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Effects of Hyperthermia and Subsequent Minocycline Treatment in Acute Ischemic Stroke

by

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Dedication

I would like to dedicate this thesis to my parents, both of whom have helped me through all the good times and the bad. They have always supported and encouraged my work and have nurtured my curiosity through my life. I would also like to dedicate this work to my wonderful brother, who puts up with all of my quirks and is always willing to listen to gripes and rants while offering sound advice.

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Abstract

Stroke, a reduction in blood flow to the brain, activates various proteins that contribute to neuronal death. The matrix metalloproteinases (MMPs), specifically MMP2 and MMP9, are important in maintaining the blood brain barrier (BBB). After a stroke the BBB permeability increases which is caused by MMP upregulation. Hyperthermia is known to exacerbate the damage caused by a stroke, but the exact mechanism is unknown. We hypothesize that hyperthermia will increase MMP expression and that minocycline, an MMP inhibitor, will decrease MMP activity and BBB permeability. Our data demonstrates that hyperthermia increases expression of both MMP2 and MMP9. Furthermore, hyperthermia after ischemia increases degradation of laminin. Minocycline can reduce both expression and activity of MMP2 and MMP9 after ischemia. Minocycline is effective in hyperthermic conditions and reduces degradation of laminin. This data shows that minocycline is a potential therapeutic treatment for reducing MMP activity and subsequent laminin degradation after a stroke.

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List of Abbreviations

ADP	Adenosine Dipoosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of Variance
Apaf-1	Apoptotic Protease Activating Factor1
ATP	Adenosine Triphosphate
BAD	Bcl-2 Associated Death Promoter
BAD	Bcl-2 Associated X Protein
BAK	Bcl-2 Homologous Antagonist/Killer
BBB	Blood Brain Barrier
BCL-2	B-Cell Lymphocyte 2
Bcl-2	B-Cell Lymphoma 2
BCL2LL	Bcl-2 Like Protein 11 (formerly Bim)
Bcl-xL	B-Cell Lymphoma Extra Large
Bid	BH3 Interacting Domain Death Activator
CAD	Caspase Activated DNAses
CBF	Cerebral Blood Flow
CCA	Common Carotid Artery
CD11b	Cluster of Differentiation 11b (Integrin Alpha M)
COX-2	Cyclooxygenase 2
DISC	Death Induced Signalling Complex
DNA	Deoxyribose Nucleic Acid
ECA	External Carotid Artery
ECM	Extra Cellular Martix
FADD	Fas Associated Death Domain
FasL	Fas Ligand
FasR	Fas Receptor
FDA	Federal Drug and Administration
HSPG	Heparin Sulfate Proteoglycan
$HgCl_2$	Mercuric Chloride
ICA	Internal Carotid Artery
ICE	IL-1β Converting Enzyme
ICH	Intra Cranial Hemorrhage
IL-1β	Interleukin 1β
IL6	Interleukin 6
iNOS	Inducible Nitric Oxide Synthase
KA-R	Kiainic Acid Receptor
LPS	Lipopolysaccaride
MAC-2	Macrophage Galactose-specific Lectin-2
MCA	Middle Cerebral Artery
MCAO	Middle Cerebral Artery Occlusion
MMP	Matrix Metalloproteinase
MT1-MMP	Membrane Type 1 MMP (also known as MMP14)
NADPH	Nicotinamide Adenine Dinucleotide Phosphate-Oxidase
NMDA	N-Methyl-D-aspartic acid

NO	Nitric Oxide
NSAID	Non-Steroidal Anti-Inflammatory Drug
O_2^-	Superoxide
$ONOO^-$	Peroxynitrite
PARP	Poly ADP Ribose Polymerase
PGE2	Prostoglandin E2
ROS	Reactive Oxygen Species
SAH	Sub-Arachnoid Hemorrhage
tBid	Truncated Bid
TIMP	Tissue Inhibitor of MMP
TNF-α	Tumour Necrosis Factor α
tPA	Tissue Plasminogen Activator

4X SB	4 Times Sample Buffer
dd H_20	Double Distilled Water
EtOH	Ethanol
HCl	Hydrochloric Acid
MeOH	Methanol
NaCl	Sodium Chloride
PVDF	Polyvinylidene Flouride
RIPA	RadioImmunoPrecipitation Assay
SDS	Sodium Dodecyl Sulphate
TTBS	Tween 20 Tris Buffer Saline
TTC	Tetrazolium Chloride
$ZnCl_2$	Zinc Chloride

µL	miroliter
µm	micrometer
g	gravity
h (hr)	hour
kDa	Kilodaltons
L	Liter
M	Molar
mg	milligram
min	minute
mM	millimolar
^oC	degrees Celsius

Chapter 1: Introduction

Introduction

Stroke

Stroke is a leading cause of disability and the third leading cause of death behind heart disease and cancer (Lloyd-Jones et al., 2010). Stroke is a common disease effecting approximately 50,000 and killing 14,000 Canadians each year. It is considered a disease of the elderly, significantly increasing in prevalence after the age of sixty and increasing in prevalence each year subsequently, but can occur in younger individuals as well. Strokes can be caused by two separate mechanisms; haemorrhage or ischemia. Haemorrhage is less common in occurrence, accounting for 5 to 15% of all strokes (Grysiewicz, Thomas, & Pandey, 2008) and can be further classified into two subgroups. Subarachanoid haemorrhage (SAH) occurs when a blood vessel bursts within the subarachanoid space, while an intracrainial haemorrhage (ICH) is caused by the bursting of a blood vessel within the brain. In both of these conditions there is a reduction in blood flow to areas within brain and neuronal cell death begins. Additionally, blood accumulates within the brain parenchyma and causes a rise in intracranial pressure which is usually lethal, having a 44% mortality rate at 30 days (Broderick, Brott, Tomsick, Miller, & Huster, 1993). Fortunately, haemorrhagic strokes are far less common than ischemic stokes (Grysiewicz et al., 2008).

Ischemic strokes, the most common form of a stroke, are caused by a blockage of a cerebral blood vessel. This blockage, or occlusion, can be from either a thrombus or an embolus. A thrombus is classified as a blood clot, while an embolus is considered to be a fragment of any other material within the vessel wall such, as an atherosclerotic plaque, which ends up blocking the vessel. These plaques are caused by the build up of cholesterol, foam cells and low density lipoproteins. This can lead to a calcification of the vessel and eventually lead to a narrowing of the artery called stenosis. Stenosis of the blood vessels of the head and neck (the left and right common carotid arteries and the left and right vertebral arteries) is a major risk factor for stroke. If arteries become too narrow blood flow is either reduced or restricted when red blood cells become trapped and collect around the plaque forming a blood clot. These blood clots can

either occlude arteries where they are formed and are named a thrombus, or they can dislodge and travel within the blood stream as an embolus, to occlude an artery elsewhere in the body (Thom et al., 2006). When these emboli occlude arteries within the brain, an ischemic stroke occurs. Many ischemic strokes are caused by formation of an embolism from the heart or other proximal arteries. Of the cardioembolic factors, left atrial thrombus formation due to atrial fibrillation is a common source of emboli causing stroke. Heart attacks and abnormalities or diseases of the heart valves can also cause the formation of cardioembolic thrombi and can lead to stroke as well.

Ischemia can be either global or focal in nature. Global ischemia is caused when the entire brain suffers from a reduction in blood flow. The cause of this condition may be due to a heart attack, hypotension, or significant stenosis of the carotid and vertebral arteries within the neck (Johansson, 1999). Focal ischemia is a localized reduction in blood flow caused by an occlusion of an artery or vein within the brain and reduces or prevents blood flow to that specific area. This reduction in blood flow, also called perfusion, in a localized portion within the brain can lead to many wide ranging symptoms and deficits including paralysis, the inability to generate or understand language, lack of motor coordination and balance, memory retention and vision problems among many others (Sinanovic, Mrkonjic, Zukic, Vidovic, & Imamovic, 2011; Timmermans, Spooren, Kingma, & Seelen, 2010; Freeman, Dawson, & Flemming, 2010). These wide ranging symptoms, many of which overlap with other more benign conditions make stroke difficult to diagnose quickly, and can lead to significant death of brain tissue and loss of and function (Lopez, Afshinnik, & Samuels, 2011). Acute ischemic stroke requires early presentation, early diagnosis, and early thrombolytic therapy to prevent disability or death due to a stroke. Although the adoption of thrombolytic therapy in acute stroke is less rapid the adoption of thrombolysis for myocardial infarction, progress is being made in developing new therapies. Due of the permanent damage to the brain after an ischemic stroke, time is a critical factor in saving as much brain tissue as possible. Therefore new treatment options must be developed to deal with the multifaceted nature of brain damage due to stroke and administered within a shorter time frame to salvage tissue at risk.

Reduction of Blood Flow and Cell Death

The reduction in perfusion to different areas of the brain begins to alter signalling pathways within the brain, as well as the metabolic activity of the cells. Under normal conditions, healthy brain cells receive an average of 50mL per 100g of brain tissue per minute (Roberts, Detre, Bolinger, Insko, & Leigh, Jr., 1994). This is termed the average cerebral blood flow (CBF) in the brain. In an ischemic brain, the CBF drops below this value causing brain cells to become metabolically stressed and vulnerable to death. At a CBF of 30mL per 100g of brain tissue per minute cells are still viable, and can survive in a stressed state but become more active and enhance their signalling pathways to activate vasodilation, angiogenesis, gluconeogenesis and anerobic respiration (Roberts et al., 1994). At 10mL per 100g of brain tissue per minute cells no longer have the basic minimum energy requirements to maintain their structure or function and lose their membrane potential and begin to undergo cell death through apoptotic signalling or tissue necrosis (Roberts et al., 1994).

There are two distinct areas within a stroke, the ischemic core region and the surrounding ischemic penumbra. The ischemic core is localized to the site of the occlusion and contains the lowest CBF (Wang, Gao, Xue, Liu, & Ma, 2010), where cells undergo predominantly tissue necrosis (Campbell et al., 2011). This region accounts for the majority of lost brain tissue within the acute phase of a stroke and these cells usually cannot be saved. Within the ischemic penumbra CBF is reduced, but this reduction is not always significant enough to cause immediate cell death (Shen, Huang, Du, & Duong, 2011). These cells are still alive and most therapeutics target this salvageable brain tissue after the initial stroke. Perfusion within the penumbra region is within 20 mL of blood per 100g of brain tissue per minute (Roberts et al., 1994) and this can be increased with removal of the occlusion and recannalization of the vessel. Conversely, a failure to remove the occlusion can cause the cells within the penumbra area to die due to a lack of both oxygen and energy from adenosine triphosphate (ATP). In this region cell death is due to apoptosis and not the necrotic cell death found within the ischemic core.

Apoptosis

Apoptosis of cells is a highly coordinated and specific method of programmed cell death. In apoptosis cells undergo death by systematically breaking down and expending energy in a process to coordinate their fragmentation and reabsorbance by other neighbouring cells (Onteniente, Couriaud, Braudeau, Benchoua, & Guegan, 2003). Apoptosis is hallmarked by changes in certain characteristics of cell morphology, namely cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmenting and fragmentation of the nuclear envelope. Apoptosis signalling involves two distinct pathways, the intrinsic pathway and the extrinsic pathway.

The intrinsic pathway of apoptosis begins with an internal signal within the cell including DNA damage, loss of cell survival factors, detachment from the extracellular matrix (ECM), loss of ion gradients, hypoxia or loss of energy. As mentioned previously, cerebral ischemia reduces blood flow to the brain tissue and reduces both oxygen and glucose that cells can use. This reduction in oxygen and glucose leads to the metabolic failure of cells, which are now deprived of an energy source. Without sufficient energy sources to maintain ion concentration gradients within the cell, the membrane potential of these cells soon collapses and the cells begin to activate their apoptotic signalling pathways. This begins with the activation of members of the Bcl2 family of proteins (18), which are both pro- and anti-apoptotic. The balance between these proteins allows cells to either continue normal function or commit to apoptosis. With the activation of BCL2 (B-cell lymphocyte 2) family members Bid (BH3 interacting domain death agonist), BCL2L11 (Bcl-2 like protein 11, formerly known as Bim), BAD (Bcl-2 associated death promoter), BAK (Bcl-2 homologolous antagonish/killer) and BAX (Bcl-2 associated X protein), pro-apoptotic proteins, and the reduction in activity of Bcl-xL (B-cell lymphoma extra large) and Bcl-2 (B-cell lymphoma 2), both anti-apoptotic factors, cells begin to undergo apoptosis.

BAX, BAK and Bid all translocate to the mitochondria (Ferrer & Planas, 2003; Ferrer, Friguls, Dalfo, Justicia, & Planas, 2003; Mattson, Culmsee, & Yu, 2000; Plesnila, 2004) where they open mitochondrial transition membrane pores which allow cytochrome c to diffuse from the inner mitochondrial membrane into the cytoplasm (Ferrer et al., 2003; Yin, 2000). Once in the cytoplasm cytochrome c can then bind to cytosolic

apoptotic protease activating factor 1 (Apaf-1) along with ATP and pro-caspase 9 to form the apoptosome (Broughton, Reutens, & Sobey, 2009). This complex will then cleave pro-caspase 9 into active caspase-9 which can then activate pro-caspase 3 by formation of caspase 3(Broughton et al., 2009). Caspase 9 can also activate other caspases such as caspase 6 and 8 (Broughton et al., 2009). Caspase 3, the effector caspase, can then go on to cleave poly ADP ribose polymerase (PARP) and activate caspase-activated DNAse (CAD) (Ferrer & Planas, 2003; Onteniente et al., 2003). CAD activation leads to DNA fragmentation and breakdown of the nuclear membrane, as will caspase 6 (Broughton et al., 2009), while PARP's functional role is to utilize ATP to repair DNA damage (Mattson et al., 2000). With the inactivation of PARP, CAD and caspase 6 can fragment DNA and complete the process of apoptosis.

In contrast to this the extrinsic pathway of apoptosis signalling involved molecules from the extracellular environment transducing their signalling pathway onto the target cell and inducing the apoptotic signalling cascade. After the onset of an ischemic stroke, microglial cells increase their expression and secretion of cytokines, specifically TNFα (tumour necrosis factor α) and FasL (Fas ligand) (Ferrer et al., 2003). These cytokines then bind to their plasma membrane receptors and initiate apoptosis through a death signal with the binding of a homotrimer of FasL to a homotrimer of Fas receptor (FasR). The Fas ligand-receptor complex will then recruit FADD (Fas associated death domain) and pro-caspase 8 (Broughton et al., 2009). This entire entity composed of FasL, FasR, FADD and pro-caspase 8 is called the DISC (death induced signalling complex). The DISC can then cleave pro-caspase 8 into active caspase 8 through autocatalysis (Broughton et al., 2009; Ferrer & Planas, 2003). Active caspase 8 can then cleave Bid into t-Bid (truncated Bid) (Durukan & Tatlisumak, 2007; Durukan & Tatlisumak, 2009) which can involve the intrinsic apoptotic signalling pathway as well. Additionally, active caspase 8 can also cleave caspase 3 (Mattson et al., 2000), the effector caspase, and can cause DNA fragmentation and breakup of the nuclear envelope.

In both the intrinsic and extrinsic pathways cells are broken down and compartmentalized into smaller vesicles and membrane blebs which can be engulfed by macrophages and microglia limiting the release of additional toxic signalling molecules, like cytokines. This limits further damage to neighbouring cells unlike necrosis.

Necrosis

Necrosis is an uncontrolled form of cell death characterized by swelling of the cell, a rupture of the plasma membrane, release of cytoplasmic contents into the extracellular environment and an inflammatory response and subsequent cytokine cascade. Necrosis is also caspase independent and occurs through changes in cell polarity, structure and osmotic pressures (Henriquez, Armisen, Stutzin, & Quest, 2008; Broker, Kruyt, & Giaccone, 2005), not signalling transduction pathways as seen in apoptosis. However, there are signalling cascades that can contribute to the initiation of necrosis.

Cells within the brain rely exclusively on glucose and oxygen to meet their energy requirements and maintain homoestasis. After cerebral ischemia, cells no longer have the necessary energy required to maintain ion gradients and osmotic pressure. This lack of ATP causes a failure of the Na^+/K^+ and Ca^{2+}/Na^+ exchangers and the ATP-sensitive K^+ channel (Durukan & Tatlisumak, 2007). The loss of function of these ionic pumps and channels causes a collapse in the ionic gradients and leads to the influx of Na^+ and Ca^{2+} and an efflux of K^+ from the cell. This loss of ionic gradients, which give neurons their membrane potential, will further cause an activation of voltage gated Na^+ and Ca^{2+} causing further influx of Na^+ and Ca^{2+} into the cell (Durukan & Tatlisumak, 2007; Deb, Sharma, & Hassan, 2010). These changes in ionic gradients cause a change in osmotic pressure and a subsequent influx of water into the cell causing cytotoxic edema within cells which begin to swell (Brouns & De Deyn, 2009). In addition the rapid increase in Ca^{2+} within the cell can lead to the production of free radicals and reactive oxidative species (ROS) (Brouns & De Deyn, 2009). These ROS are usually created within the cell in controlled reactions, such as the electron transport chain of oxidative phosphorylation, and removed by antioxidant scavenger molecules (Wang & Shuaib, 2007b; Slemmer, Shacka, Sweeney, & Weber, 2008). The excess ROS within the cell overwhelm the scavenger molecules and cause damage to DNA, oxidation of lipids and amino acids, inactivation of some enzymes and activation of others such as the matrix metalloproteinases (MMPs) (Tian, Liu, Bitterman, & Bache, 2002; Kim, Kim, & Park, 2002; Saldeen, Li, & Mehta, 1999; Violi, Marino, Milite, & Loffredo, 1999; Yavuz et al., 2003; Chakraborti, Mandal, Das, Mandal, & Chakraborti, 2003a; Galis & Khatri, 2002a).

In addition to the production of ROS and cytotoxic edema, Ca^{2+} influx can cause the release of many neurotransmitters from cells. The depolarization of neurons and the influx of Ca^{2+} will cause vesicle fusion and release of these neurotransmitters into the synapse (von & Matthews, 1994; Randic & Padjen, 1967). Glutamate is the most abundant and primary excitatory neurotransmitter of the central nervous system and upon release will bind to both NMDA (N-Methyl-D-aspartic acid) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors (Onteniente et al., 2003). This binding causes a further increase in Ca^{2+} influx and activation of calcium dependent enzymes such as calpains, endonucleases, ATP-ases and phospholipases (Durukan & Tatlisumak, 2007; Brouns & De Deyn, 2009). This further causes uncontrolled cell damage and leads to further necrosis of cells. Finally, once cells have become swollen and ruptured they release their cytoplasmic contents into the extracellular space where macrophages and microglia will phagocytose the cellular debris. This will activate phagocytotic cells and leads to a release of further pro-inflammatory cytokines and chemoattractrants to the area (Dirnagl & Schwab, 2009; Jin et al., 2010), which will further enhance the inflammatory response and can begin the extrinsic apoptotic signalling pathway in neighbouring cells.

