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Effects of estrogen in an aged animal model of stroke

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Effects of estrogen in an aged animal model of stroke

Rachel L. León

**Dissertation submitted to the
West Virginia University School of Medicine
in partial fulfillment of the requirements
for the degree of**

**Doctor of Philosophy
in
Neuroscience**

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Morgantown, West Virginia

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ABSTRACT

Effects of Estrogen in an Aged Animal Model of Stroke

Rachel L. León

In 2000, approximately 10 million women were taking hormone replacement therapy (HRT) for alleviation of menopausal symptoms. A number of prior animal studies also suggested that HRT may be neuroprotective and cardioprotective as well. Although estrogen is neuroprotective in young adult animal models of stroke, clinical trials demonstrate that estrogen increases the incidence and severity of stroke in aged women. We have previously shown that experimental stroke pathophysiology differs between young adult and aged rats. In the first study, we show that aged rats treated with 17β -estradiol had significantly increased infarct volumes compared to placebo-treated aged rats. Young adult rats treated with 17β -estradiol had significantly decreased infarct volumes and functional outcome compared to ovariectomized young adult rats. Aged female rats treated with 17β -estradiol had decreased post-ischemic GFAP-positive astrocytes and altered astrocyte morphology. In the second study, we investigate a potential mechanism involved in the deleterious effects of estrogen in the aged female rat brain. In rats chronically supplemented with estrogen or placebo, we measure a significant reduction in erythropoietin (EPO) receptor mRNA in the ischemic cortex following transient occlusion of the middle cerebral artery. We also observe decreased EPO-receptor protein expression in the penumbral cortex and striatum using immunohistochemical techniques. In a second group of rats, we confirm that EPO administration attenuates estrogen-induced increase in infarct volume, and is accompanied by decreased expression of $ER\alpha$ in the ischemic penumbra. In the third study, we assess hippocampal-dependent memory using the 8-arm radial maze in young adult and aged female rats following middle cerebral artery occlusion with fibrin clot and tissue plasminogen activator reperfusion. Aged rats required greater number of sessions to become trained and had increased trial duration, suggesting reduced locomotor activity. Although baseline differences in reference and working memory errors were observed, they did not reach significance. Following MCAO, no significant differences were found in reference or working memory errors between young adult and aged rats. Finally, we investigate the effects of estrogen supplementation on structure and function of lacrimal and Meibomian glands in aged female rats. Following 90 days of estrogen supplementation in aged female rats, a reduction in tear volume and tear break up time in estrogen treated rats was accompanied by increased connective tissue fibrosis in both extraorbital and intraorbital lacrimal glands and reduced acinar lumen in the intraorbital lacrimal glands. These functional and structural changes are likely mediated by $ER\alpha$, as estrogen treatment led to upregulation of $ER\alpha$ expression in lacrimal and Meibomian glands.

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List of Abbreviations

ANOVA	analysis of variance
AQP4	aquaporin 4
BAD	bcl-2/bcl-xl-associated death promoter
BBB	blood-brain barrier
CEE	conjugated equine estrogen
CI	confidence interval
COX-1	cyclooxygenase-1
CREB	cAMP response element binding protein
CSF	cerebrospinal fluid
E2	17 β -estradiol
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial-derived nitric oxide synthase
EPO	erythropoietin
EPO-R	erythropoietin receptor
ER	estrogen receptor
ERK1/2	extracellular signal-regulated kinase 1/2
GFAP	glial fibrillary acidic protein
gp130	glycoprotein 130
GPR30	G-protein coupled estrogen receptor 30
HERS	Heart and Estrogen/progestin Replacement Study
HIF	hypoxia-inducible factor
HRT	hormone replacement therapy
HRT	hazard ratio
Iba-1	ionized calcium binding adaptor molecule 1
IGF-1	insulin-like growth factor-1
IL	interleukin
JAK	janus kinase
KCS	keratoconjunctivitis sicca
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
MCP-1	monocyte chemoattractant protein-1
MMP	matrix metalloproteinase
mNSS	modified Neurological Severity Score
MPA	methoxyprogesterone acetate
mRNA	messenger ribonucleic acid
MTA-1	metastasis-associated protein-1
NF κ B	nuclear factor κ B

NMDA	N-methyl-D-aspartic acid
NOS	nitric oxide synthase
NOS	nitric oxide
OSM	oncostatin M
OTC	over-the-counter
OVX	ovariectomy
PI3K	phosphatidylinositol-3-kinase
PRL	prolactin
PRL-R	prolactin receptor
ROS	reactive oxygen species
SOCS	supressor of cytokine signaling
STAIR	Stroke Therapy Academic Industry Roundtable
STAT	signal transducer and activator of transcription
TBI	traumatic brain injury
TBUT	tear break-up time
TNF α	tumor necrosis factor- α
tPA	tissue plasminogen activator
TTC	2,3,5 triphenyltetrazolium chloride
VEGF	vascular endothelial growth factor
WEST	Women's Estrogen for Stroke Trial
WHI	Women's Health Initiative

Chapter One

Literature Review

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ABSTRACT

In 2000, approximately 10 million women were taking hormone replacement therapy (HRT) for alleviation of menopausal symptoms. A number of prior animal studies also suggested that HRT may be neuroprotective and cardioprotective as well. Then, in 2003, reports from the Women's Health Initiative (WHI) indicated that chronic estrogen/progestin supplementation led to increased incidence of stroke. A second branch of the WHI in women with prior hysterectomy found an even stronger correlation between estrogen supplementation alone and stroke incidence. Follow up analyses of the data, as well as data from other smaller clinical trials, has also demonstrated increased stroke severity in women taking HRT or estrogen alone. This review examines the studies indicating that estrogen is neuroprotective in animal models and explores potential reasons why this may not be true in postmenopausal women. Specifically, age-related differences in estrogen receptors and estrogenic actions in the brain are discussed with the conclusion that animal models of disease must closely mimic the human pathology in order to produce clinically relevant results.

