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The role of NADPH oxidase in blood -brain barrier dysfunction following stroke in aged rats

Kimberly Kelly
West Virginia University

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**The Role of NADPH Oxidase in Blood-Brain Barrier Dysfunction
Following Stroke in Aged Rats**

Kimberly Kelly

**Dissertation submitted to the
School of Pharmacy
at West Virginia University**

for the degree of

**Doctor of Philosophy
in
Pharmaceutical and Pharmacological Sciences**

**Jason D. Huber, Ph.D., Chair
Charles Rosen, M.D., Ph.D.
James O'Callaghan, Ph.D.
Patrick Callery, Ph.D.
James O'Donnell, Ph.D.**

Department of Basic Pharmaceutical Sciences

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NADPH oxidase (NOX), Apocynin**

ABSTRACT

The Role of NADPH Oxidase in Blood-Brain Barrier Dysfunction Following Stroke in Aged Rats

Kimberly Kelly

Stroke is the leading cause of long-term adult disability and the third leading cause of death in the United States. More than 72% of stroke victims are over the age of 65, yet most studies utilize young animals. Aged rats suffer exacerbated stroke volume and inflammation, as well as worsened functional outcome when compared to young adult rats. While the blood brain barrier (BBB) remains intact with age, it becomes more susceptible to external factors. BBB permeability in the penumbra appears acutely, 20 min after middle cerebral artery occlusion (MCAO) in aged rats. Importantly, BBB permeability is earlier and more severe in aged animals and occurs before neuronal damage. Interestingly, our permeability studies using a small vascular space marker indicated small, subtle, permeability changes in the contralateral hemisphere. Permeability changes in the contralateral hemisphere were not found in young rats and were not found in aged rats when using a large vascular space marker (Evan's blue).

The free radical theory of aging contends that age-related diseases result from accumulation of oxidative stress damage as a result of increased generation of reactive oxygen species (ROS) and decreased antioxidant enzyme activity. Of particular interest are the NADPH oxidases (NOX1, NOX2, and NOX4), which have been implicated as a damaging source of ROS during the aging process. However, the role of NADPH oxidase isoforms during the progression of cerebral ischemia in aged rats is poorly understood. Apocynin, a NOX2 inhibitor, reduces infarct volume, neuron death, activated microglia, and edema formation in young rodents following brain injury. However, when administered to aged rats, apocynin significantly increased mortality and edema formation. These results indicate an age-dependent difference in the mechanism of action of apocynin.

Our studies demonstrate that aged rats have significantly worse strokes both in gross size as well as structure as compared to young rats. Aged rats consistently have worsened and prolonged functional impairment, exacerbated BBB permeability, increased neuronal damage, and intensified inflammatory cell infiltration post MCAO and reperfusion when compared to young adult rats. Importantly, BBB permeability and increased oxidative stress markers were found in the contralateral hemisphere of aged rats after MCAO and reperfusion. These findings provide evidence for a compensatory role of the contralateral hemisphere after stroke that can only be studied in aged animals. Utilizing aged animals to study the mechanisms of brain injury after MCAO and reperfusion is vital to finding clinically applicable therapies.

This work is dedicated to my loving and supportive family.

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I had an acknowledgments page written and ready, but as I looked over it one final time it seemed generic and cliché. So you get to read this instead.

Arriving at WVU I felt scared yet invincible. I was ready to take on the health crises of the day and age. Then classes began. I soon realized that I was sitting in the room with other best and brightest students from other universities and that collectively, we were as useful as the Saran Wrap and Duct Tape protecting us from Mesothelioma. We didn't know what we didn't know.

I was an idealist when I began my work in the lab. I read any paper I could find associated with Diabetes and Stroke and believed every published word. Dr. Huber, you forced me to critically question the implied authority of a publication. The ability to think critically of not only my own research but also the work of others is essential to a true scientist and I owe this lesson to you.

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List of Abbreviations

Abbreviation	Term
3-NO ₂ -Tyr	3-nitrotyrosine
ACE	angiotensin converting enzyme
ANGII	angiotensin II
AT1	angiotensin II receptor subtype 1
AT2	angiotensin II receptor subtype 2
BBB	blood brain barrier
Bcl-2	an anti-apoptotic gene
CAT	catalase
CBF	cerebral blood flow
cGMP	cyclic guanosine monophosphate
CIV	corrected infarction volume
COX-2	cyclooxygenase 2
ECA	external carotid artery
EEIIMD	plasminogen activator inhibitor type 1 derived peptide
FDA	Food and Drug Administration
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
gp91phox	NADPH oxidase isoform 2 (NOX2)
GPx	glutathione peroxidase
H&E	hematoxylin and eosin stain
H ₂ O ₂	hydrogen peroxide
HDL	high density lipoprotein
HEPES	(4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HOCl	hypochlorous acid
ICA	internal carotid artery
IFN-γ	interferon-gamma
IL-1	interleukin-1
IL-1β	interleukin 1 beta
IL-6	interleukin-6
LD-CBF	laser Doppler monitoring of cerebral blood flow
LDL	low-density lipoproteins
LPS	lipopolysaccharide
LRP	lipoprotein receptor-related protein
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
MDA	malondealdehyde
MMP-2	matrix metalloproteinase 2
MMP-9	matrix metalloproteinase 9

MMPs	matrix metalloproteinases
Mn-SOD	manganese super oxide dismutase
mNSS	modified neurological severity score
MPO	myeloperoxidase
MRP	multidrug resistance associated protein
MVI	microvessel isolation buffer
NADPH	nicotinamide adenine dinucleotide phosphate
NF- κ B	nuclear factor kappa-light-chain enhancer of activated B cells
NO	nitric oxide
NO ₂ [·]	nitrogen dioxide radical
NOX1	NADPH oxidase isoform 1
NOX2	NADPH oxidase isoform 2
NOX4	NADPH oxidase isoform 4
NoxA1	NOX activator 1
NoxO1	NOX organizer 1
ONOO ⁻	peroxynitrite
p22phox	alpha polypeptide-accessory protein to NOX-2
p47phox	accessory protein to NOX-2
p67phox	accessory protein to NOX-2
P-gp	p-glycoprotein
PI3K/AKT	phosphoinositide 3-kinase
PIC	protease inhibitor cocktail
PCR	polymerase chain reaction
PMNs	polymorphonuclear leukocytes
PVDF	polyvinylidene difluoride
rac	GTPase
RNS	reactive nitrogen species
ROS	reactive oxygen species
rt-PA	recombinant tissue plasminogen activator
RT-PCR	real time polymerase chain reaction
siRNA	small interfering ribonucleic acid
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substances
TGF- β	transforming growth factor beta
TNF α	tissue necrosis factor alpha
tPA	tissue plasminogen activator
TS-2	tissue solubilizer
TTC	2,3,5 triphenyl tetrazolium chloride
VEGF	vascular endothelial growth factor
ZO-1	zonula occludens 1
ZO-2	zonula occludens 2

Chapter One

Literature Review

Stroke is the leading cause of long-term adult disability and the third leading cause of death in the United States. Over 750,000 individuals suffered a stroke in 2005 with mortality in 143,579 cases (Lloyd-Jones et al., 2009). Figure 1.1 depicts the incidence of stroke increasing exponentially with age. In fact, approximately 72% of stroke victims are over 65 years of age (Figure 1.1). Moreover, due to the longer life span of females stroke burden is actually greater for women than men.

Age, in particular, has been identified as an independent predictor of outcome following cerebral ischemia, influencing several aspects of stroke pathophysiology including blood brain barrier (BBB) integrity. The BBB acts as a regulatory interface between the systemic circulation and the brain parenchyma. Disruption of this critical barrier during cerebral ischemia is associated with increased morbidity and mortality. Morphological changes in the BBB, which occur during aging, make it more susceptible to injury.

The only FDA-approved therapy for ischemic stroke is the i.v. administration of tissue plasminogen activator (tPA) which promotes thrombolysis of an embolic thrombus to reestablish cerebral blood flow. However, indications for tPA administration are strict including a narrow time window of opportunity, limitations due to risk of hemorrhage and implications of possible neurotoxicity. Consequently, less than 5 percent of stroke patients receive i.v. tPA thrombolysis treatment. After a decade of searching for neuroprotective agents to treat stroke, no clinically applicable treatments have been approved. These failures may be attributed to the disregard for two important variables: advanced age and blood-brain barrier integrity. Age is the largest risk factor for stroke and has been largely ignored in experimental animal models of stroke. The BBB preserves the neuronal microenvironment and thus protecting the integrity of this barrier may lead to neuron viability.

Stroke and Animal MCAO Models

As 87% of strokes are ischemic in nature, most stroke models utilize occlusion of the middle cerebral artery (MCA) which results in reduced cerebral blood flow to the striatum and cortex (Ponten et al., 1973; Garcia, 1984). Several types of MCA occlusion (MCAO) models exist that produce either permanent (Tamura et al., 1981; Bederson et al., 1986) or transient (Longa et al., 1989; Belayev et al., 1996) occlusion via chemical (Moyanova et al., 1998; Nikolova et al., 2009) or physical (Badan I et al., 2003; Liu et al., 2009; Yao et al., 2003; Yao et al., 2009; DiNapoli VA et al., 2006) approaches. Chemical occlusion consists of administration of endothelin-1 which constricts the vessel to reduce blood flow (Moyanova et al., 1998; Nikolova et al., 2009). Physical occlusion can be achieved by lifting the MCA with a tungsten hook (Badan I et al., 2003), photothrombotic occlusion (Yao et al., 2003; Yao et al., 2009) or through the use of a nylon suture to block the vessel (Liu et al., 2009).

A more appropriate model utilizes a blood clot to create an ischemic stroke, followed by tPA administration to disrupt the clot and reperfuse tissue (Kudo et al., 1982; Kaneko et al., 1985; DiNapoli VA et al., 2006). Several advantages of a fibrin clot model include: more realistic reperfusion, ability to utilize thrombolytic therapy, and ability to screen for neuroprotectants that are effective in concert with tPA. Investigations by well established research groups administer to rodents a tPA dosage of 10-20 times the therapeutic dose for humans (0.9 mg/kg). At this supratherapeutic dose tPA has been shown to increase edema (Armstead WM et al., 2006) and excitotoxicity (Parathath et al., 2006; Tsirka, 1997; Wang et al., 1998) which lead to neuron death.

Young adult male rats are most commonly used to study stroke. With the investigation of the role of estrogen levels in stroke outcome, the use of young adult female rats has been implemented with an ovariectomized group to study post-menopausal changes. However, ovariectomy models only represent approximately 13% of women (Acosta et al., 2009).

Furthermore, a disparity exists with estrogen treatment where aged animals have worsened stroke volume (Selvamani and Sohrabji, 2008; Rosen CL et al., 2005) and young adult females have improved outcome (McCullough LD and Hurn PD, 2003).

Utilization of an embolic model of MCAO and tPA reperfusion in aged reproductively senescent female rodents allows for the investigation of mechanisms underlying stroke pathophysiology and effects of thrombolysis in a clinically relevant model.

Age & Stroke

Aging affects every organ system in the body, including the vasculature. These changes have a compounding effect in relationship to deteriorated functional performance and increased prevalence of chronic diseases. In the cerebral microcirculation, age is associated with decreased elastin and collagen (Grunnet, 1969), increased tortuosity (Hassler, 1965; Akima et al., 1986), and vascular thickening (Ay et al., 2005) causing impaired cerebral autoregulation. The ability to react to and secrete neurotransmitters such as dopamine (Freeman and Gibson, 1987; Arai and Kinemuchi, 1988; Gerhardt et al., 2002), glutamate (Dawson, Jr. et al., 1989) and nerve growth factor (Gomez-Pinilla et al., 1989) become altered in the aged brain.

Advanced age is associated with dysregulation of the nitric oxide/cyclic guanosine monophosphate (NO/cGMP) pathway (Chalimoniuk and Strosznajder, 1998), which under physiological conditions promotes angiogenesis, neurogenesis, axonal outgrowth, and synaptic plasticity (Truman et al., 1996; Koyano-Nakagawa et al., 1999; Bredt and Snyder, 1994). In agreement with this, aging has been correlated with decreased expression of VEGF and impaired angiogenesis (Black et al., 1989; Rivard et al., 1999), as well as decreased neurogenesis and neuronal progenitor cells (Kempermann et al., 1998; Kuhn et al., 1996). Furthermore, aging is characterized by vulnerability to oxidative stress (Aliev et al., 2002; Li et al., 2005b; Floyd and Hensley, 2000), excitotoxic damage (Wozniak et al., 1991; Wheeler and

Ondo, 1986), apoptotic events (Hiona and Leeuwenburgh, 2004) and traumatic injury (Hoane et al., 2004) possibly causing the cortical atrophy seen with advanced age.

Age is a risk factor predicting worsened functional performance (Lindner et al., 2003) and poor functional recovery (Rosen CL et al., 2005; Markus et al., 2005; Lindner et al., 2003; Zhang et al., 2005; Zhao et al., 2005). Likewise, with every year of age, nearly a percent more penumbral tissue turns to infarction (Ay et al., 2005). Age-related alterations in the microvasculature appear to be the cause of poor neuron viability and increased mortality associated with stroke (Rosen CL et al., 2005; Lindner et al., 2003; Zhang et al., 2005).

Structure and Function of the Blood-Brain Barrier

The BBB forms discrete microenvironments within the brain to support the optimal functioning of a diverse array of neurotransmitters (Abbott, 1987). With over 20 m² of surface area, BBB endothelial cells serve as the physical interface between the systemic circulation and brain parenchyma (Butt et al., 1990). The BBB is a semi-permeable membrane with unique characteristics that confer distinct properties that differentiate the BBB from peripheral capillaries, including a well-defined basement membrane, presence of tight junctions, absence of fenestrations, and close apposition to other brain cell types, including pericytes, astrocytes, microglia, and neurons (Hawkins et al., 2007a; Huber et al., 2001). Several reviews detail the transport properties of the BBB as they pertain to both drug delivery and pathology (Ohtsuki and Terasaki, 2007; Pardridge, 2007; Hawkins et al., 2007b; Persidsky et al., 2006; Pan and Kastin, 2004).

Once considered a static, rigid wall, the BBB is now considered a dynamic, complex structure capable of rapid modulation and response to stimuli (Huber et al., 2001). The BBB is able to maintain and regulate brain homeostasis and compensate for fluctuations in the systemic circulation and increased metabolic functions within the brain as it functions like a

dynamic barrier. The active nature of the BBB also has important implications for the development and progression of central nervous system diseases, including HIV encephalitis (Toborek et al., 2003), meningitis (Winkler et al., 2001), multiple sclerosis (Plumb et al., 2002), Alzheimer's and Parkinson's disease (Zlokovic, 2002; Ariga et al., 1998), epilepsy (Saija et al., 1992), and stroke (Latour LL et al., 2004). Previous studies have shown that the BBB is responsive to external stimuli and systemic-based diseases (Brooks et al., 2005; Witt et al., 2003; Huber et al., 2002). Viewing the endothelium of the blood-brain barrier as part of a larger, integrated functional unit (neurovascular unit) has opened up exciting avenues for translational and collaborative research in which the blood-brain barrier is viewed as susceptible to pathology and a potential therapeutic target.

