°C for 1min; 58°C for 1 min and 72°C for 1min. Degenerate primers were made to detect VEGF; Forward Primer: 5' CTGGTGGANATNTTCCAG 3' where "N" could be A/G/C/T and Reverse primer: 5' TCTTTCTTTGGTCTGCATT 3' with a 233 bp PCR product . For all the reactions actin was run as an internal control that does not change with change in experimental conditions (Prentice et al, 2003). All the data was normalized with actin and expressed as percent control.

To confirm the identity of the PCR fragment obtained from VEGF amplification, the PCR product was run on 2% agarose gel and extracted from the gel. The PCR product was purified using Qiagen gel extraction kit (Qiagen, Valencia, CA) and sequenced (Davis Sequencing, Davis, CA).

Protein extraction and Western blot analysis

Protein was extracted from whole brain and cell cultures using RIPA lysis buffer (0.15 M NaCl; 5 mM EDTA, pH 8; 1% Triton X100;10 mM Tris-Cl, pH 7.4 and 5M DTT; 100mM PMSF; 5 M mercaptoethanol at a ratio of 1:1000 of the working lysis buffer). Brain tissue was homogenized in a glass douncer and cells were scraped using sterile cell scrapers. Homogenized tissue and cells were centrifuged at 13,000 rpm for 10 min at 4°C and the supernatant collected. Proteins were quantified using standard BCA protein analysis. 50 μg of protein was loaded on to 12 % acrylamide gels with actin used

as a loading control (Prentice et al., 2004, Milton et al, 2008). Proteins were separated at 150 v for 1 h and transferred to nitrocellulose membrane at 0.3 A for 1 h. The membrane was blocked in 5% milk for 2 h and incubated overnight at 4°C with primary antibodies: rabbit polyclonal VEGF (1:1000, Sc 152, Santa Cruz, CA) and rabbit polyclonal HIF 1α (1:1000, Novus Biologicals, Littleton, CO) and mouse monoclonal beta- actin (Sigma, St Louis, MO). Membranes were washed three times in TBS/Tween and incubated with HRP anti-rabbit and HRP anti - mouse secondary antibodies(Southern Biotech, Birmingham, AL) at 1:3000 dilution for 2 h at room temperature. Proteins were visualized by ECL chemiluminescence (Amersham Biosciences, NJ, USA) and quantified by densitometry using NIH Image J. All the data were standardized with actin and expressed as percent control.

Immunohistochemistry

Controls and animals exposed to 24 h of anoxia (n=2 each) were anesthetized and brains perfusion-fixed with 4% buffered paraformaldehyde. Cryostat sections were prepared at 40 micron thickness and reacted free-floating with rabbit-anti VEGF (anti-body A-20, sc-152; Santa Cruz) at 1:500 dilution in PBS/0.3% Triton X-100 at 4°C overnight. Bound antibody was visualized with swine anti-rabbit IgG (1:100) and rabbit-PAP complex (1:200) followed by DAB reaction for permanent labeling. To study expression of VEGF in neurons with double labeling were reacted with rabbit anti VEGF (1:100) and mouse anti-NeuN (1:200) followed by goat anti-rabbit Cy2 ™ and goat anti-mouse Alexa594.

Electrophoretic mobility shift assay (EMSA)

Probes/oligonucleotide primers for the Electrophoretic mobility shift assay contained HIF-1 binding sites (Hypoxia response elements, HRE) obtained from the rat iNOS(iNOS :TGACTACGTGCTGC CTA GGG GCC ACT GCC and iNOS mut GTGACTAAAAGCTGC CTA GGG GCC ACT GCC) (Matrone etal., 2004). Equimolar forward and reverse primers were annealed (annealing conditions: 940C for 1 min followed by incubation for 1 h at room temperature) in Klenow buffer (NEB, Ipswich, MA). The primers were radioactively labeled by using 50 pM / µl of annealed primers in T4 poly nucleotide kinase buffer (15 units, NEB) with γ32P (166 µci) and were incubated at 370C for 1 h. Excess label is removed by passing through sephadex G25 column. HIF-1 binding reaction was performed in a total reaction volume of 20 µl. Nuclear extracts were isolated from the primary neuronal cell culture using a nuclear extraction kit as per manufacturer's protocol (Panomics, Fremont, CA). The binding reaction mix containing 1x NEBB buffer (20 mM HEPES pH7.5, 1 mM DTT, 10% glycerol, 0.5 mM MgCl2), 250 mM NaCl, 2 µg of nuclear extract and 0.5 µg of Poly poly-deoxyinosine deoxycytidine (dIdC). The reaction mixture was incubated at room temperature for 10 min before adding the probe (1 µl, 106 cpm) and then incubated for 20 min further at room temperature. Hypoxia inducible DNA-protein complexes were separated at 300 V for 3 h on a 5% poly acrylamide gel. The probe was competed away with 40 fold excess of unlabelled primer. The gel was transferred, dried and analyzed by autoradiography.