Age-related differences in brain injury

Stroke is the leading cause of long term disability and the third leading cause of death in the United States. According to the American Heart Association, approximately 72% of people who suffer a stroke are over 65 years old, almost 25% of the population over 75 years old will suffer a stroke, and for people over the age of 55 years, the incidence of stroke more than doubles each successive decade (Figure 1.1). Due to the increased incidence of traumatic brain injury (TBI) in young adults, young animal models have a role in understanding the mechanisms involved in the injured brain, particularly in the traumatically injured brain. However, this review argues that young animal models do not accurately represent the response to injury unique to the aged brain; thus, aged animals must have a place in the study of neural injury, particularly stroke.

Both basic science (Popa-Wagner et al., 2007) and clinical studies (Marquez de la Plata et al., 2008) indicate that older age decreases the ability of the brain to recover from neural injury. Neural injury causes an initial region of cell death followed by a secondary phase of injury characterized by release of intracellular glutamate and calcium, an inflammatory response, activation of apoptotic pathways, formation of reactive oxygen species (ROS), and blood-brain barrier (BBB) disruption. Animal studies of stroke have shown unique, age-related differences in both phases of ischemic injury (Badan et al., 2003b, Baltan et al., 2008). Specifically, aged rats have larger infarct volume (Popa-Wagner et al., 2007) and less functional recovery (Badan et al., 2003a) than young adult rats after transient focal cerebral ischemia. Morphological changes in the brain of aged rats following neural injury include massive infiltration of polymorphonuclear neutrophils, pettichiae, and necrosis formation (Rosen et al., 2005). These markers of inflammation are reduced in the post-ischemic brain of young adult rats (Dinapoli et al., 2006). Clinically, age-related differences in functional recovery from neural injury are well established. Results from a recent clinical study assessing over 400 TBI victims (ages ranging from 16 to 85 yr and divided into three groups: 16-26, 27-39, and 40-85) at one and four years after injury showed

similar improvements initially between age groups, but vast differences at the five-year assessment (Marquez de la Plata et al., 2008). These findings, along with a number of other investigations, cite age as a predictive factor in functional outcome from TBI (Flanagan et al., 2005). Older age also leads to greater disability following cerebral ischemia including decreased ability to perform activities of daily living, higher percentage of use of assistive devices for ambulation, and increased use of institutionalized care facilities (Kelly-Hayes et al., 2003).

Several hypotheses exist to explain the mechanisms at work in the aging brain: increased inflammation, decreased metabolic reserve, and accumulation of reactive oxygen species. The “inflammation hypothesis of aging”, also termed “Inflam-Aging”, suggests that increased inflammation during the aging process results from deregulation of the immune system and a progressive inability to handle pathological stimuli (Chung et al., 2001). Aging in the brain is characterized by cortical atrophy, and an increased number of activated astrocytes and microglia (Rosenberg, 2005). Another prominent idea is that aged brains have an accumulation of reactive oxygen species along with reductions in the enzymes to control them. Specific genes (i.e. *age-1*, *daf-2*, *methuselah*, and *shc*) have been identified that demonstrate a definitive connection between accumulation of ROS and longevity (Finkel and Holbrook, 2000). Although aging is a complex process influenced by many factors, the aged brain is clearly unique from its younger counterpart in both health and disease.

Animal models of stroke

Eighty-seven percent of strokes are ischemic in nature, with the most common site of occlusion the middle cerebral artery (MCA). For this reason, most stroke models utilize occlusion of the MCA, which results in reduced cerebral blood flow to areas supplied by that artery including the lateral surface of the hemisphere, parts of the basal ganglia, and the internal capsule (Ponten et al., 1973, Garcia, 1984). Models of MCA occlusion (MCAO) can be divided into two main categories: physical occlusion and chemically-induced occlusion. Methods of physical

inclusion include lifting the MCA with a tungsten hook (Badan et al., 2003a) photothrombotic occlusion (Yao et al., 2003), or through the use of suture (Liu et al., 2009) or fibrin clot occlusion (DiNapoli et al., 2006) inserted into the vessel lumen. Chemical occlusion consists of administration of endothelin-1 directly onto the exposed vessel which leads to vessel constriction and reduced blood flow (Moyanova et al., 2003, Nikolova et al., 2009). Methods of physical occlusion have the benefit of either being permanent (Bederson et al., 1986) or transient (Longa et al., 1989, Belayev et al., 1996), while chemical occlusion is always transient.

The most clinically relevant model of stroke utilizes a fibrin-rich blood clot to create an ischemic stroke, followed by tissue plasminogen activator (tPA) administration to disrupt the clot and permit re-opening of the vessel for reperfusion of ischemic tissue (Kudo et al., 1982, Kaneko et al., 1985, Dinapoli et al., 2006). While the use of tPA has some caveats, there are several advantages in using the fibrin clot model, including smaller, more precise infarctions, a mortality more representative of the clinical setting, slower, more clinically relevant reperfusion, the ability to utilize thrombolytic therapy, screen for neuroprotectants and develop clinically useful combination therapies. In addition, the use of laser Doppler monitoring of blood ensures proper ischemic depth and allows the reperfusion with tPA to be monitored. In addition to using a fibrin-rich clot, the use of aged animals plays a crucial role in creating a clinically relevant model for stroke research.

The guidelines for preclinical stroke research have been debated and discussed with initial recommendations provided by the Stroke Therapy Academic Industry Roundtable (STAIR) in 1999 (Fisher et al., 2009). In those guidelines, members of the STAIR group recommended that preclinical stroke models study potential stroke therapies in blinded, physiologically controlled experiments with rodents initially. They also recommended that studies create dose response curves, evaluate effectiveness in permanent occlusion first, then move to transient occlusion models, and assess functional and histological outcomes both acutely and long term. In a 2006 systematic review of nearly 3500 articles published from 1957 until 2003, O'Collins et al.

found that only 5 of 550 drugs tested as stroke therapeutics adhered to the STAIR I recommendations (O'Collins et al., 2006). The quality score, based upon level of adherence to STAIR I recommendations was inversely correlated with the efficacy of the drug reported, meaning that poorer quality studies routinely reported greater efficacy of the study drug than more rigorous studies. Recently, the STAIR committee updated their recommendations for preclinical stroke research and added additional criteria. First, studies with positive results should be repeated in an independent laboratory for validation. STAIR also specifically recommends that following initial studies in young, healthy animals, studies should be conducted in aged animals and animals with comorbidities such as hypertension, diabetes, obesity, and hypercholesterolemia. Other criteria include the use of both male and female animals and the use of relevant biomarker endpoints.