A particularly unique aspect of BBB structure is the presence of tight junctions (Figure 1.2), which create a rate-limiting barrier to paracellular diffusion of solutes between adjacent endothelial cells. The tight junction consists of the transmembrane proteins junctional adhesion molecule, occludin, and claudins, linked via accessory proteins including zonula occludens (ZO)-1 and -2 to the actin cytoskeleton. ZO-1 is a cytoskeletal scaffolding protein that recruits the transmembrane proteins occludin and claudin-5. Occludin and claudin-5 homotypically bind the adjacent endothelial cell to seal the paracellular pathway. Altered protein expression of occludin and claudin reflect tight junctional changes indicative of increased paracellular permeability. Claudin-5 is essential for tight junction formation and the decreased expression of occludin at the cytoskeleton indicates increased permeability. The BBB possesses an 'epithelial-like' resistance ($1500\text{--}2000\ \Omega\cdot\text{cm}^2$) to paracellular diffusion, which helps to maintain brain homeostasis (Crone and Christensen, 1981).

Tight junctions are dynamic structures, in which multiple signaling pathways and factors regulate the expression, localization, and protein-protein interactions (Bazzoni and Dejana, 2004; Harhaj and Antonetti, 2004). Studies have shown that changes in total expression and

subcellular localization of the tight junction proteins have been associated with alterations in paracellular permeability (Huber et al., 2001) and may play an important role in communicating the state of cell–cell contacts to the nucleus and participating in the regulation of growth, differentiation, and gene expression (Matter et al., 2005).

Disruption of this critical barrier during cerebral ischemia is associated with increased morbidity and mortality. The time course of BBB disruption during stroke and its role in ischemia and reperfusion injury is poorly understood. Morphological changes in the BBB, which occur with aging, make it more sensitive to insult. Activation of the barrier to ischemia is well studied; however, the role of aging in altering the dynamic properties and functionality of the BBB has direct implications for the majority of stroke victims.

The Inflammation and Free Radical Theories of Aging Combined

The free radical theory of aging (Harman D, 1994;Harman D, 1956) contends that as aging progresses oxidative stress ensues due to accumulation of reactive substances as by-products of metabolic processes and decreased activity of antioxidant enzymes. This theory has been widely substantiated by findings of decreased antioxidant enzyme activity (Mayhan WG et al., 2008;Abete P et al., 1999;Harvey BH and Nel A, 2003;Kolossova NG et al., 2006;Azhar S et al., 1995;Carney JM et al., 1991;Azhar S et al., 1995;Reaven PD et al., 1999), increased reactive oxygen species (ROS) (Hamilton CA et al., 2002;park L et al., 2007;Hamilton et al., 2001), and accumulation of products of oxidative stress (Azhar S et al., 1995;Reaven PD et al., 1999;Carney JM et al., 1991;Knight JA et al., 1987) with advanced age. Also, a species life-span directly correlates to its ability to adapt to and neutralize ROS formation (Cutler RG, 1991).

Accumulation of ROS and reactive nitrogen species (RNS) correlates with increased inflammatory processes (Chung et al., 2001;Reale et al., 2003;Rodriguez et al., 2007). Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) responds to pro-oxidants by

activating pro-inflammatory cytokine gene expression (Chung et al., 2001;Salvioli et al., 2006). Likewise, the aging process correlates with a basal level of inflammation (Muller-Werdan, 2007;Krabbe et al., 2004;Perry et al., 2007). Known as the inflammatory hypothesis of aging, increased age is associated with increased interleukin-6 (IL-6) (Miller and Cappuccio, 2007;Krabbe et al., 2004;Muller-Werdan, 2007;Chung et al., 2001), interferon-gamma (IFN- γ) (Rodriguez et al., 2007;Chung et al., 2001), tumor necrosis factor-alpha (TNF α) (Rodriguez et al., 2007;Krabbe et al., 2004;Siren et al., 1993;Chung et al., 2001), interleukin-1 (IL-1) (Chung et al., 2001), and transforming growth factor beta (TGF- β) (Chung et al., 2001). The inflammation hypothesis of aging has become a widely known theory into the relationship between the co-morbidity of aging and neurodegenerative diseases (Chung et al., 2001;Muller-Werdan, 2007;Krabbe et al., 2004).

Pro-inflammatory cytokines can also induce formation of ROS (Madamanchi et al., 2005). In particular, TNF α has been shown to modulate NADPH oxidase to increase ROS production (Basuroy et al., 2008;St et al., 2008;Anilkumar N et al., 2008;Jung Y et al., 2008;Guggilam et al., 2007;Chenevier-Gobeaux et al., 2006). Thus, a positive feedback loop of inflammation and oxidative stress ensues. While the aging body is able to keep the basal inflammation and oxidative stress under control under physiological conditions, an injury or pathogen could disrupt the balance and exacerbate damage.

The Blood-Brain Barrier and Aging

BBB disruption plays a prominent role in the progression and onset of several neurodegenerative diseases, including Alzheimer's and Parkinson's diseases, as a result of a damaged endothelial barrier (Ariga et al., 1998). Increased transport of circulating amyloid beta peptides across the BBB has been linked to increased age in nonhuman primates, potentially leading to development of Alzheimer's disease (Mackic et al., 2002). The BBB remains intact

during aging; however, it becomes more susceptible to external factors. Changes in BBB composition in aged animals include alterations in lipid and protein composition (Mooradian and Smith, 1992;Mooradian, 1994), changes in nutrient transport (Shah and Mooradian, 1997), and decreased reactivity to β -adrenergic neurotransmitters (Mooradian, 1994). Results have also demonstrated changes in tight junction proteins with decreased occludin expression and increased ZO-1 expression in 24 month old rats as compared to 3 and 12 month old rats (Mooradian et al., 2003).

The BBB expresses efflux proteins responsible for preventing toxic substances that gain entry into the brain from accumulating. Two such proteins are P-glycoprotein (P-gp) and multidrug resistance associated protein (MRP). P-gp acts as an active efflux pump by shuttling potentially toxic substances from the brain to the blood. Decreased P-gp expression has been shown in Parkinson's (Kortekaas et al., 2005;Bartels et al., 2008b) and Alzheimer's diseases (Cirrito et al., 2005). Aging has also been shown to decrease P-gp expression and function in rodents (Matsuoka et al., 1999;Rosati et al., 2003;Tsai et al., 2002) and humans (Bartels et al., 2008a).

Age affects several critical BBB parameters, and recent evidence suggests that altered tight junction protein expression in aged rats could disrupt the functional and structural integrity of the BBB. In a recent report, both ipsilateral and contralateral hemispheres of aged rats had increased BBB permeability to sucrose (342 Da) 24 h after MCAO and reperfusion (Kelly et al., 2009). Often in stroke studies utilizing young animals, the contralateral hemisphere is either ignored or considered control. However, previous clinical studies have observed changes in the contralateral hemisphere following stroke (Carey et al., 2002;Blank et al., 2003). These results highlight a role for the contralateral hemisphere as a compensatory mechanism following stroke. Utilizing aged rodents in stroke models provides a unique opportunity to gain a better understanding of changes in the contralateral hemisphere following stroke.

The Neurovascular Unit

The periendothelial accessory structures of the BBB include pericytes, astrocytes, neurons and a basal membrane. Pericytes can contribute to the regulation of endothelial cell proliferation, survival, migration, differentiation and vascular branching (Hellstrom et al., 2001). Astrocytes are glia that surround more than 99% of the BBB endothelium affecting its function and morphology (Beck et al., 1984;Arthur et al., 1987). Astrocytes also serve as a scaffold for neurons and microvessels during development. The basal lamina, lying between the capillaries and the rest of the neurovascular unit, provides support for cell attachment and migration, as well as preventing the passage of macromolecules into the brain parenchyma. Neuronal involvement in the neurovascular unit remains unclear. However, the ability of the brain to increase the blood supply to neuronally active areas suggests at least an indirect link between the neurons and the BBB.

Glial cells, specifically astrocytes, are involved in BBB development and activation during stress (Abbott, 1987). Transformation of astrocytes to the reactive phenotype is characterized by increased expression of GFAP (glial fibrillary acidic protein) (Stoll et al., 1998) which has been shown to be a biomarker of neuronal damage (O'Callaghan and Sriram, 2005). Following cerebral ischemia there is increased expression of GFAP (Petito et al., 1990;Kindy et al., 1992;Petito and Halaby, 1993). Constitutive expression of GFAP is increased in 24 month old rodents, as well as 60-79 year old humans, in the cortex and hippocampus (Nichols et al., 1993). Sex steroids (estrogen, testosterone, and dihydrotestosterone) have been implicated in maintaining GFAP levels, the ramifications of this are a loss of plasticity correlated with aging (Schroeder E et al., 2003;Sapolsky, 1992;Day et al., 1993). In aged animals, the astroglial and microglial activation, along with glial scarring is abnormally accelerated in pathological conditions (Popa-Wagner A et al., 2007;Badan I et al., 2003).

Microglia exist in two forms: ramified and reactive. Ramified microglia are present in the brain throughout life, while reactive microglia are found during nervous system damage (Giulian and Baker, 1986; Lin et al., 1998). Following ischemic onset, microglia become activated within minutes (Gehrmann et al., 1995; Gehrmann et al., 1992; Banati et al., 1993) with peak activation at 2 weeks (Lin et al., 1998). Microglia can release cytokines, chemokines and other neurotoxic agents which can cause inflammation and exacerbate injury. However, microglia are also responsible for scavenging cellular debris and releasing growth factors (Sriram and O'Callaghan, 2005). In aged animals microglia become activated faster in response to stroke than young adult animals (Popa-Wagner A et al., 2007; Badan I et al., 2003).

Reactive Oxygen Species & Reactive Nitrogen Species

Schematic representation of reactive oxygen and nitrogen species generation and breakdown appears in Figure 1.3. Superoxide (O_2^-) is formed by NADPH oxidases. Superoxide is normally converted by superoxide dismutase (SOD) into H_2O_2 . When pathological conditions cause increased superoxide concentration (either by increased production or decreased metabolism) it reacts with nitric oxide (NO^-) to form peroxynitrite ($ONOO^-$).

Hydrogen peroxide is thought to be mainly produced by the breakdown of superoxide by SOD. However, NOX4 containing NADPH oxidase, xanthine oxidase, and glucose oxidase also produce hydrogen peroxide (Martyn KD et al., 2006; Ruiz-Gines JA et al., 2000; Cai H, 2005). Catalase and glutathione peroxidase reduce hydrogen peroxide into water and molecular oxygen. Under pathological conditions, H_2O_2 production can overwhelm these enzymes. Hydrogen peroxide can spontaneously form hydroxyl radical or can react with myeloperoxidase (MPO) to produce hypochlorous acid (HOCl) or nitrogen dioxide radical (NO_2^-) which can cause nitration of tyrosines (3- NO_2 -Tyr) (Miller AA et al., 2006).

The effects of ROS on cerebral microvasculature

The direct effects of superoxide on the vasculature have not been clearly defined, but many speculate that the effects are concentration dependent (Miller AA et al., 2006). When superoxide is produced in excess, it reacts with the vasodilator nitric oxide, decreasing its bioavailability and therefore possibly causing vasoconstriction. Peroxynitrite formation inactivates Mn-SOD (Guo W et al., 2003), and leads to peroxidation of lipids, protein oxidation and DNA damage (Chan PH, 2001).

Hydrogen peroxide has been shown to cause cerebral vasodilation (Iida Y and Katusic ZS, 2000; Paravicini et al., 2004; Wei EP et al., 1996; Yang ZW et al., 1998b; You J et al., 2005). Dilation can also be caused by amelioration of contractions by angiotensin II (Miller AA et al., 2005). Hypochlorous acid (HOCl) can oxidize low-density lipoproteins (LDLs) which then get taken up by macrophages, build up in atherosclerotic plaques, and react with proteins to inhibit the synthesis of nitric oxide (Zhang C et al., 2003). The 3-nitrotyrosine (3-NO₂-Tyr) produced by the reaction of nitrogen dioxide radicals with tyrosine residues can lead to impaired protein catalysis and dysregulated tyrosine kinase pathways, as phosphorylation of tyrosine is prevented by nitrogen dioxide (Bigelow DJ, 2008).

Hydrogen peroxide has been shown to dose-dependently increase permeability (Kahles T et al., 2007). Hydroxyl radical, being highly reactive, can damage nuclei and cell membranes which can directly impact the integrity of the BBB (Bonomini F et al., 2008). Peroxynitrite exposure has been shown to decrease claudin-5 content (Haorah et al., 2005). ROS have also been shown to redistribute occludin (Schreibelt et al., 2007; Lee et al., 2004) and ZO-1 (Lee et al., 2004). The changes in these tight junction proteins cause dysregulation of the BBB allowing paracellular diffusion and therefore disruption of the homeostasis of the brain parenchyma. With most studies of BBB functional integrity looking at Evan's blue extravasation, it is not

surprising that few studies show changes in permeability. The passage of Evan's blue bound to albumin into the brain parenchyma marks the entry of a 65,000 dalton molecule past the BBB. As the barrier is physiologically able to prevent passage of molecules more than 200 times smaller than albumin, the extravasation of Evan's blue into the brain marks irreparable damage to the integrity of the BBB. The use of small vascular space markers, i.e. sucrose (342 daltons), allows investigation of small, possibly reparable, damage to the BBB, or alternately controlled areas in the integrity.

NADPH Oxidases

Several isoforms of NADPH oxidases are expressed in vascular cells. While the NOX2 isoform is widely considered the predominant isoform, it is the least expressed isoform in the brain vasculature. In the brain vasculature, NADPH oxidase isoform expression varies with NOX1 being the most abundant, followed by NOX4 and lastly NOX2 (Ago et al., 2005). The NOX1 isoform of NADPH oxidase consists of an enzymatic subunit which complexes with p22phox. Cytosolic subunits NOX organizer 1 (NoxO1) and NOX activator 1 (NoxA1) are required for activation of NOX1 (Takeya R et al., 2003). Conversely, the NOX4 isoform does not require activation by recruitment of cytosolic subunits and is thus constitutively active (Martyn KD et al., 2006). The NOX2 isoform consists of a membrane bound enzymatic subunit (gp91phox) complexed with p22phox. Phosphorylation of p47phox recruits the complex of p47phox, p67phox and rac to the membrane, activating NOX2 (Bedard K and Krause K, 2007). Both NOX1 and NOX2 isoforms require activation to produce superoxide. However, the NOX4 isoform constitutively produces hydrogen peroxide (H_2O_2) (Martyn KD et al., 2006).

NOX2 is widely considered the isoform of NADPH oxidase to cause oxidative damage after cerebral ischemia (Kahles T et al., 2007; Tang et al., 2007; Wang Q et al., 2006). However, in several studies NOX2 has been shown to be unchanged not only between the genders (Miller

AA et al., 2007) despite a well known difference in stroke risk between women and men, but also in saphenous veins following heart failure (Dworakowski R et al., 2008). In basilar arteries NOX1 mRNA expression is 2.4 fold higher in males than in females (Miller AA et al., 2007) which correlates to the higher stroke risk in men when compared to pre-menopausal women. The role of NOX1 in stroke pathology has yet to be studied. In vitro studies have shown upregulated NOX4 after serum deprivation (Lassegue et al., 2001). In vivo, permanent occlusion of the MCA resulted in increased NOX4 mRNA at 24 h (Vallet P et al., 2005;Badan I et al., 2003) whereas transient occlusion resulted in increased NOX4 at 6 h but not 24 h (McCann SK et al., 2008). NOX4 was also found to be upregulated in the contralateral hemisphere 24 h post-MCAO (Badan I et al., 2003). Finally, NOX4 was increased in the saphenous veins following heart failure compared to healthy controls (Dworakowski R et al., 2008). While NOX1 and NOX4 have been largely ignored in stroke research, evidence suggests they play an important role in the progression of ischemic damage.