4.3. Results

The VEGF sequence is highly conserved between the freshwater turtle and chicken. VEGFA Sequence analysis of VEGF revealed 84% homology with Gallus gallus

(Chicken) vascular endothelial growth factor VEGF A, mRNA transcript, NM_001110355.1.

Anoxia upregulates transcript levels of vascular endothelial growth factor. To determine whether exposure to anoxia and reoxygenation inducesVEGF, we analyzed the expression of VEGF mRNA levels both in the turtle brain in vivo and in neuronal cell cultures in vitro. Exposure to anoxia for 24 h increased VEGF transcript levels two- fold compared to normoxic controls both in vivo and in vitro (Fig 1a and 1b), while 4 h of anoxia increased the RNA levels in vivo by 150±23.2% and by 128±3.2% in vitro. By contrast, the RNA levels of VEGF after 4 h post-anoxia reoxygenation did not increase further but returned instead to basal levels.

Coordinated increase of VEGF mRNA and protein both in vivo and in vitro. To determine if transcriptional induction translated into regulation of protein levels, we analyzed VEGF protein expression.

Unlike mammals, VEGF is expressed at high levels in normoxic conditions in the turtle brain. The VEGF protein increase was nearly identical to the changes observed in VEGF mRNA. At 24 h anoxia VEGF levels had increased 2.5-fold over control in vivo and by two fold in vitro (Fig 1c and 1d). VEGF levels also increased by 4 h anoxia exposure; this increase was statistically significant in vivo but not in vitro. Matching events at the transcriptional level, VEGF protein levels also returned to basal upon reoxygenation in both the whole brain and in cell culture.

VEGF protein is localized in cortical neurons under normoxia and anoxia. To further understand the pattern and localization of VEGF expression during normoxia and anoxia, we performed immunhistochemical analysis. A distinct pattern of immunoreactivity was

found in sections of the normal turtle brain reacted with the rabbit-anti VEGF antibody. Prominent immunostaining was found in the cortex as a band of pyramidal neurons. In other brain areas, only scattered neurons were VEGF-positive. Animals fixed at the end of 24 h of anoxia showed more VEGF-positive cortical neurons and these had stronger labeling of their cytoplasm than controls (Fig. 2). Fibers with varicose swellings were seen in the ventral brain areas following anoxia. Double labeling experiments showed colocalization of VEGF and NeuN immunoreactivity in cortical regions (data not shown).

HIF-1 alpha protein levels are not triggered by anoxia.

Earlier studies in the freshwater turtle did not find anoxia-induced alteration in the levels of Hif 1-alpha transcripts (Prentice et al., 2003). As reported for mRNA, expression of HIF -1 alpha protein was observed with only moderate and statistically insignificant increases in anoxia and reoxygenation (Fig 3a), reaching 131±3.3 % above basal over 24 h anoxia.

High constitutive HIF-1 binding activity decreases in anoxia

Electrophoretic mobility shift assays were performed on nuclear extracts obtained from neuronal cell cultures (Fig 3b). HIF1 alpha in the nuclear extracts was detected using a DNA probe, iNOS that contains the HIF 1 alpha binding site. The specificity of this binding activity was assessed by the addition of excess cold mutated probe, which did not diminish the binding activity, while excess cold wild type (wt) probe diminished binding activity in a dose-dependent manner. We found a strong binding signal for HIF-1 and the DNA probe complex in normoxic conditions that decreased during anoxia.

4.4. Discussion

This is the first report showing detectable constitutive expression of VEGF in both the whole brain and in primary neuronal cultures in turtles, in contrast to post-natal mammals. In this study, semiquantitative RT-PCR, western blot, and immunohistochemistry all showed high basal expression of VEGF and evidence for further upregulation in anoxia. Immunohistochemical labeling showed neuronal expression in the cortex of the normoxic turtle but only scattered neurons in other brain regions. Such a differentiated expression pattern suggests a highly regulated expression of VEGF in the turtle with important functions in cortical neurons.

VEGF is known primarily for its role in angiogenesis and vasculogenesis during embryonic development, and its activity is then relatively reduced in adult mammals where constitutive expression of VEGF is very low and restricted mainly to the glial cells (Ogunshola et al., 2000). However, adult mammals show an upregulation of VEGF in the brain under pathophysiological conditions like prolonged hypoxia (Marti and Rissau., 1998), stroke (Hayashi et al., 1997) and cerebral hypoperfusion (Hai et al., 2003) that suggest a possible protective role in maintaining neuronal homeostasis.

While a role for VEGF in angiogenesis has been reported in other non-mammalian vertebrates, including Xenopus (Koibuchi et al., 2006) and zebrafish (Covassin et al., 2006) no such study has been done in turtles. However, the diving turtle would have little use for angiogenesis following each hypoxic episode, lest organs be overrun by blood vessels. On the other hand, stresses like hypoxia or ischemia induce VEGF differentially in several organs in adult mice (Kuo et al., 1999) and in the anoxia tolerant Crucian carp (Rissanen et al., 2006).