Age-related differences in estrogen activity in the brain

There is substantial evidence that estrogen is an effective neuroprotectant. The secondary phase of neural injury activates numerous inflammatory, pro- and anti-apoptotic, and angiogenic factors. These processes respond to estrogen differentially depending on age. Studies performed by a variety of investigators clearly show that estrogen reduces infarct size (Dubal et al., 1998), neuronal cell death (Rau et al., 2003), and mortality (Simpkins et al., 1997) following neural injury. One common variable in almost all studies showing neuroprotection by estrogen is the use of *young* animals. The few studies that have examined age-related differences in estrogenic effects have used models of injury that do not mimic stroke or methods of estrogen supplementation that do not mimic its clinical use. One study that investigated age-related differences in estrogenic actions used a 10-minute period of global ischemia – an injury that mimics cardiac arrest, rather than stroke (Wappler et al.). A study concluding that estrogen supplementation attenuates stroke injury in aged female rats used only one week of estrogen exposure before middle cerebral artery occlusion by intraluminal filament (Alkayed et al., 2000). Clinical

trials, however, have shown a clear relationship between increased stroke incidence and severity with estrogen supplementation in postmenopausal women (Wassertheil-Smoller et al., 2003, Anderson et al., 2004).

Clinical trials show estrogen increases stroke risk and stroke severity

The Women's Health Initiative (WHI) randomized controlled trials investigated the benefits and risks of 0.625 mg/day conjugated equine estrogen (CEE) plus 2.5 mg/day medroxyprogesterone acetate (MPA) in 16,608 women. The trials were terminated before the planned end date due to elevated breast cancer risk along with other risks defined in the global index as time to incident coronary heart disease, stroke, pulmonary embolism, hip fracture, or death from other causes (Wassertheil-Smoller et al., 2003). The average follow-up of women in the CEE/MPA trial was 5.6 years. In that time the CEE/MPA group experienced 151 strokes (1.8%) while the placebo-treated group had 107 strokes (1.3%). The hazard ratio (HR) for all stroke subtypes was 1.31 (95% CI, 1.02-1.68). For ischemic stroke, however, the HR was 1.44 (95% CI, 1.09-1.90) indicating an approximate 31% increase in stroke risk with CEE/MPA compared to placebo. The elevated risk of stroke with CEE/MPA could not be attributed to any other risk factor and did not appear until after the first year of treatment.

A second branch of the WHI simultaneously studied the effects of CEE alone in 10,739 women with prior hysterectomy (Anderson et al., 2004). Researchers also found an increased risk of ischemic stroke with CEE, warranting the early termination of those trials as well. When the CEE trial was stopped, the participants had an average of 6.8 years of follow-up. The HR for stroke in those taking CEE vs. placebo was 1.39 (95% CI 1.10-1.77) with an absolute excess risk of 12 additional strokes per 10,000 person-years. Although this elevation of stroke risk may be associated with an observed increase in systolic blood pressure, there are likely more global effects of CEE requiring further investigation.

Smaller-scaled clinical trials have shown increased stroke severity with estrogen treatment in women with known vascular disease. The Women's Estrogen for Stroke Trial (WEST) was a randomized, double-blind, placebo-controlled trial of 664 postmenopausal women that demonstrated increased risk of fatal stroke and worse neurological outcome from stroke in women taking estrogen (17 β -estradiol, 1 mg/day) after a first cerebral ischemic event (Viscoli et al., 2001). The Heart and Estrogen/progestin Replacement Study (HERS) investigated the use of CEE/MPA (0.625 mg/day CEE, 2.5 mg/day MPA) in 2,763 women with coronary heart disease and found that treatment was not *significantly* associated with risk of stroke (HR 1.23; 95% CI 0.89-1.70), but it also did not reduce the incidence of coronary or cerebrovascular events as expected (Hulley et al., 1998). Interestingly, HERS (Grady et al., 2000) as well as the unblinded follow-up study (HERSII) did find a significant increase in the risk of thromboembolic events (deep vein thrombosis and pulmonary embolism) in women taking HRT compared to those taking placebo (HR 2.08, 95% CI 1.28-3.40) (Hulley et al., 2002), indicating a possible role of estrogen in thrombus creation. Two separate meta-analyses combining data from each of these three clinical trials as well as many other smaller trials have shown a clearly increased risk of stroke incidence and severity with HRT (Bath and Gray, 2005, Sare et al., 2008).

Timing of initiation hypothesis

Researchers have responded to these reports by arguing that variations in the timing of initiation of estrogen treatment, is the probable reason for differences between animal studies and the clinical trial data. They contend that a period of hypoestrogenicity following menopause leads to irreversible vascular changes that are not mimicked in ovariectomized animals immediately supplemented with estrogen. Participants in the WHI trials were between 50 and 79 years old with an average age of 63 years – approximately 12 years after the average age of menopause. However, subsequent analyses of WHI data of women with prior CEE and/or CEE/MPA use that began within 5 years of menopause has not supported the “timing of initiation hypothe-

sis” (Prentice et al., 2009). However, basic science research from proponents of the “timing of initiation hypothesis” *has* supported the idea that a period of hypoestrogenicity negates the neuroprotection afforded by constant estrogen exposure. In particular, one study examining the “timing of initiation hypothesis” showed a significant difference between infarct volumes of mice supplemented with estrogen immediately following ovariectomy and a placebo-treated control group. A second comparison was made between mice undergoing a 10-week period of no estrogen exposure compared to a second control group, showing no significant differences in infarct volumes (Suzuki et al., 2007). The key comparison, however, was not presented in that study – namely, differences in infarct volumes between mice immediately supplemented with estrogen and those undergoing a period of hypoestrogenicity. By comparing each treatment group to a control (placebo-treated) group rather than to each other, no conclusions regarding the “timing of initiation hypothesis” can be made. Furthermore, these studies, among others, utilizing young animals cannot be used to predict the role estrogen plays in the aged brain.