Microvasculature

Cerebral blood vessels are compromised during stroke and are a site of dysfunction after ischemia. These blood vessels make up the cerebral vasculature and are composed of the arterioles, venues and capillary beds where oxygen and carbon dioxide exchange occurs to facilitate cellular respiration. The capillary beds are important to the structure and function of the blood brain barrier (BBB) due to the large amount of surface area that they create within the brain. They are also associated with perivascular cells such as astrocyte endfoot processes, pericytes and neuronal processes (del Zoppo & Hallenbeck, 2000). The blood vessel wall, which is made up of the endothelial cell layer, the internal basal lamina between the endothelial cells and the surrounding smooth muscle cell layers compromise the capillary beds (del Zoppo & Hallenbeck, 2000; Wang & Shuaib, 2007a; Takahashi & Macdonald, 2004a).

The microvasculature is composed of an endothelial layer which is surrounded by a structural support wall named the basal lamina. There are also layers of structural proteins and cells surrounding the basal lamina including the myointimal layers of smooth muscle and advential tissue which include pericytes and the endfoot processes of astrocytes and some neuronal processes. The capillaries, which do not posses any structural support aside from the endothelial layer and the basal lamina, are the most vulnerable to damage during as ischemic event. The capillaries are also the location where the most damage is done by endogenous proteases and the site where there is the greatest dysfunction in the permeability of the BBB.

Little is currently known about how microvasculature signalling is affected by either ischemia or hyperthermia. The microvasculature is clinically relevant as the only applicable therapy for stroke intervention tissue plasminogen activator (tPA) is administered as a thrombolytic agent to facilitate reperfusion of the microvasculature. With a collapsed or highly permeably microvasculature the efficacy of tPA as a therapeutic agent decreases as reperfusion to the affected area is now reduced. In the case of increased microvasculature permeability, reperfusion can lead to secondary effects such as edema within the surrounding tissue, causing more damage than benefit from the thrombolytic therapy of tPA.

The microvascular wall behaves like an elastic tube that can expand and contract depending of the hydrostatic and structural pressure surrounding the vessel. Mechanically, the vessel can also collapse with enough pressure applied across the vessel wall (Lambert, 1991). Different components of the ECM and basal lamina provide different structural elements and support to the vessel. Collagen IV, the stiffest component, is organized in a structured network of parallel and perpendicular fibers that provide a high mechanical resistance to sheer and compression forces to the basal lamina (Dehan et al., 1997). Laminin, being the largest non-collagen component of the basal lamina also provides a functional role, being a connection between the more robust collagen layer and the astrocyte end-foot processes and the pericytes cells (Schnittler, Franke, Akbay, Mrowietz, & Drenckhahn, 1993). Structurally, laminin also contributes to the resistance of the microvasculature to mechanical stress forces (Schnittler et al., 1993) and reduced laminin content after degradation leads to a

reduced mechanical resistance of the microvasculature (Hamann, Okada, Fitridge, & del Zoppo, 1995).

Basal Lamina

The basal lamina, a sublayer within the basement membrane, is derived from the extracellular matrix (ECM) and is a collection of many proteins, proteoglycans and epithelial cells (del Zoppo & Hallenbeck, 2000; Takahashi & Macdonald, 2004b). Cerebral microvessels consists of type IV collagen, laminin, fibronectin, heparan sulfate proteoglycans (HSPG), and other glycoproteins (Petty & Wettstein, 2001; Yurchenco & Schittny, 1990). Type IV collagen is the major component of the basal lamina, and is organized in a manner that enhances and increases the structural stability and integrity of the vessel wall. Laminin is the second most important protein to the structure of the vessel wall and forms another crosslinked oligomer to further strengthen the vessel wall (McMillan, Akiyama, & Shimizu, 2003). Entactin connects laminin to collagen IV further reinforcing the basal lamina. HSPG is a protein crosslinked to a unique glycosaminoglycan chain which has a repeating array of alternating dissacharide units (Iozzo, 2001). The protein domain of HSPG can bind to collagen IV and the glycoslyated chain binds to laminin, further strengthening the structure of the basal lamina. The major components of the ECM in the microvasculature and those in the surrounding brain parenchyma are vastly different. Laminin, collagen IV and fibronectin are nearly absent from brain parenchyma and undetectable around neurons and glial cells, while being prevalent in the microvasculature (Lo, Wang, & Cuzner, 2002a; Sobel, 1998).

The external basal lamina envelops the entire blood vessel and prevents any leakage of blood, either plasma or cellular components, into the brain parenchyma. Astrocyte endfoot processes and pericytes, which complete the formation of the BBB, further seal the blood vessels from the brain parenchyma and allow the brain to become an immune privileged organ (Palmer, 2011). The BBB prevents any substances from crossing from the systemic circulation into the brain and affords the brain a second form of immunity, one from the native humoral and cell mediated immunity, and another by preventing transmission of infection via the circulation, as both bacteria and other infectious agents cannot cross the BBB (Schwartz & Ziv, 2008). The BBB also prevents the passage of

many other molecules into the brain, such as antibiotics and other therapeutic compounds, which makes treatment of the brain directly a difficult proposition under normal circumstances (Silva, 2008; Morris & Abrey, 2009; Banks, 2009; Wilson, Weninger, & Hunter, 2010).

Little is known of about the function and molecular signalling pathways of the microvasculature during hyperthermia or ischemia. There is some work demonstrating that as little as 10 minutes after the onset of ischemia, the microvasculature begins to break down and increase in permeability (Hamann 2004, Yepes and Lawrence 2004, Yepes 2003). In rodent models of transient stroke, the basal lamina begins to shown signs of degradation 2 hours after ischemia and this continues during reperfusion (Hamann 1995). Immunohistochemical studies detecting changes in blood vessels of the microvasculature demonstrate a reduction in staining for components of the basal lamina (laminin, collagen IV, fibronectin) after transient ischemia (Hamann 2003, Vosko 2003). There is still a reduction of detection of these markers up to 24 hours after reperfusion and this signifies a continued degradation of these components after the reestablishment of blood flow to the affected areas. Collagen IV has also been shown to be increased in degradation 3 hours after a stroke. It is these components of the basal lamina which make up part of the protective layer around the brain which are most susceptible to degradation during an ischemic attack. One of the major endogenous protease families that are thought to degrade components of the basal lamina are the MMPs.

MMPs

There are currently 28 known members of the MMP family. MMPs, first characterized as a collagenase in tadpole tail development (Stolow et al., 1996), have grown into an expanded role, influencing tumour growth (Zhi, Song, Wang, Zhang, & Yin, 2009; Jackson et al., 2009; Chakraborti, Mandal, Das, Mandal, & Chakraborti, 2003b) arthritis (Jackson et al., 2009), angiogenesis (Su, Mendoza, Kwak, & Bayless, 2008), wound healing (Su et al., 2008; Imai et al., 1996; Gill & Parks, 2008) and edema (Betz, 1996). Because of the complex and varied role MMPs play throughout body much work is still being done to elucidate both the signalling pathways which cause MMP activity and the roles of MMPs

in their tissue specific locations (Chakraborti et al., 2003b). MMPs are zinc dependent endopeptidases which are classified based on their substrates. These can be grouped into collagenases (MMP1, MMP8, MMP13, and MMP18), gelatinases (MMP2 and MMP9), stromelysins (MMP3 and MMP10), matrilysins (MMP7 and MMP26) and the membrane type MMPs (MT-MMPs) (MMP14, MMP15, MMP16 and MMP24) (Visse & Nagase, 2003). In addition to these five major grouping, seven MMPs (MMP12, MMP19, MMP20, MMP21, MMP23, MMP27, MMP28) cannot be grouped in this way and so are not assigned a specific category (Visse & Nagase, 2003).

MMPs are known for their ability to cleave the constitutive proteins of the ECM. Because of their important role in degrading and remodelling the ECM the regulation of MMPs become an important task for a cell. All MMP family members posses both a catalytic and propeptide domain, though the specific structures vary depending on the present domains (Woessner, Jr., 1991; Werb, Alexander, & Adler, 1992). This propeptide domain is one of the major areas of MMP regulation, as the endopeptidase can be inactivated and sequestered until required. Sequestering is itself another form of regulation of MMP expression and activity. Compartmenting the MMPs allows for a controlled release of the protease when required.

Another method of limiting MMP activity is to degrade them via catalysis. Removal of certain domains of MMPs will diminish their ability to localize with the cellular interface with the ECM (Imai et al., 1996). This in addition to complete degradation will attenuate MMP activity in the ECM. In addition to the regulation of the mature polypeptide, either the propeptide or the endopeptidase, there is the possibility of regulation at the genomic level which is covered in detail in elsewhere (Brenneisen, Sies, & Scharffetter-Kochanek, 2002; Clark, Krekoski, Bou, Chapman, & Edwards, 1997a).

The first step in MMP activation is the removal of propeptide domains on all isoforms. Activation of the zymogen can vary from direct cleavage of the pro domain by proteases to degradation by chemical agents, like glutathione (oxidized), SDS, reactive oxygen species and $HgCl_2$ (and other thiol modifying agents) (Visse & Nagase, 2003). In addition

to these factors, pH and heat can also cause an activation of MMPs (Koklitis, Murphy, Sutton, & Angal, 1991; Chen, Noelken, & Nagase, 1993). The proteolytic activation of MMPs occurs in a step wise fashion (Nagase, 1997) and is covered elsewhere (Nagase, 1997). ProMMP activation can occur by other peptidases as well as other members of the MMP family. Tissue plasminogen can form plasmin which activates the zymogens. These newly activated MMPs can then in turn activate more MMPs in an enzyme cascade that can quickly increase proteolytic activity several fold in a short amount of time.

The mode of action of MMPs is complex, to the degree where spatial and temporal expression may have different effects on the same tissue (Sandoval & Witt, 2008). Further complicating this is the fact that each MMP subfamily is specific for the cleavage of a certain section of the ECM and that these have not been studied in all tissues. Indeed, no single study has looked at broad MMP activity in a single tissue. The general mode of action of MMPs is to cleave parts of the ECM and to help in various stages of tissue growth and repair. As stated above, this can range from tumour invasiveness, (Zhi et al., 2009; Jackson et al., 2009; Chakraborti et al., 2003b) to angiogenesis (Su et al., 2008) and wound healing (Su et al., 2008; Imai et al., 1996; Iwanami, Ishizaki, Fukuda, & Takahashi, 2009). MMP activity can also lead to dysfunction in tissues when not correctly expressed (Sawicki et al., 2005; Schulz, 2009). Overall, the general pattern for MMP activity leads to a degradation of the ECM and cleavage of the specific target proteins, though much further research needs to be done to evaluate both the temporal and spatial expression patterns in such a complex family of proteases.

It has been known that MMPs can cleave ECM proteins since their first discovery, but few studies looked beyond the heart as an area of MMP expression. The brain has become a new area of research for MMP study as it is well supplied with blood vessels which are subject to MMP targeted degradation. Components of the ECM, notably collagen and laminin, make up the basement membrane (Visse & Nagase, 2003; Galis & Khatri, 2002b) and can be targeted for degradation by MMPs (Sole, Petegnief, Gorina, Chamorro, & Planas, 2004; Gurney, Estrada, & Rosenberg, 2006). Additionally, MMPs can degrade the tight junctions within the blood brain barrier (BBB) and can cause

edema (Simard, Kent, Chen, Tarasov, & Gerzanich, 2007). Combing these two can lead to major complications in the brain due to the permeability of the BBB and resulting edema.

Under normal conditions MMPs in the brain are used for angiogenesis and vascularisation of the brain and are readily present during development of the brain (Zhao, Tejima, & Lo, 2007). Pathologically MMP expression and activation in the brain is quite low and is used to maintain the support structures of the vasculature, namely the basement membranes and ECM. Under ischemic conditions MMPs are robustly expressed (Fukuda et al., 2004a) and contribute to the weakening of the basal lamina and the ECM components.

Under ischemic conditions MMP expression is increased (Fukuda et al., 2004a) and this exacerbates the vasogenic edema found as a secondary characteristic of stroke. Furthermore, during a stroke MMP expression also degrades the basal lamina, making the blood brain barrier more permeable to the leakage of fluids and proteins. The mode of action of MMPs is complex, to the degree where spatial and temporal expression may have different effects on the same tissue (Sandoval & Witt, 2008). The general mode of action of MMPs is to cleave parts of the ECM and to help in various stages of tissue growth and repair.

Ischemic insult to the brain is the most commonly studied complication when looking at the expression and activity of MMPs. MMP activity in the brain is almost undetectable (Dzwonek, Rylski, & Kaczmarek, 2004) but the two most common ones studied are MMP2 and MMP9 due to their gelatinolitic activity. It has long been hypothesised that MMPs degrade components of the ECM which lead to the dysfunction of the signalling within the neurovascular unit, summarized nicely in a figure from Rosell (Rosell et al., 2006). MMPs are known to cleave the BBB (Lo, Wang, & Cuzner, 2002b; Rosenberg, Navratil, Barone, & Feuerstein, 1996) which can lead to edema and cellular death (Simard et al., 2007). This opening of the BBB by MMPs have been found in many studies (Rosenberg, 1995; Rosenberg, Estrada, & Dencoff, 1998a) and can be inhibited

with the use to TIMPs (Rosenberg et al., 1992) or with MMP knockout mice (Asahi, Sumii, Fini, Itohara, & Lo, 2001). Both studies draw a concrete link between the activity and expression of MMPs and an overall deterioration of the BBB.

MMP activation degrades the components of the ECM as well as those of the basal lamina increasing vessel permeability (Hamann et al., 2002; Hamann, del Zoppo, & von, 1999). Additionally, the BBB is dependent on structural proteins that make up the tight junctions between endothelial cells to maintain its integrity (Watson, Anderson, Vanltallie, & Doctrow, 1991; Furuse, Fujita, Hiiragi, Fujimoto, & Tsukita, 1998). A degradation of these proteins will lead to edema (Simard et al., 2007). MMP inhibition has already been widely studied and various inhibitors have gone through clinical trials, only to prove clinically unsuccessful. A new drug has made resurgence in the field of MMP inhibition, and is currently undergoing a clinical trial, which will be discussed in a later section.

TIMPs

Tissue inhibitor of MMPs (TIMPs), a family with four isoforms, are relatively small molecular weight proteins (between 20 to 30 kDa). As their name suggests, they inhibit MMPs by forming a stiochiometric ratio of 1:1 with MMPs and binding to the catalytic site. All TIMPs posses 12 conserved cysteine residues forming six disulfide bonds (Douglas, Shi, & Sang, 1997). TIMP1 (Gomis-Ruth et al., 1997), TIMP2 (Tuuttila et al., 1998) and TIMP3 (Wisniewska et al., 2008), but not TIMP4 have all been successfully crystallized. The structures show TIMPs as wedge shaped proteins, with a central helical region, surrounded by a five stranded β-barrel and a C-terminus β-sheet (Gomis-Ruth et al., 1997). They are good inhibitors of MMPs *in vitro*, but yet have to be proven *in vivo,* despite over a decade of research their *in vivo* inhibiting effect has not been proven (Zucker, Cao, & Chen, 2000). TIMPs interact with MMPs at both the N-terminal domain and C-terminal domain (Murphy et al., 1991; Williamson et al., 1990). The N-terminal domain is capable of inhibiting MMPs by way of binding to the active site of MMPs (Murphy et al., 1991). The C-terminal domain also plays a critical role in MMP inhibition by increasing the rate of TIMP association with MMPs many fold (Kleiner, Jr., Tuuttila,

Tryggvason, & Stetler-Stevenson, 1993; Willenbrock et al., 1993). TIMPs have been found to inhibit all classes of MMPs, with one exception; TIMP1 is unable to inhibit MT1-MMP (MMP 14) (Will, Atkinson, Butler, Smith, & Murphy, 1996).

TIMPs remain an alternative therapeutic method for treating the various conditions caused by MMP degradation of the ECM. Other than their major role of MMP inhibition, TIMPs also have many smaller functions in the cell, linked to cell growth, apoptosis, cell regulation and migration. The vast majority of work on TIMPs stems from their work in inhibiting MMPs readily expressed in tumour invasion and metastasis. However, TIMPs also play a critical role in many other tissues such as the blood brain barrier and degradation of the ECM because of their close association with MMPs. As more roles for MMPs are being identified, TIMPs have also become a topic of interest.

TIMPs, being specific MMP inhibitors, are integrally related to the destruction of tissue in human malignancies. They act to impede the degradation of the ECM by MMPs, especially during inflammatory processes and tissue remodelling. TIMP 1 being the most commonly expressed TIMP in the human body, is able to limit tumour metastasis and invasiveness in preclinical studies (DeClerck et al., 1992). However due to the discouraging results from the use of small molecule inhibitors (Zucker et al., 2000) TIMPs were not considered a viable alternative, but recent work has begun to displace this view (Memtsas, Zarros, & Theocharis, 2009; Overall & Kleifeld, 2006).

Other than the inhibition of MMPs, many other roles of TIMPs have been identified and are not limited to erythroid-potentiating activity (Stetler-Stevenson, Bersch, & Golde, 1992; Gasson et al., 1985) and cell growth promoting activities (Hayakawa, Yamashita, Ohuchi, & Shinagawa, 1994; Hayakawa, Yamashita, Tanzawa, Uchijima, & Iwata, 1992). TIMPs have been found to translocate to the nucleus of breast carcinoma cells after binding to the surface (Ritter, Garfield, & Thorgeirsson, 1999), whether they influence gene expression though remains to be seen. TIMP 2 also inhibits endothelial cell growth induced by basic fibroblast growth factor, while TIMP 1 does not perform this same function (Murphy, Unsworth, & Stetler-Stevenson, 1993) denoting a difference within the TIMP family itself.

TIMPs are primarily studied as inhibitors of MMPs and thus much of their pathophysiology is related to the regulation of MMPs and the reduction, or sometimes induction, of the degradation of the ECM. In the brain the major role of TIMPs is to mitigate the early degradation of the BBB and reduce edema and cell death in the brain after an ischemic stroke. Thus we would expect TIMPs to be upregulated to reduce MMP degradation of the BBB, but this is not the case (Shuaib et. al, unpublished data). Conversely, in the heart MMPs are initially upregulated while TIMPs are not (Peterson, Li, Dillon, & Bryant, 2000), so that MMPs are able to remodel the myocardium in cardiac dysfunction and, like TIMPs, are only upregulated later to inhibit MMPs during the second phase of MMP regulation.

TIMPs have recently been studied in the brain and seem to be involved in neuroprotective mechanisms. TIMP 1 is induced after transient global ischemia in rat brain in both neurons and astrocytes (Rivera et al., 2002). TIMP 1 performs its neuroprotective function independently of MMPs (Tan et al., 2003), as pharmacological inhibition within a synthetic broad range MMP inhibitors did not produce the same effects. Also the induction of point mutations of MMPs and the removal of TIMP 1 produced similar results, a loss of the neuroprotective effects (Tan et al., 2003). TIMP 1 is also upregulated in transient ischemia in hippocampus (Rivera et al., 2002). TIMP 1 acts rather quickly to specific glutamate dependent neuronal loss but not ischemia induced with neurotoxins (Tan et al., 2003).