Modulation of NADPH oxidases

Inhibition of NOX2 with apocynin has recently gained momentum as a therapeutic option for treatment of acute ischemic stroke (Tang et al., 2007;Wang Q et al., 2006;Kahles T et al., 2007) as studies demonstrate that administration of apocynin (5-50 mg/kg; i.p.) in young rodents prior to MCAO, attenuated superoxide production, infarct volume (Tang et al., 2007), neuronal death, activation of microglia (Wang Q et al., 2006) and edema formation (Kahles T et al., 2007).

In non-phagocytic cell culture, apocynin is not an inhibitor of NADPH oxidase (Heumuller S et al., 2008). However, apocynin has been extensively used as a selective inhibitor of NADPH oxidase in studies utilizing both in vitro and in vivo methodologies. Apocynin is activated by oxidation by peroxidases into products that have higher efficiencies with faster initiation of NADPH oxidase inhibition (Johnson et al., 2002). The oxidation of apocynin forms a

radical (Kanegae et al., 2007). Two of these radicals can form a apocynin dimer which can also be oxidized by myeloperoxidase and bind to a radical to become a trimer (Ximenes et al., 2007). The mechanism of NADPH oxidase inhibition by apocynin is thought to be the interaction of apocynin with a thiol group on the membrane bound subunit of NADPH oxidase which then prevents the recruitment of the cytosolic subunits and therefore prevents superoxide production (Stefanska and Pawliczak, 2008). Apocynin, as well as the dimer and trimer, were found to be incapable of binding GSH (thiol group) (Ximenes et al., 2007). The radical and dimer radical, however, did react with GSH (Ximenes et al., 2007). Thus the radical forms of apocynin are probably the inhibitors of NADPH oxidase.

In pathologic inflammatory conditions, MPO is elevated (Tsimikas, 2008). In particular, aged animals exhibit exacerbated inflammatory cell infiltration (DiNapoli VA et al., 2008) and worsened stroke volume, which produces increased MPO expression (Romanos et al., 2007). The positive correlation between MPO expression and apocynin activation suggests enhanced NOX2 inhibition with age. Data gathered from young animals suggest that increased NOX2 inhibition results in a favorable outcome. In direct contrast, our data show that aged rats exhibit exacerbated edema formation and increased mortality (Kelly et al., 2009). We conclude that inhibition of NOX2 exacerbates stroke injury in the aged rat and would be a poor choice for clinical treatment of stroke.

Plumbagin is a specific inhibitor of NOX4 (Ding et al., 2005). It has been shown to have anti-carcinogenic (Ahmad et al., 2008; Kuo et al., 2006; Thasni et al., 2008), anti-atherosclerotic (Sharma I et al., 1991) and anti-thromboembolic effects (Shen Z et al., 2003). In a study utilizing cholesterol fed rabbits, subsequent oral dosing of plumbagin reduced LDL cholesterol, increased HDL cholesterol, and decreased plaque formation on the aortic wall (Sharma I et al., 1991). In breast cancer cells, decreased estrogen receptor signaling increases NF- κ B expression (Ahmad et al., 2008). NF- κ B promotes cell survival by inducing Bcl-2 (an anti-

apoptotic gene) (Wang et al., 2007) and upregulating the PI3K/AKT pathway (promotes autophagic cell death). Plumbagin has been shown to inhibit the activation of NF- κ B caused by TNF, IL-1 β , okadaic acid, LPS, H₂O₂, phorbol myristate acetate, and cigarette smoke (Sandur et al., 2006). Also, TNF α induced COX-2, MMP-9 and VEGF were attenuated with plumbagin treatment (Sandur et al., 2006). To date, plumbagin has not been studied in ischemia.

Angiotensin II (ANGII) can be actively metabolized in the cerebrovasculature (Zhou et al., 2006). Recent evidence suggests that ANGII provides the link between synaptic activity and blood flow, also known as functional hyperemia (Kazama K et al., 2004). ANGII has been shown to increase the expression of gp91phox (Imanishi et al., 2005), and the effects of ANGII can be reversed by ROS scavengers (Kazama K et al., 2004;Didion and Faraci, 2003), NADPH oxidase inhibitors (apocynin and gp91ds-tat) (Kazama K et al., 2004;Rodriguez-Pallares et al., 2008;Didion and Faraci, 2003;Pendergrass et al., 2009) and the use of gp91phox-null mice (Kazama K et al., 2004). Also, administration of AT1 (angiotensin II receptor subtype 1) antagonists prevents the increased ROS associated with increased ANGII (Rodriguez-Pallares et al., 2008;Imanishi et al., 2005). Animal models of cerebral ischemia have shown that administration of AT1 receptor antagonists reduce the expression of NADPH oxidase, decrease lipid peroxidation, improve functional outcome and reduce stroke volume (Kusaka I et al., 2004;Faure et al., 2008). However, the use of ACE (angiotensin converting enzyme) inhibitors or AT2 (ANGII receptor subtype 2) receptor antagonists have been shown to worsen ischemic damage (Faure et al., 2008;Dalmay et al., 2001;Li et al., 2005a), suggesting the effects of ANGII acting at non-AT1 receptors is neuroprotective. In patients receiving AT1 receptor antagonists for treatment of hypertension, a meta-analysis found a 13% reduction in cardiovascular morbidity and mortality which included cardiovascular death, stroke, and myocardial infarction (Thomas et al., 2006).

Matrix Metalloproteinases and Tissue Plasminogen Activator

Tissue plasminogen activator (tPA) is the only FDA approved treatment of ischemic stroke. The object of tPA treatment is to break up the clot and reperfuse the tissue affected by the occlusion. While tPA does effectively lyse blood clots, its effectiveness is questioned due to the increased risk of hemorrhage and deleterious effects on the neurovascular unit associated with its use. However, at a dose of 5 mg/kg i.v., administered 2 h post MCAO, tPA decreases BBB permeability in both young adult and aged rats 24 h after embolic MCAO (DiNapoli VA et al., 2008). Furthermore, a recent study utilizing EEIIMD (a hexapeptide that binds non-thrombolytic sites on tPA) provides evidence that while some cortical damage could be due to non-thrombolytic actions of tPA; largely, striatal and total stroke volume are not exacerbated by tPA (Tan et al., 2009).

During physiological conditions tPA is produced endogenously in the CNS during learning of complex motor tasks (Harris-White and Frautschy, 2005). Extracellular matrix remodeling, vascular permeability and excitotoxicity are some of the deleterious effects of tPA that most likely occur during pathological increase in tPA production (Harris-White and Frautschy, 2005; Yepes et al., 2003). tPA is a ligand for low density lipoprotein receptor-related protein (LRP). Recently, the relationship between tPA and LRP has been implicated in the permeability of the BBB after an ischemic injury (An et al., 2008). Both tPA (Yepes et al., 2003) and LRP (An et al., 2008) are upregulated after ischemic insult and inhibition of LRP (An et al., 2008) decreases edema formation. Specifically, tPA binds to LRP to phosphorylate Akt which activates NF- κ B and increases the expression of MMP-9 (An et al., 2008; Wang et al., 2003b; Herz and Strickland, 2001).

Matrix metalloproteinases (MMPs) are a family of Ca^{2+} or Zn^{2+} containing endopeptidases that have three similar structural domains: an aminoterminal propeptide, a

catalytic metal-containing domain and a hemopexin-like domain at the carboxy-terminal end. A subtype of MMPs is known as the gelatinases (MMP-2 and MMP-9) which are specialized for degrading basement membrane (i.e. laminin and heparan sulfate proteoglycans) and extracellular matrix (i.e. collagen IV and fibronectin) proteins (Uemura et al., 2001; Thrall et al., 2007). Under physiological conditions, expression of MMPs remains low. However, MMP expression can be upregulated by reactive oxygen species (Shin MH et al., 2008; Haorah et al., 2007; Gasche et al., 2001). During pathologies that involve increased oxidative stress, such as stroke, MMP-2 and MMP-9 can become upregulated (Nagel S et al., 2007; Gasche et al., 2001).

The fact that tPA upregulates MMP-9 is widely accepted (Tsuji et al., 2005; Montaner et al., 2001b; Wang et al., 2003b; Sumii and Lo, 2002; Cuadrado et al., 2008). The actions of tPA and MMP-9 are implicated in the hemorrhagic transformation that casts risk onto the use of tPA as a stroke treatment. Over 24 h, administration of tPA is associated with a time and dose dependent increase in active MMP-9 (Wang et al., 2003b). Inhibition of MMPs attenuated incidence of tPA induced cerebral hemorrhage (Sumii and Lo, 2002). In a model of focal cerebral ischemia MMP inhibitors reduced hemorrhage and improved functional outcome after tPA administration (Tsuji et al., 2005).

MMP-9 is elevated after stroke (Zhao et al., 2006; Tsuji et al., 2005; Yepes et al., 2003; Montaner et al., 2001b; Montaner et al., 2001a; Wang et al., 2003b; Gidday JM et al., 2005; Sumii and Lo, 2002; Clark et al., 1997; Cuadrado et al., 2008; Gu et al., 2002) by the actions of inflammatory cytokines (Akool et al., 2003; Rosenberg et al., 1995; Jian and Rosenberg, 2005; Harkness et al., 2000; Zhou et al., 2009) and free radicals (Jian and Rosenberg, 2005; Akool et al., 2003; Tsuji et al., 2005; Haorah et al., 2007; Gu et al., 2002). As early as 4 hours (Gasche et al., 2001) and from 1 to 3 d post-cerebral ischemia MMP-9 is increased in the endothelial cells of the ischemic striatum and cortex (Zhao et al., 2006; Montaner et al., 2001a; Jian and Rosenberg, 2005; Wang et al., 2003b; Gidday JM et al., 2005; Clark et al., 1997).

At later time points, 7 to 14 d, MMP-9 is increased in the penumbral neurons and astrocytes (Zhao et al., 2006). MMP-2, while constitutively active, becomes upregulated several months after stroke (Montaner et al., 2001a; Clark et al., 1997). Levels of MMP-2 are increased in patients with previous history of stroke (Montaner et al., 2001a). Increased MMP-9 is associated with worsened neurological outcome (Montaner et al., 2001a), activation of macrophages (Haorah et al., 2007), degradation of BBB substrates (Tsuji et al., 2005; Rosenberg et al., 1995; Haorah et al., 2007; Gidday JM et al., 2005; Asahi et al., 2001; Van and Libert, 2007; Gu et al., 2002) and BBB permeability (Rosenberg et al., 1995; Haorah et al., 2007; Montaner et al., 2001b; Gidday JM et al., 2005; Asahi et al., 2001), increased inflammatory cell infiltration (Tsuji et al., 2005; Rosenberg et al., 1995; Montaner et al., 2001a), parenchymal damage (Tsuji et al., 2005; Gidday JM et al., 2005), edema formation (Tsuji et al., 2005; Haorah et al., 2007; Montaner et al., 2001b; Gidday JM et al., 2005) and hemorrhage (Montaner et al., 2001b; Lee et al., 2007) after cerebral ischemia.

Use of MMP knockout mice caused decreased BBB degradation (Yepes et al., 2003), specifically attenuation of ZO-1 degradation (Asahi et al., 2001) after brain injury. MMP inhibitors have decreased capillary permeability (Rosenberg et al., 1995) and hemorrhage (Lee et al., 2007) after cerebral ischemia. Finally, treatment with MMP-9 siRNA 60 min after MCAO resulted in significant reduction in mortality, infarct volume, edema formation, and functional outcome (Hu et al., 2009). It has been suggested that MMPs could be a possible therapeutic target (Hu et al., 2009) or surrogate marker for stroke damage (Montaner et al., 2001a). This may be possible if focusing on MMP-9 at early time points. However, MMP-2 is associated with angiogenesis (Rosenberg et al., 1995) and MMPs in general have been shown to be active in tissue repair (Jian and Rosenberg, 2005). Due to the beneficial effect of MMPs at late time points, more investigation into a safe time window for inhibition is necessary.

CONCLUSION

As promising neuroprotectants screened in animal models fail to translate into clinically relevant stroke therapies, the need for better models becomes increasingly imperative. In this review of the current literature we have discussed the role of aging and the blood-brain barrier in stroke pathophysiology. Age is the largest risk factor for stroke; yet, an overwhelming majority of animal models continue to utilize young adult animals to understand the mechanisms and find therapies for cerebral ischemia. Results from models utilizing aged animals are increasingly divergent from those in young adult animals. This disparity suggests that age is an important and yet overlooked variable in the study of stroke mechanism and the search for clinically applicable therapies.

Blood-brain barrier functional and structural integrity is extremely important to maintaining the microenvironment of the brain. Studies have focused on gross breakdown of BBB functional integrity. Utilizing small vascular space markers has allowed fine changes in BBB dysregulation to be elucidated. Investigation of subtle changes in BBB functional and structural integrity will give better insight into the time frame and mechanism of the pathophysiology of ischemic damage.

Figure 1.1 Stroke Prevalence by age and sex (Lloyd-Jones et al., 2009).

Figure 1.2 Schematic representation of intercellular junction between two endothelial cells. Cerebral endothelial cells possess cadherins, JAM, and tight junctions. However, the tight junction provides the cerebral endothelium with its unique phenotype. Zona occludens serve as the cytoskeletal scaffolding for the transmembrane proteins claudin-5 and occluden, which forms the cell-to-cell junction. Claudin 5 is essential for tight junction formation. Occluden exists as a cytosolic or plasma membrane protein and location within the cell reflect changes in paracellular permeability (Huber et al., 2001).

Figure 1.3. Mechanisms of formation and breakdown of reactive oxygen species. Schematic modified from Miller et al., 2006. (Miller AA et al., 2006).

Chapter Two

Early disruptions of the blood-brain barrier may contribute to exacerbated neuronal damage and prolonged functional recovery following stroke in aged rats

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ABSTRACT

We examined the effects of age on stroke progression and outcome in order to explore the association between blood-brain barrier (BBB) disruption, neuronal damage, and functional recovery. Using middle cerebral artery occlusion (MCAO), young (3 months) and aged (18 months) rats were assessed for BBB disruption at 20 min post-MCAO, and 24 h post-MCAO with tissue plasminogen activator induced reperfusion at 120 min. In addition, young and aged rats were assayed for functional recovery out to 14 d post-MCAO and reperfusion. Results showed that BBB disruptions in aged rats occurred early and increased nearly two-fold at both the 20 min and 24 h time points when compared to young animals. Neuronal damage in aged rats was increased two-fold as compared to young rats at 24 h, and differed qualitatively in the degree of inflammatory cell infiltration, hemorrhage and edema. No difference in neuronal damage was observed between young and aged rats at 20 min. Young and aged rats exhibited neurological deficits when compared to sham-controls out to 14 d following MCAO and reperfusion; however, aged rats exhibited more severe onset of deficits and prolonged recovery. Results indicate that aged rats suffer larger infarcts with increased BBB disruption, greater neuronal damage, and reduced functional recovery. Moreover, BBB disruption at 20 min post-MCAO in aged rats, in the absence of significant neuronal damage, suggests a potential mechanism by which alteration in BBB functional integrity during stroke contributes to a greater degree of subsequent neuronal injury.