Age, not timing, determines response to estrogen

Age is the likely variable accounting for the contrast between animal studies and clinical trial findings. The vast majority of basic science studies in neural injury, including studies on the effects of estrogen, utilize young animals. This research is based on the assumption that aging is a linear process, whereas studies on aging do not support that claim (Bishop et al.) Therefore, assessment of the response to neural injury in young or middle-aged rats cannot be used to extrapolate data to predict a post-injury response in an aged animal or, more importantly, in an aged patient. Together, these factors indicate that more studies on estrogenic effects on stroke, using clinically relevant animal models, are warranted. Evidence presented in this review suggests that estrogenic down regulation of the post-ischemic inflammatory response may contribute to worsened stroke injury by attenuating beneficial downstream anti-apoptotic and angiogenic pathway activation, which will be discussed more fully.

Estrogen receptors within the CNS

Estrogen mediates many of its actions in the periphery via two forms of the estrogen receptor (ER), ER α and ER β , encoded on chromosomes six and fourteen, respectively. There are at least three known ER α isoforms and five known ER β isoforms, with varying degrees of transactivation function (Ishunina et al., 2005). Some ER agonists bind preferentially to one form of the ER. Estrone binds with greater affinity to ER α , while estriol and reloxifene preferentially bind ER β . 17 β -estradiol binds equally well to both forms of the ER, thus is used as the most common form of estrogen supplementation in research. Upon activation, these receptors act as ligand-dependent transcription factors by either binding directly to estrogen response elements on target DNA sequences or by interacting with nuclear proteins to alter gene expression. Estrogen-induced genomic actions are well understood and occur over hours or days (McDonnell and Norris, 2002).

Neuroprotection by estrogen in young animals is thought to be partially mediated through ER α -dependent activity. ER α and ER β are constitutively expressed in cortical (Mannella and Brinton, 2006) and hippocampal (Mehra et al., 2005) neurons in addition to cells in many other brain regions, yet only ER α is upregulated in astrocytes and microglia following injury (Garcia-Ovejero et al., 2002). Estrogen-induced neuronal sparing from ischemia is abolished in ER α knockout mice compared to exacerbated infarcts in ER β knockout mice (Dubal et al., 2001). However, studies utilizing both the ER α and ER β knockout mice are likely confounded by the fact that ER β knockout mice have been shown to be in a state of systemic hypoxia (Morani et al., 2006). ER β is highly expressed in type I and type II pneumocytes and the deletion of this gene leads to fewer alveoli (Patrone et al., 2003) and reduced lung volume (Massaro and Massaro, 2006). In treadmill tests, ER β knockout mice are highly susceptible to ischemic injury compared with wild-type littermates (Morani et al., 2006), thus making this transgenic model less than ideal for the study of cerebral ischemia.

A G-protein coupled estrogen receptor (GPR30) has also been found, and cited as the possible mediator of rapidly occurring, nongenomic estrogenic action (Revankar et al., 2005). Specifically, estrogen has been shown to directly stimulate calcium mobilization, activate cAMP-mediated signaling, and extracellular signal-regulated kinase 1/2 (ERK1/2) activity in multiple cell types (Prossnitz et al., 2008). However, debate still exists regarding the role of GPR30. One study found that the receptor was not activated by radioactive estradiol nor did estradiol exposure lead to elevations in intracellular cAMP or calcium levels (Otto et al., 2008). Another hypothesis is that rapidly occurring, nongenomic actions of estrogen are mediated through membrane-bound ER. Some reports have shown evidence for an association between a membrane-bound ER and insulin-like growth factor I (IGF-I) receptors. Colocalization studies indicate that IGF-I is present in neurons expressing ER α and in neurons expressing ER β in hypothalamus, hippocampus, and cerebral cortex (Cardona-Gomez et al., 2000). Although these studies provide important information on ER-independent activity, to date a complete understanding of the mechanisms of estrogenic effects on the brain are still unclear, and warrant further investigation.

Estrogen receptors differ in the aged brain

Evidence of a differential effect of estrogen with age has been demonstrated in the brain as well as other tissues. Animal models show decreased nuclear ER concentrations in selected brain regions of middle-aged rats compared with young adult counterparts (Wise et al., 1984). Further investigation has shown differential response to estrogen supplementation with age in hypothalamus and pituitary, although it is unknown if the same age-related changes occur in other brain regions. Specifically, ovariectomized, young animals supplemented with estrogen showed increased ER expression, while middle-aged and aged animals had little or no increase in ER in cytosolic or nuclear fractions of hypothalamus and pituitary, despite similar increments in plasma estrogen levels in all age groups (Belisle et al., 1985).

More recently, post-mortem analysis of specific human brain regions has demonstrated age-related changes in levels of a certain ER α splice variant known as mamillary body, exon 1 (MB1) (Ishunina and Swaab, 2008). Discovered in 2005, this receptor protein variant lacks exon 1, giving it an expected four-fold decrease in transactivation function, which would alter its response to estrogen (Ishunina et al., 2005). Similarly, aging has been associated with reduced expression and activity of ER α -transactivation domain interacting protein, metastasis associated protein 1 (MTA1), an important regulator of histone deacetylation and transcriptional control (Thakur and Ghosh, 2009). These findings suggest that the aged brain may respond differently to circulating estrogen (Table 1.1). Although these studies do not provide a complete understanding of age-related differences in estrogenic activity in the brain, emerging evidence supports the idea that estrogen has differential effects depending on age in both health and following neurologic insult.

Estrogen produced endogenously by aromatase

Local production of estrogen by aromatase may play an important role in recovery from neural injury, particularly in postmenopausal females and males. Aromatase is a cytochrome P450 enzyme that converts testosterone to estradiol and androstenedione to estrone, and provides the only source of estrogen in males. Aromatase is bound to the endoplasmic reticulum and under physiological conditions is expressed at low levels in the brain after the perinatal period (Tsuruo et al., 1994). Studies have shown the presence of aromatase in both neurons (Cecchetti et al., 1992) and, following insult, in glia (Saldanha et al., 2005). Testosterone, administered before or immediately after penetrating brain lesion, has been shown to decrease expression of vimentin immunoreactive astrocytes in male orchidectomized rats (Barreto et al., 2007); however, this effect is likely due to a local conversion of testosterone to estrogen by aromatase. Both *in vitro* (Liu et al., 2008) and *in vivo* (McCullough et al., 2003) studies with male aromatase knockout mice and cell cultures from these transgenic animals, show greater cell death from

experimental neural injury compared to wild type counterparts. These reports suggest a significant role for the endogenously produced estrogen that arises in response to neural injury. Levels and activity of neural aromatase have not been reported in aged animals.