TIMP 2 is the most abundantly expressed TIMP in the adult CNS (Fager & Jaworski, 2000). TIMP 2 does not seem to be upregulated by the same conditions, pathological or neuronal depolarization, that cause the activation of TIMP 1 (Khuth et al., 2001; Lorenzl, Albers, Narr, Chirichigno, & Beal, 2002; Pagenstecher, Stalder, Kincaid, Shapiro, & Campbell, 1998). TIMP2 is expressed at the same time as MMP14 during mouse embryological development (Apte, Fukai, Beier, & Olsen, 1997) as co-regulated genes. This is an addition to its tight functional synergy with MMP14 in the activation and control of MMP2 (Itoh et al., 2001).

TIMP 3 is expressed at low levels in the brain natively (Fager & Jaworski, 2000), but is over expressed after cerebral ischemia in a short (90 min) ischemic model (Rosenberg et al., 2001; Wallace et al., 2002a). TIMP 3 differs from TIMP 1 in that the neuroprotective mechanism is not as vigorous as blocking MMP activity using synthetic MMP inhibitors. However, treatment with TIMP-3 offered no neuroprotection against exocytotoxicity (Tan et al., 2003). TIMP 3 has been found to be over expressed in brain tissue after transient ischemic insult (Wallace et al., 2002b).

TIMP 4, the most recently discovered TIMP, has had little study devoted to it. This may be due to the limited expression of TIMP 4 in the body. TIMP4 is expressed in the heart, kidney, pancreas, colon, testes, brain and adipose tissue (Greene et al., 1996). Most of the work in TIMP 4 has been done in cancer models, utilizing TIMP 4 as an inhibitor of MMPs to limit invasiveness and growth (Wang et al., 1997). Melendez-Zajgla et al. has a current review on TIMP 4 function and activity (Melendez-Zajgla, Del, Ceballos, & Maldonado, 2008).

Hyperthermia

Hyperthermia, an elevation of the core body temperature, is one complication observed after the initial onset of an ischemic stroke (Phipps, Desai, Wira, & Bravata, 2011a). Hyperthermia differs from fever in that it is a pathological increase in body temperature whereby the ventromedial preoptic area and the anterior hypothalamic nucleus, the thermo regulation centers of the brain, are damaged or unable to properly regulate body temperature. In contrast to this, fever is caused by an increase in activity in the VMPO and an increase in the internal core temperature as part of the body's response to a fever in order to further increase blood circulation, mobility of phagocytes and leukocytes and profileration of T cells (Mace et al., 2011; Nemoto et al., 2005). None of these effects will occur with only hyperthermia, as the signalling mechanisms of fever proceed though pyrogens, in essence cytokines, TNFα, IL-1β and IL-6, and the production of prostaglandin E2 (PGE2) (Roth, Rummel, Barth, Gerstberger, & Hubschle, 2009; Hou, Lin, Chang, Huang, & Lin, 2011a). These mechanisms at both the vascular and cellular levels occur through multiple pathways to produce the deleterious effects of hyperthermia. There is little research published on the effects of hyperthermia on the

microvasculature and the blood vessels of the brain. Some work has been published on hyperthermia and focal embolic strokes (Noor, Wang, & Shuaib, 2003; Noor, Wang, & Shuaib, 2005a), specifically focusing on the effects of the microvasculature and an increased permeability of the BBB. Hyperthermia also causes a reduction of the effectiveness of tPA and a subsequent failure of thrombolysis to protect the brain.

Hyperthermia and Stroke

As mentioned previously, ischemia does cause an upregulation of various cytokines, including both TNFα and IL-1β (Roth et al., 2009; Hou, Lin, Chang, Huang, & Lin, 2011b). These cytokines are also pyrogenic molecules, that can increase core body temperature, which leads to hyperthermia. Thus, hyperthermia is found to be commonly associated with inflammation, which also occurs during an acute ischemic stroke (Phipps, Desai, Wira, & Bravata, 2011b). Hyperthemia is commonly observed with stroke, indeed between 20-25% of all patients diagnosed with a stroke will have concurrent hyperthermia ((Phipps et al., 2011b; Dinarello, 2004; Zaremba, 2004). Hyperthermia is also well established to have a poor outcome on stroke, increase stroke size and neuronal damage, decrease outcome of good prognosis and increasing mortality and morbidity (Wong et al., 2007). Hyperthermia is also correlated with an increase of a patient's National Institutes of Health Stroke Score (NIHSS) (Wong et al., 2007).

Additionally, hyperthermia also has been found to lead to further disruption of the BBB and can cause complications in the treatment of stroke with tPA (tissue plasminogen activator) which can lead to hemorrhagic transformations in effected patients (Rosell, Foerch, Murata, & Lo, 2008). Previous research from our lab indicates that hyperthermia also further increases damage to the blood brain barrier and enhances perfusion deficits within the ischemic region of the brain (Noor et al., 2003; Noor, Wang, & Shuaib, 2005b). It has been recently been revealed that temperature in the brain is elevated further than the increase in core temperature. Some reports place this difference at 0.1 to 0.76^{o}C while others place it at 1.0^{o}C in cases of severe brain trauma (Mellergard & Nordstrom, 1991; Henker, Brown, & Marion, 1998). This would further increase any adverse effects hyperthermia has on ischemia.

Experimental and clinic studies consistently demonstrated the critical importance of the time of onset of hyperthermia and the extent of damage caused by a stroke. In human acute stroke patients, a worsening outcome is strongly correlated with an earlier onset of hyperthermia (Castillo, Davalos, Marrugat, & Noya, 1998). For each degree increase in core body temperature there in a corresponding increase in poor outcome by 2.2% (Reith et al., 1996). In addition to severe hyperthermia, mild hyperthermia can also induce significant neuronal death and brain tissue damage. Mild hyperthermia can enhance the ischemic core and reduce the penumbral tissue and correspondingly can increase necrotic death in the core region of an infarct (Yip, Koh, Lin, & Chen, 1998; Castillo et al., 1998). Hyperthermia, induced in animal models, can also increase ischemic injury to structures not normally affected (Favero-Filho et al., 2008; Dietrich, Busto, Valdes, & Loor, 1990) and reduce the effectiveness of normally effective neuroprotective strategies (Noor et al., 2005b; Ginsberg & Busto, 1998).

The mechanism of damage due to hyperthermia is still under investigation, but a few mechanisms have been proposed. First there is a possibility that an increase in temperature will increase the basal metabolic rate of cells. This increase in brain temperature will cause a larger metabolic strain on tissue already deprived of oxygen and glucose and may exacerbate ischemic injury. A second proposed mechanism is that hyperthermia increases the release of free radicals and reactive oxygen species furthering exacerbating cell death (Roth et al., 2009). A final proposed mechanism is the increased release of excitatory neurotransmistters which can lead to neuronal excitotoxicity (Castillo, Davalos, & Noya, 1999) and increased ischemic injury.

Animal Models

There are many experimental models of ischemia currently used in research and they all posses advantages and disadvantages, with each model better suited for certain research aims than others. These models are varied in both the way they mimic the mechanism and pathology of a stroke (Table 1). As with all models, these are tools useful for researchers to elucidate mechanisms of injury after stroke of potential therapies to aid in recovery after stroke.

Table 1. Models of Ischemia

Model	Classification of Induction	Compounds and Methods Used
Culture	Apoptosis	C2 ceramide, paclitaxel, ptoposide, tunicamycin, staurosporine, serum deprivation, low potassium, S-nitroso-A-acetylpenicillamine (SNAP), SIN-1, sodium nitoprusside (SNP), 3-morpholinosydnonimine bleomycin sulfate
	Calcium	Thapsigarrin
	Inflammation	Lipopolysaccharide, chromogranin A
	Oxidation	H_2O_2, iron, heme, superoxide, rotenone, 3-morpholinosydnonimine, sodium nitoprusside (SNP), S-nitroso-A-acetylpenicillamine (SNAP), glutathione depletion, antimycin A, photochemical stress, naphthazarin
	Ischemia	Hypoxia, oxygen/glucose deprivation, sodium cyanide, anoxia, sodium azide, endothelin-1, veratridine, iodoacetic acid
Global Ischemia	Mechanical	Tourniquet, clip, ligation, balloon compression, intracranial pressure, decapitation
	Respiratory	Potassium cyanide, carbon dioxide, nitrogen, asphyxia
	Thermal	Electrocauterization
Focal Ischemia	Chemical	Rose Bengal photosensitive dye, arachidonic acid, endothelin-1, $FeCl_3$, adenosine 5' phosphate
	Thermal	Electrocauterization
	Mechanical	Internal filament, ligation, clip, inflatable cuff
	Embolic	Autologous clot, fibrin clot, microsphere, macrosphere, polyvinyl acetate

Adapted from O'Collins et al. (2006).

Table 1. Different models used to induce ischemia. Models fall into three types: culture, global ischemia and focal ischemia. Different compounds and methods used for inducing ischemia are listed on the right.

The study of stroke, as with most diseases and conditions, began in humans afflicted with it. Animal models were then used to understand different aspects of stroke, from the formation of an ischemic event to the lysis of a clot to provide benefits after a stroke had occurred. Small rodents are the ideal model to study stroke experimentally due to their small size, low cost of maintenance, ethical considerations and the large overlapping similarity between the human and rat cerebrovasculature and anatomy (Durukan & Tatlisumak, 2007; Durukan & Tatlisumak, 2009). Additionally, rats have a very homogenous population within each strain and which aids in reduction of experimental variability and increased reproducibility of results (Durukan & Tatlisumak, 2009). Mice are more commonly used in the investigation of certain genes and proteins in the mechanism of stroke, with respect to the molecular pathophysiology of a stroke and are usually less suitable for studies involving drug applications. Due to costs restrictions and the ethical implications, non-human primates, while the best model for testing drug therapies, are not widely employed but are still recommended before any potential therapeutic applications begin clinical evaluation (O'Collins et al., 2006a).

Focal Ischemia model

Though global ischemia was commonly used to study the pathophysiology of stroke, most current models focus on focal ischemia, rather than global ischemia, which is more clinically relevant to the understanding and treatment of stroke. Focal ischemia may be either transient or permanent, and can be modulated accordingly, depending on the focus of the research and the variables being investigated. Transient ischemia is commonly used when there is a desire to study reperfusion and thrombolytic agents, while permanent ischemia is used to study mechanisms of limiting neuronal death and tissue loss. The permanent ischemia model also allows for the investigation of ischemia without the confounding variable of reperfusion, which can be both beneficial and harmful in varying circumstances.

Embolic Models

The embolic model of focal ischemia is subdivided into the non-clot embolus and the thromboembolic models. Non-clot embolic models utilize a variety of different materials

to occlude the vessel, usually the middle cerebral artery (MCA) using silicone coated silk sutures or microsphere beads (Lauer et al., 2002; Durukan & Tatlisumak, 2007; Yang, Yang, Li, Wang, & Shuaib, 2002). These methods produce reproducible and controlled ischemia and can be permanent or transient, but lack the ability to test for thrombolysis and reperfusion. Thromboembolic models of ischemia are most closely linked to the clinical condition of stroke, whereas a thromboembolus is the cause of stoke within the vast majority of stroke patients (Baldwin & McCoy, 2010). Thromboembolic models also allow for the testing of thrombolytics and reperfusion to the affected lesion area and can be combined with other therapeutic compounds to test for combination therapies. Unfortunately, this model is both highly variable and there is a chance of spontaneous recannalization and reperfusion within the model, providing another confound to most experimental paradigms. The middle cerebral artery occlusion (MCAO) model is commonly used to mimic the human etiology and conditions of a stroke. Stroke is induced with the injection of an autologous thrombus within a small gauge catheter into the external carotid artery (ECA) (Wang, Yang, & Shuaib, 2001a; Wang, Yang, Yang, & Shuaib, 2001). The catheter is then advanced towards the junction between the MCA and the anterior cerebral artery (ACA) where the thrombus is released into the lumen of the vessel. The clot is then allowed to remain with the vessel until a thrombolytic agent, other therapeutic compound or other treatment is utilized to lyse it. As stated previously, this model has a wide degree of variability in the size and location of the lesion due to the variability of the clot, its placement and the strength of the clot. Spontaneous recannalization is another problem for this model, as this will allow reperfusion earlier than was set by the experimental paradigm and increase variability and decrease reproducibility. Nonetheless, this model of experimental ischemia is the closest mimic to the clinical situation and is heavily favoured in studies testing new potential therapeutics.

Other MCAO Models

The intra luminal suture model is used to produce highly controlled, reproducible and specific lesions while also allowing the testing of reperfusion within the stroke area. In this model the ECA is transected and a 4-0 monofilament silk suture is placed within the lumen of the ECA and advanced to the junction between the MCA and the ACA

(Carmichael, 2005). The suture can then be removed at a designated time point and reperfusion will occur, or the suture can be left within the lumen of the artery to form a permanent occlusion. There are some sources of variability within this model, as it requires correct placement of the suture, which generally requires the assistance of laser doppler flowmetry. Additionally, the diameter of the suture, the coating used, the length of time of occlusion and the placement can all lead to variability of the lesion size (Durukan & Tatlisumak, 2007; Zarow, Karibe, States, Graham, & Weinstein, 1997). Indeed, placement too deep within the MCA can lead to subarachnoid haemorrhage and variable diameters and coatings used can increase or decrease the size of the lesion (Carmichael, 2005; He et al., 1999).

Photothrombotic models are also used to induce cerebral ischemia localized to the cortex (Carmichael, 2005; Watson, Dietrich, Busto, Wachtel, & Ginsberg, 1985). This model utilizes the injection of a photosensitive dye into the systemic circulation of the animal. A craniotomy is then performed and specific blood vessels are photoirradiated by a specific wavelength of high intensity light (Carmichael, 2005). This wavelength will excite the photoactive dye to produce reactive oxygen species which will then inflame the endothelium and produce platelet activation and clot formation at this specific location. The photothrombotic model is highly specific and targeted and reproducible and used extensively in studies of collateral blood flow and axonal regeneration and remapping of the cortex. Unfortunately, due to the nature of the lesion produced, predominantly cortical with little penumbra (Carmichael, 2005), this model is not ideal for testing of neuroprotective agents.

Treatment

Because of the wide range of symptoms and deficits associated with stroke, it is a very costly condition to recover from, often requiring months of treatment and rehabilitation. There is only one approved form of treatment for stroke, tissue plasminogen activator (tPA), which must be given within a strict time frame (4.5 hours after a stroke), thus limits the number of people who receive it. Indeed, only 5% or all patients eligible for tPA treatment will receive it due to this time window and the

uncertainty of diagnosing the precise time of onset of a stroke (Kaur, Zhao, Klein, Lo, & Buchan, 2004; Wang et al., 2004b).

Due to the nature of the damage caused to the brain after a stroke, the long term effects of the stroke, the lengthy recovery, living with a permanent disability and the short time window for stroke, there is a desperate need for alternative therapies which can be given to stroke patients. One potential therapy for consideration is the drug minocycline. Minocycline is a second generation tetracycline antibiotic commonly prescribed against acne. Its primary mechanism of action is through inhibition of protein synthesis, specifically through inhibiting tRNA from binding to the A (Zhanel et al., 2004; Hubbell, Hobbs, Rist, & White, Jr., 1982; Chopra & Roberts, 2001) site on the ribosome during translation. Minocycline also posses other pleiotropic effects including its anti-inflammatory properties and its ability to inhibit Matrix Metalloproteinases (MMPs) (Fong, Rae-Grant, & Huang, 2008). It has been approved by the Food and Drug Administration (FDA) in the US to be given orally. Given the specificity of Minocycline for MMP inhibition it might be a viable candidate for therapeutic application. Currently, there is a clinical trial of Minocycline ongoing to test this potential therapeutic benefit which is in the early stages, currently testing dosages for minocycline treatment acutely (Fagan et al., 2010).

On the subject of clinical trials, there have been more than 1,000 such drugs and other compounds that have been used and tested as potential therapeutic strategies for neuroprotection in animal models (O'Collins et al., 2006b). In human acute stroke patients, 114 of these compounds have been tested for safety, efficacy and neuroprotection (O'Collins et al., 2006b). Results from these various trials have been variable, ranging from a twofold increase in damage to 94% protection. These results demonstrate the wide variety of drugs and nonphamracological compounds as effective neuroprotective agents. Common in all of these potential therapeutic agents is the wide ranging variability in the predictive potential of animal models for human patients, specifically relating other factors (conditions such as diabetes and hypertension and other drugs patients might be taking) to one specific compound in tightly controlled laboratory settings.

No single mechanism of neuroprotection was established as superior to another in the wide variety of therapies and drugs used based on the effectiveness of animal models. This reflects the nature of damage after a stroke, the wide variety of mechanisms recruited in neuronal damage provides many possible mechanisms to block neuronal death and brain damage. Unfortunately this complexity also possess a challenge, that multiple mechanisms can cause damage and so therapies must either be combined to block these multiple pathways or be broad range in design. In either case, new strategies for combating damage caused by ischemic stroke are always being tested and refined and must be improved in order to treat stroke more effectively.

Neuroprotective effects of Minocycline

As mentioned previously minocycline posses neuroprotective effects reported in a wide variety of neurodegerative diseases and including Parkinson's disease, Alzheimer's disease, multiple sclerosis, spinal cord injury, and Huntington's disease (Wu et al., 2002; Seabrook, Jiang, Maier, & Lemere, 2006; Popovic et al., 2002; Stirling et al., 2004; Wang et al., 2003). It has also been demonstrated to provide neuroprotection by inhibition of the inflammatory cascade in ischemia (He, Appel, & Le, 2001a). Indeed, minocycline provides neuroprotection through inhibition of microglial activation excitotoxicity (Zhang, Goetz, & Duncan, 2003), glutamate (Tikka & Koistinaho, 2001a), nitric oxide release (Yrjanheikki, Keinanen, Pellikka, Hokfelt, & Koistinaho, 1998a) and ischemic injury (Yrjanheikki et al., 1999a). Minocycline can also decrease apoptosis through Bcl-2/cytochrome c pathways is the ischemic kidney (Wang et al., 2004a). In vascular smooth muscle it also decreases NO release and excitotoxicity (Amin et al., 1996) decreases inflammation in the spinal cord (Wells, Hurlbert, Fehlings, & Yong, 2003). In stroke models minocycline looks to be a promising therapeutic treatment, reducing the lesion size and decrease clinical scores of behavioural deficits (Wang, Yang, Noor, & Shuaib, 2002; Xu et al., 2004a; Arvin et al., 2002a). This is mechanistically thought to occur through the inhibition of microglial activity and a reduction in the production and secretion of pro-inflammatory cytokines (Stirling, Koochesfahani, Steeves, & Tetzlaff, 2005a). It is believed that the neuroprotective effects of minocycline stems from its microglial inhibitory properties and in stroke decreases lesion size.