INTRODUCTION

Stroke is a disease of the elderly. Approximately 72% of people who suffer a stroke are over the age of 65, 25% of the population over the age of 75 will have a stroke, and the incidence of stroke more than doubles with each successive decade for people over the age of 55 (Lloyd-Jones et al., 2009). Increased infarction volumes and altered physiologic response has been demonstrated in aged rodents following middle cerebral artery occlusion (MCAO) (Rosen CL et al., 2005; Sutherland et al., 1996; DiNapoli VA et al., 2006). Young male animals and filamentous methods of occlusion commonly utilized in laboratory investigations may not accurately represent the stroke patient, as these methods have consistently identified promising neuroprotective candidates that have failed in clinical trials (Gladstone DJ et al., 2002; Fisher, 1999; Fisher and Ratan, 2003).

The disparity between laboratory models and clinical studies may be secondary to changes which occur in the blood-brain barrier (BBB). A number of morphological and functional changes to the BBB occur during the aging process, including remodeling of the extracellular matrix, decreased occludin expression, changes in lipid and protein composition (Burns EM et al., 1981; Mooradian AD, 1988; Mooradian and Meredith, 1992; Mooradian and Smith, 1992), alterations in nutrient and protein transport (Shah GN and Mooradian AD, 1997; Banks and Kastin, 1985; Kleine et al., 1993), and decreased reactivity to β -adrenergic neurotransmission (Mooradian, 1994). These alterations make the barrier more vulnerable to insult, such as ischemia. Cessation of cerebral blood flow and the subsequent lack of tissue oxygenation can lead to increased BBB permeability by a progressive loss of component antigens from the basal lamina and extracellular matrix, as well as alterations in endothelial cell-cell and cell-matrix interactions. The active hypoxia/ischemia induced following a stroke stimulates the surface expression of adhesion molecules (Haring et al., 1996; Mao et al., 2000) and changes in integrin

expression (Wagener et al., 1997) in the first few hours. Activation of the BBB leads to endothelial dysfunction, characterized by increased permeability, extravasation of plasma components, and edema formation (Okada et al., 1996). What effect aging has in this process and how it may affect stroke outcome are not well characterized.

We utilized a clinically relevant model of stroke, based upon selective embolization of the middle cerebral artery (MCA) combined with recombinant tissue plasminogen activator (rt-PA) induced reperfusion, and assessed tissue loss, functional outcomes, histopathology and changes in BBB permeability in young (3 mo.) and aged (18 mo.) Sprague-Dawley rats. Our data demonstrated that the BBB in aged animals is disrupted earlier and to a greater degree compared to young animals at 24 h post-MCAO and rt-PA treatment, and 20 min post-MCAO. Aged rats suffer strokes which are larger in size and have more severe functional consequences, providing a potential mechanism by which accelerated breakdown of the BBB during acute stroke leads to worsened outcomes.

MATERIALS AND METHODS

Animals

This study was conducted in accordance with NIH guidelines for the care and use of animals in research. All protocols were approved by the West Virginia University Animal Care and Use Committee. Three groups of female, age-matched, Sprague-Dawley rats were investigated. Young (3-4 months; n=68), and aged (17-18 months; n=77) animals underwent an embolic middle cerebral artery occlusion (MCAO).

Surgical Procedure for MCAO

Rats were anesthetized with an intraperitoneal injection of 2.5 mg/kg flunixin, 90 mg/kg ketamine and 5 mg/kg xylazine, or with inhaled isoflurane (4% induction; 1% maintenance) for

BBB objectives. A servo-controlled homeothermic heating blanket, utilizing a rectal thermometer, was used to maintain body temperature at 37°C. A cartridge based arterial blood gas machine (GEM Premier 3000, Instrumentation Laboratory, Lexington MA) was used to monitor arterial P_{aO_2} , P_{aCO_2} , pH and hematocrit. Blood from the femoral artery was analyzed just prior to embolization, during ischemia, and following recanalization. The femoral vein was catheterized for fluid replacement (1.4 ml/h 0.9% saline) and drug delivery.

Rats underwent selective embolization of the right middle cerebral artery (MCA), producing 120 min of ischemia, followed by rt-PA induced reperfusion utilizing a technique recently described (DiNapoli VA et al., 2006). Briefly, a microcatheter, outer diameter 0.3 mm, was inserted into the ICA via the ECA stump and advanced until its tip occluded the ipsilateral MCA. This mechanical occlusion was verified by laser Doppler (LD-CBF) monitoring of the MCA perfusion territory. The catheter was retracted until MCA flow was restored around the microcatheter. A 25 mm fibrin-rich, autologous blood clot was then injected directly into the MCA. Ischemia was monitored continuously for 120 min, rt-PA was administered via the femoral vein, and restoration of MCA flow was verified by LD-CBF.

Functional Testing

Rats were handled and habituated to testing procedures in order to establish stable baselines on all measures. Animals were then tested repeatedly in a battery of functional assessments described below, beginning 24 h post-MCAO or sham operation and continuing daily for 1 week. Animals were then allowed to recover for an additional week and final assessments made at 14d post-insult. Scores were obtained by an observer blind to treatment of subject in order to reduce bias.

Composite Functional Score. The mNSS is a composite score of motor, sensory, balance and reflex measures and ranges from 1 to 17, with higher scores indicating greater neurological injury (Chen J et al., 2001; Germano et al., 1994; Seyfried et al., 2004).

Bracing. Postural adjustments were assessed according to methods previously reported (Schallert et al., 1979). Animals were gently pushed laterally across a stainless steel bench top over a distance of 90 cm, at a rate of approximately 20 cm/s, by the experimenter's hand. Rats will generally adjust their posture, making numerous adjustments with their forelimb on the side to which they are being moved. These adjustments were tallied over two trials and analyzed.

Placing. This is a test of sensorimotor function previously reported to be effected by normal aging in rodents (Marshall, 1982). Animal were held suspended by the investigator, allowing free movement of the unsupported limbs. The animals were then brought to the edge of a bench top, such that its body was parallel to the table's edge its whiskers brushed against the top surface. The number of times the animal successfully raised its forelimb to the table top was tallied over a series of 10 trials for each forelimb.

Tactile adhesive-removal test. Somatosensory function was tested as previously reported (Schallert et al., 1982; Schallert et al., 1983). A small adhesive paper circle ("Color Coding Labels", 1/4 inch in diameter, ACCO brands, Lincolnshire, IL) was placed on the distal-radius area of each forelimb and placed back in cage. The time taken for the animals to remove to stimulus with its mouth was recorded over a series of three trials, with an inter-trial interval of 1-2 min, and a maximum trial length of 180 s. The means from three trials were analyzed.

Akinesia test. This test assesses the animal's ability to initiate movements with the paretic forelimb (Schallert et al., 1992). The rat was held by the experimenter such that one forelimb was allowed to rest on the table top and move freely. The number of steps taken with the limb over a 30 s trial was tallied for each forelimb.

TTC staining

Animals were allowed to recover 24 h following MCAO and rt-PA reperfusion, anesthetized with Sleepaway (Fort Dodge Animal Health), and the brains were removed immediately and sliced coronally at 2 mm intervals. Slices were placed in 2%-2,3,5-triphenyltetrazolium chloride (VWR International, West Chester PA) in 0.1 M PBS for 20 min at 37°C, scanned on a flatbed scanner and analyzed using Adobe Photoshop software (v7.0). Infarction volumes were determined according to the methods described by Yang et al (Yang ZW et al., 1998a).

Tissue collection for frozen sections

Animals were allowed to recover for designated period, anesthetized with Sleepaway (Fort Dodge Animal Health), and transcardially perfused with 100 ml of 0.09% saline followed by perfusion solution (4.0% w/v paraformaldehyde in 1X SPB). Brains were removed, placed in fixative for 24 h, cryoprotected in a series of 10, 20 and 30% sucrose w/v in DPBS solution for 24 h per solution and sectioned at 35 µm.

Hematoxylin and eosin staining. Slide-mounted sections from three sham-controls and three randomly selected animals in each age group 24 h post-MCAO and rt-PA reperfusion, were rehydrated in DPBS for 5 min, and incubated in hematoxylin and eosin (Fischer Scientific) stain for 5 min at room temperature. Sections were rinsed for 5 min in running tap water and dehydrated for 5 min each in 75%, 95% and 100% ethanol, cleared in xylene, and coverslipped with Permount (Fischer Scientific). Sections were taken serially at 250 µm intervals, and examined utilizing light microscopy for areas of polymorphonuclear infiltration, hemorrhage and edema.

Fluoro-Jade B staining. Slide-mounted sections from three sham-controls and three randomly selected animals in each age group 24 h post-MCAO and rt-PA reperfusion were stained with Fluoro-Jade B. Slides were immersed in a solution of 1% sodium hydroxide in 80%

ethanol for 5 min, followed by immersion in 70% ethanol for 2 min, and distilled water for 2 min. The slides were transferred to 0.06% potassium permanganate for 10 min on a shaker to suppress background staining. Following a rinse in distilled water for 2 min, the slides were transferred to the staining solution for 20 min (stock FJB solution: 10 mg dye in 100ml distilled water; 0.004% working solution: 4 ml stock solution in 96 ml 0.1% acetic acid). The slides were washed three times for 1 min each in distilled water, and air-dried overnight. The slides were coverslipped directly with DPX (Fisher Scientific)

Confocal Microscopy

At 23 h following MCAO and rt-PA administration, 1 mg each of lysine fixable dextran (70,000 Da; AlexaFlour 488) and fibrinogen (300,000 Da; AlexaFlour 568) dissolved in 0.5 ml of 0.9% saline were injected into the femoral vein. After 1 h, rats were anesthetized and transcardially perfused with 0.9% saline for 5 min then 4% paraformaldehyde/ 0.1 M sodium cacodylate buffer (pH 7.2). For 20 min assessments, fluorescent markers were injected 40 min prior to clot injection and animals were perfused, as stated above, 20 min post-MCAO. Following perfusion, brains were removed and post fixed in 4% paraformaldehyde overnight. Areas around infarction were cut into 4 mm blocks, sliced into 60 μ m sections, and assessed for fluorescent activity using a Zeiss LSM 510 confocal microscope and analyzed using Optimas analysis system.

Evans blue albumin extravasation

Albumin extravasation was quantified 24 h following MCAO and rt-PA reperfusion. Rats were anesthetized with 2.5mg/kg flunixin, 90 mg/kg ketamine and 5 mg/kg xylazine and the infused with 2% Evans blue (4 ml/kg) via the femoral artery. The Evan's blue was allowed to circulate for 1 h and the rats were perfused with cold phosphate buffered saline (pH 7.4) for 15 min via the left ventricle. The brains were excised; meninges and ependymal organs removed, hemispheres excised, separated, weighed, and homogenized in 500 μ l of 50% trichloroacetic

acid. The tissue was incubated for 24 h at 37°C, and then centrifuged at 13,000 X g for 10 min. The supernatants were diluted four-fold with absolute ethanol; fluorescence intensity was measured using a fluorometer at 620 nm excitation, 680 nm emission (Beckman DU 640). Calculations were based on external standard readings and extravasated dye was expressed as ng EB/mg brain tissue.

Statistical Analysis

Data and figures are presented as means $\pm$ SE. Physiologic parameters and functional data were compared by two-way ANOVA with groups of age (young and aged) by treatment (pre- or post-stroke), followed by Tukey's post-hoc evaluation. Infarction volumes and EB data were compared by one-way ANOVA. Post-hoc t-tests were performed as directed by ANOVA outcomes. $P < 0.05$ was considered statistically significant.

RESULTS

Physiologic Variables

Physiologic data for groups subjected to MCAO are presented in Table 2.1. Significant differences between groups were not observed, and values are within normal physiologic ranges.

Mortality

Aged animals exhibited a 33% mortality rate at 24 h post-MCAO and rt-PA reperfusion with 12 of 36 animals dying during the first day of recovery, young animals suffered 17% mortality (5 of 29). In the group receiving MCAO only, 4 of 9 aged animals and 2 of 7 young animals died during the 24 h recovery period. There were no deaths amongst sham-operated animals or prior to 20 min sacrifice. The final analyses included 42 sham-operated controls, 65 rats with 120 min MCAO and rt-PA reperfusion, 16 rats with 120 min MCAO and 22 rats with 20 min MCAO.

Tissue loss is exacerbated in aged animals

Infarction volumes were significantly affected by animal age in both the cortical ($F[2,27]=8.5$, $P=0.001$) and striatal ($F[2,27]=6.9$, $P=0.004$) regions, 24 h following 120 min MCAO and rt-PA induced reperfusion (Figure 2.1, C). The percentage of infarcted cortex relative to contralateral hemisphere was significantly higher in the aged (Figure 2.1, B) group ($63\pm 9\%$) when compared to the young (Figure 2.1, A) ($26\pm 6\%$, $P=0.002$). Striatal infarction volumes were also significantly greater in the aged group ($53\pm 5\%$) relative to the young ($33\pm 4\%$, $P=0.006$).

Increased hemorrhage and infiltration of inflammatory cells into the infarct is observed in aged animals

At 24 h following MCAO and rt-PA reperfusion, brains from three animals in both age groups were harvested for pathological examination. Figure 2.2 displays representative photomicrographs (H&E) of slices from a young and aged rat. In the young animal (Figure 2.2, A), a central area of coagulation necrosis is evident. This area contains sparse aggregates of acute inflammatory cells (PMNs). Penumbra contains swollen neurons, some with vacuolated neuropil. In the aged animal (Figure 2.2, B), the area of coagulation necrosis is partially obscured by dense inflammatory cell infiltration and petechial hemorrhages. Increased vacuolation of the neuropil is evident.

Functional deficits are more severe with prolonged recovery in aged animals

All animals were assessed for gross neurologic competence after awakening from anesthesia. Behavioral deficits were grossly apparent in both age groups throughout the 14 d testing period, with the persistence of circling in 4 of 10 young animals and all aged animals. Impairment in normal feeding and grooming behavior was observed by 1 day post-MCAO. A red Harderian gland secretion surrounding the eyes and nostrils was present in most animals during the initial 48-72 h period, indicating significant stress following the insult. Feeding and grooming behavior

returned in all young animals by 7 d following MCAO, however, 4 of the 10 aged animals remained severely impaired until time of sacrifice.

Consistent with gross observations, behavioral assays detected significant functional impairment compared to baseline in both the young and aged animals throughout the recovery period (Figure 2.3). Animals were tested for neurological competence utilizing the modified neurologic severity scale (mNSS) (Chen J et al., 2001), sham-operated animals are represented as day 0. ANOVA demonstrated a significant effect of age on functional severity of stroke ($F[2,27]=17.07$, $P<0.001$) (Figure 2.3, A), with elevated mNSS scores correlating to increased severity of functional impairment.