Estrogen reduces inflammation

Neuropoietic cytokines and the JAK/STAT pathway

Inflammation contributes to neural damage in ischemic injury models. Injury leads to increased nuclear factor κ B (NF κ B) activation, which causes induction of many mediators of inflammation. TBI causes elevation of interleukin (IL)-6, IL-1 β , and tumor necrosis factor- α (TNF- α). IL-1 is also a key component of the inflammatory response to neural injury and has been shown to cause neuronal cell death (Basu et al., 2004). IL-1 receptor-1 null mice subjected to neural injury demonstrate decreased edema, leukocyte infiltration, and overall cell death as well as decreased astrocytic reactivity (Lin et al., 2006), indicating that IL-1 receptor activation may be detrimental to recovery from neural injury.

Yet, not all inflammatory factors have adverse effects. In some types of neural injury, such as cerebral ischemia, the inflammatory response contributes to the induction of genes that aid in microvessel regrowth and the salvage of cells through activation of anti-apoptotic pathways (Dziennis et al., 2007). Specifically, IL-6 and other neuropoietic cytokines act on type I cytokine receptors, composed of a ligand-binding domain, CD126, and a signal-transducing domain, glycoprotein 130 (gp130). Intracellular regions of the transmembrane gp130 molecule are coupled to the Janus kinase (JAK)/signaling transducer and activator of transcription (STAT) pathway – most notably STAT3 (Hirano et al., 2000). STAT3 is activated by phosphorylation on a tyrosine residue on its C terminus. Upon phosphorylation, STAT3 forms a hetero- or homodimer and translocates to the nucleus, where it activates transcription of target genes, including proteins that are involved in cell survival and proliferation, such as *bcl-2*, *bcl-xL*, *mcl-1*, *Fas*, *survivin*, *cyclin D1*, *cyclin E1*, and *p21* (Hodge et al., 2005). In addition, other transcription factors,

including *c-myc*, *c-jun*, and *c-fos*, are STAT3 targets (Yang et al., 2003). Vascular endothelial growth factor and brain derived neurotrophic factor have also been shown to be targets of STAT3 and contribute to angiogenesis (Xu et al., 2005).

Interleukin-6 signaling pathways, specifically JAK2/STAT3, play a major role in modulating the complex relationship between aging and pathology. Research demonstrates that not all actions mediated by IL-6 are beneficial. Elevated CNS levels of IL-6 leads to increased astrogliosis and BBB permeability and IL-6 activation of the JAK2/STAT3 pathway contributes to neuronal loss after transient focal cerebral ischemia (Satriotomo et al., 2006). Data from our lab indicate that dysfunction of the JAK2/STAT3 pathway plays a significant role in the increased injury and decreased functional recovery observed in studies of ischemic injury in aged animals. Specifically, during the acute phase after injury, JAK2/STAT3 signaling contributes to a robust pro-inflammatory response that leads to an early, more severe BBB disruption. As the injury response continues, JAK2/STAT3 signaling is attenuated by desensitization or increased negative regulation by the suppressor of cytokine signaling 3 (SOCS3) resulting in decreased angiogenesis and increased apoptosis (DiNapoli et al.). Data from clinical studies confirm that elevated plasma IL-6 is predictive of greater stroke severity and worsened functional outcome in older adults (Nakase et al., 2008) indicating a connection between the inflammatory response, stroke severity, and aging. However, studies on aging show that STAT3 expression is decreased in the rodent brain with increasing age. The counterpart of STAT3, known as STAT1, is associated with detrimental effects following neural injury, and remains unchanged with age (De-Fraja et al., 2000). Studies have shown that estrogen increases activation of STAT3 in young adult rats after ischemic injury (Dziennis et al., 2007), but it is unknown if this occurs in aged rats following neural insult. Other studies indicate that estrogen also increases inhibitors of the JAK2/STAT3 pathway (Wong et al., 2003). Figure 1.2 indicates known effects of estrogen on gp130 signaling through JAK2/STAT3 pathway. It is unknown what the ultimate results of estrogenic actions are on STAT3 activation in aged animals following neural injury.

Other neuropoietic cytokines also utilize gp130 and activate STAT3. Leukemia inhibitory factor (LIF) and oncostatin M (OsM) are two neuropoietic cytokines of particular interest in the injured brain. LIF has a myriad of actions throughout the body including enhancing migration of inflammatory cells to damaged neuronal tissue (Sugiura et al., 2000). During mammary gland involution, LIF induces expression of the two phosphatidylinositol-3-kinase (PI3K) regulatory subunits p50 and p55 via STAT3, resulting in diminished levels of Akt activity and eventually leading to apoptosis (Abell et al., 2005). OsM reduces excitotoxic damage like that which occurs in the peri-infarct region following ischemia/reperfusion injury (Weiss et al., 2006) and activates STAT3 to induce apoptosis and cell cycle arrest in certain tumor cells. Data from our lab suggest that aged rats have a more robust post-ischemic increase in LIF, OsM and SOCS3 compared to young adult rats, indicating a potential role of these neuropoietic cytokines and SOCS3-mediated inhibition of STAT3 in the worsened recovery from experimental stroke observed in aged animals (DiNapoli et al.).