MMP Inhibitory Properties

Minocycline is a specific MMP inhibitor in addition to its neurprotective and anti-inflammatory effects. The specific inhibition of MMP comes from the ability of minocycline to bind to the catalytic site of MMPs and prevent MMP from degrading any other substrates. The family of drugs known as the tetracyclines are known inhibitors of the MMPs (Golub et al., 1984). Doxycycline, at low doses was the first FDA approved MMP inhibitor within this family and is currently used in periodontal infection and disease (Greenstein, 2003). In an arthritic rat model, tetracycline and doxycycline, two compounds very similar to minocycline, in addition to a nonsteroidal anti inflammatory drug (NSAID), decreased swelling and inflammation within the joint. It was suggested that the suppression of MMP8 decreased the inflammation within the joint (Lauhio et al., 1994). Minocycline has been shown to inhibit MMP9 in a model of experimental autoimmune encephalomyelitis (Brundula, Rewcastle, Metz, Bernard, & Yong, 2002). Intracerebral hemorrhage, caused by collagenase injection, was used to demonstrate that minocycline reduced MMP-12 and improved functional outcome (Wasserman & Schlichter, 2007). Genetic studies have shown that minocycline is specific to MMP inhibition as minocycline reduces infarct size in wild-type but not in MMP9 knockout mice (Koistinaho et al., 2005a). Other work has shown that minocycline is a potent inhibitor of MMP9 at very low doses of 3 mg/kg (Xu et al., 2004a).

Anti-Inflammatory Effects

Minocycline possesses anti-inflammatory properties that are entirely distinct from its antimicrobial action. Current evidence has begun to accumulate that minocycline can attenuate neuroinflammation by inhibiton of microglia and the inhibition of induction of IL-1b-converting enzyme (ICE) mRNA which was expressed mostly in microglia (Yrjanheikki, Keinanen, Pellikka, Hokfelt, & Koistinaho, 1998b; Yrjanheikki et al., 1999b). In mixed spinal cord cultures and in pure microglial cultures as well minocycline prevents NMDA mediated excitotoxicity (Tikka & Koistinaho, 2001b) and NMDA activated p38 MAPK, NO and IL-1β release (Stirling et al., 2005a). Therefore, in addition to its neuroprotective effects and ability of inhibit MMPs, minocycline also has a effect

by inhibition of microglial activation, proliferation and secretion of pro-inflammatory cytokines.

Ischemic stroke has a multifocal effect of cell signalling and survival pathways activated after it occurs. As described previously, the release of glutamate, pro-inflammatory cytokines, ROS and activation of calcium dependent phospholipase (Durukan & Tatlisumak, 2007; Brouns & De Deyn, 2009) and proteases all contribute to the damage ultimately attributed to a stroke . Minocycline also has anti-inflammatory pleiotrophic effects in addition to its neuroprotective effects which make it an attractive potential therapy for use after a stroke. This dual role gives this class of drug a distinct advantage over drugs used solely for the purpose of neuroprotection or anti-inflammation (O'Collins et al., 2006b). Another advantage of minocycline is that unlike doxycycline and tetracycline, minocycline is highly lipophillic and can cross the BBB (Fagan et al., 2004; Guan, Kozak, & Fagan, 2011a; Vojtova & Urbanek, 2009). This removes a large hindrance that most therapeutic agents posses; that they cannot cross the BBB in any significant quantities and are not effective in the low concentrations that do cross into the brain parenchyma.

Hypothesis

As has been shown previously, dysfunction of the BBB after a stroke is a major cause of perfusion deficits and can increase the overall damage caused by a stroke. Due to the sudden onset of a stroke, the delay in properly diagnosing it and the strict time window in providing the drug necessary to treat an acute stroke there is a dire need for an alternative therapeutic method to treat acute stroke. The work of this thesis is to measure the effectiveness of Minocycline to prevent degradation of the BBB, specifically by inhibition of MMPs in both normothermic and hyperthermic conditions. Little is known about giving minocycline to acute stroke patients intravenously, and what effects an elevation of body temperature has on this drug. In this thesis, I have utilized current models and techniques to investigate the effects of minocycline inhibition of MMPs in

acute stroke, both in normothermia and hyperthermia in an animal model. I hypothesize that hyperthermia will increase activity of MMP2 and MMP9 and subsequently will cause a greater breakdown in the components of the BBB, namely laminin. With the administration of minocycline the basal lamina will remain intact through the inhibition of MMP2 and MMP9 activity. The inhibition of MMP2 and MMP9 will prevent an increased permeability of the BBB and keep the microvasculature intact and preserve collateral blood flow limiting the damage caused by ischemia and hyperthermia.

Chapter 2: Methods

Materials and Methods

Changes in MMP expression after hyperthermia

In this study MCAO was used in conjunction with either normothermia or hyperthermia to determine changes to MMP2 and MMP9 levels within the brain parenchyma at four and 24 hours. Animals were anesthetized and the MCAO procedure was performed. Afterwards animals were either recovered for four or 24 hours at room temperature or at 39^{o}C.

Animals

Thirty two male Sprague Dawley rats, weighting 300 to 350 g (Charles River, St Constant, Canada), were used in the focal stroke model. Of these 32 animals, six died after induction of hyperthermia but before reaching 24 hours of recovery. The entire hyperthermia group at 24 hours recovery time was removed from analysis owing to this large mortality rate. Focal cerebral ischemia was induced by embolizing a preformed clot into the middle cerebral artery (MCA) as described later. Surgery was performed in rats anesthetized with 1.5-2.5% isoflurane in a mix of O_2 and NO_2 (30:70; O_2:NO_2). In rats where hyperthermia was induced, treatment lasted for two hours at 39^{o}C using a thermal regulated heating blanket. The core temperature of the animals was kept constant with a feedback controlled regulator using a temperature probe. Animals were housed in pairs in standard cages with a 12 hours light-dark cycle. Animals were allowed free access to both food and water before and after surgical procedures.

Treatments

Animals were anesthetized and the MCAO procedure was performed. Afterwards animals were either subjected to room temperature for four hours of recovery (normothermia) or at 39^{o}C for two hours, followed by two hours at room temperature (hyperthermia). Animals within the 24 hour treatment group were either subjected to 24 hours at room temperature (normothermia) or two hours at 39^{o}C and then 22 hours at room temperature (hyperthermia). This final group, hyperthermia at 24 hours

recovery, had a large mortality rate due to the effects of hyperthermia and an extended recovery time period and was not included in the final analysis.

MCAO Induction

Middle cerebral artery occlusion (MCAO) was performed as reported previously (Wang et al., 2001a) with some modifications to reduce mortality rates. In brief, animals were anaesthetized with isoflurane (1.5-2.5 %). Body temperature was maintained at 37^{o}C during surgery, unless stated otherwise. A midline incision was made on the ventral side of the neck to expose the left common carotid artery. The external carotid artery (ECA) was ligated and dissected distally. 100 uL of blood was collected through a small incision in the ECA, using a PE-50 catheter prefilled with bovine thrombin. A clot was formed by letting the blood coagulate, and allowed clot by keeping it at room temperature for 15 minutes. A 15 to 20 mm section of the blood clot was then attached to a 100 uL Hamilton syringe filled with saline. A PE-10 catheter was used as an adapter to inject the blood clot. The tip of the PE10 was advanced 17 mm into the internal carotid artery towards the middle cerebral artery (MCA) and the clot was injected. The incision was closed and animals were allowed to recover after the surgery. Rats were then sacrificed after four or 24 hrs.

2,3,5-triphenyltertazolium-Chloride Staining

Animals were sacrificed at either four or 24 hours after induction of stroke by MCAO. Brains were then dissected out *en bloc* and placed within a dissecting matrix. Infarct volume was quantified using 2% 2,3,5-triphenyltetrazolium chloride (TTC)-stained brain sections four hours after the MCA occlusion (Wang, Yang, & Shuaib, 2001b; Shuaib, Xu, Yang, & Noor, 2002). Brains were sectioned into coronal sections at 2mm intervals and the first three slices were used for infarct verification. The rest of the brain was bisected and each hemisphere was stored at -80^{o}C until further use.

Western analysis

Western blot analyses were performed as described in the earlier studies (Song et al., 2005). Brain tissues were homogenized in cold lysis buffer (1x RIPA buffer, Upstate

Biotechnologies) and supernatants were collected. Cell lysates were loaded onto an SDS–polyacrylamide electrophoresis gel at 60mg per lane and transferred to PVDF membranes. The membranes were blocked in Tris-buffered saline with 0.05% Tween-20 containing 5% milk and then blotted overnight with primary antibodies. The membranes were washed and incubated with horseradish peroxidise conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA). The membranes were washed and developed by chemiluminescence. Densitometric analyses of protein levels were performed with NIH image software. There were eight rats per group. Actin was used as loading controls for each western blot.

Zymography

Gelatin gel zymography was used to determine the MMP2 and MMP9 activities (Kelly, Shuaib, & Todd, 2006). In brief, brain samples were homogenized in lysis buffer and centrifuged. Protein concentration was determined using Bio-Rad (Hercules, CA, USA) protein assay reagent; equal amounts of proteins were loaded onto an 8% acrylamide gel containing 0.1% gelatin as a substrate. Purified human MMP9 (R&D system, Minneapolis, MN, USA) was also included as a standard control. Upon the completion of protein separation, the gels were incubated in Triton-X 100 for one hour and then in incubation buffer at 37^{o}C for 24 to 72 hours. Gels were then stained with Coomassie blue and destained accordingly. MMP activation appeared as transparent bands on blue background. Images of the gels were captured by scanning on an HP ScanJet flatbed scanner (Boise, ID, USA) and analyzed with NIH image software. In these experiments, we examined the MMP2 and MMP9 activity in the brain when animals were sacrificed at four and 24 hours after the MCA occlusion. Each group consisted of eight animals.

Treatment with Minocycline in Normothermia and Hyperthermia

In this study animals were randomly assigned to either to one of four groups after induction of ischemia. The four groups were normothermia with saline treatment, normothermia with minocycline treatment, hyperthermia with saline treatment and hyperthermia with minocycline treatment. Biochemical analysis was done to determine the change in both expression and activity of MMP2 and MMP9 as well as expression of TIMP1 and laminin. Minocycline treatment was compared to control saline treatment in both the normothermic and hyperthermic conditions.

Animals

Thirty two male Sprague Dawley rats, weighting 250 to 300 g (Charles River, St Constant, Canada), were used as the focal stroke model. Focal cerebral ischemia was induced as previously described. In animals where hyperthermia was treatment lasted for two hours at 39^{o}C using a thermal regulated heating blanket, as described previously. Animals were housed in pairs in standard cages with a 12 hour light-dark cycle. Animals were allowed free access to both food and water before and after surgical procedures.

Minocycline administration and time course

After the induction of stroke by embolization of a preformed clot, animals were all kept anesthetized for another hour until the beginning of their treatment. Animals then received either saline or minocycline at 3mg/kg intravenously one hour after the induction of stroke. Animals in the normothermic group were returned to their home cages after this and allowed to recover for three hours. Animals in the hyperthermia groups were immediately placed in the heating blanket to raise their core temperature and kept there for one hour. After one hour they were treated, either saline or minocycline, and then kept at 39^{o}C for another hour. After this second hour, these animals in the hyperthermia group were then returned to their home cages and allowed to recover for two hours. Four hours after the induction of stroke, all animals were

anesthetized and decapitated. Their brains were carefully dissected out *en bloc* and placed within a dissecting matrix. As previously described brains were sectioned into coronal sections at 2mm intervals and the first three slices were used for infarct verification. The rest of the brain was bisected and each hemisphere was stored at -80^{o}C until further use.

Western analysis

Western blot analyses were performed as described in the earlier studies (Song et al., 2005). Frozen brain tissue was allowed to thaw then and was subsequently homogenized in cold lysis buffer (1x RIPA buffer, Upstate Biotechnologies) and centrifuged to remove cellular debris. Supernatants were collected and cell lysates were loaded onto an SDS–polyacrylamide electrophoresis gel at 60 or 80 mg per lane and transferred to PVDF membranes. The membranes were blocked in Tris-buffered saline with 0.05% Tween-20 containing 5% milk and then blotted overnight with primary antibodies. The membranes were washed and incubated with horseradish peroxidise conjugated secondary antibodies. The membranes were washed and developed using chemiluminescence detection kits. Densitometric analyses was performed with NIH image software. There were eight rats per group. Actin was used as loading controls for each western blot.

Zymography

Gelatin gel zymography was used done as described above. In these experiments, we first examined the MMP2 and MMP9 activity in the brain when animals were sacrificed at four and 24 hours after the MCA occlusion. Each group consisted of eight animals.

Solutions and Reagents

10X Tween-20 Tris Buffered Saline (10X TTBS)

In 1 L ddH_2O dissolve 24.2 g Tris, 80.0 g NaCl and 10mL Tween 20. Adjust pH to 7.6.

1X TTBS

To 100mL 10X TTBS add 900 mL dd H_2O.

5% Nonfat Skim Milk

To 100 mL 1X TTBS dissolve in 5g of nonfat dried skim milk.

5X Running Buffer

In 1 L ddH_2O dissolve 15.1 g Tris , 94.0 g Glycine and 5.0 g SDS. Check that the pH is between 8.3 and 8.8.

5X Transfer Buffer

In 800 mL ddH_2O dissolve 29.0 g Tris, 14.5 g Glycine and 1.85 g SDS. Adjust pH to 8.3.

1X Transfer Buffer

To 160 mL 5X Transfer Buffer add 640 mL cold dd H_2O and 200 ml methanol.

4X Sample Buffer (4X SB) (reducing)

Add 4 mL beta mercapto ethanol,4 mL glycerol and 1.6 mL 1.25 M Tris (pH 6.8) together. Dissolve in 0.85 g SDS and 0.04 g Bromophenol Blue.

4X Sample Buffer (4X SB) (non-reducing)

Add 4 mL glycerol, 4 mL dd H_2O and 1.6 mL 1.25 M Tris (pH 6.8) together. Dissolve in 0.85 g SDS and 0.04 g Brompphenol Blue.

Saline Solution

To 1L ddH_2O dissolve 9 g NaCl. This solution was then filter sterilized using a 0.22 μm filter.

Minocycline

Minocycline hydrochloride was dissolved in dd H_2O. 20 mg of Minocycline HCl was dissolved into 10mL of saline solution to form a 2mg/mL working solution.

Statistical Analysis

Statistical analysis and level of significance for changes in MMP2 and MMP9 expression in normothermia and hyperthermia at four and 24 hours were calculated by two analysis of variance (ANOVA) followed by Tukey's multiple comparison procedure. Statistical analysis and level of significance of changes in MMP2, MMP9, laminin, TIMP1 and actin at normothermia and hyperthermia in both the minocycline treatment and saline treatment groups was done with two way ANOVA followed by a student Neuman-Keuls test. An asterix above datum denotes a p value <0.05.

Chapter 3: Results

Parts of this Chapter have been previously published by Alam M, Mohammad A, Rahman S, Todd K and Shuaib A under the title Hyperthermia up-regulates matrix metalloproteinases and accelerates basement membrane degradation in experimental stroke in the Journal Neuroscience Letters. (2011) May 16;495(2):135-9.

Results

Effects of Hyperthermia on Stroke

In this first study we investigated the effects of hyperthermia on stroke. We also investigated the temporal aspect of changes in MMP2 and MMP9 expression, degradation of the BBB and the proteolytic activity of both MMP2 and MMP9 as well as the degradation of laminin.

In this study an experimental design was utilized where animals were randomly placed within one of four groups (normothermia with recovery to four hours, hyperthermia with recovery to four hours, normothermia with recovery to twenty four hours and hyperthermia with recovery to twenty four hours) to determine the effects of hyperthermia on stroke. As previously described, animals in this last group (hyperthermia with twenty four hours recovery) experienced a high mortality (62.5%) and this group was excluded for any further analysis.

Infarction Area

After undergoing the MCAO surgical procedure to induce strokes and recovering to the designated time points brain tissue was extracted and staied with TTC to confirm the infarction. In this study we did not use laser Doppler flowmetry to confirm the infarction, and so TTC was used to confirm the infarction instead. Figure 1 shows representative coronal sections of animals in each experimental group. Areas of white indicate the infarcted area, while healthy tissue is unstained and appears red.

Figure 1. TTC Staining of Brain Slices after MCAO.

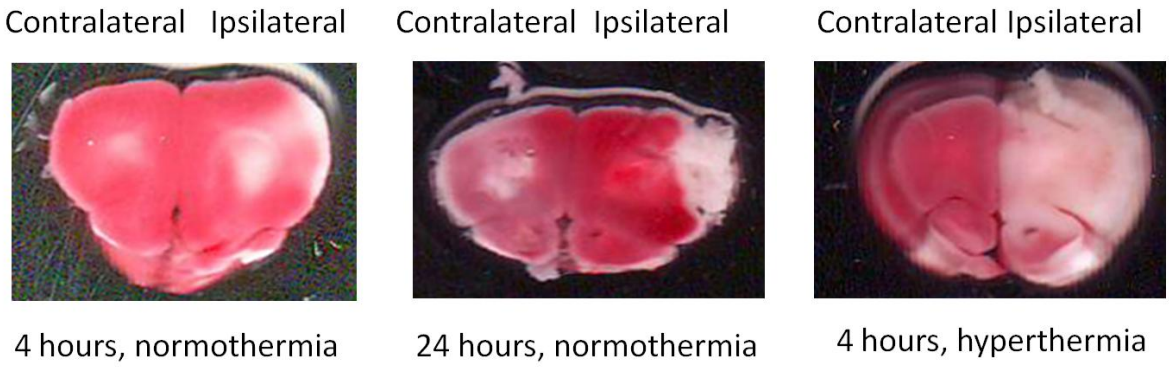

Figure 1. The left panel shows a brain slice from an animal that had four hours of recovery. The pink area is healthy tissue and the while area is the infarct area. The middle panel shows an animal that underwent 24 hours or recovery. The white area is noticibly larger and clearer in this group. The right panel shows an animal that underwent hyperthermia. In this panel the whole hemisphere is infarcted four hours after induction of a stroke, considerably more than either of the other groups.

Western analysis

Western blotting was performed on proteins MMP2, MMP9 and laminin to determine any change in their expression at the translational level after stroke and hyperthermia. Total protein levels were determined for each experimental condition in both the contralateral and ipsilateral hemispheres. By using this experimental paradigm each animals served as its own control, increasing the reproducibility and decreasing the variability of the data.

MMP2

MMP2 expression was significantly increased in both the normothermic condition at 24 hours and the hyperthermic condition at four hours in the ipsilatreal hemisphere as see in Figure 2. The increase in expression in the hyperthermic group was not significantly different from the normothermia group at 24 hours. This suggests that two hours of hyperthermia is as severe as 24 hours at normothermia in terms of the increase in MMP2 expression. In all groups the contralateral hemisphere, designated as the control hemisphere, did not show any change in MMP2 expression regardless of temperature or time. Ischemia at 24 hours of normothermia and four hours of hyperthermia did significantly increase MMP2 expression. There was no difference in the ipsilateral and contralateral hemispheres at four hours of normothermia. Taken together, this suggests that early changes to MMP2 expression do not begin until after 4 hours, but can be detected at 24 hours. In hyperthermia MMP2 expression is drastically increased and can be detected at four hours in contrast to the normothermic condition.