Several studies on neuronal cell lines also indicate a protective role for VEGF (Jin et al., 2000; Rosenstein et al., 2003): exogenous addition (Jin et al., 2000) or endogenous elevation of VEGF (Bernaudin et al., 2002) increase neuronal survival following hypoxia/ischemia (Ogunshola et al., 2000). As a neuroprotectant, our finding of high basal levels of VEGF would thus support earlier studies in the turtle, in which it was suggested that high constitutive levels of heat shock proteins (Prentice et al., 2004) favor turtle survival by providing "constitutive preconditioning" (Lutz and Milton, 2004) for recurring episodes of anoxia and reoxygenation.

Interestingly, Kilic et al. (2006) showed a protective pattern of signaling induced by VEGF in the transgenic mouse brain (overexpression human VEGF 165) that resembles the pattern of MAPK expression found in the turtle brain during anoxia. Neuroprotective effects of VEGF were found to be mediated by phosphorylation of the Akt pathway (Jin et al., 2000; Wick et al., 2002) and ERK pathway (Ogunshola et al, 2000, Feng et al, 2008) that could further activate the NF-kappaB pathway and inhibit caspase-3 pathways (Sun and Guo, 2005; Jin et al, 2001) thereby increasing cell survival and preventing apoptosis. In the turtle, both Akt and ERK are upregulated in the initial hour of anoxia (Milton et al., 2008), though this has been linked to adenosine signaling and both Akt and ERK levels decrease to basal by 4 h anoxia. NFkB and BCl-2 are also upregulated, while Bax is upregulated as well increasing the Bax/Bcl2 ratio in long term hypoxia (Lutz and Prentice, 2002; Haddad 2007a; Haddad 2007b). In this study VEGF expression increases continuously over 24 h anoxia, suggesting an additional role in long-term survival unrelated to Akt or MAPK activation. One recent study reported that exogenous application of recombinant VEGF suppresses synaptic activity in the

adult rat hippocampus, suggesting a functional role in neuronal synaptic transmissions (McCloskey et al., 2005). It would be of interest to see if such a role for VEGF functions in the turtle brain, where both synaptic and electrical activity are known to be downregulated in anoxia (Fernandes et al., 1997; Bickler and Buck, 1998; Milton et al., 2002; Thompson et al., 2007). While neurotransmitter release and reuptake continue during anoxia, albeit at reduced rates (Milton and Lutz, 1998; Milton et al., 2002), the initial regulation by adenosine gives way to other regulatory mechanisms during long term anoxia (Milton et al., 2002; Thompson et al., 2007). Upregulation of VEGF in turtle neurons might thus be one of the many molecular mechanisms that preserve the structural and functional integrity of the brain in anoxia.

VEGF, however, is also known to the increase the permeability of vessels, including brain vessels, which has limited its clinical utility as a treatment for stroke. It has been noted in other studies that even the brains of highly anoxia tolerant vertebrates do swell, including the Crucian carp (Van der Linden et al., 2001); in the turtle, we have noted swelling of the cortical layer in anoxic and post-anoxic animals (Cserr et al., 1988; Kesaraju et al., in prep). While the skulls of both the carp and turtle can apparently accommodate this swelling without damaging the brain (Van der Linden et al., 2001), it seems unlikely that there is an adaptive benefit to cerebral edema in anoxia/reoxygenation. Thus the continued increase in VEGF, if this also mediates cerebral edema, is even more curious, particularly in light of its rapid return to baseline upon reoxygenation, and deserves further study.

Reoxygenation or reperfusion results in a burst of reactive oxygen species (ROS) that become lethal to neurons damaging proteins, lipids and DNA (Valko et al, 2006,

Calabrese et al, 2007). Several studies in ischemic (Jin et al, 2000) and retinal pigment epithelial cells models (Kuroki et al, 1996) have shown induction of VEGF in response to ROS stress, and this induction can occur rapidly, within 1 h of reperfusion in a stroke model, and return to undetectable basal levels by 3 h (Hayashi et al.,1997). ROS generated from NADPH oxidase1 (Nox 1) in endothelial cells (EC) stimulates VEGF that aids in EC migration and proliferation (Ushio – Fukai, 2007). Hayashi et al. (1998) have also shown that exogenous application of VEGF reduces the infarct size and edema in rats after 90 min of MCAO, which suggests that anoxia-induced increases in the turtle may be a form of VEGF "banking" against reoxygenation toxicity. Freshwater turtles survive repeated anoxia/reoxygenation with little damage either in vivo (Rice at al., 1995; Lutz et al., 2003) or in vitro (Lutz et al., 2003; Milton et al., 2007, Kesaraju et al., submitted). ROS damage in turtles is prevented by a combination of high antioxidant levels (Rice et al., 1995; Wilmore et al, 1997) and the suppression of excess ROS production (Milton et al., 2007; Pamenter et al., 2007). Anoxia-induced VEGF increases may then aid in ROS protection upon reoxygenation when coupled to these additional ROS defense mechanisms, without need to further upregulate VEGF during reoxygenation.