Estrogen attenuates inflammation in vivo and in vitro

Estrogen plays a significant role in regulation of inflammatory pathways (Maggi et al., 2004). In young animals treated with estrogen prior to middle cerebral artery occlusion (MCAO), there are reductions in IL-6 and monocyte chemoattractant protein-1 (MCP-1) (Suzuki et al., 2007). Further evidence of estrogen-mediated attenuation of neuroinflammation indicates specific estrogenic effects on microglia following exposure to lipopolysaccharide (LPS) in female ovariectomized rats (Vegeto et al., 2006). It is unknown if such a reduction in microglia reactivity occurs in aged animals following neural injury. The anti-inflammatory action of estrogen is also supported in cell culture models of neural injury. Primary rat microglia cell lines exposed to LPS show reduced microglial superoxide release and phagocytic activity when pre-treated with estrogen. These effects are abolished by both ER inhibitor and mitogen-activated protein kinase (MAPK) inhibitor (Bruce-Keller et al., 2000). However, no effect is seen in NFkB levels, sug-

gesting that estrogen-induced anti-inflammatory actions in microglia are likely dependent on ER-mediated activation of MAPK. MAPK promotes cell survival through cAMP response element binding protein (CREB) transcription pathways.

Therefore, the ability of estrogen to attenuate inflammation may only occur in young animals, or may occur to a lesser extent or by different mechanisms in aged animals. In aged brains, the inflammatory response may serve a vital purpose that becomes weakened by anti-inflammatory activity of estrogen. Following ischemic injury, gp130-mediated upregulation of angiogenic and anti-apoptotic factors serves to protect the peri-infarct region from cell death and aid in regrowth of microvessels to the damaged area. If these processes are inhibited by estrogen in aged animals, perhaps due to differential expression of ER α splice variants and their responsiveness to circulating estrogen, then exacerbated injury may result. Further research is needed to fully characterize the role estrogen plays in the inflammatory response following neural injury in the aged brain.

Estrogen attenuates reactive gliosis

Reactive astrocytes undergo gliosis following neural injury, which involves a continuum of specific molecular and morphological changes. Transformation of astrocytes to reactive phenotype is characterized by increased expression of glial fibrillary acidic protein (GFAP) and vimentin (Stoll et al., 1998). Increased expression of GFAP protein and mRNA is well documented following cerebral ischemia (Petito et al., 1990, Petito and Halaby, 1993). In patient studies of ischemic stroke, there is severe reactive astrogliosis accompanied by proliferation of astrocytes forming a glial scar at the infarct border. Quantitative and qualitative analysis has shown GFAP expression to be a biomarker of neuronal injury, which is both sensitive and specific (O'Callaghan and Sriram, 2005). Induction of GFAP in reactive astrocytes following MCAO provides a powerful tool in assessing glial reaction to ischemic injury.

Basal levels of GFAP mRNA and protein are elevated in both the human and rodent brain with increased age. Constitutive expression of GFAP mRNA is increased in 24 month old rodents, as well as in humans age 60-79, in the hippocampus, frontal and temporal cortices (Nichols et al., 1993). GFAP protein has been shown to be elevated in all brain regions, and is associated with an age-linked increase in the number hypertrophic astrocytes related to accumulation of glial filaments (Scheff et al., 1980, Hansen et al., 1987, O'Callaghan and Miller, 1991). Evidence also exists for increased levels of neurofilament protein and gene expression in aged animals following transient focal ischemia (Schroeder et al., 2003). Glial activity is augmented in the aging brain. Time course of astroglial and microglial activation, as well as glial scarring is abnormally accelerated in normal aged rodents (Badan et al., 2003b). This irregular activity has correlated to impairment in functional recovery following MCAO. The expression of neurofilament (GFAP) mRNA and protein has been shown to be decreased in the penumbra of aged animals compared to young adults following MCAO. Although some evidence suggests that glial scar formation serves to limit and contain post-ischemic neuronal death, gliosis has also been shown to inhibit axonal regeneration and remyelination thereby hindering recovery from injury (Rosen et al., 1989). Likewise, astrocytes release a host of pro-inflammatory cytokines that may contribute to neuronal damage.

Estrogen may also exert effects on astrocytes and their response to neural injury. Studies have shown that astrocytes express both nuclear ER as well as membrane-associated ER (Kuo et al., 2009). Several *in vivo* models of neural injury, including penetrating brain lesion, excitotoxic-induced neurodegeneration, and cholinergic basal forebrain lesion demonstrate estrogen-mediated reduction in GFAP and vimentin expression, indicating estrogenic attenuation of post-injury activation of astrocytes (Garcia-Ovejero et al., 2002, Djebaili et al., 2005). This activity appears to be preserved with age, as chronic estrogen treatment also attenuates age-related astrocyte activation in hippocampus of 20-month old mice (Lei et al., 2003).

Estrogenic effects on microglia

Microglia comprise between 5% and 15% of all brain cells. Their role is primarily immunologic as evidenced by their macrophage-like properties. The actions of microglia were first described by del Rio Hortega in 1932 in experiments involving brain injury. In those studies, del Rio Hortega described microglia migration to the site of injury, morphologic changes, proliferation and phagocytic activity (del Rio-Hortega, 1993). In homeostasis, microglia exhibit a ramified morphology, while in reaction to injury they become ameboid often containing phagocytosed cellular debris (Giulian and Baker, 1986, Lin et al., 1998). Ramified microglia act in surveillance fashion, continuously sampling the extracellular microenvironment (Kreutzberg, 1996). The role of microglia in ischemia is multifaceted (Lees, 1993). Microglia activation is initiated within minutes following ischemic onset noted by the expression of distinct cell surface molecules, notably MHC-I and MHC-II. Following ischemia, microglia release cytokines, chemokines and other neurotoxic agents, which are thought to exacerbate neuronal injury and instigate inflammation. Specifically, the cytokines IL-1, MCP-1 and TGF β -1 are released following ischemic (Gehrmann et al., 1992, Banati and Kreutzberg, 1993, Gehrmann et al., 1995). Expression of MCP-1 in microglia seems to be a sensitive and early response to CNS insult, even in the absence of elevated cytokines (Little et al., 2002). Microglia expression of MCP-1 peaks at around 2 weeks following ischemia, coinciding with peak astrogliosis (Lin et al., 1998).