Results of the bracing test showed a significant effect of treatment on performance of both young and aged rats throughout the 14 d testing period ($F[8,72]=5.83$, $P<0.001$) (Figure 2.3, B). Additionally, age had a significant affect on performance ($F[1,72]=223.06$, $P<0.001$). Sham-controls in the aged group (10.4 ± 0.6) demonstrated decreased baseline performance when compared to young sham-controls (13.6 ± 0.24) ($P<0.01$).

Results of the placing test showed a significant effect of treatment on performance of both young and aged rats throughout the 14 d testing period ($F[8,72]=24.69$, $P<0.001$) (Figure 2.3, C). Additionally, age had a significant affect on performance ($F[1,72]=933.11$, $P<0.001$). Young animals fully recovered in this task by day 14, with a peak deficit of 8.6 ± 0.74 responses/10 trials. Aged animals recovered to a mean of 4.4 responses/10 trails by day 14, with a peak deficit of 0 responses on days 1-3.

Results of the tactile removal test showed a significant effect of treatment on performance of both young and aged rats throughout the 14 d testing period ($F[8,72]=22.64$, $P<0.001$) (Figure 2.3, D). Additionally, age had a significant affect on performance ($F[1,72]=13.2$, $P<0.001$). Young animals showed significant recovery in this task by day 7 with a

mean removal time of 96.3 ± 29.6 s, and a peak deficit of 170.6 ± 9.3 s on day 1. Aged animals did not show meaningful recovery.

Results of the akinesia test showed a significant effect of treatment on performance of both young and aged rats throughout the 14 d testing period ($F[8,72]=21.5$, $P<0.001$) (Figure 2.3, E). Additionally, age had a significant affect on performance ($F[1,72]=98.8$, $P<0.001$). Young animals showed moderate recovery in this task by day 14 with a mean of 26 ± 2.7 steps, a baseline of 43.6 ± 0.9 and a peak deficit of 16.4 ± 3.3 steps on day 1. Aged animals did not show meaningful recovery with a mean of 10.6 ± 1.3 steps on day 14, a baseline of 40.4 ± 1.9 and a peak deficit of 7 ± 1.6 steps on day 1.

BBB permeability 24h post-MCAO is two-fold greater in the aged brain

BBB permeability in young and aged rats was assessed at 24 h post-MCAO by quantifying extravasation of albumin (Figure 2.4, A) and visualization of fluorescent vascular markers by confocal microscopy (Figure 2.4, B frames 3-6). Administration of rt-PA (5 mg/kg) at 2 h after MCAO significantly ($p<0.01$) reduced albumin extravasation in infarcted hemispheres of both young and aged rats at 24 h post-MCAO (Figure 2.4, A). Aged animals demonstrated a 212% increase in BBB permeability to albumin in the infarcted hemisphere as compared to young animals at 24 h post-MCAO and rt-PA reperfusion (Figure 2.4, A; $p<0.01$). No significant ($p>0.05$) difference in albumin extravasation at the BBB was noted between contralateral hemispheres of young and aged rats (Figure 2.4, A) nor in comparing contralateral hemispheres of young and aged rats to age-matched sham control hemispheres (data not shown).

Figure 2.4 B (frames 3-6) depict a visualization of cerebral microvessels in an area adjacent to the infarct (penumbra) of young (frame 3) and aged (frames 4-6) rats at 24 h post-MCAO and rt-PA reperfusion using two vascular space markers (dextran-70 kDa and fibrinogen-300 kDa). Frame 3 shows fibrinogen outlining the circuitous pattern of microvessels

with no apparent detection of dextran in the microvessels or extracellular space. Frame 4 shows fibrinogen outlining microvessels and the appearance of punctate regions of dextran in the extracellular space. Frame 6 is a brightfield image that clearly shows fibrinogen immunofluorescence is confined to the luminal side of the microvasculature and dextran is found only in the extracellular fluid. Frame 5 is an image of the contralateral side of an aged rat 24 h post-MCAO and rt-PA reperfusion.

Functional disturbance of the BBB by 20 min post-MCAO

BBB permeability in young and aged rats was assessed at 20min post-MCAO by quantifying extravasation of albumin (Figure 2.5, A) and visualization of fluorescent vascular markers by confocal microscopy (Figure 2.5, B frames 3 and 4). Aged animals demonstrated a 175% increase in BBB permeability to albumin in the infarcted hemisphere as compared to young animals at 20 min post-MCAO (Figure 2.5, A; $p < 0.001$). No significant ($p > 0.05$) difference in albumin extravasation at the BBB was noted between contralateral hemispheres of young and aged rats (Figure 2.5, A) or in comparing contralateral hemispheres of young and aged rats to age-matched sham control hemispheres (data not shown).

Figure 2.5 B (frames 3 and 4) depict a visualization of cerebral microvessels in an area adjacent to the infarct of young (Frame 3) and aged (Frames 4) rats at 20 min post-MCAO using two vascular space markers (dextran-70 kDa and fibrinogen-300 kDa). Frame 3 shows fibrinogen outlining the microvessels with no apparent detection of dextran in the microvessels or extracellular space. Frame 4 shows fibrinogen outlining microvessels and the appearance of dextran in the surrounding extracellular space. No difference in dextran extravasation was noted between contralateral hemispheres of young and aged rats or in comparing contralateral hemispheres of young and aged rats to age-matched sham control hemispheres (data not shown).

Neuronal damage is preceded by BBB disruption

Neuronal damage in young and aged rats was assessed at 24 h post-MCAO and rt-PA reperfusion (Figure 2.4, B frames 1 and 2) and 20 min post-MCAO (Figure 2.5B frames 1 and 2) by Fluoro-Jade B staining. Aged (Figure 2.4, B frame 1) animals demonstrated increased severity of injury, characterized by increased density and intensity of staining, as well as a greater degree of vacuolation compared to young (Figure 2.4, B frame 1) rats. The transition from severe neuronal injury within the MCA distribution to survival within the penumbra can be observed.

No difference in intensity of staining was seen between aged (Figure 2.5, B frame 1) and young (Figure 2.4, B frame 2) animals at 20 min post-MCAO. Early neuronal damage could not be revealed utilizing the Fluoro-Jade B staining technique in the young or aged animals.

DISCUSSION

We demonstrated that aged rats suffered more severe infarcts with increased disruption of the BBB, a greater degree of neuronal damage, and reduced functional recovery following MCAO. In addition, we found that rt-PA (5 mg/kg) induced reperfusion at 120 min post-MCAO produced a significant decrease in disruption of the BBB to albumin in young and aged animals at 24 h following MCAO. Finally, we report data that suggests BBB disruption occurs prior to observable neuronal damage during MCAO; thus, suggesting a potential mechanism by which alterations in BBB functional integrity during stroke contribute to the degree of subsequent neuronal damage.

A primary concern of this study was the use of rt-PA in rodents. Prior investigations have administered an rt-PA dosage 10-20 times the therapeutic dose for humans. A primary reason for this discrepancy in dose was that rt-PA is produced by human recombinant DNA, and species variations decrease rt-PA efficacy in rodents. In humans, rt-PA is administered at a dosage of 0.9 mg/kg and has a narrow therapeutic window. Since introduction in 1995, rt-PA

has remained the only approved method of drug induced thrombolysis for acute ischemic stroke (1995). These breakthroughs have been hindered by unwanted consequences of rt-PA treatment, most prominently the increased risk of hemorrhagic transformation and neurotoxicity (1995;Jiang et al., 2003).

Successful and reproducible thrombolysis was achieved in rodents at half the rt-PA dose which is commonly administered, limiting the deleterious effects. The drug was only administered according to NIH suggested guidelines, and both young and aged rats were routinely screened for intracranial hemorrhage following administration (DiNapoli VA et al., 2006). Young and aged rats underwent 120 min of stable MCA occlusion, followed by administration of rt-PA for recanalization. Duration of occlusion and successful reperfusion of the MCA territory was verified by laser Doppler; furthermore, the reperfusion profile was similar to that seen in a clinical setting. Previous studies have reported mortality rates (up to 80%) amongst aged animals receiving 60-120 minutes of filamentous occlusion, which would preclude further use in MCAO models (Futrell et al., 1991;Hachinski et al., 1992;Wang et al., 1995;Wang et al., 2003a). This model produced a 33% mortality rate in the aged animals. Administration of rt-PA at 120 min post-occlusion resulted in significantly decreased disruption of the BBB and reduced mortality in both young and aged animals.

Studies have produced varied results concerning the effect of age on stroke outcome. In comparison to young rats, the aged rats in this study showed nearly a two-fold increase in infarction volume. The principal difference in infarction size can be attributed to larger involvement of the cortical brain region in aged animals. The current study produced larger infarctions volumes in all age groups and brain regions, compared to suture occlusion (Rosen CL et al., 2005). In addition, histopathology of the infarcted region in aged animals distinctly revealed advanced vacuolation of the neuropil, increased infiltration of polymorphoneutrophils and pettichial hemorrhaging not seen in younger animals. These findings demonstrate that

cerebral ischemia in aged rodents creates infarcts which are structurally different than those generated by equivalent insults in younger animals.

The intact BBB has negligible transport of albumin into the brain; however, susceptibility of the BBB has been documented in a number of diseases. Evidence has suggested that a damaged endothelial barrier results in BBB disruption contributing to the progression and onset of several neurodegenerative diseases, including Alzheimer's and Parkinson's diseases (Ariga et al., 1998; Mackic et al., 2002). Under homeostatic conditions, the BBB remains functionally intact during aging (Mooradian AD, 1988); however, becomes more susceptible to disruption by external factors with increasing age (Daniel et al., 1978; Burns EM et al., 1981; Shah and Mooradian, 1997).

In this study, we measured Evan's blue albumin extravasation into the brain parenchyma of infarcted and non-infarcted hemispheres in young and aged rats at 20 min and 24h post-MCAO, and 24 h post-MCAO with rt-PA induced reperfusion. Young and aged rats exhibited a significant increase in BBB permeability to albumin 24 h post-MCAO and rt-PA reperfusion, and this disruption was two-fold greater in the aged compared to the young rats. Confocal microscopy confirmed leakage of albumin sized molecules across the BBB in aged animals. Microscopy demonstrated extravasation of dextran (70 kDa) in an area adjacent to, but not within, the infarct of aged animals. The failure to observe dextran in the penumbral region of the infarct in young rats suggests that the BBB is still functionally intact. Fluoro-Jade B staining assessed the degree of neuronal injury present, and demonstrated grossly observable damage within the MCA fed territory of young and aged animals. In addition, the aged rats exhibited increased intensity of staining within injured neurons, as well as vacuolation of the neuropil in agreement with histopathology.

A significant increase in albumin extravasation was also noted at 20 min post-MCAO in both age groups. The disruption in aged animals was again nearly 2-fold that seen in the young group and was greater than 3-fold that observed in the contralateral hemisphere. Confocal microscopy demonstrated the presence of dextran (70 kDa) in the extracellular space surrounding the MCA perfusion territory at 20 min post-MCAO in aged rats, while Fluoro-Jade B staining did not reveal neuronal injury at this time point. These data demonstrate a functional alteration in the barrier's ability to maintain a separation between systemic circulation and the brain parenchyma, ultimately contributing to cytotoxic changes in the extracellular environment. The presence of this disruption preceding observable neuronal injury suggests that exacerbated breakdown of the BBB in aged rats during acute stroke may contribute to the worsened outcomes observed. Furthermore, it may be important to consider the stabilization of the barrier in investigating new therapeutic interventions for stroke.

Evaluation of functional impairment has become a prominent focus in development of preclinical models, as results gained can be correlated to subsequent clinical assessment in stroke victims. Inclusion of aged animals in long-term functional assays has proven problematic due to impractically high mortality rates. Previous reports have addressed this issue by creating an incomplete filamentous occlusion, limiting the amount of tissue loss and lowering long term mortality in the aged animals to approximately 24% (Lindner et al., 2003). Their experiments produced long-lasting functional deficits amongst the aged rats, and allowed for functional evaluation in the aged group (age 16 months) out to 90 days post-occlusion. This approach certainly provides a compliment to the traditional use of young animals, which show less initial deficit and recover more quickly; however, the previously mentioned shortcomings of filamentous occlusion remain, as well as the inability to study rt-PA reperfusion and infarctions representative of larger strokes.

In utilizing functional assessments previously reported (Lindner et al., 2003), the current study examined functional deficits in young and aged animals following 120 min embolic MCAO and rt-PA induced reperfusion. Functional deficits seen in young animals were very similar to previous reports, showing a moderate initial deficit followed by significant recovery over a two week period. Aged animals in this study, however, showed severe initial deficits, with a dense and profound paresis of the limbs on the affected side and significantly poorer performance on a battery of functional challenges. Additionally, aged animals never exhibited functional competence equivalent to the young animals at any time point and recovery was prolonged. Thus aged rats had a functional status very similar to that seen clinically in patients having suffered a stroke.

The need for an accurate preclinical MCAO model that mimics the stroke population and allows for effective, reliable reperfusion is imperative for the successful translation of therapeutic strategies and improved stroke outcomes. Hope for potential neuroprotective agents has spurred intense research interest in basic science investigation and clinical trials, yet no effective agent has been found. This study clearly demonstrated that age has a profound influence on acute brain damage and subsequent functional recovery following an MCAO and rt-PA reperfusion. These data further the consensus that stroke models should be developed in older animals, and that use of aged animals in preclinical models may increase their clinical relevance and predictive validity (Millikan, 1992). To our knowledge, this is the first study to report reproducible reductions in MCA flow using an embolic technique, with consistent rt-PA induced reperfusion and acceptable mortality rates in aged animals. Additionally, we begin to explore the role of the aging BBB and how alterations to this structure during acute stroke affect outcome. Early disruption of the BBB in aged animals during stroke provides a mechanism by which this breakdown contributes to subsequent neuronal damage, and is a critical element to

consider in the development and implementation of effective therapeutic strategies for treatment of elderly stroke victims.

Figure 2.1. TTC stained sections representative of young (A) and age (B) infarction distribution 24 h post-MCAO and rt-PA reperfusion. C, Volumetric analysis of cerebral infarctions. Infarction volumes are expressed as a percentage of the contralateral structure $\pm$ SE. * Significant difference in 18 month vs. 3 month age group ($P \leq 0.006$). N=10 for all groups.

Figure 2.2. Representative photomicrographs of slices from young (A) and aged (B) animals, 24 h post-MCAO and rt-PA treatment. In the young rats (A), a central area of coagulation necrosis is evident (closed arrowhead). This area contains sparse aggregates of acute inflammatory cells (*) (PMNs). The margin of the infarct contains swollen neurons, some with vacuolated neuropil (arrow). In the aged animals (B), the area of coagulation necrosis (closed arrowhead) is partially obscured by dense inflammatory cell infiltration (*) and petechial hemorrhages (open arrowhead). Vacuolation of the neuropil is evident (arrow) (Scale bar = 100 μ m).