MMP9

MMP9 expression was also increased in a similar pattern to MMP2 expression as shown in Figure 3. There was a significant increase in expression of MMP9 in both the 24 hour normothermic and four hour hyperthermic groups in the ipsilateral hemisphere. There was also a significant difference between the four hour hyperthermia and the 24 hour normothermia groups. Thus, MMP9 expression is increased at 24 hours of

normothermia, and this change is greater than that seen in four hours of hyperthermia. Hyperthermia did not seem to induce as robust an expression in MMP9 as MMP2, but still significantly increased overall expression. In all contralateral hemispheres there was no change to the expression levels, but there was a significant difference between the ipsilateral and contralateral hemispheres within the 24 hour normothermia and four hour hyperthermia groups. This data demonstrates that MMP9 expression is increased in the ischemic ispilateral hemisphere and cannot be detected at four hours but can be detected at 24 hours. In conditions of hyperthermia MMP9 expression in increased more than at normothermia, but this increase is not as great as the 24 normothermic time point.

Figure 2. MMP2 Protein Expression in Hyperthermia.

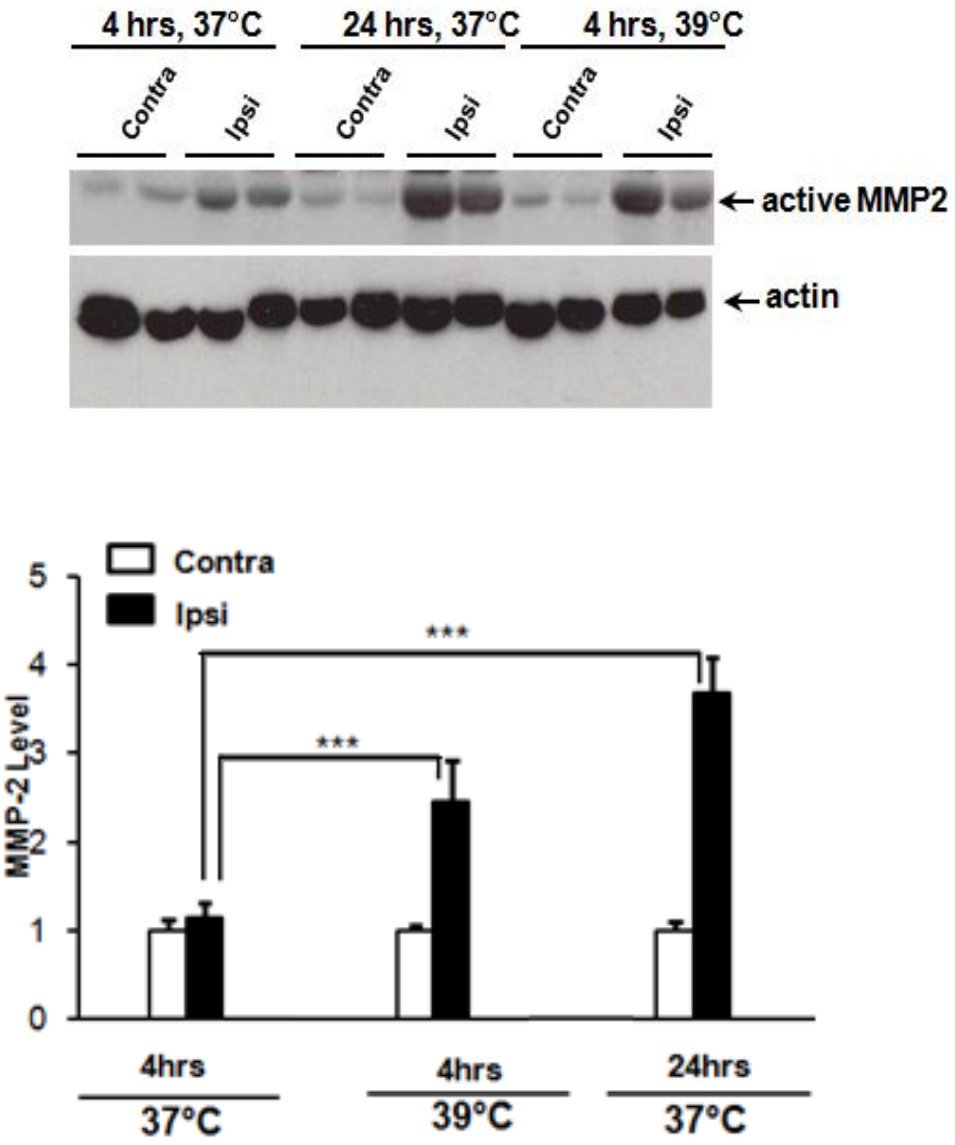

Figure 2. The top panel shows the protein expression of MMP2 (active form) after normothermia (at 4 and 24 hours) and hyperthermia (4 hours) by western blot. The bottom panel quantifies this data. There is a significant increase in MMP2 protein expression after hyperthermia and 24 hours of normothermia as compared with four hours of normothermia. There is no significant difference in MMP2 expression between hyperthermia and 24 hours of normothermia.

Figure 3. MMP9 Protein Expression in Hyperthermia.

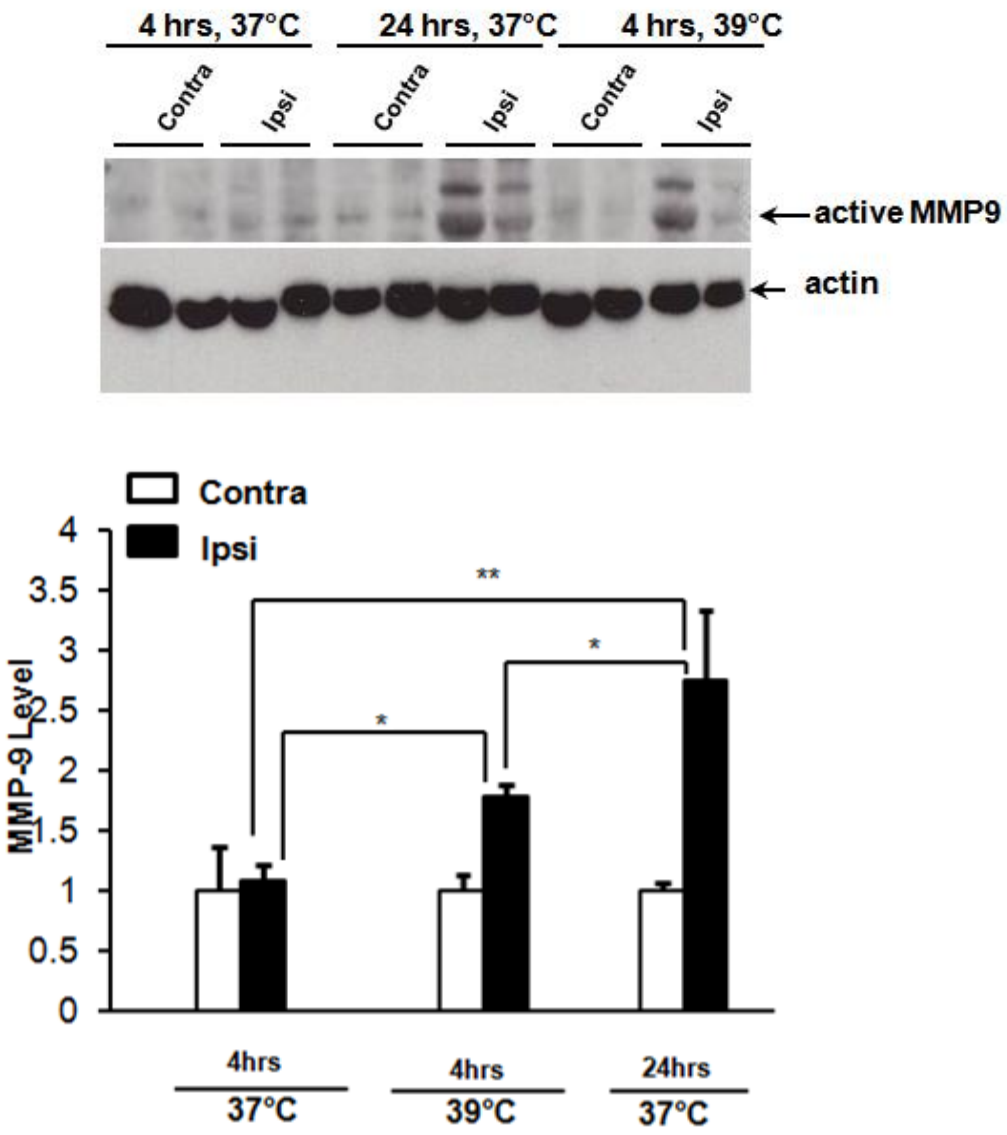

Figure 3. The top panel shows the protein expression of MMP9 via western blot after normothermia (at 4 and 24 hours) and hyperthermia (after 4 hours). The bottom panel is a quantification of this data. There is a significant increase in MMP9 protein expression in both hyperthermia and normothermia at 24 hours as compared with normothermia at 4 hours. There is also a significant difference in MMP9 protein expression between hyperthermia and normothermia at 24 hours.

Laminin

Laminin degradation was determined by western blot analysis and revealed a pattern of laminin degradation that was greatest in the four hours hyperthermia group shown in Figure 4. The laminin degradation band at 72 kilodaltons (kDa) was used to determine the extent of degradation, and was corroborated by a decrease in the intensity of the mother band seen at 250 kDa. At 24 hours of normothermia laminin degradation was also significantly elevated as compared to the four hour normothermia group, but this increase was also significantly less than the amount found in the four hours hyperthermia group. Laminin degradation was increased in both the 24 hours normothermia group and the four hour hyperthermia group, but not in the four hour normothermia group. This demonstrates that laminin degradation follows a pattern of MMP2 and MMP9 expression, whereby an increase in MMP expression correlates to a increase in laminin degradation. To further study this correlation, the activity of MMP9 was determined with zymographic analysis.

Zymography

Zymography was performed to determine the activity of MMP2 in both groups of normothermia and hyperthermia at four hours. An internal standard of MMP2 was used to differentiate between MMP2 activity and any other gelatinolytic activity within the experimental conditions. As shown in Figure 5 hyperthermia significantly increased MMP2 activity in the ipsilateral hemisphere as compared to the normothermic condition. There was no change in the activity of MMP2 in normothermia at 4 hours. MMP9 activity significantly increased in the hyperthermia group at four hours between the contralateral and ipsilateral hemispheres. This data suggests that hyperthermia increased MMP2 activity in the ischemia area, but not in unaffected tissue. Furthermore, hyperthermia alone does not increase MMP2 activity as observed by a lack of difference between the contralateral normothermia and hyperthermia groups. Some MMP9 activity is present in the four hour hyperthermia group, but in all other groups MMP9 activity was too low to be appreciably measured and was not included in the analysis.

Figure 4. MMP2 Activity after Hyperthermia.

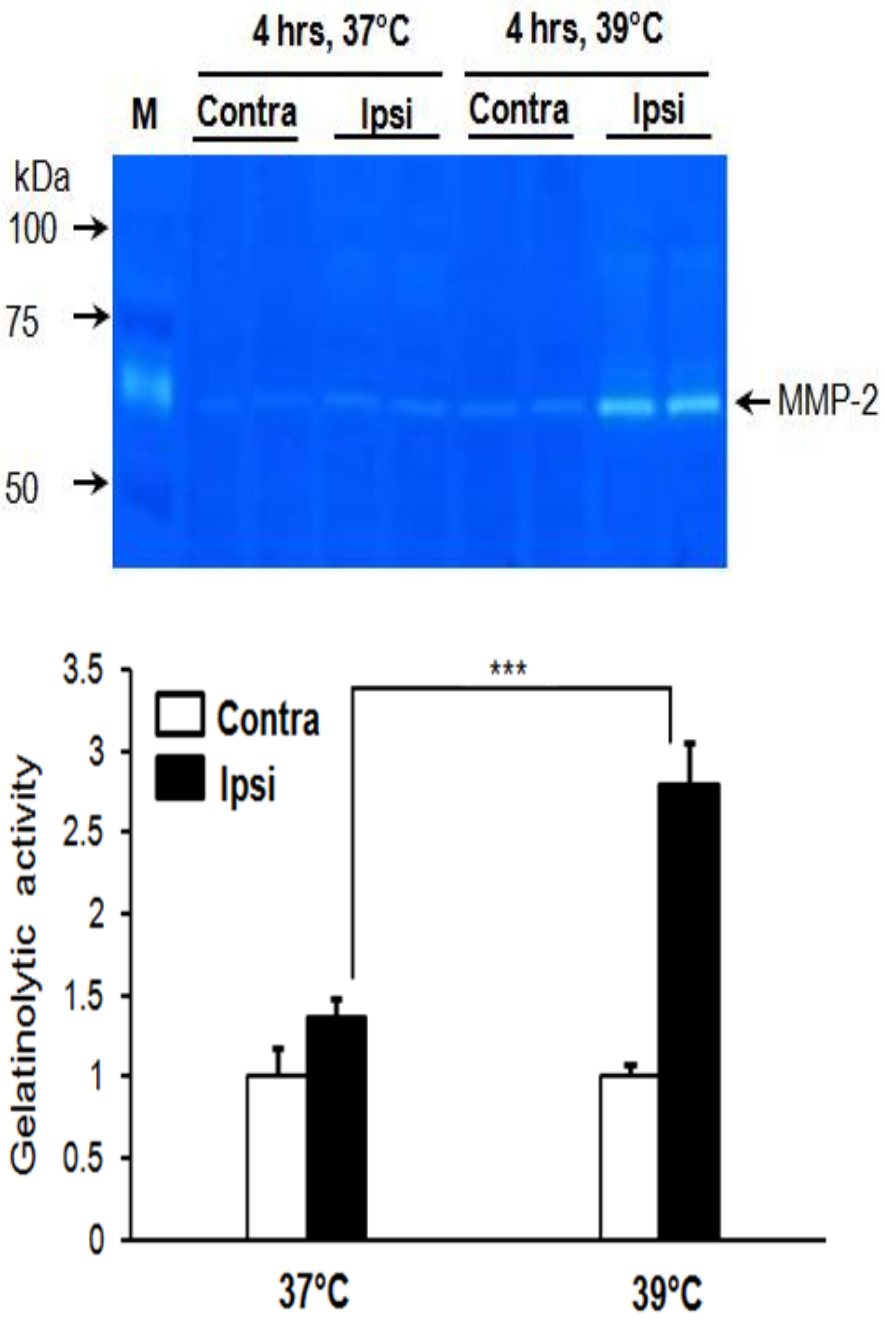

Figure 4. The top panel shows MMP2 gelatinolytic activity by zymography. MMP2 activity appears as a light band on a blue background. The bottom panel, a quantification of this data, shows a significant increase in MMP2 activity in hyperthermia as compared to normothermia. (M = marker lane with MMP2 standard)

Figure 5. Laminin Degradation after Hyperthermia.

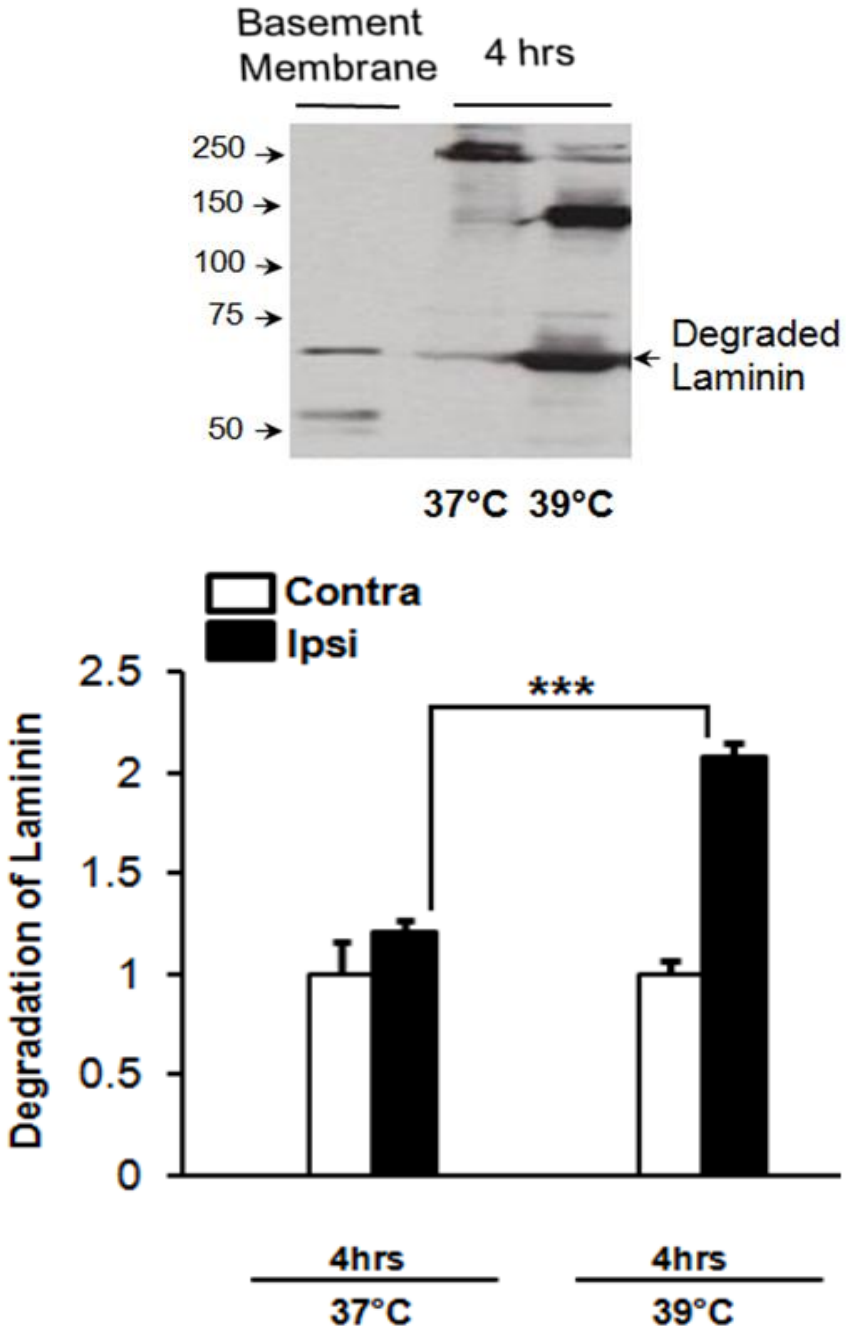

Figure 5. Degradation of laminin after hyperthermia. In the top panel laminin was detected relative to a sample of basement membrane containing only dissociated laminin. At normothermia (37^{o}C) laminin does not show any degradation into fragments where as this is evident in hyperthermia (39^{o}C). The bottom panel shows a significant difference between degraded laminin present at normothermia and hyperthermia.