Cytokines and chemokines released by microglia, including MCP-1 have been shown to regulate, at least in part, astroglial scarring and can impede re-establishment of neuronal axons and synapses. Some studies suggest that suppression of microglia improves recovery from cerebral ischemia (Giulian and Baker, 1985). However, evidence also exists for a neuroprotective role of microglia. They also participate in release of beneficial growth factors and are responsible for scavenging cellular debris (O'Callaghan and Sriram, 2005). Microglial expression of os-

teopontin by microglia occurs at approximately 24 hours following ischemia and has been thought to define the peri-infarct region. This new glial-pial boundary may play a role in healing of the brain, perhaps through limiting spread of excitotoxic glutamate released from dying cells (Ellison et al., 1998). Microglial response to stroke is accelerated in aged animals. The microglia response in aged animals at 24 hours correlates well to that seen in young adult animals as late as 2 to 4 weeks following insult (Badan et al., 2003b).

Estrogenic effects on microglia have been documented in health and disease. Estrogen has been shown to inhibit microglia proliferation *in vitro*. Microglial cell cultures treated with estrogen also have reduced basal levels of TNF- α and reduced TNF- α following exposure to LPS (Dimayuga et al., 2005). However, in animal models using N-methyl-D-aspartic acid (NMDA)-induced cytotoxic injury, the effects on IL-1 β expression are dependent on age and hormonal status (Nordell et al., 2003). The same inhibitory effect of estrogen has been found in animals administered LPS intracerebroventricularly (Vegeto E, Belcredito S 2003). Evidence is accumulating that this anti-inflammatory effect of estrogen is mediated through ER α . Specifically, ER α activation by E2 has been shown to inhibit the transport of nuclear factor κ B to the nucleus where it acts as a transcription factor of genes involved in the inflammatory response.

Estrogen affects the blood-brain barrier

The BBB is a functional element of the 'neurovascular unit,' an extensive network comprised of endothelium, glia, pericytes, neurons, and extracellular matrix. The BBB partitions the systemic circulation from brain parenchyma and serves to establish, maintain, and regulate discrete microenvironments within the brain for optimal neuronal function (Huber et al., 2001). The neurovascular unit undergoes a number of morphological and functional changes during the aging process that make the BBB more vulnerable to insult (DiNapoli et al., 2008).

Following injury, there are two principle mechanisms by which brain edema develops: cytotoxic and vasogenic. Cytotoxic edema results from a loss of ionic homeostasis and can occur as astrocytes increase glutamate uptake in an attempt to limit excitotoxicity, which requires glucose utilization and leads to intracellular water and sodium accumulation. Neurons can undergo cytotoxic swelling when ionic exchangers dysfunction or as part of the metabolic consequences of excitotoxicity. Vasogenic edema occurs when there is a breakdown in the tight junctions between the endothelial cells of the BBB or upregulation of water transport channels in perivascular cells, allowing fluid to enter the brain parenchyma. While relatively little is known about the specific mechanisms by which estrogen attenuates post-injury edema, evidence suggests it inhibits BBB breakdown following neural insult (Cipolla et al., 2009).

Estrogen inhibits post-injury edema via multiple mechanisms

Some evidence suggests that estrogen-mediated attenuation of BBB breakdown may be related to inhibition of matrix metalloproteinases (MMPs), the enzymes responsible for extracellular matrix remodeling (Liu et al., 2005). Although the effects of estrogen on the family of MMPs have long been studied in regards to reproductive physiology as well as pathologies of the endometrium, recent research has focused on the effects of estrogen on centrally located MMPs, particularly in relation to BBB disruption. This research has led to potential mechanisms by which estrogen helps maintain functional integrity of the BBB following insult. In particular, estrogen has been shown to significantly decrease Evans blue extravasation into the brain (Liu et al., 2005), a common assay for assessment of BBB disruption. Evans blue complexes to albumin making it a 68kDa marker, the passage of which through the BBB indicates marked disruption. More subtle changes are better assayed with dextrans (10-60kDa), inulin (5kDa), or sodium fluorescein (376 Da), but the effects of estrogen on BBB permeability to those markers has not been measured to date. Estrogen also decreases local MMP2 and MMP9 activity following ischemia/reperfusion injury (Liu et al., 2005). By inhibiting the enzymes responsible for injury-

induced BBB breakdown, estrogen indirectly preserves BBB integrity. Several reports have also indicated that estrogen can modulate injury-induced ROS generation (Behl et al., 1997, Culmsee et al., 1999), which may contribute to the maintenance of BBB functional integrity following insult. Since ROS contribute to BBB breakdown through lipid membrane peroxidation, attenuation of ROS production should protect BBB integrity. F(2)-isoprostanes result from free radical oxidation of arachidonic acid and are lower in cerebrospinal fluid from female patients with severe TBI than in male counterparts (Bayir et al., 2004), thus, antioxidant activity may account for estrogen-mediated protection of the BBB. Estrogen decreases ROS-related cell death through a nongenomic mechanism of action in organotypic hippocampal slice cultures (Behl et al., 1997). Alternatively, this BBB-sparing effect may be attributable to antioxidant capacity of progesterone, which decreases F(2)-isoprostanes *in vitro* (Roof et al., 1997).

In addition to the mechanisms by which estrogen inhibits BBB disruption, the hormone is also involved in formation and clearance of the subsequent edema, through its regulation of the major water transport channel in the brain, aquaporin 4 (AQP4). In LPS-induced BBB disruption models, estrogen attenuates the induction of AQP4 in perivascular glial cell processes, thereby decreasing the associated edema (Tomas-Camardiel et al., 2005). Together, these studies demonstrate that estrogen has potential use in the acute treatment of neural injury. Some studies on estrogenic neuroprotection in young animal models have used acute treatment after neural insult and found promising results (Liu et al., 2005, Lebesgue et al., 2009). In terms of treatment for stroke, however, aged animal models must also have a place in validating these results.