In mammalian systems, VEGF activation occurs primarily, though not exclusively, through HIF-1α. Ischemic tolerance can be conferred by elevated levels of HIF-1 alpha and its target genes in animals exposed to brief periods of hypoxia (Sharp et al, 2004), and Li et al. (2007) suggested that induced HIF-1 alpha might protect from apoptosis. Other studies contradict the protective role of HIF -1 alpha, however, with Chen et al (2008) showing that suppression of HIF-1 alpha prevents edema and BBB

leakage by inhibition of VEGF. Recent studies have also suggested a proapoptotic role for HIF-1α both in vivo and in vitro, especially for more prolonged or severe hypoxic episodes (Helton et al., 2005).

Hypoxia increases apoptosis in wild type embryonic stem cells compared to HIF knockout cells (Carmeliet et al., 1998), while HIF-1 dominant negative cortical neurons showed reduced apoptosis following hypoxia (Halterman and Federoff, 1999). HIF-1α has been found to induce pro-apoptotic genes including caspase-9 (Nishiyama et al., 2001), BNIP and NIX (Bruick, 2000; Sowter et al., 2001; Kothari et al., 2003; Schmidt-Kastner et al., 2004) and caspase-3 (Van Hoecke et al., 2007). In the turtle, the high constitutive expression of HIF-1alpha in the brain and stabilization with no further upregulation by anoxia is distinctly different from the pattern seen in mammals (Schmid-Brunclik et al., 2008). The decrease in binding activity in the anoxic turtle brain makes it appear unlikely, then, that anoxic VEGF increases are totally regulated by HIF-1α in this model, though the constitutive binding of HIF-1 alpha may explain elevated basal VEGF levels and stabilization of HIF –1 alpha might be enough to trigger VEGF during anoxia.

There are likely to be significant differences between general vertebrate responses to hypoxia and adaptations to complete anoxia. Arterial oxygen partial pressures reported in *Trachemys* range from 60 to 80 torr (Milton, 1994). Diving or periodic apneic breathing patterns could result in a degree of hypoxia as the usual state in turtles, with some induction of HIF-1α and HIF regulated genes. While beyond the scope of this work, HIF-1 α is also activated by a number of other factors, including the Ras/MEK/MAPK and PI3K/Akt pathways, growth factors, and cytokines (Bilton and Booker, 2003) and activators present in normoxia or hypoxia may be lost in anoxia. ERK1/2 and Akt, for

example, both increase significantly in the first hour of anoxia in the turtle brain, but decrease to basal levels by 4 h anoxia (Milton et al., 2008). HIF-1α is also activated by ROS, and while ROS production is relatively high in the normoxic turtle brain, this too disappears by 4 h anoxia (Milton et al., 2007). Prentice et al.(2003) have reported temporary HIF binding activity in response to transient ischemia. HIF activity is also regulated by inhibitory factors such as asparaginyl hydroxylase (Factor Inhibiting HIF, FIH), which prevents its interaction with a transcriptional coactivator (Dann et al., 2002). Continued HIF activity during extended anoxia in the turtle, in fact, would be maladaptive. As the anoxic turtle enters a state of deeply depressed metabolism, with a reverse Pasteur effect (suppression of glycolytic pathways) and reduced heart rate, it would be counterproductive in the long term to upregulate many key enzymes affected by HIF, e.g. erythropoeitin, transferrin, or the enzymes of glucose metabolism. Studies have in fact shown a suppression of glycolytic enzymes in turtles and other good facultative anaerobes, including the HIF targets phosphofrucktokinase and pyruvate kinase (Brooks and Storey, 1988a, 1988b).

Anoxia/ glucose deprivation in astrocytes led to higher accumulation of HIF-1 alpha than hypoxia/glucose deprivation while blocking of HIF-1 alpha resulted in only 50% decrease in the expression of VEGF, suggesting that HIF-1 alpha only partially regulates VEGF (Schmid-Brunclik et al., 2008). Schmid-Brunclik et al. (2008) indicated the possible role of constitutive HIF-2 alpha in VEGF regulation. Arany et al. (2008) recently demonstrated HIF-independent regulation of VEGF by the transcriptional coactivator PGC-1 (peroxisome-proliferator-activated receptor-γ coactivator-1α), which

does not involve HIF-1α but strongly induces VEGF during oxygen or oxygen and nutrient deprivation.

The present study, therefore adds a new aspect of "constitutive preconditioning" to the hypoxia/ischemia research and provides insight into anoxia resistance.

Figures

Figure 1: mRNA and protein levels of VEGF on exposure to anoxia and reoxygenation.