Minocycline treatment of Hyperthermia induced MMP expression

The previous study has demonstrated that there is an increase in the expression of MMP2 and MMP9 at earlier time points that normothermia. We have also determined that this increase in early expression is concominant with an increase in activity of MMP2 and the subsequent degradation of laminin. In this current study minocycline was used to inhibit MMP2 and MMP9 activity and reduce degradation of laminin.

Minocycline administration and timecourse

Minocycline was administered one hour after the induction of stroke via intravenous injection at a 3mg/kg dose. Animals receiving no treatment were given an intravenous injection of saline. Animals in the hyperthermia group were kept at 39^{o}C core temperature for two hours. All animals were allowed to recover for four hours after stroke induction before tissue was extracted and analysed.

Brains were divided into two hemispheres. Since the right MCA was occluded the stroke area should occur on the right hemisphere. The left hemisphere was designated as the contralateral hemisphere and suffered no stroke while the right hemisphere was designated the ipsilateral hemisphere and suffered a stroke. This section analyses the changes in MMP2, MMP9 and TIMP1 expression in the contraleral hemisphere. Laminin degradation was also investigated as was MMP2 and MMP9 proteolytic activity in all conditions.

MMP2 expression

MMP2 expression in the contralateral hemisphere increased between the normothermia and hyperthermia groups receiving saline as seen in Figure 6. With minocycline treatment there was no significant difference in expression between the normothermia and hyperthermia groups in the contralateral hemisphere. When comparing normothermia between saline treatment and minocycline treatment, there was no difference in the expression of MMP2. This was the same in hyperthermia, where there was no change in expression comparing hyperthermia with saline

treatment and minocycline treatment. This data demonstrated that hyperthermia increases MMP expression alone and minocycline alone does not induce MMP2 expression. When comparing within the same temperature groups but with different treatments, there is no change in MMP2 expression, which suggests that minocycline does not change MMP2 expression.

When comparing the ipslateral hemispheres for each group a different pattern arises. MMP2 expression is significantly increased with hyperthermia as compared to normothermia when treated with saline. With minocycline treatment there is no change in MMP2 expression profiles between the normothermia and hyperthermia groups. There is also no significant difference between normothermia with saline treatment and minocycline treatment. However, there is a decrease in MMP2 expression with minocycline treatment as compared to saline treatment. This suggests that hyperthermia induces MMP2 expression with saline treatment, but with minocycline treatment there is no change in MMP2 expression and so minocycline decreases MMP2 expression. This is further supported when comparing the hyperthermia group between the two treatments, where minocycline treatment significantly reduces MMP2 expression as compared to the saline treatment.

Finally, when comparing the contralateral and ipsilateral hemispheres of each experimental group, there is no significant change between the normothermia saline groups, but a significant increase in the hyperthermia saline groups. This supports the data from the previous study (see Figure 2) demonstrating the same expression pattern. When treatment with minocycline was given to the normothermia group there was no change, which is due to the fact that there is no change in MMP2 expression in the normothermia group at this early time point of four hours. In hyperthermia, where there is an early change in MMP2 expression there is a trend towards an elevation in MMP2 expression but this is not significant. Taken together, ischemia can increase MMP2 expression and that hyperthermia but not minocycline will increase MMP2 expression further. Minocycline reduced MMP2 expression when it becomes overexpressed in the hyperthermic condition with concurrent ischemia.

Figure 6. MMP2 Protein Expression after Hyperthermia and Minocycline Treatment

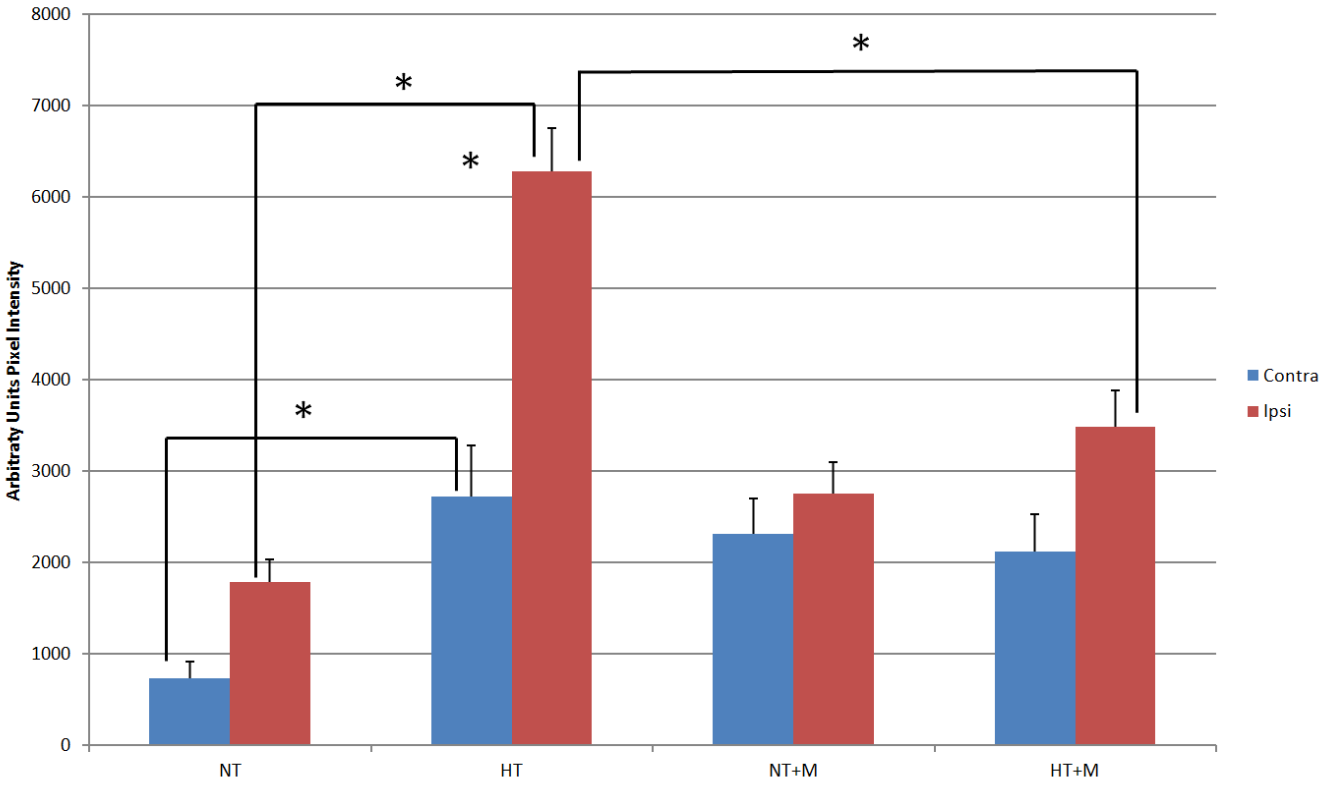

Figure 6. The expression of MMP2 significantly increases with hyperthermia as compared to normothermia. Minocycline treatment in normothermia did not change MMP2 expression. However, minocycline treatment after hyperthermia significantly decreased MMP2 protein expression compared to untreated hyperthermia. (contra = contralateral hemisphere, ipsi = ipsilateral hemisphere, NT = normothermia, HT = hyperthermia, NT+M = normothermia with minocycline treatment, HT+M = hyperthermia with minocycline treatment) n = 8.

MMP9 expression

Expression of MMP9 in the contralateral hemisphere of the normothermia and hyperthermia groups was not significantly changed when treated with saline, shown in Figure 7. Treatment with minocycline did not change expression either. In the normothermia group when comparing saline to minocycline treatment there was also no change. There was a trend to a decrease in expression in the minocycline treated hyperthermia group compared with the minocycline treated normothermia group. Overall, there was no changes in MMP9 expression in the contralateral hemisphere with either hyperthermia or minocycline alone, or both together.

However in the ipsilateral hemispheres for these same experimental groups there was a significant increase in MMP9 expression in hyperthermia compared with normothermia when given saline treatment. With the treatment of minocycline there was no difference between the normothermia and hyperthermia groups, though there is a trend towards an increase in the hyperthermia group. In normothermia, with either saline or minocycline treatment, there is no change in MMP9 expression, as is the same with hyperthermia in both saline and minocycline treatment. This data shows that MMP9, like MMP2, is upregulated with hyperthermia, but there is no decrease in the expression with the application of minocycline.

When comparing the contralateral hemisphere to the ipsilateral one there are no significant differences in any of the experimental groups. However, there is a trend in each condition for the ipsilateral hemisphere to have a higher level of MMP9 expression, but this is not significant. Overall, these results demonstrate that minocycline does not have as great an effect on MMP9 expression as MMP2, but this was as hypothesized as minocycline is an MMP inhibitor, not a transcriptional regulator.

Figure 7. MMP9 Protein Expression after Hyperthermia and Minocycline Treatment

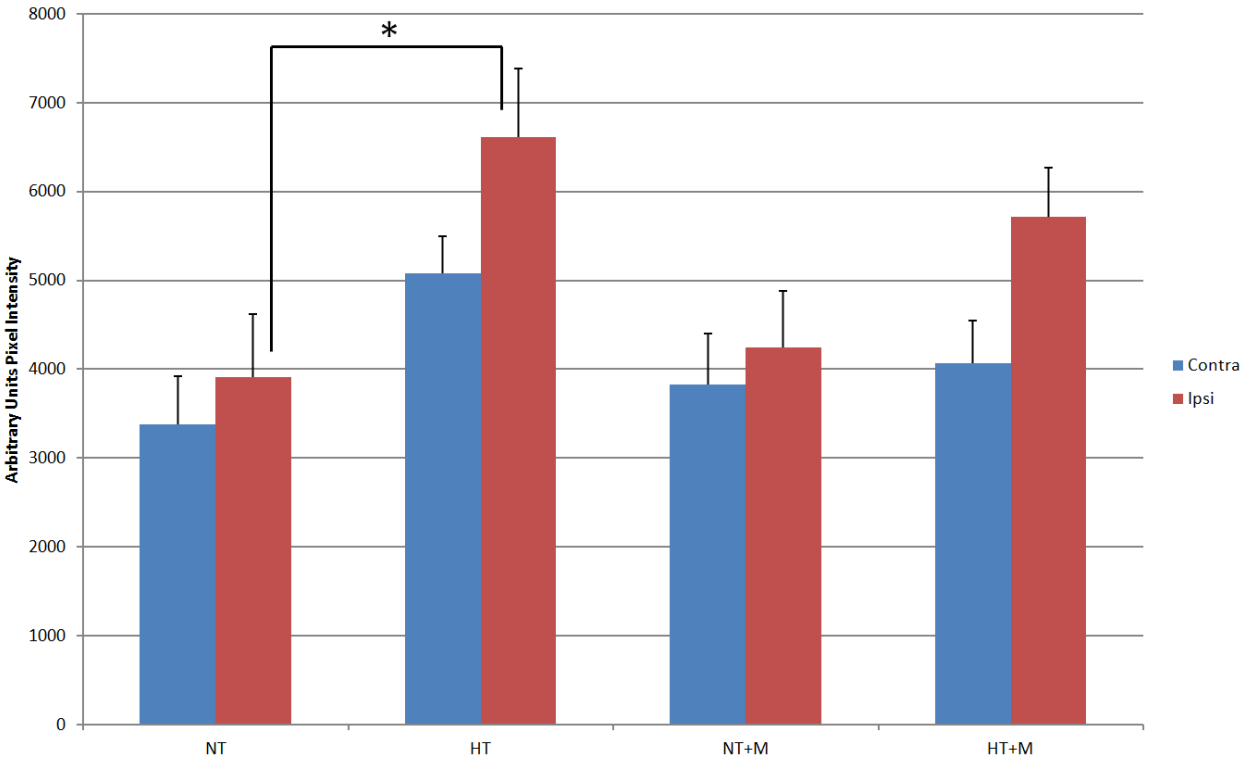

Figure 7. MMP9 expression significantly increases with hyperthermia as compared with normothermia. Minocycline treatment in either normothermia or hyperthermia does not significantly change protein expression of MMP9 when compared to untreated normothermia. (contra = contralateral hemisphere, ipsi = ipsilateral hemisphere, NT = normothermia, HT = hyperthermia, NT+M = normothermia with minocycline treatment, HT+M = hyperthermia with minocycline treatment) n = 8.

TIMP1 expression

The contralateral hemisphere in each experimental condition was unchanged wither the induction of hyperthermia, the treatment of minocycline or a combination of both factors as seen in Figure 8. Furthermore, in the ipsilateral hemisphere the same pattern was present, with no changes between any groups regardless of treatment group. This demonstrates that neither hyperthermia nor minocycline alone affect TIMP1 expression. However there was a significant difference in each experimental group when comparing the contralateral hemisphere to the ipsilateral hemisphere. In each case, the ipsilateral hemisphere had a significantly higher level of TIMP1 expression. This data shows that ischemia, as shown in the ipsilateral hemisphere, can induce TIMP1 expression at four hours after a stroke and that this expression is independent of either hyperthermia or minocycline treatment. It can be speculated that TIMP1 may have a different recruitment and expression pathway than MMP2 and MMP9, one that is separate from the pathways activated by hyperthermia in stroke.

Figure 8. TIMP1 Expression after Hyperthermia and Minocycline Treatment.

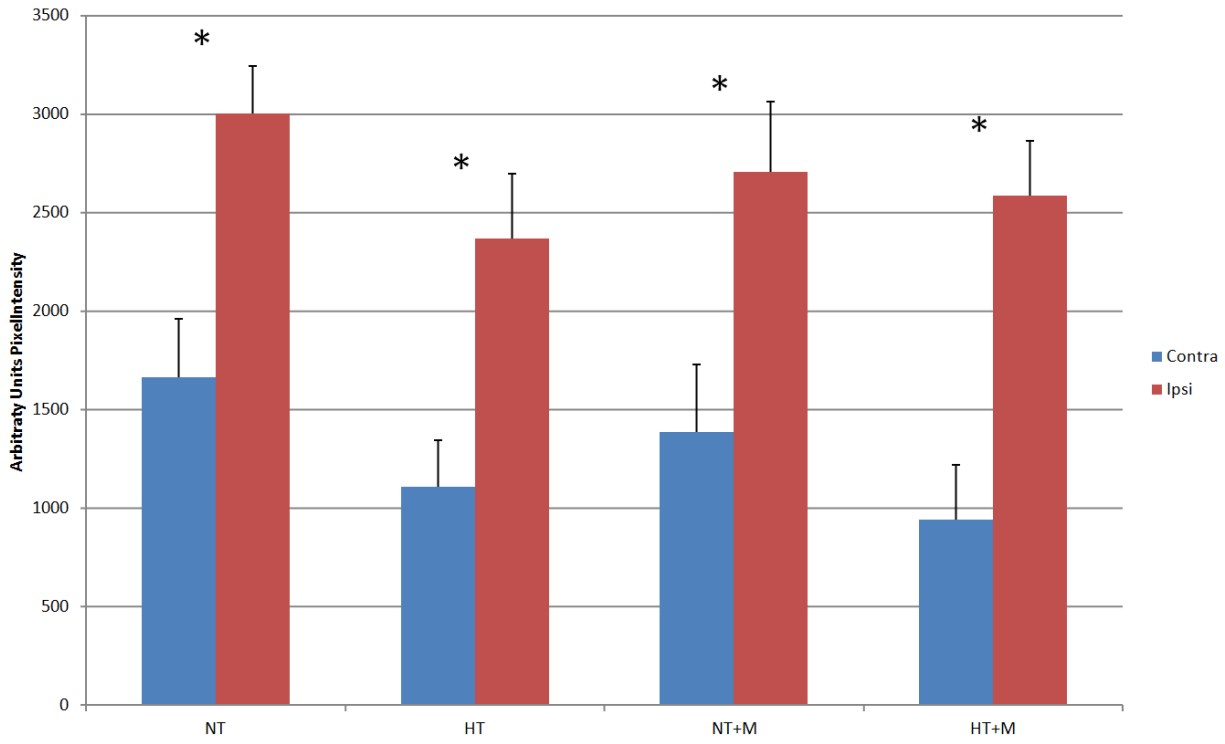

Figure 8. TIMP1 expression is significantly increased after ischemia with normothermia and hyperthermia. Treatment with minocycline has no effect on TIMP1 expression in either normothermic or hyperthermic conditions after ischemia. (contra = contralateral hemisphere, ipsi = ipsilateral hemisphere, NT = normothermia, HT = hyperthermia, NT+M = normothermia with minocycline treatment, HT+M = hyperthermia with minocycline treatment) n = 8.

Laminin degradation

Measurement of laminin degradation in the contralateral hemisphere in all experimental conditions showed no change between groups. Figure 9 shows the increase in laminin degradation in hyperthermia as compared to normothermia in the ipsilateral hemisphere with saline treatment. Minocycline treatment reduced this increase in laminin degradation to levels where the was no significant difference between the normothermia and hyperthermia groups. This data demonstrates that minocycline can reduce the degradation of laminin in hyperthermia conditions after ischemia. There is no change in the normothermia groups treated either with saline or minocycline, which corroborates previous data showing that four hours after a stroke is too early to detect changes to laminin degradation. However there is a significant reduction in laminin degradation between the hyperthermia groups receiving saline and minocycline treatment. Minocycline significantly reduces laminin degradation in hyperthermia in the ipsilateral hemisphere four hours after a stroke. This data demonstrated that minocycline is an effective treatment to hyperthermia induced damage to the basal lamina early after a stroke.

When comparing the contralateral and ipsilateral hemispheres for degradation of laminin there is no difference in the normothermia groups, whether treated with saline or minocycline. This is most probably due to the fact that there is no laminin degradation present at four hours after a stroke, so treatment of minocycline through inhibition of MMPs will not have any effect. Hyperthermia with saline treatment increases degradation of laminin, but this can be significantly reduced with the treatment of minocycline. In the hyperthermia groups, where there is an early and detectable change in both MMP2 and MMP9 expression and a correlated increase in laminin degradation, minocycline treatment significantly reduces laminin degradation. Thus, minocycline is effective in reducing laminin degradation and may possess functional benefits for reducing prefusion deficits in the early phase after a stroke with simultaneously hyperthermia.