Figure 2.3. Performance on functional tests performed from 1-14 d post-occlusion to assess neurological deficit. A, mNSS, A composite functional score, judged on a scale of 0 to 17 with higher scores correlating to increased severity of functional deficits (N=10/group). B, Bracing test reflects number of postural adjustments made by the animals while being pushed laterally toward the paretic side over 90 cm (N=5/group). C, Placing test reflects the number of directed paw placements made in response to somatomotor stimulus over 10 trials (N=5/group). D, Tactile removal test reflects the time taken for animal to remove somatosensory stimulus placed on distal forearm (N=5/group). E, Akinesia test reflects the number of steps made with usable forearm over 30 s trial (N=5/group). Scores are means $\pm$ SE.

B.

Figure 2.4

Figure 2.4. A, Extravasation of albumin across the BBB assessed by quantification of Evan's blue dye 24 h post-MCAO. Aged group untreated and treated with rt-PA exhibited greater BBB permeability in relation to corresponding young animals and contralateral hemisphere (*, $P < 0.01$) (N=5). B, Photomicrographs of Fluoro-Jade B stained sections from a young (frame 1) and aged (frame 2) animals 24 h post-MCAO and rt-PA reperfusion. Damaged neurons within the infarcted region are fluorescently stained (green), while surviving neurons within the penumbra are left unstained (Scale bar = 100 μm). Frames 3-6, Confocal microscopy of vascular permeability markers dextran (70 kDa) and fibrinogen (300 kDa) 24 h post-MCAO and rt-PA reperfusion, taken in the penumbral region of the infarct. Frames 3 and 4, Confocal images of fibrinogen (red) and dextran (green) reactivity in the ipsilateral cortex of young (frame 3) and aged (frame 4) animals. No dextran extravasation is observed in the young animal (frame 3) and is similar to images from contralateral hemisphere of the aged animal (frame 5). Dextran is present in the extracellular space of aged (frame 4) animals at the same time point. Panel 6, Image in bright field that depicts lumen of cerebral microvessel. Fibrinogen can be seen remaining within the lumen of the vessel, while dextran reactivity is present along the outer surface in an aged animal that underwent MCAO and rt-PA reperfusion. (Scale bar = 5 μm)

Figure 2.5

Figure 2.5. A, Extravasation of albumin across the BBB assessed by quantification of Evan's blue dye 20 min post-MCAO. Aged group exhibited greater BBB permeability in relation to corresponding young animals and the contralateral hemisphere (*) ($P < 0.001$). B, Photomicrographs of Fluoro-Jade B stained section from a young (frame 1) and aged (frame 2) animal 20 min post-MCAO. Damaged neurons were not observed within the MCA perfusion territory in either young or aged animals (Scale bar = 100 μm). Frames 3 and 4, Confocal images of fibrinogen (red) and dextran (green) reactivity in the ipsilateral cortex of young (frame 3) and aged (frame 4) animals, taken in the penumbral region of the infarct. No dextran extravasation is appreciated in the young rats (3) and is similar to images from contralateral hemisphere of the aged rats (not shown). Dextran is present in the extracellular space of aged (4) animals at the same time point. (Scale bar = 5 μm)

Supplemental Figure 2.1. All animals experienced an equivalent reduction and restoration of cerebral blood flow. Laser Doppler cerebral blood flow (LD-CBF) was monitored throughout the experiment. Chart represents mean percentage of baseline LD-CBF values $\pm$ SE, at equivalent time points, throughout the experiment. There are no significant differences between the means at any time point. The 3 month old group demonstrated a higher LD-CBF with initial reperfusion than the aged animals (time point 7); however, this difference was not significant ($P=0.41$). (N=10)

Table 2.1. Summary of Physiological Values Pre-, During and Post-Middle Cerebral Artery Occlusion.

		PaCO2	PaO2		
Groups	pH	(mm Hg)	(mm Hg)	% Hct	MAP
Young					
Pre (10)	7.44±0.07	32±8.1	109±6.8	36±3.2	80±7.8
During (10)					89±11.5
Post (10)	7.39±0.05	37±4.9	97±5.3	35±3.3	102±18.1
Aged					
Pre (10)	7.43±0.07	34±6.8	114±7.3	34±2.5	84±13.7
During (10)					79±13.0
Post (10)	7.41±0.03	32±9.6	116±6.3	32±3.9	98±19.5

Chapter Three

NOX2 inhibition with apocynin worsens stroke outcome in aged rats.

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ABSTRACT

Background and Purpose: This study utilized middle cerebral artery occlusion (MCAO) with tissue plasminogen activator (tPA) to assess inhibition of the NOX2 isoform of NADPH oxidase on brain injury and functional recovery in aged rats.

Methods: Effects of NOX2 on the degree of brain injury and functional recovery following MCAO and tPA reperfusion was assessed in young adult and aged rats. Rats received apocynin (NOX2 inhibitor; 5 mg/kg) or saline 30 min prior to MCAO. At 24 h following MCAO, blood-brain barrier permeability (BBB), stroke infarct volume, edema formation, and oxidative damage were measured.

Results: Apocynin treatment in aged rats increased mortality rate and failed to improve functional outcome, total infarct volume, edema formation, and BBB permeability. Aged rats displayed increased BBB permeability to sucrose in the contralateral hemisphere following MCAO and diminished antioxidant capacity in the brain as compared to young adult rats.

Conclusions: We conclude that inhibition of NOX2 in the aged rat exacerbates stroke injury and diminishes functional outcome. These results suggest age is an important factor in stroke damage and more rigorous examination of apocynin as a therapeutic agent for treatment of stroke must be done.

Introduction

NADPH oxidases are a damaging source of reactive oxygen species during the aging process (Park L et al., 2007; Hamilton CA et al., 2001). Inhibition of the NOX2 isoform of NADPH oxidase reduces superoxide formation (Tang et al., 2007), prevents oxidative damage in the vasculature (Godbole et al., 2009; Park et al., 2008), and was recently touted as a promising therapeutic target for treatment of stroke (Kahles T et al., 2007). Studies using embolic models of stroke have identified many neuroprotective agents; however, none of these drugs have improved stroke outcome in humans (Gladstone DJ et al., 2002). Most preclinical studies have been conducted in young rodents. We contend that aging impacts brain damage and functional recovery following stroke and that by not focusing on age-related processes, a vast area of potential therapeutic growth has been overlooked. This study utilized middle cerebral artery occlusion (MCAO) with tissue plasminogen activator (tPA) reperfusion to assess inhibition of NOX2 on brain injury and functional recovery in aged rats. Our results demonstrated that age is an important factor in stroke damage and suggest more rigorous examination of NOX2 inhibition as a therapeutic strategy for treatment of acute ischemic stroke is needed.

EXPERIMENTAL PROCEDURE

MCAO and Apocynin Treatment

Female rats were used in this investigation due to the exaggerated sexual dimorphism of size and weight in young adult versus aged males. Female Sprague-Dawley rats [(3-4 months, 260-280 g, n=92) and (18-20 months, 275-350 g, n=120)] were received from Harlan (Indianapolis, IN) and housed under natural light/dark conditions with food and water available ad libitum. Procedures involving animals abided by the West Virginia University Animal Care and Use

Committee. Before MCAO or sham surgery, rats from both age groups were randomly divided into apocynin (5 mg/kg; i.p.) and vehicle (0.9% saline; i.p.) treated groups. Treatment was administered 30 min prior to surgery. Rats were anesthetized with ketamine (90 mg/kg; i.p.; Webster Veterinary; Sterling, MA) and xylazine (5 mg/kg; Webster Veterinary) prior to surgery and then supplemented as needed. Rats underwent MCAO for 2 h followed by tPA (Genentech; San Francisco, CA)-induced reperfusion as previously described (DiNapoli VA et al., 2006). Briefly, a micro-catheter was inserted into the internal carotid artery (ICA) and advanced until its tip occluded the ipsilateral MCA. This mechanical occlusion was verified by laser Doppler (LD-CBF) monitoring of the cerebral blood flow in the MCA perfusion territory. A 25 mm fibrin-rich, autologous blood clot was then injected directly into the MCA. Sham surgery consisted of all steps above except for occlusion of the MCA. Ischemia was monitored continuously. At 2 h, tPA (5 mg/kg) was administered via the femoral vein, and restoration of blood flow through the MCA was verified by LD-CBF. Ischemia was defined as a perfusion drop across the MCA territory of >80% as determined by laser Doppler and successful reperfusion was denoted as a return to >80% of baseline perfusion rate by 30 min after tPA administration. At a dose of 5 mg/kg i.v., tPA decreases BBB permeability in both young adult and aged rats 24 h after embolic MCAO (DiNapoli VA et al., 2008). Furthermore, a recent study from our lab utilizing EEIIMD (a hexapeptide that binds non-thrombolytic sites on tPA) provides evidence that while some cortical damage could be due to non-thrombolytic actions of tPA; largely, striatal and total stroke volume are not exacerbated by tPA (Tan et al., 2009).

Functional Testing

At 24 h following MCAO, functional testing was performed using the modified Neurological Severity Scores (mNSS), which is a composite score of motor, sensory, balance and reflex measures ranging from 1-17, with higher scores indicating greater neurological injury (Chen J et al., 2001).

Determination of Infarct Volume and Edema Formation

At 24 h following MCAO, rats (n=6) were anesthetized with ketamine and xylazine, sacrificed by decapitation, brains removed and sliced coronally at 2 mm intervals. Sections were incubated in 2% 2,3,5-triphenyltetrazolium chloride (TTC) for 20 min at 37°C. Following TTC staining, infarct volumes were quantified according to method previously described (Yang ZW et al., 1998a).

For each brain section, ischemic area was outlined and infarct volume calculated. To avoid overestimation of infarct volume, the corrected infarction volume (CIV) was calculated: $CIV = (LA - [RA - RI]) \times d$, where LA was area of the left hemisphere (mm^2), RA was area of the right hemisphere (mm^2), RI was the infarcted area (mm^2), and d was slice thickness (2 mm). Edema index (%) was calculated according to previously described method (Maier et al., 1998). Briefly, $\text{edema index} = (RV - LV) / LV \times 100\%$, where RV was volume of right hemisphere (mm^3) and LV was volume of left hemisphere (mm^3).

Blood-brain barrier (BBB) permeability

At 24 h following MCAO, *In situ* brain perfusion and capillary depletion were carried out as described previously (n=6) (Preston JE et al., 1995; Triguero D et al., 1990). Briefly, rats were anesthetized with ketamine (90 mg/kg; i.p.) and xylazine (5 mg/kg; i.p.) and heparinized (10,000 U/kg; i.p.). The common carotid arteries were exposed, cannulated and perfused with modified Krebs-Henseleit Ringer's (117 mmol/L NaCl; 4.7 mmol/L KCl; 0.8 mmol/L MgSO_4 ; 24.8 mmol/L NaHCO_3 ; 1.2 mmol/L KH_2PO_4 ; 2.5 mmol/L CaCl_2 ; 10mmol/L D-glucose; dextran (70,000 Da) 29 g/L; bovine serum albumin 10 g/L), aerated with 95% O_2 /5% CO_2 and warmed to 37°C. With start of the perfusion jugular vein was sectioned to allow for drainage. Once both arteries were cannulated, [^{14}C]-sucrose was infused (flow rate: 0.5 ml/min) into the inflowing Ringer's solution (total flow rate: 3.6 ml/min/hemisphere). After 20 min, the brain was flushed for 20 s with unlabeled Ringer's solution and the rat sacrificed by decapitation. The brain was removed,

dissected into ipsilateral and contralateral hemispheres and the choroid plexis and meninges excised. Brain tissue samples (~150 mg wet weight) and 100 µl of perfusate samples were prepared for radioactive counting by addition of 1 ml of tissue solubilizer (TS-2; Research Products Inc.; Mount Prospect, IL). After 72 h in TS-2, 250 µl glacial acetic acid and 4 ml scintillation cocktail (Bio-Safe NA; Research Products Inc.) were added and samples analyzed by liquid scintillation counting (Beckman LS5801; Beckman Coulter; Fullerton, CA). Amount of [¹⁴C] radioactivity in the brain (C_{tissue} ; dpm/g) was expressed as a percentage of that in artificial perfusate ($C_{\text{perfusate}}$; dpm/ml) and termed $R_{\text{tissue}}\%$ (µl/g) as follows: $R_{\text{tissue}}\% = (C_{\text{tissue}} / C_{\text{perfusate}}) \times 100\%$.

Capillary depletion was carried out after *in situ* perfusion, the brain was removed dissected into ipsilateral and contralateral hemispheres and choroid plexis and meninges excised.

Hemispheres were homogenized in 1.5 ml capillary depletion buffer (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid (HEPES); 141 mM NaCl; 4 mM KCl; 2.8 mM CaCl₂; 1 mM MgSO₄; 1 mM NaH₂PO₄; 10 mM D-glucose; pH 7.4) maintained at 4°C. An equal volume of ice-cold 26% dextran (60,000 Da) solution was added to homogenate. Aliquots of homogenate were taken and centrifuged at 5,400 x g for 15 min. Capillary depleted supernatant was separated from the vascular pellet. Homogenate, supernatant, and resuspended vascular pellet were assayed for radioactivity using a liquid scintillation counter.

Markers of oxidative stress

At 24 h following MCAO, rats (n=6) were anesthetized with ketamine and xylazine and transcardially perfused with 0.9% saline with heparin (2 U/ml). Brains were removed, and the ipsilateral and contralateral hemispheres separated. Brain sections were weighed and stored at -80°C. Measurement of superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) activities as well as, thiobarbituric acid reactive substances (TBARS) in the form

of malondialdehyde (MDA) and protein carbonyl were carried out according to manufacturer's protocols (Cayman Chemicals; Ann Arbor, MI).

Statistics

Data are presented as mean $\pm$ S.E. BBB permeability and oxidative stress assays were compared by three-way (ANOVA) with Tukey's post hoc analysis. Functional score, stroke infarct volume and edema formation were compared by two-way ANOVA with Tukey's post hoc analysis. Statistical significance was set at $p < 0.05$.

RESULTS

A total of 92 young adult and 120 aged rats were used in this study. At 24 h following MCAO, apocynin treatment led to increased mortality in aged rats from 14% (7 of 51) to 36% (25 of 69). Vehicle-treated aged rats suffered higher mortality than young adult rats (6%; 3 of 45). In young adult rats, treatment with apocynin (10%; 5 of 47) had no effect on mortality. No rats were excluded from the study for not meeting the criteria for ischemic depth and reperfusion. No deleterious signs of hemorrhage from tPA use were observed in the study. These findings were consistent with prior studies showing that administration of tPA at 5 mg/kg at 2 h after start of vessel occlusion did not worsen and, in fact, improved infarct volume compared to ischemic injury following suture occlusion and reperfusion (DiNapoli VA et al., 2006; DiNapoli VA et al., 2008; Tan et al., 2009).

Functional Score

At 24 h following MCAO, neurological function was assessed using a modified mNSS (Figure 3.1). Aged rats (11 ± 1) suffered worsened functional outcome compared to young adult rats (8 ± 0.5). While apocynin improved functional outcome in young adult rats (5 ± 1), no improvement from apocynin was noted in aged rats (10 ± 0.5).