(a) mRNA levels analysed by RT-PCR in vivo (b) mRNA levels in vitro (c) protein levels analysed by western blot in vivo and protein levels in vitro (d). Representative agarose gel images and western blots with densitometric analysis are shown. The samples from the left are: Normoxia, 4 h anoxia, 24 h anoxia and 4 h anoxia/4 h reoxygenation. * Significantly different (sd) from control, # sd from 4 h anoxia/reoxygenation.

Figure 1

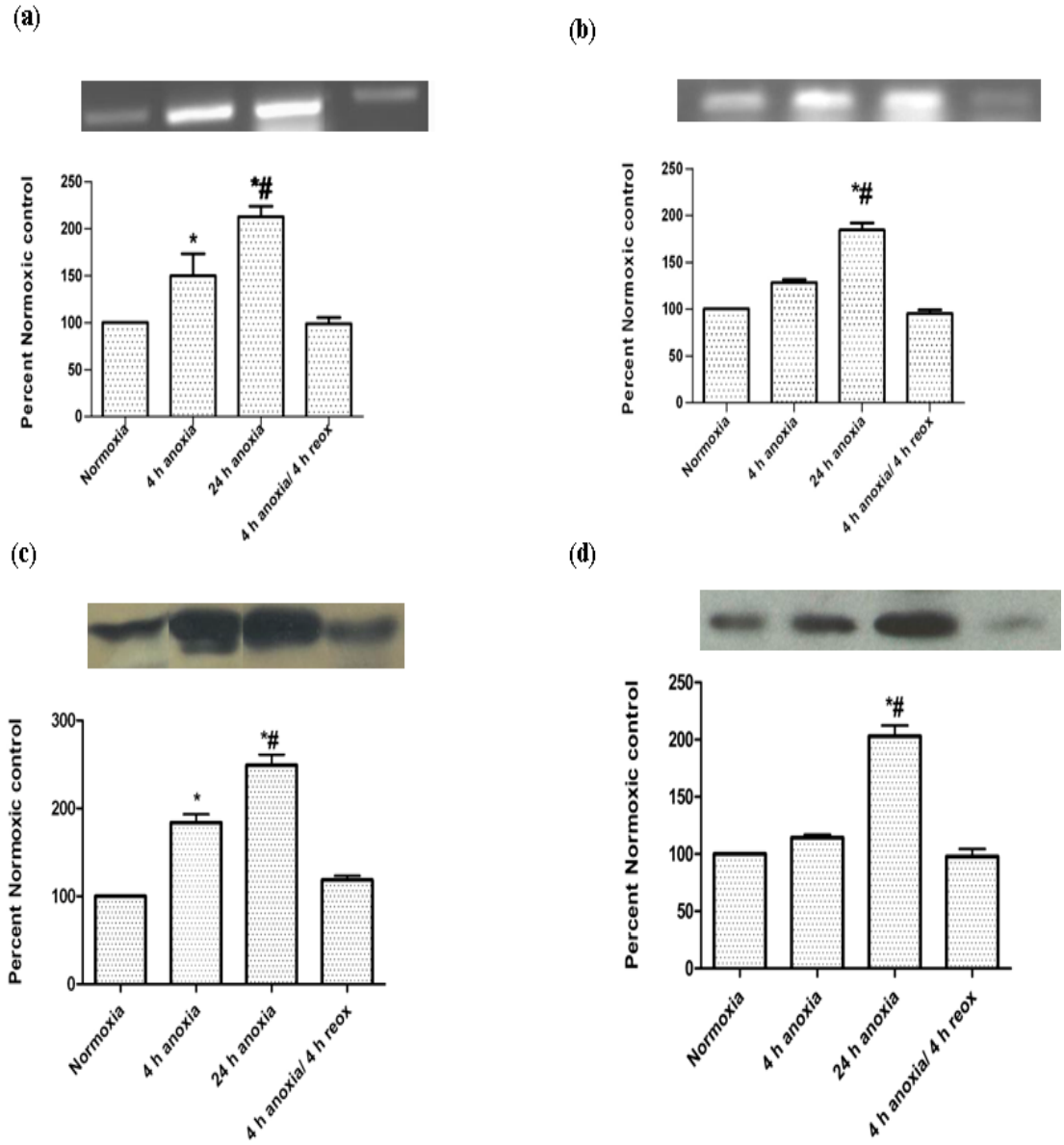

(a)
(b)
(c)
(d)
Percent Normoxic control
Normoxia
4 h anoxia
24 h anoxia
4 h anoxia/ 4 h reox
*
*#

Figure 2: Imunohistochemical labeling of vascular endothelial growth factor (VEGF). VEGF labeling in the frontal sections of the turtle brain showing high basal expression in the cortical neurons of a control animal (a), (b) enhanced expression at the end of 24 h anoxia and (c) a decrease at 3d reoxygenation after anoxia.