Figure 9. Laminin degradation after Hyperthermia and Minocycline Treatment

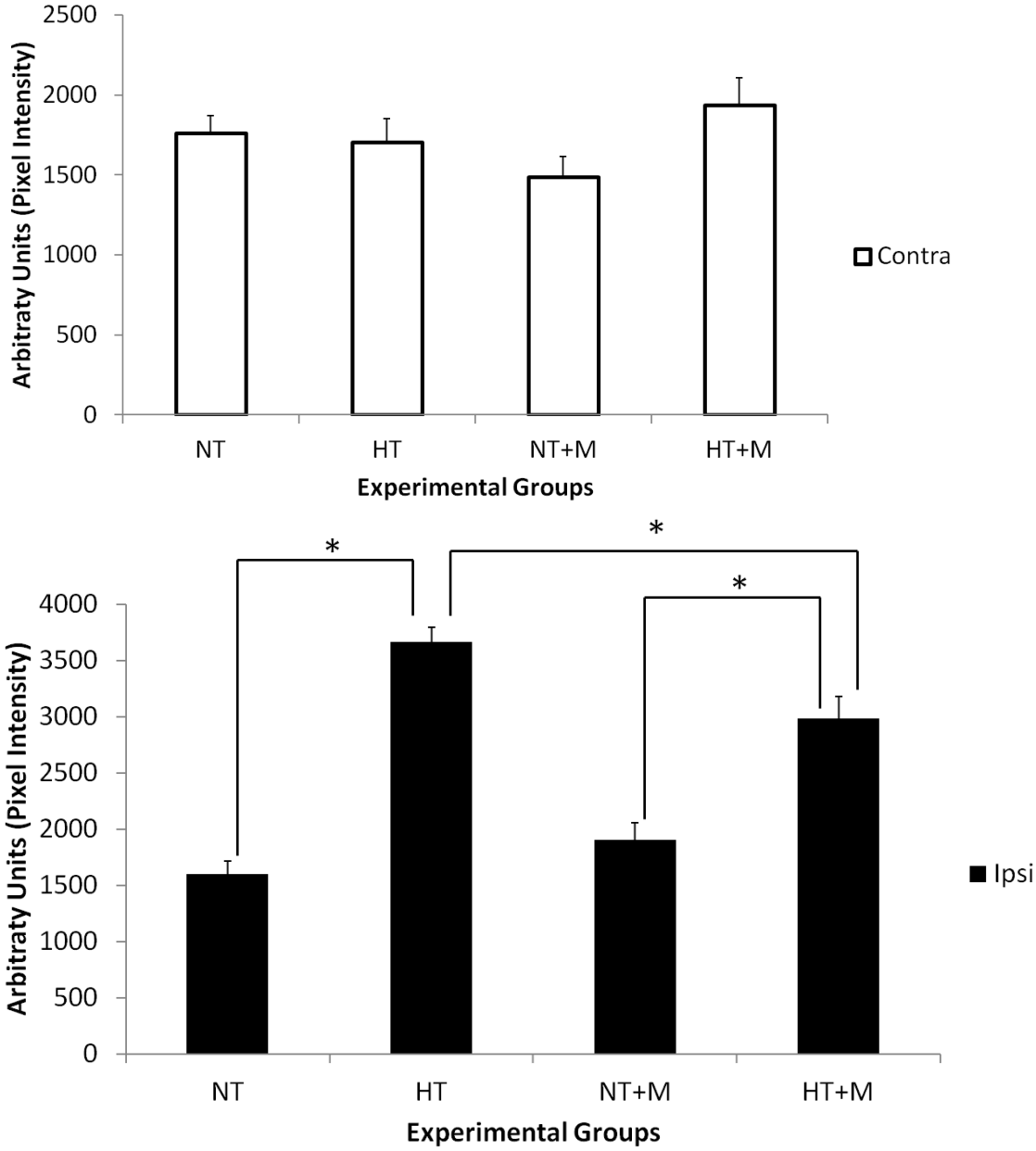

Figure 9. While there are no differences in laminin degradation in the contralateral hemisphere (top panel), the ipsilateral hemisphere shows a significant increase in laminin degradation (bottom panel). Hyperthermia alone significantly increases laminin degradation after ischemia, with or without minocycline treatment. Minocycline treatment does significantly decrease laminin degradation in hyperthermia as compared to hyperthermia untreated hyperthermia(contra = contralateral hemisphere, ipsi = ipsilateral hemisphere, NT = normothermia, HT = hyperthermia, NT+M = normothermia with minocycline treatment, HT+M = hyperthermia with minocycline treatment) n = 8.

Zymography analysis of MMP activity

Lastly, MMP2 and MMP9 activity was measured using gelatin zymography to determine if minocycline did indeed have a functional effect by inhibition of MMPs. MMP9 was used as a loading standard to differentiate between MMP9 and MMP2 activity as shown in Figure 10 and 11. The upper clear band on the blue background, corresponds to the same molecular weight as the MMP9 internal standard. The lower band present on the same gelatin zymography gel was determined to be MMP2 due to its molecular weight and gelatinolytic activity.

MMP2 activity

In the contralateral hemisphere there is a significant increase in MMP2 activity between the saline treated normothermia and hyperthermia groups shown in Figure 10. There is also a significant difference between normothermia and hyperthermia in the minocycline treated groups. This data shows that hyperthermia alone increases MMP2 activity and that minocycline does not decrease it. When comparing normothermia in the saline and minocycline treatment groups, there is no difference in the activity of each sample, which is the same with the hyperthermia groups for the same two treatment conditions. This data suggests that minocycline does not have any effect in inhibition of MMP2. Though there are trends towards a decrease in MMP2 activity there is still a significant difference between the minocycline treated group and the saline treated group in both normothermia and hyperthermia though this in located within the non infarcted contralateral hemisphere.

Within the ipsilateral hemisphere, there is a large increase in MMP2 activity in hyperthermia compared to normothermia in saline treated groups. This large increase is also seen in the corresponding minocycline treated groups, demonstrating that ischemia with hyperthermia greatly increases MMP2 activity and that minocycline treatment cannot reduce this back to a basal level. Within the normothermia group, there is again a trend towards a slight decrease in MMP2 activity when treated with minocycline as compared to saline treatment, but this is not significant. In hyperthermia minocycline significantly reduced MMP2 activity compared to the saline treated hyperthermia group.

This demonstrates that minocycline can be effective in decreasing MMP2 activity via inhibition.

When comparing the contralateral hemisphere to the ipsilateral hemisphere there is no change in either the normothermia saline treated or minocycline treated groups, as there is no increase in MMP2 expression or activity in these groups. In the hyperthermia group treated with saline, there is a significant increase in MMP2 activity, which corroborates the previous study. With hyperthermia and minocycline treatment there is a significant difference between the groups, suggesting that minocycline alone cannot reduce MMP2 levels back to those seen in the contralateral hemisphere. There is a significant decrease in the activity from hyperthermia though, so minocycline is effective in inhibition of MMP2 in hyperthermia.

Figure 10. MMP2 Activity after Hyperthermia and Minocycline Treatment

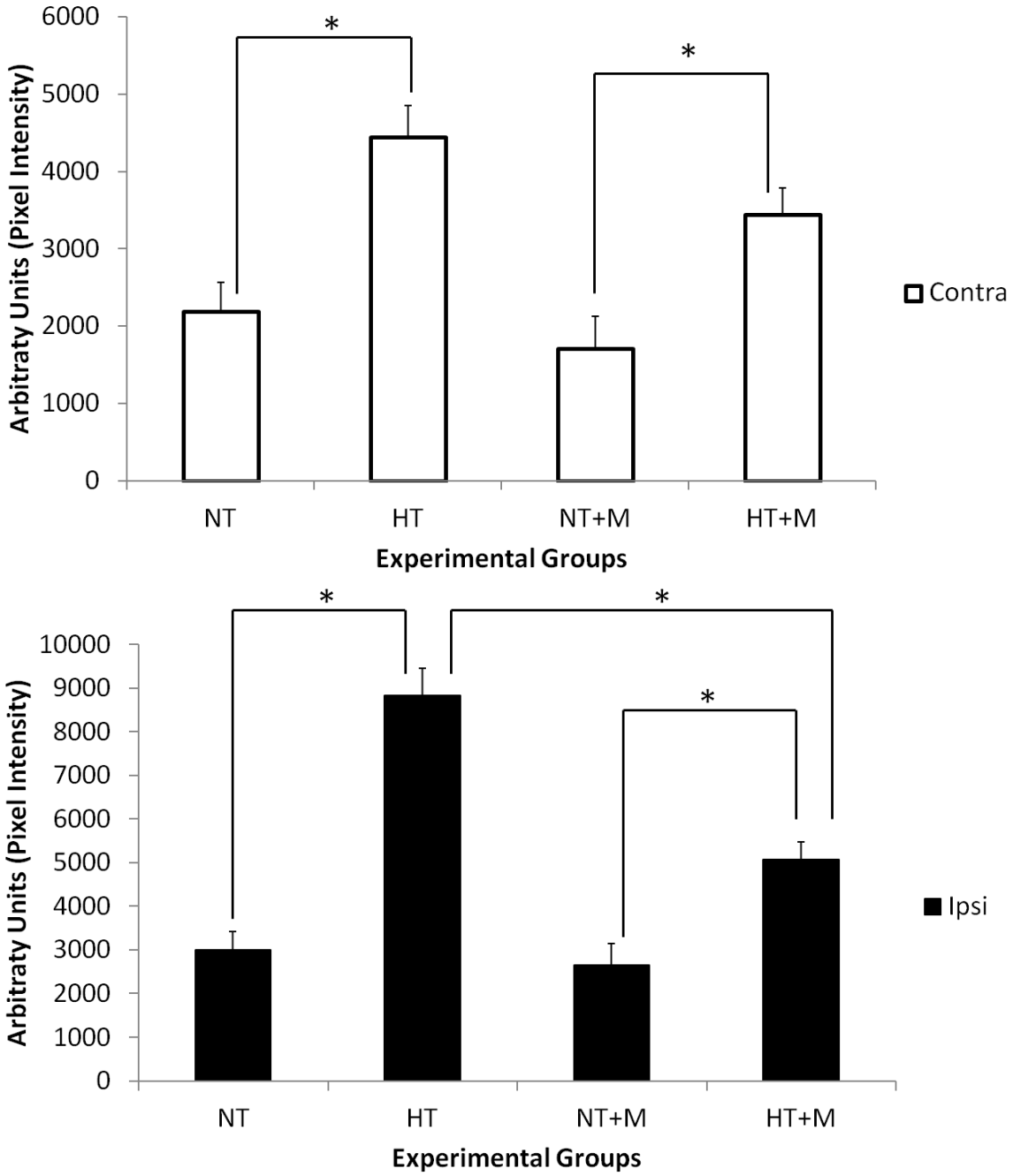

Figure 10. MMP2 activity was increased in after hyperthermia after ischemia regardless of minocycline treatment (top panel). With minocycline treatment MMP2 activity was significantly decreased in hyperthermia after ischemia. However, this was still significantly increased as compared to minocycline treatment of normothermia after ischemia. (contra = contralateral hemisphere, ipsi = ipsilateral hemisphere, NT = normothermia, HT = hyperthermia, NT+M = normothermia with minocycline treatment, HT+M = hyperthermia with minocycline treatment) n = 8.

MMP9 activity

As seen in Figure 11 the activity pattern of MMP9 is also altered by both hyperthermia and minocycline treatment. In the contralateral hemisphere there is a significant increase in saline treated normothermia compared to hyperthermia, but no difference in minocycline treated normothermia compared to hyperthermia. This data demonstrates that hyperthermia increases MMP9 activity, and that minocycline can reduce this activity to basal levels. In the normothermia group, there was no change in saline treatment compared to minocycline treatment. As before, this is most likely due to the lack of MMP9 expression and activity so early after a stroke. In the hyperthermia group, there is a strong trend towards a decrease in MMP9 activity compared to the hyperthermia group alone in the contralateral hemisphere. This data shows that minocycline can inhibit MMP9 activity induced hyperthermia alone.

In the ipsilateral hemisphere there is a significant increase in hyperthermia compared to normothermia alone with saline treatment, which was seen in MMP2 activity as well. There is no difference in the minocycline treated normothermia and hyperthermia groups. This is the same pattern seen in the contralateral hemisphere and also with MMP2, further supporting the notion that MMPs have increased activity after hyperthermia and minocycline can reduce this activity via inhibition of MMPs. In the normothermia group, comparing saline to minocycline treatment showed no change, again due to the lack of early increase in MMP9 activity. In the hyperthermia group, where there is an early upregulation of both expression and activity of MMP9 there is a significant difference in MMP9 activity between saline treated and minocycline treated hyperthermia.

Finally, when comparing between the contralateral and ipsilateral hemispheres in each experimental group there is no change in the normothermia group, either treated with either saline or minocycline. Once again, this is most likely due to the absence of early expression of MMP9 in normothermia. In hyperthermia with saline treatment, there is a significant increase in MMP9 activity, and this can be reduced with minocycline treatment. Treating with minocycline in hyperthermia shows a strong trend towards the reduction of MMP9 activity, though this is only significantly reduced from the hyperthermia saline treatment group and is still significantly elevated from the

contralateral hyperthermia minocycline treatment group. Thus, minocycline is beneficial in reducing MMP9 activity in hyperthermia alone, and can reduce MMP9 activity after ischemia, but not to basal activity levels.

Figure 11. MMP9 Activity after Hyperthermia and Minocycline Treatment

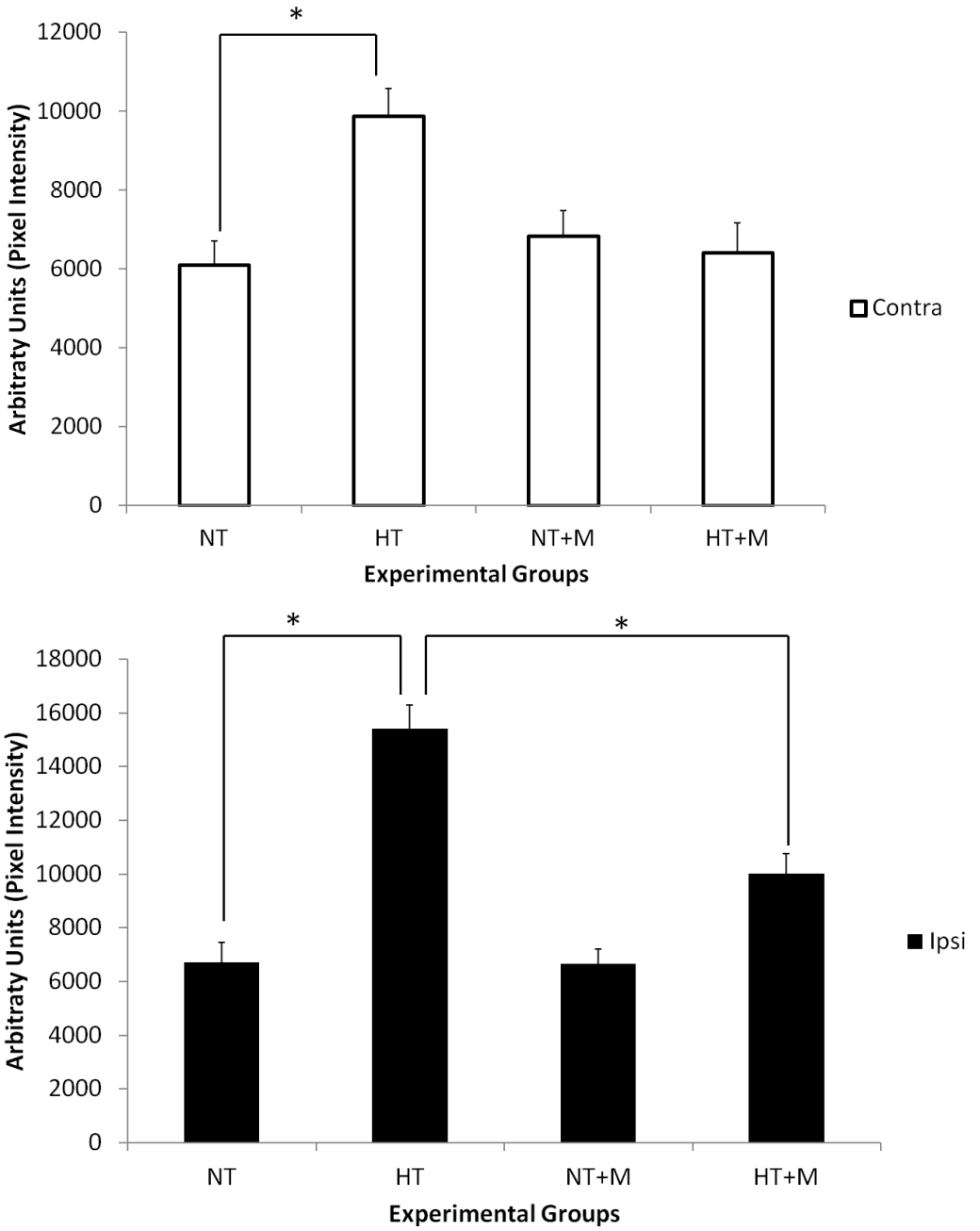

Figure 11. MMP9 increased in activity after hyperthermia and minocycline decreased this activity to control levels without ischemia (top panel). With ischemia, hyperthermia increased MMP9 activity significantly in control groups. With minocycline treatment, MMP9 activity was significantly decreased to below temperature controls levels. This MMP9 activity was no significantly different from normothermia untreated ischemia. (contra = contralateral hemisphere, ipsi = ipsilateral hemisphere, NT = normothermia, HT = hyperthermia, NT+M = normothermia with minocycline treatment, HT+M = hyperthermia with minocycline treatment) n = 8.

Chapter 4: Discussion

Discussion

Summary of Results

In this thesis experiments were proposed to identify the role of MMP2 and MMP9 after an ischemic stroke in conditions of normothermia and hyperthermia. Further experiments were performed to investigate a potential therapeutic application of minocycline via inhibition of MMP2 and MMP9 and the subsequent degradation of laminin. In the first set of experiments the data showed that hyperthermia induced MMP2 and MMP9 protein expression significantly greater than those in matched normothermia conditions at an early time point of four hours. This early increase in MMP2 expression was as great as that seen in normothermia 24 hours after the induction of a stroke. MMP9 levels also increased, but this was not to the same extent as seen in MMP2. Activity of MMP9 increased in hyperthermia, while MMP2 levels were not detected. Finally, the data also demonstrated that laminin degradation occurs with the increase in expression and activity of MMPs and that the most severe degradation of laminin occurred in the hyperthermia group at the early time point, greater than even that of the normothermia group at 24 hours post stroke.

The second group of experiments sought to investigate the role of minocycline inhibition of MMP2 and MMP9 and the subsequent degradation of laminin. The major findings of this study were that minocycline can indeed decrease laminin degradation via a reduction in MMP2 and MMP9 activity. Another interesting observation from this study was that minocycline can also decrease the protein expression of MMP2, but not MMP9 in hyperthermia. Additionally, minocycline can be beneficial in hyperthermia alone, reducing MMP2 and MMP9 activity in hyperthermia alone, without any associated ischemia. Minocycline can be given quickly after stroke, via an intravenous injection to facilitate the efficacy of this drug in inhibiting MMP activity, which has not been shown in previous studies. Finally, minocycline does not itself alter MMP2 or MMP9 expression or activity in normothermia where expression and activity is natively low.