Stroke Volume

At 24 h following MCAO, cortical, striatal and total stroke infarct volumes were calculated from TTC stained brain sections (Figure 3.2 A-C). Apocynin-treated aged rats displayed no improvement compared to vehicle-treated aged rats in cortical ($27\pm4\%$ and $21\pm4\%$, respectively), striatal ($62\pm7\%$ and $80\pm5\%$, respectively) or total ($33\pm3\%$ and $31\pm3\%$, respectively) stroke volumes. Aged rats exhibited significantly worsened cortical ($21\pm4\%$ and $10\pm2\%$, respectively) and total ($31\pm1\%$ and $19\pm2\%$, respectively) stroke volumes compared to young adult rats. Treatment with apocynin significantly decreased striatal ($33\pm10\%$ and $72\pm7\%$, respectively) and total ($6\pm2\%$ and $19\pm2\%$, respectively) stroke volume in young adult rats compared to vehicle-treated young adult rats.

Edema Formation

At 24 h following MCAO, edema formation was measured (Figure 3.2D). Apocynin treated aged rats ($20\pm2\%$) had exacerbated edema as compared to vehicle-treated aged rats ($6\pm3\%$). No difference in edema formation between young adult, young adult apocynin treated, and aged rats was observed.

BBB Permeability

At 24 h following MCAO, BBB permeability was measured utilizing a 20 min *in situ* brain perfusion with [^{14}C]sucrose (Figure 3.3). Aged rats treated with apocynin showed no difference in BBB permeability compared to vehicle-treated aged rats. The ipsilateral hemisphere of aged rats ($4.2\pm0.2\%$) showed increased permeability of [^{14}C]sucrose compared to both young adult MCAO rats ($2.9\pm0.4\%$) and aged sham rats ($2.0\pm0.1\%$). Following MCAO, aged rats also demonstrated increased permeability to [^{14}C]sucrose in the contralateral hemisphere ($2.9\pm0.2\%$) as compared to aged sham rats ($2.0\pm0.1\%$). The ipsilateral hemisphere of young rats ($2.9\pm0.4\%$) exhibited increased permeability of [^{14}C]sucrose as compared to young adult sham

rats ($1.6 \pm 0.1\%$). When treated with apocynin 30 min prior to MCAO, young adult rats exhibited attenuated BBB permeability ($2.0 \pm 0.2\%$).

Enzyme activity

At 24 h following MCAO, aged rats treated with apocynin showed reduced SOD activity compared to respective young adult rats (Figure 3.4). Following MCAO, young adult rats had reduced GPx in the contralateral hemisphere (321 ± 66 nmol/min/ml) compared to young adult sham rats (471 ± 36 nmol/min/ml). Treatment with apocynin in young adult rats reduced GPx in the ipsilateral hemisphere (205 ± 55 nmol/min/ml and 605 ± 57 nmol/min/ml respectively), and GPx (138 ± 35 nmol/min/ml and 321 ± 68 , respectively) and CAT (71 ± 0.6 mmol/min/ml and 115 ± 15 mmol/min/ml, respectively) in the contralateral hemisphere compared to vehicle-treated young adult rats. When compared to young adult sham rats, aged sham rats had decreased SOD (805 ± 80 U/ml and 388 ± 82 U/ml respectively) and GPx (471 ± 36 nmol/min/ml and 348 ± 66 nmol/min/ml respectively).

Oxidative Damage

At 24 h following MCAO, lipid peroxidation was assessed by measurement of its byproduct, MDA (Figure 3.5A). No significant difference was found between sham and MCAO rats. Apocynin treatment significantly decreased MDA concentration in aged rats as compared to aged sham rats.

Protein oxidation was measured by assessment of protein carbonyl content (Figure 5B). In aged rats, apocynin treatment significantly increased protein carbonyl content in the ipsilateral (13 ± 1 nmol/mg) and contralateral (11 ± 2 nmol/mg) hemispheres as compared to aged sham rats (6 ± 1 nmol/mg). Aged rats showed significantly higher protein carbonyl content in the contralateral hemisphere post MCAO (11 ± 2 nmol/mg) than aged sham rats (6 ± 1 nmol/mg). When treated with apocynin there was a significant increase in protein carbonyl content in

young adult rats in both the ipsilateral (10 ± 0.5 nmol/mg and 7 ± 1 nmol/mg, respectively) and contralateral (8 ± 1 nmol/mg and 6 ± 0.7 nmol/mg, respectively) hemispheres as compared to young adult vehicle-treated rats. At 24 h following MCAO, young adult vehicle-treated rats showed no change in protein carbonyl content as compared to sham rats.

DISCUSSION

The primary finding of this study was that inhibition of the NOX2 isoform of NADPH oxidase prior to MCAO elicited distinctly different brain injuries between young adult and aged rats. We report, for the first time, that treatment with apocynin prior to MCAO in aged rats resulted in increased mortality at 24 h with no improvement in total or regional infarct volume, edema formation, or functional outcome. We showed that at 24 h following MCAO, both ipsilateral and contralateral hemispheres of aged rats had increased BBB permeability to sucrose (342 Da) and apocynin treatment did not improve BBB functional integrity. Often in stroke studies utilizing young animals, the contralateral hemisphere is either ignored or considered control. However, previous clinical studies have observed changes in the contralateral hemisphere following stroke (Carey et al., 2002; Blank et al., 2003). In addition to changes in BBB permeability in the contralateral hemisphere, significant changes in catalase activity, GPx activity, and protein carbonyl content after MCAO were found. This study, in correlation with others (Buga et al., 2008; Carey et al., 2002; Blank et al., 2003), highlights a potential role for the contralateral hemisphere as a compensatory mechanism following stroke. Utilizing aged rodents in stroke models provides a unique opportunity to gain a better understanding of changes in the contralateral hemisphere following stroke.

Our study confirmed the findings of previous studies that in young adult rats, NOX2 inhibition with apocynin prior to MCAO significantly improved total stroke volume and functional outcome. We demonstrated that much of the improvement in brain injury in young adult rats

following MCAO was due to improved striatal infarct volume, lessened edema formation, and decreased BBB disruption. Understanding the effects of ischemic brain injury in reproductively senescent females is an understudied yet important area of research as females have an increased overall stroke burden (Lloyd-Jones et al., 2009). Recent studies indicate that gender plays a role in some mechanistic pathways following brain injury and recovery after stroke (De Silva et al., 2009; McCullough LD et al., 2005). Our current results argue against gender-related differences in NADPH oxidase activity, as results obtained from young adult female rats in our study were comparable to young adult male rats used in previous studies (Tang et al., 2007; Tang et al., 2008; Yenari MA et al., 2006; Kahles T et al., 2007); however, further investigation will need to be done to ascertain if these findings hold true in aged rats.

The decreased SOD and GPx activity observed in aged rats supports the “free radical theory of aging”, which contends that age-related diseases result from increased generation of reactive oxygen species (Harman D, 1994) and decreased antioxidant enzyme activity (Azhar S et al., 1995; Abete P et al., 1999). However, in concurrence with other studies (Meng et al., 2007; Goto et al., 1999), catalase activity and protein oxidation were unchanged due to the aging process. The brain, having a high metabolic rate and lack of metabolic reserves, possesses an increased concentration of mitochondria compared to other organs. Thus the brain is more resistant to lipid peroxidation (Meyer et al., 2004), perhaps due to break down of MDA by mitochondrial aldehyde dehydrogenase (Siu and Draper, 1982). Hypoxia and brain injury show elevated MDA, however, this increase is only seen at early time points, between 1 and 3 h (Lo W et al., 2007; Wang Q et al., 2006; Pantke et al., 1999). Due to the early formation and stability of protein carbonyl (Pantke et al., 1999), we propose that it is a better indicator than TBARS of oxidative damage in the brain at 24h after MCAO.

Inhibition of NOX2 with apocynin has recently gained momentum as a therapeutic option for treatment of acute ischemic stroke (Tang et al., 2007; Wang Q et al., 2006; Kahles T et al.,

2007) as several studies demonstrate that administration of apocynin (5-50 mg/kg; i.p.) in young rodents prior to MCAO attenuated superoxide production, infarct volume (Tang et al., 2007), neuronal death, activated microglia (Wang Q et al., 2006) and edema formation (Kahles T et al., 2007). Interestingly though, route of administration may play a role in apocynin toxicity as a recent study reported that when apocynin (5 mg/kg) was administered intravenously prior to MCAO, rodents exhibited larger cerebral hemorrhages in both hemispheres and increased mortality (Tang et al., 2008). These opposing findings suggest that the activation, mechanism of action, and toxicity of apocynin are still poorly understood and further studies are necessary, especially before any therapeutic use of NOX2 inhibition is pursued in clinical trials.

In this study, young adult apocynin-treated rats exhibited decreased GPx activity and increased protein oxidation after MCAO and reperfusion, possibly as a consequence of unactivated apocynin, which acts as a pro-oxidant by decreasing the glutathione to glutathione disulfide ratio (Riganti et al., 2006). Unactivated apocynin promotes hydrogen peroxide production and increases protein carbonyl formation (Riganti et al., 2006; Ciolino and Levine, 1997). Unactivated apocynin must be activated to inhibit NOX2 (Stefanska and Pawliczak, 2008). Activation of apocynin by myeloperoxidase (MPO) forms an apocynin dimer, which prevents recruitment of the cytosolic proteins and thereby inhibits NADPH oxidase activation (Stefanska and Pawliczak, 2008). In pathologic inflammatory conditions MPO is elevated (Tsimikas, 2008). In particular, aged animals exhibit exacerbated inflammatory cell infiltration (DiNapoli VA et al., 2008) and worsened stroke volume which produces increased MPO expression (Romanos et al., 2007). The positive correlation between MPO expression and apocynin activation suggests enhanced NOX2 inhibition with age. Data gathered from young animals suggest that increased NOX2 inhibition results in a favorable outcome. In direct contrast, our data show that aged rats exhibit exacerbated edema formation and increased

mortality. We conclude that inhibition of NOX2 with apocynin exacerbates stroke injury in the aged rat and would be a poor choice for clinical treatment of stroke.

Results from models utilizing aged animals are increasingly divergent from those in young adult animals. Evidence collected from aged animal models (DiNapoli VA et al., 2008; DiNapoli VA et al., 2006; Badan I et al., 2003; Popa-Wagner et al., 2007; Schroeder E et al., 2003) suggests that, in addition to exacerbated brain injury and persistent functional deficit after stroke, changes are occurring in the contralateral hemisphere. Disregarding the aging process in stroke research may be an important and yet overlooked mechanism resulting in failure to produce clinically applicable therapies.

Figure 3.1: Functional Outcome was measured utilizing the modified Neurological Severity Score (mNSS). Values are expressed as mean $\pm$ SEM (n=42). Statistical significance was determined as * $p < 0.05$ as compared to vehicle treated MCAO young adult rats and # $p < 0.05$ compared to apocynin treated young adult rats using two-way ANOVA with tukey's post hoc analysis.

Figure 3.2: TTC stained brain sections were used to calculate (A) cortical, (B) striatal, and (C) total stroke volume and (D) edema formation at 24 h following MCAO in vehicle and apocynin-treated young adult and aged rats. Bars represent mean $\pm$ S.E. (n=6). Statistical significance was set at *p<0.05 compared to vehicle-treated young adult rats, #p<0.05 as compared to apocynin-treated young adult rats using two-way ANOVA with Tukey's post hoc analysis.

Figure 3.3. At 24 h following MCAO, (A) *in situ* brain perfusion using [¹⁴C]sucrose (342 Da) was used to determine the degree of BBB permeability and (B) the effect of apocynin treatment on changes in BBB permeability in young adult and aged rats. Brains were separated into infarcted and non-infarcted hemispheres. Bars represent mean $\pm$ S.E. (n=6). Statistical significance was set at *p<0.05 compared to vehicle-treated age-matched sham, #p<0.05 compared to respective hemisphere of the vehicle-treated young adult MCAO rats, ‡p<0.05 compared to the respective non-infarct hemisphere, and †p<0.05 as compared to apocynin-treated young adult rats using three-way ANOVA with Tukey's post hoc analysis.

Figure 3.4. (A) Superoxide dismutase (SOD), (B) glutathione peroxidase (GPx), and (C) catalase (CAT) activities were spectrophotometrically measured in infarcted and non-infarcted hemispheres at 24 h following MCAO in vehicle and apocynin-treated young adult and aged rats. Bars represent mean $\pm$ S.E. (n=6). Statistical significance was set at * $p < 0.05$ compared to age-matched sham, # $p < 0.05$ compared to vehicle-treated young adult MCAO rats, † $p < 0.05$ compared to respective non-infarct hemisphere, ‡ $p < 0.05$ compared to apocynin-treated young adult rats and § $p < 0.05$ compared to respective young adult group using three-way ANOVA with Tukey's post hoc analysis.

Figure 3.5: Downstream products of lipid peroxidation (A) malondialdehyde (MDA) and (B) protein carbonyl were spectrophotometrically measured to assess the effect of apocynin treatment on oxidative stress at 24 h following MCAO in young adult and aged rats. Brains were separated into infarcted and non-infarcted hemispheres. Bars represent mean $\pm$ S.E. (n=6). Statistical significance was set at * $p < 0.05$ compared to age-matched sham, # $p < 0.05$ compared to vehicle-treated young adult MCAO, and \$ $p < 0.05$ compared to respective young adult group using three-way ANOVA with Tukey's post hoc analysis.

Chapter Four

NADPH oxidase isoform expression in aged female rats after MCAO and reperfusion.

ABSTRACT

NADPH oxidases (NOX) are a family of enzymes that play a role in the aging process, progression of atherosclerosis, and inflammatory response. However, the role of NOX isoforms during stroke-related ischemia and reperfusion in aged rats has yet to be elucidated. In this study, middle cerebral artery occlusion (MCAO) with tissue plasminogen activator (tPA) was used to assess time-dependent changes in NOX1, NOX2, NOX4, and catalytic subunits P22PHOX and P47PHOX in aged female rats. Real time (RT) PCR was used to determine transcriptional regulation of NOX isoforms 2 and 24 h post MCAO and reperfusion. NOX1 transcriptional regulation was decreased in the contralateral hemisphere 24 h after MCAO. Downregulation of NOX4 24 h after MCAO and reperfusion was found in the ipsilateral cortex. NOX2 was upregulated at 24 h after stroke and reperfusion, a finding consistent with young animals. Catalytic subunit P22PHOX, binds to all NOX isoforms, and is upregulated at 24 h. Post-ischemia molecular mechanisms in aged rats are poorly understood, which may explain differences in functional stroke outcomes and failed clinical treatments.

Introduction

Stroke is a disease of the elderly, yet most preclinical studies have been conducted in young rodents. Evidence suggests that altered NOX expression may play a role in the aging process (Krause, 2007; Geiszt et al., 2000; Park L et al., 2007). NOX2 containing NADPH oxidases are a damaging source of reactive oxygen species during the aging process (Park L et al., 2007; Hamilton CA et al., 2001) and progression of ischemic damage (Walder et al., 1997; Wang Q et al., 2006). Several studies demonstrate that inhibition of NOX2 with apocynin in young rodents prior to MCAO attenuated superoxide production, infarct volume (Tang et al., 2007), neuronal death, activated microglia (Wang Q et al., 2006) and edema formation (Kahles T et al., 2007). In contrast, inhibition of NOX2 with apocynin in aged animals caused increased edema and mortality 24 h after middle cerebral artery occlusion (MCAO) with tissue plasminogen activator (tPA) reperfusion (Kelly et al., 2009).