Figure 2

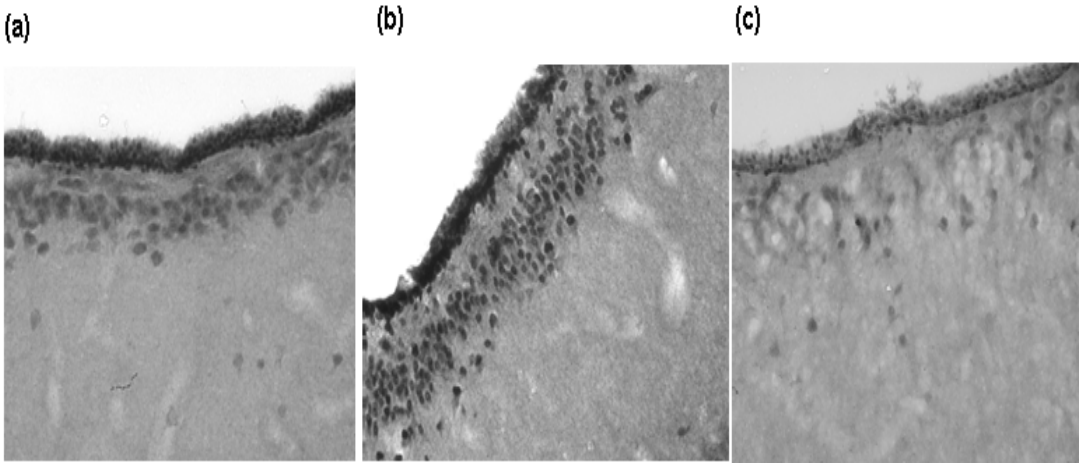
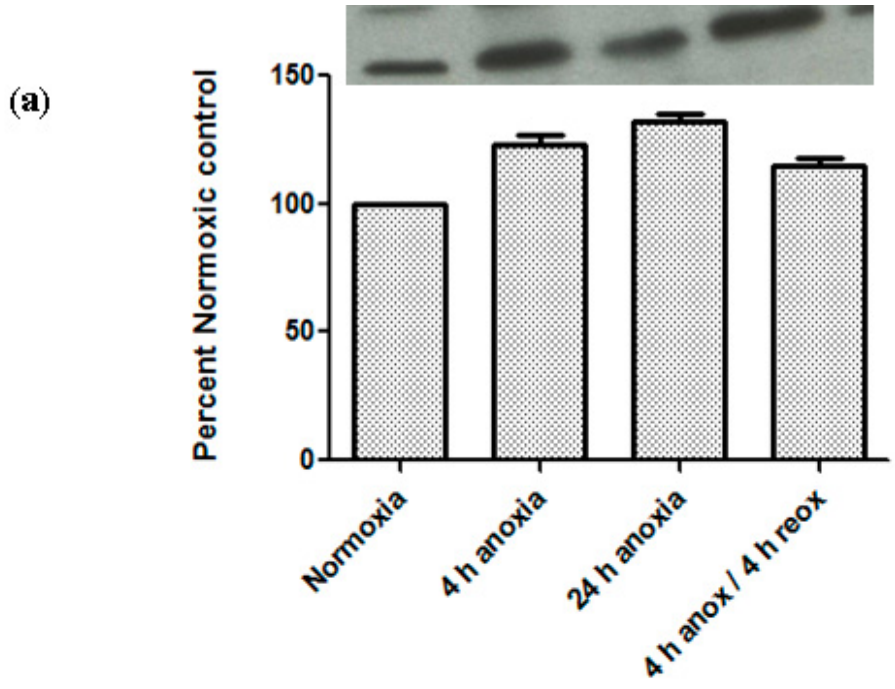


Figure 3: HIF-1 alpha protein levels and HIF-1 binding assay (a) Effect of anoxia and reoxygenation on the stability of HIF-1 alpha protein levels, in vivo. A representative western blot showing HIF-1 alpha protein under normoxia, 4 h anoxia, 24 h anoxia and 4h anoxia/4 h reoxygenation. Quantification by image J analysis is also shown.
(b) Demonstration of HIF-1 binding to HRE. Electrophoretic mobility shift assay on nuclear extracts prepared from normoxic cell cultures (Lane 1-5) and from 6 h anoxic cell cultures (Lane 6-10). Binding was competed away with excess of unlabelled/cold wild type probe; 40X (Lane 3 and 9), 10X (Lane 4 and 10) and mutated probe; 40X (Lane 1and 7), 10X (Lane 2 and 8). Lane 5 is normoxic nuclear extract with labeled wild type probe. Lane 6 is anoxic nuclear extract with labeled wild type probe.