Upregulation of MMPs in Ischemia with Hyperthermia

MMPs, specifically MMP2 and MMP9, have been implicated in the progression of stroke (Lee, Park, Kim, Lo, & Lee, 2009; Liguz-Lecznar et al., 2012; Copin et al., 2011; Yang & Rosenberg, 2011a). Many glial cells, as well as neutrophils, vascular smooth muscle cells, endothelial cells and even neurons, can synthesize and secrete MMPs and are involved in the pathology of stroke (del Zoppo et al., 2012; Hwang et al., 2011; Mairuae, Connor, & Cheepsunthorn, 2011; Mattila et al., 2011). Accumulating evidence points to the upregulation of MMP expression after ischemia at the cellular level, which is both a beneficial and detrimental to the patient. Due to the temporal nature of expression of MMPs, where early expression causes degradation of the BBB, and later expression allows for the establishment of new blood vessels through angiogenesis (Rosenberg, Estrada, & Dencoff, 1998b; Rosenberg & Yang, 2007).

Laminin, an integral component of the basal lamina, is important as a functional unit to provide structural support and integrity to the BBB. Degradation of laminin, and other basal laminal components, are a likely candidate for the disruption of the BBB and their degradation may be responsible for the progression of neuronal death (Hamann et al., 1999; Wang & Shuaib, 2007a). Some studies have even reported that MMPs are involved in the breakdown of the BBB (Asahi et al., 2001; Hallmann et al., 2005) however the specific mechanism of activation and degradation is still unclear at this time. In experimental models of focal cerebral ischemia, the basal lamina of the blood vessels and the ECM is affected (del Zoppo, von, & Hamann, 1998). Several reports suggest that in acute ischemic stroke, the loss of microvascular basal laminal antigens coincides with rapid increase in the expression of endogenous tPA which in turn activates the latent MMP2 activity and disrupts the integrity of basement membrane, indicating a potential role of tPA in the regulation and activation of MMPs. Both tPA and MMPs are known to breakdown components of the BBB (del Zoppo et al., 1998) and this provides another potential mechanism for MMP activation after a stroke. Experimentally, there is limited evidence to suggest that recombinant tPA treatment of stroke increases vascular permeability and BBB leakage through a receptor-mediated event and not through generalized degradation of the basement membrane (Yepes et al., 2003). Clinically this it is well known that the major complication of recombinant tPA is a hemorrhage, which

has a vascular origin and so this might be another possible link between activation of MMPs and stroke.

Laminin, a large multidomain protein, is susceptible to proteolytic degradation at the interdomain linker or hinge region (15,16). It is possible that laminin is more susceptible to degradation at this site, breaking down into discrete fragments or domains rather than at multiple random sites. Considering the complex nature of multidomain protein structure and the large size of laminin, partial degradation rather than complete degradation might be increased by proteases like MMPs. This is supported by Figure 4, which shows discrete bands of laminin degradation, rather than a smear within the lane. It is possible the laminin is only degraded partially by MMPs and it is this fragment that is the most commonly cleaved target site for degradation. It is also possible that the antibodies used in this study were specific to an eiptope of laminin that was fragmented in this manner, and antibodies to different epitopes can yield different degradation patterns. This becomes even more relevant to the stroke outcome because it has been suggested that apoptotic cell death in ischemic stroke is mediated through partial and not complete degradation of laminin (Chen & Strickland, 1997). This was further corroborated with laminin knockout mice (Lamgamma1-KO), where it was shown that in an ischemic stroke partial degradation of endogeneous laminin is indeed more harmful than the complete absence of laminin in ischemia. Partially degraded laminin signals through the kainite receptor (KA-R) which is responsible for increased neuronal death (Chen, Yu, Yu, Pawlak, & Strickland, 2008). Previous studies investigating basement membrane degradation used immunohistochemical analysis, where detection of partial degradation products of the basement membrane is difficult (Fukuda et al., 2004b; Hamann et al., 1995; Rosell et al., 2008). This study used immunoblotting, which provides more precision in detecting fragments not possible with other methods.

These results demonstrate that in hyperthermia with stroke increases the degradation laminin (Figure 4) after the increase in expression and activity of MMP2 and MMP9 activity. From earlier studies it is known that following a focal ischemic stroke, there is an increase in the permeability of the BBB and a subsequent increase in haemorrhage and edema occur. This also causes an increase of MMPs but their direct relationship to laminin degradation has not been established. Though there are some reports that in

human colorectal cancer and liver metastases MMP2 and MMP9 can degrade type IV collagen to yield N-terminal and C-terminal fragments (Zeng & Guillem, 1995; Collier et al., 1988) no previous work has investigated laminin as a target of MMP activity after ischemia. To our knowledge there is no biochemical data showing the degradation of the basement membrane by MMP upredulation. These findings demonstrate that increased MMP2 and MMP9 expression and concomitant degradation of laminin in part explains the biochemical mechanism of increased BBB permeability and edema commonly observed in ischemic stroke (Mun-Bryce & Rosenberg, 1998; Rosenberg, Mun-Bryce, Wesley, & Kornfeld, 1990; Heo et al., 1999). Our results can also explain the mechanism of severe complication observed in hyperthermia and stroke when compared to normothermia and stroke, namely that hyperthermia increases MMP2 and MMP9 expression significantly and increases BBB disruption. This data also provides an insight into the mortality in the 24 hour hyperthermia group. Specifically, that at 24 hours after a stroke with hyperthermia at the onset, damage to the BBB is to great and very little of the tissue within the penumbra, or indeed the brain can be saved. The stroke lesion size then grows too much larger sizes and ultimately becomes fatal.

Our results support the hypothesis that in hyperthermic stroke early upregulation of proteases (mainly MMP2 and MMP9) accelerates degradation of basement membrane components in ischemic hemisphere leading to perfusion deficits causing a more severe ischemic insult. Currently, we cannot exclude the possibility of other proteases (other MMPs, cathepsins or calpains) being involved in these critical and complicated neuropathological events (Jian & Rosenberg, 2005). Therefore, more data is required to conclusively prove that in acute ischemic stroke, degradation of laminin is exclusively regulated by increased MMP2 and MMP9 in acute ischemic strokes.

Effects of Minocycline on MMPs during Hyperthermia and Ischemia

As stated earlier, MMPs in the brain also undergo a temporal expression profile was well as a localized pattern. There is an early increase in MMP2 which degrades Claudin 5 (Yang & Rosenberg, 2011b). This leads to an early increase in the permeability of the BBB but is reversible depending on the severity of the injury. After 24 hours tight

junction proteins are no longer present in the BBB (Jiao, Wang, Liu, Wang, & Xue, 2011). It has reported that there is a second opening of the BBB between 24 and 48 hours (Willis, Meske, & Davis, 2010) where MMP9 expression is increased and vessels are more prominently damaged. MMP inhibitors have proven to be useful in decreasing infarct size and preventing further degradation of the BBB (Guan, Kozak, & Fagan, 2011b; Wang, Xue, Jiao, Liu, & Wang, 2011; Copin et al., 2011) in animal models.

In ischemic stroke there are two phases of MMP release, the first is early MMP2 expression. MMP2 is tethered to the cell surface by MT1-MMP (MMP 14) where they are activated together (Murphy et al., 1999). TIMP2 is required in this activation process and so MMP2 is restricted to the cell surface. Conversely, MMP9 is released into the interstitial fluid and there is able to degrade proteins in the extracellular matrix. MMP9 and not MMP2 is the critical protease in BBB breakdown. Current work has shown that a MMP9 and not MMP2 gene knockout is associated with a decrease in BBB breakdown and infarct reduction (Svedin, Hagberg, Savman, Zhu, & Mallard, 2007; Koistinaho et al., 2005b). Finally, human studies have found that MMP2 expression is increased in a short time period (2-5 days) after a stroke, but increased MMP9 expression can be four months after the stroke (Clark, Krekoski, Bou, Chapman, & Edwards, 1997b). There is also a positive correlation between MMP9 concentrations in the plasma and NIHSS scores of stroke patients (Montaner et al., 2001).

This evidence supports our data that MMPs are upregulated in expression and activity early after a stroke and cause damage to the BBB. Knockouts of MMPs are also beneficial in reducing the damage caused by an ischemic stroke and can decrease the ischemic lesion size. In clinical practice this is neither feasible nor practical, and so the focus shifted to the inhibition of MMPs. Inhibition of MMP can be neuroprotective was recent research shows. It has been found that overexpressing TIMP1 carries some neuroprotective effects in reducing infarct size and decreasing the degradation of the BBB (Tejima et al., 2009) which was looked at over a period of days, but another study found that an increase in TIMP1 expression was correlated to an increase in infarct volume and a decrease in neurological function after middle cerebral artery occlusion (Maddahi, Chen, & Edvinsson, 2009). These two studies seem at odds with each other, but taking the temporal and localized nature of MMP expression and activity and the

tight association of TIMPs with MMPs into consideration, it is possible that these two studies demonstrate the complexity of the system. Little work has been done on TIMP expression after an ischemic insult, but the work presented in this thesis shows an increase in TIMP1 (Figure 8).

In addition to using an endogenous inhibitor of MMP activity in TIMPs, it is also possible to inhibit MMPs through the use of synthetic inhibitors. Minocycline was chosen as a model therapeutic agent owing to both its specific inhibitory role of MMPs and its neurprotective effects. Additionally minocycline posses a pleiotrophic anti-inflammatory property which further aids in preventing neuronal damage after an ischemic stroke. Previous approval by the FDA to be given orally at large doses (100 mg/kg) was also considered, when selecting between potential therapeutic agents. In this current study, minocycline was given at a dose of 3 mg/kg via an intravenous route, following previous work of the administration and absorbance of minocycline in various tissues throughout the body (Xu et al., 2004b). It is important to note that minocycline is rapidly absorbed into the brain after intravenous injection, due to its lipophillic properties, and that intravenous injection is a much quicker route for therapeutic administration as compared to oral administration. This is crucial in the treatment of stroke, where “time is brain” and the speed of treatment is of the essence.

The results of this study conclusively demonstrate that minocycline inhibits MMP2 and MMP9 activity in hyperthermia (Figure 10 and 11 respectively) and also reduce the degradation of the basal lamina component laminin (Figure 9). In addition minocycline also decreased the expression of both MMP2 and MMP9 while leaving TIMP1 unchanged in expression. There are many possible explanations of how minocycline provides these benefits in ischemia with hyperthermia, chief among them is the inhibition of MMP2 and MMP9. Minocycline has been found to be effective in focal ischemia in rats but through an intraperitoneal route which reduced ischemic lesion size (Yrjanheikki et al., 1999b). Unfortunately for the clinical situation minocycline was administered before the onset of stroke, but later work has shown that minocycline is also neuroprotective when either one or four hours after the onset of ischemia (Wang et al., 2002). In that study, infarct volume was significantly reduced in normothermia

when minocycline was administered by intraperitoneal injection (Wang et al., 2002). As a comparison, a similar period of delayed hypothermia was not protective.

In the same vein, a single dose of minocycline, given intraperitoneally, in a neonatal hypoxic-ischaemic model reduced ischemia injury one week after the onset of hypoxia-ischemia (Arvin et al., 2002b). In this study, minocycline was given immediately after the onset of hypoxia-ischemia. Lastly, minocycline given intravenously has been reported to provide a more predictable plasma concentration and half-life than that achieved by intraperitoneal injection (Fagan et al., 2004) and was neuroprotective after middle-cerebral-artery occlusion in rats. This came with the additional benefit that lower doses were required then administered intravenously (3mg/kg) than those given intraperitoneally (45mg/kg, daily) (Xu et al., 2004a).Taken together, these studies utilizing minocycline in animal models of ischaemic provide strong support for the application of minocycline as a potential therapeutic tool for acute ischemic stroke patients.

In addition to being a neuroprotective agent and an MMP inhibitor, minocycline also possess anti-inflammatory effects which can further reduce damage caused by a stroke. Minocycline exerts its anti-inflammatory actions by inhibition of microglia, immune cell activation and the release of cytokines, chemokines, NO and MMPs (Stirling, Koochesfahani, Steeves, & Tetzlaff, 2005b). Pro-inflammatory cytokines, such as TNFα, IL-1β and IL-6 are synthesized and secreted by microglia cells, astrocytes, and blood borne cells such as neutrophils within the brain parenchyma after a stroke (Stirling et al., 2005b). These cytokines further increase the apoptotic signalling of cells with the ischemic area and increase subsequent immune responses. Minocycline reduces both the proliferation and activation of resting microglia cells as evidenced by a CD11b (a microglial marker), MAC-2 (a macrophage marker) or isolectin-B4 (an endothelial marker) staining in hypoxic-ischemic brain injury models in rats (Dommergues, Plaisant, Verney, & Gressens, 2003). Minocycline has also been reported to be effective in mice models of hypoxia-ischemia showing a decrease in neuronal injury after injection of 6-hydroxydopamine (He, Appel, & Le, 2001b). Results from various studies indicate that minocycline exhibits a broad spectrum of anti-inflammatory actions by inhibiting enzymes such as COX-2, iNOS, NAPDH-oxidase, and P38 MAPK.

Future Studies

The results of this research lay the groundwork for the understanding of MMP2 and MMP9 degradation of laminin after a stroke and also provide a potential therapeutic application of combating this increase in MMP activity. But there is still much work to do before this avenue of research can be used in clinical practice. Some of these potential studies are outlined below as future directions for this project, as steps to further clarify and investigate the benefits of MMP inhibition and expression in relation to perfusion deficits and stroke treatment.

Detection of Functional Improvement

Among all the work that lies ahead in this field, perhaps no study is more important that one designed to test the functional benefits of minocycline in reducing perfusion deficits and maintaining the BBB. One possible method to investigate this to use the same experimental paradigm established here, namely the induction of stroke through the use of the MCAO model with subsequent hyperthermia and minocycline treatment coupled with the injection of Evan's Blue Dye to determine the permeability of the BBB. If minocycline truly does prevent laminin degradation and maintains the BBB, then the dye should be localized to the cerebrovasculature and not the brain parenchyma. It should also be evident that without minocycline treatment the dye will extravasate into the parenchyma. This simple set of experiments will provide evidence of the functional significance of minocycline treatment in maintenance of the BBB.

Another method of measuring the permeability of the BBB is to use sectioned brain tissue to stain for Immunoglobulin G. IgG is a protein found exclusively within the circulatory system and in an intact BBB should be located within the cerebrovasculature. The amount of IgG immunostaning within the brain parenchyma should increase with ischemia, and should be even greater with ischemia and hyperthermia. Minocycline treatment should degrease the staining of IgG within the parenchyma, and keep any staining localized to blood vessels. Either of these methods can demonstrate the functional improvement of minocycline treatment in ischemia.

Combinatorial Use of Minocycline and tPA

Another major investigation that must be carried out to aid in the translational aspect of this work is to use minocycline in combination of tPA. As stated previously, tPA is the only approved therapeutic for the treatment of stroke and a few patients actually receive it. One of the reasons that tPA is not immediately given to any patients showing signs of a stroke is to prevent hemorrhagic transformation, the process of an ischemic stroke becoming hemorrhagic in nature and causing the accumulation of blood in the brain. Minocycline should be tested to see if it raises the rate of hemorrhagic transformation with given in conjunction with tPA. Studies may also be undertaken to investigate if there is any benefit in given minocycline immediately to stroke patients before any tPA is given. Indeed, there is a current clinical trial underway to investigate such a therapeutic application of minocycline is beneficial in stroke patients. Finally, minocycline should also be investigated to determine if the pleiotropic anti-inflammatory provide any benefits to stroke patients. Much work has been done on animal models and cellular models showing that microglia are inhibited by minocycline application and that microglia are major contributors to the damage after an ischemic stroke.

Mechanism of Action of MMPs

Much is still left unsolved in the specific mechanism of activation of MMPs. Current research points to the activation of cytokines that activate an inflammatory response which will provide ROS which can activate MMPs. Research can be directed to investigate the specific mechanism of activation of MMPs, listing cytokines, ROS and other proteases as possible candidates. By further refining the mechanism of activation of MMPs, research can begin on other methods to target and prevent the activation of MMPs to begin with, acting higher upstream than a specific MMP inhibitor. This will also allow another layer of therapeutic approaches to target MMPs. There is also an overlap in the mechanisms of activation for TIMPs and MMPs, so perhaps these studies will uncover novel mechanisms for MMPs activation independent of TIMP activation. Perhaps methods for targeting MMPs and TIMPs independently will arise and can be used to tip the balance towards reducing MMP activation and increasing TIMP

activation, at least in the early stages after a stroke. It is important to remember that MMP expression after a stroke is biphasic and while early expression is related to breakdown of the BBB, later expression is actually beneficial and promotes revascularization and angiogenesis to the penumbra area. Thus complete removal or inhibition of MMPs for extended time periods are not in the best interest in the treatment of acute ischemic stroke.

ECM Components

Finally, the other components of the basal lamina, including collagen type IV, elastin, and fibronectin are potential targets of degradation. As previously stated, MMPs degrade all these molecules which are integral parts of the BBB. It is possible that laminin is the dominant degradation target of MMP activity, but other basal laminal components can also be investigated to determine if they also play a role in any further breakdown of the BBB. It is possible that collagen IV, or other ECM components, that are crosslinked together or to laminin can provide structural integrity to the BBB. In the event of laminin breakdown it might be possible for these other components to maintain the structural integrity of the basal lamina and reduce perfusion deficits after an acute ischemic stroke.

Conclusions

The experiments presented in this thesis provide evidence that MMP2 and MMP9 expression is increased after a stroke, and that hyperthermia further upregulated both the expression and activity of these protein. This increase in expression is as great as normothermia for 24 hours in the case of MMP2, and slightly less than that in the case of MMP9. Furthermore, laminin degradation is the greatest in ischemia with subsequent hyperthermia, greater than even ischemia for a prolonged period of 24 hours at normothermia.

Minocycline treatment for ischemia with hyperthermia showed a decrease in MMP2 expression, but not MMP9 expression. More importantly, minocycline treatment decreased MMP2 and MMP9 activity significantly in ischemia and ischemia with hyperthermia. It is interesting to note that minocycline can reduce MMP2 and MMP9

activity in hyperthermia alone without ischemia. Minocycline was effective in significantly reducing laminin degradation in ischemia after hyperthermia via inhibition of MMP activity, but whether this has functional benefits remains to be seen. Additionally, minocycline did not alter MMP activity in normothermia, either in the contralateral or ipsilateral hemisphere. This is most likely due to the low level of expression and activity of MMPs early after a stroke.

Lastly, the work presented in this thesis provides a strong case for the consideration of minocycline as a therapeutic agent in the treatment in acute ischemic stroke. Minocycline, via the inhibition of the MMP2 and MMP9, can prevent degradation of laminin, a large contributor to the structural integrity of the BBB. By preserving the structure of the BBB to maintain perfusion to the penumbra area after a stroke more tissue is supplied with blood and can maintain both metabolic systems and ionic gradients, allowing cells to survive for longer, reducing the lesion size and minimizing the overall impact of a stroke.

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