Several isoforms of NADPH oxidases are expressed in vascular cells. While NOX2 is widely considered the predominant isoform, it is the least expressed isoform in the brain vasculature. In the brain vasculature, NADPH oxidase isoform expression varies with NOX1 being the most abundant, followed by NOX4 and lastly NOX2 (Ago et al., 2005). As NOX1 and NOX4 have yet to be extensively studied, their roles are not yet fully understood. After cerebral ischemia, NOX1 knockout mice had greater cortical infarct volume when compared to wild type mice (Jackman et al., 2009), suggesting that NOX1 may play a neuroprotective role in stroke progression. Following ischemic stroke and reperfusion, expression of NOX4 is increased at early time points but after reperfusion returns to baseline levels (McCann SK et al., 2008), possibly impacting reperfusion injury. The relationship between NOX isoform expression, ischemia and age has yet to be determined. This study utilized MCAO with tPA reperfusion in aged rats to assess the differences in expression of NOX isoforms.

Methods

MCAO

Female Sprague-Dawley rats aged 18-20 months (275-350 g) were received from Harlan (Indianapolis, IN) and housed under natural light/dark conditions with food and water available ad libitum. Procedures involving animals abided by the West Virginia University Animal Care and Use Committee. Rats were anesthetized with 2% isoflurane (Webster Veterinary). Rats underwent MCAO for 2 h followed by tPA (Genentech; San Francisco, CA)-induced reperfusion as previously described (DiNapoli VA et al., 2006). Briefly, a micro-catheter was inserted into the internal carotid artery (ICA) and advanced until its tip occluded the ipsilateral MCA. This mechanical occlusion was verified by laser Doppler (LD-CBF) monitoring of the cerebral blood flow in the MCA perfusion territory. A 25 mm fibrin-rich, autologous blood clot was then injected directly into the MCA. Cerebral blood flow (CBF) was monitored continuously. After 2 h of ischemia, tPA (5 mg/kg) was administered via the femoral vein, and restoration of blood flow through the MCA was verified by LD-CBF. Ischemia was defined as a perfusion drop across the MCA territory of >80% as determined by laser Doppler and successful reperfusion was denoted as a return to >80% of baseline perfusion rate by 30 min after tPA administration. Animals not meeting this criteria were excluded from analysis.

Microvessel Isolation

At 2 and 24 h after MCAO rats were anesthetized with ketamine (90 mg/kg; i.p.) and xylazine (5 mg/kg; i.p.) and transcardially perfused with 0.9% saline. Brains were removed and microvessel isolations were performed as previously described (VanGilder et al., 2009). Briefly, brains were separated into ipsilateral and contralateral hemispheres and the meninges and choroid plexus removed. Samples of cortex (0.75 g) and striatum (0.75 g) were taken for RNA and protein extraction. The remaining

cortex was homogenized in 2 ml microvessel isolation buffer (MVI1; 103 mM NaCl; 4.7mM KCl; 2.5 mM KH₂PO₄; 1.2 mM MgSO₄; 15 mM HEPES; 2.5 mM NaHCO₃; 10 mM D-glucose; 1 mM Na pyruvate; 10 g/l dextran (64,000 Da)) with protease inhibitor cocktail (PIC; 1:1000). Dextran (26%) was added and the homogenate was centrifuged at 4°C for 10 min at 5,800 x g. The supernatant was aspirated and the pellet resuspended in 2.5 ml of a second microvessel isolation buffer (MVI2; 103 mM NaCl; 4.7 mM KCl; 2.5 mM CaCl₂; 1.2 mM KH₂PO₄; 1.2 mM MgSO₄; 15 mM HEPES). The homogenate was then filtered through a 100 µm filter and centrifuged at 4°C for 5 min at 5,800 x g. The supernatant was decanted and the microvessel pellet used for RNA extraction.

Real Time – Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from cerebral microvessels (n=4) using TriReagent®. Concentration of RNA was determined and only considered for use if A260/A280 was ≥1.80. Total RNA was reverse transcribed into cDNA using a 20 µl reaction. RT-PCR analyses of NOX1, NOX2, NOX4, p22phox, and p47phox using a StepOne™ detection system (Applied Biosystems; Foster City, CA) in combination with TaqMan® chemistry. Specific primers and dual labeled internal (FAM/TAMRA) probe sets were used according to manufacturer's recommendation (Applied Biosystems). All PCR amplifications were run in a 20 µl reaction volume consisting of 1µl cDNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control to normalize for differences in amount of cDNA added to reactions. Negative controls were monitored simultaneously within each run. Thermocycling conditions were set to holding stage at 50° C for 2 min and 95°C for 10 min and 40 cycles of 95° C for 15 s

and 60° C for 1 min. According to manufacturer's instructions, relative quantification of mRNA transcripts was carried out using the comparative threshold ($\Delta\Delta C_T$) method.

Statistics

Data for mRNA are presented as $\Delta\Delta C_T$ and was compared by one-way (ANOVA) with Tukey's post hoc analysis. Statistical significance was set at $p < 0.05$.

Results

RT-PCR analysis of NOX1 was performed at 2 h after MCAO and 24 h after MCAO and reperfusion (Figure 4.1). NOX1 transcription was decreased 24 h after MCAO and reperfusion in the contralateral hemisphere of the cortex (1.7 fold; $p = 0.05$) and striatum (2.3 fold; $p = 0.025$) as compared to aged matched sham rats.

RT-PCR analysis of NOX4 was performed at 2 h after MCAO and 24 h after MCAO and reperfusion (Figure 4.2). Transcription of NOX4 was decreased 2 fold in the ipsilateral hemisphere of the cortex at 24 h post-MCAO and reperfusion ($p = 0.006$) as compared to aged matched sham rats.

At 2 and 24 h after MCAO and reperfusion RT-PCR analysis of NOX2 (Figure 4.3) revealed significant increases in transcription after 24 h in the ipsilateral hemisphere. In the cerebral microvessels of the ipsilateral hemisphere NOX2 transcription was increased 4 fold as compared to aged matched sham rats ($p = 0.012$). NOX2 transcription was increased 3 fold in the ipsilateral hemisphere of the striatum ($p = 0.003$) as compared to aged matched sham rats.

The transcription of the P22PHOX membrane bound subunit of NADPH oxidase was measured by RT-PCR 2 and 24 h after MCAO and reperfusion (Figure 4.4). In the ipsilateral hemisphere, transcription of P22PHOX was increased 2 fold in the cerebral microvessels

($p=0.05$), 2.4 fold in the cortex ($p=0.018$), and 2.7 fold in the striatum ($p=0.011$) as compared to age matched sham rats.

Finally, P47PHOX transcription was measured by RT-PCR at 2 and 24 h after MCAO and reperfusion (figure 4.5). In the contralateral hemisphere at 2 h, P47PHOX transcription was decreased 2.4 fold ($p=0.01$) as compared to age matched sham rats.

Discussion

The primary finding of this study is that although the NOX2 isoform and P22PHOX subunit are increased at 24 h post-MCAO and reperfusion, conversely the NOX4 and NOX1 isoforms exhibit either unchanged or decreased transcription at both time points studied.

Without selective inhibitors of NOX1, its direct effect on stroke outcome is poorly understood. One study using NOX1 knockout mice found that cortical infarct volume was four fold greater in young adult NOX1 knockout mice when compared to wild type mice with 30 min MCAO and 23.5 h reperfusion (Jackman et al., 2009). As NOX1 may play a potentially beneficial role in stroke outcome, the decreased mRNA expression found in the cortex and striatum of the contralateral hemisphere of aged rats could explain the greater stroke damage found in aged rats after MCAO and reperfusion.

In young adult animals NOX4 mRNA is increased at 24 h after permanent cerebral ischemia peaking between 7 and 15 days in the ischemic hemisphere (Vallet P et al., 2005). In contrast, a model of MCAO and reperfusion found increased NOX4 mRNA expression as early as 6 h after MCAO with a return to baseline within 24 h (McCann SK et al., 2008), indicating a clear difference in NOX4 expression depending on reperfusion. Interestingly, immunohistochemical analysis of NOX4 at 7 days post-MCAO revealed the location of the NOX4 expression to be in the new capillaries (Vallet P et al., 2005). There was a trend towards increased NOX4 expression in the ipsilateral cortex at 2 h after MCAO and significantly

decreased expression at 24 h indicating a similar sequence of NOX4 regulation after MCAO as Vallet et al., 2005. This suggests that age does not play a significant role in transcriptional regulation of NOX4 after MCAO and reperfusion.

Tumor necrosis factor alpha (TNF α) has been shown to increase activity of NOX4 (Basuroy et al., 2008; Jung Y et al., 2008; Chenevier-Gobeaux et al., 2006). After MCAO, TNF α is increased as early as 1 h (Wang et al., 1994) and progressing out to several days and even weeks in humans (Sairanen et al., 2001). TNF α quickly activates NOX4 suggesting post-translational mechanism of the increased activity (Basuroy et al., 2008). As TNF α expression is increased in aged animals as compared to young adult animals after stroke (DiNapoli, unpublished data) it is possible that NOX4 activity but not translation is increased in aged animals after MCAO and reperfusion.

Increased mRNA expression of NOX2 was found in both the ipsilateral cortex and striatum of young adult animals 24 h post MCAO and reperfusion (McCann SK et al., 2008). In mice with dysfunctional NOX2 transient focal ischemia resulted in decreased infarct volume than wild type mice (Walder et al., 1997). Age has been shown to increase superoxide production by NOX2 causing impaired endothelium dependent relaxation and functional hyperemia (park L et al., 2007). Inhibition of NOX2 with apocynin has recently gained momentum as a therapeutic option for treatment of acute ischemic stroke (Tang et al., 2007; Wang Q et al., 2006; Kahles T et al., 2007) as studies demonstrate that administration of apocynin (5-50 mg/kg; i.p.) in young rodents prior to MCAO, attenuated superoxide production, infarct volume (Tang et al., 2007), neuronal death, activation of microglia (Wang Q et al., 2006) and edema formation (Kahles T et al., 2007). In direct contrast, our data show that aged rats exhibit exacerbated edema formation and increased mortality (Kelly et al., 2009). The present study found increased NOX2 mRNA expression not only in the striatum, where most of the stroke damage is found, but also in the cerebral microvasculature.

P22PHOX is a membrane bound subunit of NADPH oxidase. The NOX1, NOX2 and NOX4 isoforms of NADPH oxidase all bind to P22PHOX at the plasma membrane (Bedard K and Krause K, 2007; Clempus and Griendling, 2006). NADPH oxidase activity has been linked to transcription changes in P22PHOX (Manea et al., 2007). The present study found increased P22PHOX mRNA expression throughout the ipsilateral hemisphere in aged rats 24 h after MCAO and reperfusion. This suggests that activity of NOX1, NOX2 and NOX4 may be increased as they all bind to P22PHOX.

In conclusion, the direct affects of NOX1 and NOX4 activity after MCAO in aged animals need to be elucidated. The present data suggest that NOX1 transcriptional regulation is altered with age, which may exacerbate stroke damage. Transcriptional regulation of NOX2 and NOX4 mimic that seen in young adult animals, however since the present study did not measure activity of these enzymes, post-transcriptional modification could play a role. Changes in the contralateral hemisphere support recent evidence (Kelly et al., 2009; Buga et al., 2008; Carey et al., 2002; Blank et al., 2003) that investigation of changes in the contralateral hemisphere is important and understudied in stroke research.

Figure 4.1. NOX1 mRNA from sham, 2 h MCAO, and 24 h MCAO and reperfusion rats was measured by real-time PCR. Samples were taken from isolated microvessels (A and B), cortex (C and D) and striatum (E and F) of the ipsilateral (A, C and E) and contralateral (B, D, and F) hemispheres. Data are expressed as $\Delta\Delta C_T$.

Figure 4.2. NOX4 mRNA from sham, 2 h MCAO, and 24 h MCAO and reperfusion rats was measured by real-time PCR. Samples were taken from isolated microvessels (A and B), cortex (C and D) and striatum (E and F) of the ipsilateral (A, C and E) and contralateral (B, D, and F) hemispheres. Data are expressed as $\Delta\Delta C_T$.

Figure 4.3. NOX2 mRNA from sham, 2 h MCAO, and 24 h MCAO and reperfusion rats was measured by real-time PCR. Samples were taken from isolated microvessels (A and B), cortex (C and D) and striatum (E and F) of the ipsilateral (A, C and E) and contralateral (B, D, and F) hemispheres. Data are expressed as $\Delta\Delta C_T$.

Figure 4.4. P22POX mRNA from sham, 2 h MCAO, and 24 h MCAO and reperfusion rats was measured by real-time PCR. Samples were taken from isolated microvessels (A and B), cortex (C and D) and striatum (E and F) of the ipsilateral (A, C and E) and contralateral (B, D, and F) hemispheres. Data are expressed as $\Delta\Delta C_T$.

Figure 4.5. P47PHOX mRNA from sham, 2 h MCAO, and 24 h MCAO and reperfusion rats was measured by real-time PCR. Samples were taken from isolated microvessels (A and B), cortex (C and D) and striatum (E and F) of the ipsilateral (A, C and E) and contralateral (B, D, and F) hemispheres. Data are expressed as $\Delta\Delta C_T$.

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KIMBERLY A. KELLY, Ph.D.

Pharmaceutical and Pharmacological Sciences

West Virginia University

Health Sciences Center

PO Box 9530

Morgantown, WV 26506

Office #: 304-293-3042

khouser@hsc.wvu.edu

Education:

- B.S. in Toxicology, Ashland University, Ashland, OH, 2004
- Ph.D. in Pharmaceutical and Pharmacological Sciences, West Virginia University, Morgantown, WV 2004-2009

Professional Experience:

- Graduate Teaching Assistant, Pharmaceutical Care Lab, West Virginia University School of Pharmacy, Morgantown, WV. Spring 2006 & 2007

Memberships in Professional Societies:

Society for Neuroscience, 2007

Publications:

Huber JD, VanGilder R, **Houser K**: Streptozotocin-induced diabetes progressively increases blood-brain barrier permeability in specific brain regions in rats. *Am J Physiol.* (2006) 291:2660-8.

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References:

Jason D. Huber, PhD

Assistant Professor

Department of Basic Pharmaceutical Sciences

West Virginia University School of Pharmacy

PO Box 9530, Robert C Byrd Health Science Center

Morgantown, West Virginia 26506

Work: (304) 293-1474

E-mail: jhuber@hsc.wvu.edu

Charles L. Rosen, M.D., PhD

Associate Professor

Departments of Neurosurgery, Anatomy and Neuroscience

West Virginia University School of Medicine

PO Box 9183, Robert C Byrd Health Science Center

Morgantown, West Virginia 26506

Work: (304) 293-0424

E-mail: crosen@hsc.wvu.edu

Patrick S. Callery, PhD

Professor

Department of Basic Pharmaceutical Sciences

West Virginia University School of Pharmacy

PO Box 9530, Robert C Byrd Health Science Center

Morgantown, West Virginia 26506

Work: (304) 293-1482

E-Mail: pcallery@hsc.wvu.edu