Figure 3


(a)
Percent Normoxic control
150
100
50
0
Normoxia
4 h anoxia
24 h anoxia
4 h anox / 4 h reox
(b)
1
2
3
4
5
6
7
8
9
10

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CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Very little is known about the molecular mechanisms behind anoxia and reoxygenation tolerance in the freshwater turtles. Decades of research in turtle anoxic brain physiology and reports from mammalian endogenous neuroprotective mechanisms have recently shifted the focus of turtle research to understanding these cellular and molecular mechanisms in the turtle. The end effectors of ischemic preconditioning such as heat shock proteins and vascular endothelial growth factor that may aid in preventing or repairing potential neuronal damage were chosen as subjects for the present study. In this study, we have shown rapid induction of several stress proteins (Hsp72, Hsp27, HO-1, Grp94) within the first hour anoxia that is maintained at higher levels in long term anoxia in the turtle brain. The stress proteins were induced in brain tissue that also did not show any overt damage (Immunohistochemical evidence) as expected in this model of anoxia tolerance. This resistance of cells to cell death, apoptosis in particular , can be explained in part by increases in protective factors including high constitutive levels and further upregulation of stress proteins, and the absence of increased activation of pro-apoptotic proteins. The functional role of Hsp72 was analyzed utilizing siRNA against Hsp72. Increased cell death and induced release of ROS due to knockdown of Hsp72 shows that Hsp72 is critical for cell survival and ROS suppression. Data on knockdown experiments shows the presence of apoptotic machinery that is not aggressive and warrants further study on apoptosis inhibitors and necrotic regulators.

Studies on VEGF have shown (1) high basal expression of VEGF in cortical neurons in normoxic conditions and (2) induction of VEGF during long term anoxia (24 h) in mRNA and protein levels (both in vivo and in vitro). Induction of VEGF at 24 h anoxia was also evidenced in cortical sections. In addition, experiments analyzing HIF-1 alpha have shown that anoxia and reoxygenation stabilized HIF-1 alpha protein levels but did not upregulate. Binding of HIF-1 to Hypoxia response elements during normoxia and anoxia was established by performing mobility shift assay. All these data put together suggests VEGF is neuroprotective by providing "constitutive preconditioning" and also by maintaining the functional integrity of the neuronal network in long term anoxia. VEGF analysis entails identifying its functional role in neuroprotection which can be performed by siRNA against VEGF.

The adaptations of freshwater turtle to anoxia have evolved through millions of years, allowing one to assume that the pathways induced during the energy stringent conditions of anoxia are likely to be protective to the brain. The results of this study, therefore, show that the effectors of preconditioning are evolutionarily conserved and the induction of Hsp72 in particular offers neuroprotection to the anoxic turtle brain.

Future Directions

Cerebral ischemia initiates multiple cell death pathways with neurons committing mainly to the apoptotic pathway of death, although neurons do die through autophagy and necrosis (Nakka et al., 2008; Rami et al., 2008; Qin et al., 2008). The present study shows that stress proteins are critical for neuroprotection in the turtle, and the turtle has developed mechanisms to prevent the activation of apoptotic machinery in anoxia and reoxygenation. The limited changes in the caspase-pathway of apoptosis in cell cultures

where Hsp72 was knocked down significantly, however, suggest that this HSP is not solely responsible for the suppression of this apoptotic pathway. Hsp72 was found to be neuroprotective through suppression of ROS and AIF, however. The future directions of this study would be, therefore, to understand the possible role of caspase-independent pathways and apoptosis inhibitors and also the pathways that prevent the permeabilization of the mitochondrial membrane. As one HSP associated with the endoplasmic reticulum also increased significantly, the role of the ER in avoiding anoxia/reoxygenation stress is another research area of great interest.

5.1. Mitochondria and Neuroprotection

Results from our laboratory have shown suppression of ROS release and restoration of mitochondrial membrane integrity after reoxygenation, while Hsp72 knockdown induced ROS in the reoxygenation phase in the turtle brain. Preliminary evidence has also shown that knockdown of Hsp72 in the neuronal cell culture resulted in loss of mitochondrial membrane potential (MMP) not only during anoxia but during reoxygenation, when turtle neurons usually recover. In mammalian ischemic models, accumulation of Ca^{2+} and increase in ROS precede opening of the mitochondrial transition pore (Mergenthaler et al., 2004). A key component of the mitochondrial transition pore is Cyclophilin D (Cyp D), a prolyl isomerase enzyme that regulates the opening of the pore; transgenic mice lacking Cyp D have shown reduced infarct after ischemia/reperfusion (Schinzel et al., 2005). It would be interesting to observe the role of CypD in the reoxygenation resistant turtle cells.

Another key target to study are the regulatory effects of the mitochondrial K_{ATP} channels in the turtle. Mayanagi et al (2007) have shown that mito K_{ATP} channel opener,

BMS191095 suppressed the release of ROS while mito K_{ATP} blocker 5-hydroxydecanoate (5HD) abolished the protective effect in transient ischemic model. If blocking mito K_{ATP} during reoxygenation in the controls induce ROS production and opening of mito K_{ATP} channels in the knockdown cultures inhibit ROS release, that would confirm the protective function of mito K_{ATP} channels in the turtle. It has already been reported that K_{ATP} channels open in the turtle during the initial hour of anoxia, when they block the release of excitatory neurotransmitters including dopamine and glutamate (Milton et al., 2002; Milton and Lutz, 2005) and thus aid in brain survival. However, these cytoplasmic channels closed by 4 hr anoxia, and the effects of reoxygenation on K_{ATP} channels is unknown.

5.2. Apoptosis Inhibitors and Caspase-independent Pathway

In addition to Bcl2 family members and caspases, the inhibitors of family of proteins (IAP's) are found to be important regulators of apoptosis in cerebral ischemia/reperfusion injury (Nakka et al., 2008). IAP's are an apoptosis suppressing family of proteins that inactivate caspases by directly binding to Caspase 3, 7 and 9 (Deveraux et al., 1997; Deveraux and Reed, 1999). The family of IAP proteins include cIAP(cellular IAP), NAIP (neuronal IAP) and XIAP (X-linked IAP) (Roy et al., 1997). Tanaka et al (2004) have shown that global ischemia in rats upregulates both cIAP and IAP inhibitor proteins (SMAC, Second mitochondrial activator of caspases). However, preconditioning induced the expression of cIAP and suppressed the expression of SMAC, and thereby rescued the neurons from cell death cascade (Tanaka et al., 2004). Thus, it would be of great interest to analyze the expression of IAP's in the anoxic turtle brain,

particularly in the face of unaltered expression of Caspase 3 during anoxia and reoxygenation.

Caspases and Calpains constitute the family of proteases involved in neuronal damage (Rami, 2003). Increase in intracellular calcium during ischemia and excitotoxicity activates calpains, and the substrates for calpains are found in multiple locations such as synapse, mitochondria, endoplasmic reticulum and lysosomes (Beyer and Neumar, 2008; Blomgren et al., 2001). Although the action of calpains are independent of caspases, recent study has shown that Procaspase 3 was activated on incubation with m-Calpain and the activation was inhibited by the presence of calpastatin, a calpain inhibitor (Blomgren et al., 2001) in hypoxic rat brain. In vivo analysis of turtle brain revealed upregulation of Procaspase-3 while the levels of Caspase-3 remained unaltered. An interesting aspect would be to analyze the regulation of caspase inhibitors and calpain inhibitors that might provide clues for unusual resistance to Procaspase cleavage or Caspase activation. While it is not yet known if calpains are active in the anoxic turtle brain, one aspect of survival is maintenance of low intracellular calcium levels and blockade of excitotoxin release, suggesting that calpains would only be likely to be activated if the cell is already dying, without any special mechanism of suppression.

Chaitanya and Babu (2008) have shown sequential activation of Calpain, Cathepsin and Caspase respectively over time in a rodent focal ischemic model in which cell death ensued. Cathepsin is a lysosomal protease that is activated via PERK (PKR like endoplasmic Reticulum kinase) at low pH (Park et al., 2008). Induction of Hsp70 was able to prevent the release of cathepsin from lysosomes in a PERK (-/-) null cells and

thereby suppress autophagy of cells (Park et al., 2008). Data from our lab suggests that turtle is able to prevent the catastrophic events of intrinsic apoptotic pathway by maintaining the mitochondrial membrane integrity. However, to escape from alternate routes of cell death, it becomes important to maintain lysososmal membrane integrity and to avoid ER stress.

5.3. Endoplasmic Reticulum Stress (ER stress)

In the turtles during anoxia as seen from our study, there is selective upregulation of proteins and protein levels return to basal upon recovery. Global ischemia in mammals, though halts the synthesis of proteins and prolonged suppression of protein synthesis results in cell death (Paschen et al., 2007). ER is a sub cellular organelle that aids in synthesis and proper folding of proteins. Dysfunction of ER is one of the major reasons for neuronal death during ischemia/reperfusion (Morimoto et al., 2007).

ER stress acts as a defense mechanism temporarily by initiating UPR (unfolded protein response): UPR primarily involves activation of PERK that inhibits protein synthesis (Mamady and Storey, 2008), induction of ER stress proteins /chaperones Grp78 and Grp94 that help in protein folding (Hayashi et al., 2001; Kim et al., 2003) and induction ATF4 (activated transcription factor) that triggers the activity of ER chaperones (Oida et al., 2008). However, ER stress also activates caspase-12 that further activates caspase 3 in focal ischemic rodent model (Shibata et al., 2003). Mamady and Storey (2008) have shown induction of ATF4 and ATF6 in hibernating ground squirrels, suggesting the activation of UPR to resist hibernation stress. Our results have shown differential regulation of ER stress proteins: induction of Grp94 with Grp78 levels unchanged during anoxia and reoxygenation. Paschen et al (2007) have suggested that

understanding the mechanisms of protein synthesis restoration in ER stress tolerant cells would serve in providing clues to several neurodegenerative disorders and the turtle would therefore be an ideal model to study ER stress.

To conclude, the results of this study emphasizes that T*rachemys scripta*, has the advantage to survive the stress of anoxia and reoxygenation by modulation of molecular mechanisms and gives a promise to open new avenues in the research of hypoxia/ischemia and reperfusion.

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