Estrogen receptors in cerebral vasculature

Following TBI, signs of post-injury neuronal hypoxia, including hypoperfusion and jugular venous desaturation have been observed. Estrogen may have a role in reducing injury-induced hypoxia through actions on cerebral vasculature, as the vasorelaxing property of estrogen has

been demonstrated. Middle cerebral arteries isolated from female ovariectomized rats supplemented with estrogen show greater increase in diameter in response to elevated transmural pressure compared to untreated and male rats; yet, differences in vascular responsiveness between groups are abolished by removal of the vascular endothelium or by inhibition of both nitric oxide synthase (NOS) and cyclooxygenase (Geary et al., 2000). These studies and others suggest that activation of ER α leads to cyclooxygenase-1 (COX-1) induction of prostacyclin (Geary et al., 2000) and enhanced nitric oxide (NO) synthesis in cerebral vasculature (Stirone et al., 2005a) (Figure 1.3). Cerebral blood vessels isolated from estrogen-treated rats have increased COX-1, prostacyclin synthase and prostacyclin expression (Ospina et al., 2002). Prostacyclin is a prostanoid product of COX-1 that acts on both platelets and endothelial cells and activates the protein kinase A pathway, resulting in smooth muscle relaxation and subsequent vasodilation.

Estrogen-treated animals also show elevated levels of endothelial-derived NOS (eNOS) mRNA and protein suggesting a role for eNOS-dependent vasodilation (Stirone et al., 2003). This mechanism of estrogen-mediated vasodilation is further supported by studies using mouse cerebral arteries that demonstrate a reversal of estrogen-mediated changes in vascular tone when eNOS is inhibited (Geary et al., 2000) (Figure 1.3). Similar results are found in cultured cerebral vessels, where eNOS levels are significantly depressed by ER antagonist treatment (McNeill et al., 2002). Modulation in local eNOS levels may be the predominant vascular effect of estrogen. Evidence from eNOS knockout mice show elevated COX-1 and prostacyclin compared to control, estrogen-treated animals, although the vascular responses in both groups are similar (Li et al., 2004). However, in studies using young adult female rats, ER α immunoreactivity is not only present in vascular smooth muscle cells; rather, ER α immunoreactivity is also found in the nucleus, mitochondria and on the membrane of endothelial cells (Stirone et al., 2005b). Any potential differences in the effects of estrogen on cerebral blood vessels in the aged brain have not been elucidated, but may be an interesting area for future investigation due

to the vasogenic actions of estrogen and the known age-related changes in cerebral vasculature.

Effects of estrogen on excitotoxicity

Most data regarding the effects of estrogen on excitotoxicity rely on cell culture models. Evidence from these models suggest that estrogen affects the extent of excitotoxic cell death by modulating glutamate-induced cellular influx of calcium (Ciriza et al., 2004, Numakawa et al., 2007). Research demonstrates that estrogen acts directly on NMDA receptors to enhance excitatory amino acid activity (Smith, 1989). which would enhance glutamate toxicity; however, in cultured hippocampal neurons, estrogen can decrease excitotoxicity (Goodman et al., 1996). Other evidence indicates that estrogen down-regulates ionotropic glutamate receptor subunits in neurons, which would attenuate the effects of excess extracellular glutamate like that which occurs following neural injury (Numakawa et al., 2007). Estrogen also acts to increase glutamate uptake by astrocytes, thus limiting excitotoxic insult to neurons (Liang et al., 2002), although this effect was observed in cultured human astrocytes of Alzheimer's disease patients, so it is unknown if the same response to estrogen would occur in acute neural injury. Together, these studies suggest that estrogenic inhibition of excitotoxicity does not play a key role in its post-injury actions.

Estrogen modulates apoptotic signaling pathways

Following direct, excitotoxic, oxidative, or hypoxic injury, cells in the brain parenchyma may undergo apoptosis or necrosis. Research has shown that estrogen inhibits apoptotic cell death following neural injury in young animal models. Specifically, estrogen activates MAPK by triggering calcium influx through L-type calcium channels (Cheng et al., 2001) and increases levels of activated bcl-2 present in the nucleus of neurons in the peri-ischemic area of young adult rats subjected to focal cerebral ischemia (Dziennis et al., 2007). Further evidence of neu-

ronal sparing by estrogen comes from immortalized hippocampal cell lines, which show rapid estrogen-induced CREB phosphorylation that is blocked by both ER inhibitors and MAPK inhibitors (Wade and Dorsa, 2003).

Estrogen also modulates levels of IGF-1 (Cheng et al., 2001), which is a potent activator of the Akt signaling pathway. Akt inhibits apoptosis by inactivating pro-apoptotic proteins such as bcl-xl/bcl-2-associated death promoter (BAD) and activating NFkB for transcription of survival-promoting genes. Studies demonstrate estrogenic activation of Akt and the family of MAPK known as ERK1/2 independent of IGF-1 signaling (Mannella and Brinton, 2006), although estrogen-mediated increases in IGF-1 may bolster this response. Furthermore, estrogen activates the PI3K signaling cascade through an ER-mediated binding with PI3K regulator, p85 (Mannella and Brinton, 2006). The interaction between ER and p85 leads to activation of both Akt and ERK1/2 in cortical neurons in a time-dependent manner (Mannella and Brinton, 2006). Overall, estrogenic effects on apoptotic pathways are complex, a complete understanding of which has yet to be elucidated.

Estrogen and the erythropoietin/erythropoietin-receptor system

Like estrogen, the role of erythropoietin (EPO) was thought to be neuroprotective, which was supported by numerous studies in animal models of several types of neural injury (Bernaudin et al., 1999, Genc et al., 2004, Zhang et al., 2006a, Gonzalez et al., 2007, Xiong et al., 2009). Initial small-scale clinical trials of EPO for the treatment of acute stroke seemed promising (Ehrenreich et al., 2002). Then, in 2009, EPO was used in a larger clinical trial and EPO treatment was not associated with favorable outcome. In fact, patients receiving EPO had a significantly higher death rate compared to those receiving placebo (Ehrenreich et al., 2009). Estrogen is a known modulator of both EPO and its receptor, and the interaction between these two systems may have an important role in the effects of estrogen in the brain. As the mecha-

nism of estrogenic action in the brain remains unclear, estrogenic effects on the EPO/EPO-R system is a prime candidate for further investigation.

EPO is a glycoprotein well known for its effects on erythropoiesis. The *EPO* gene is encoded on chromosome 7 and consists of five exons and four introns. EPO protein is a 165-amino acid peptide with four glycosylation sites, which serve to regulate the biological half-life in the blood. Its biological activity also relies on two disulfide bonds between cysteine residues (Chong et al., 2002). EPO is primarily produced by the peritubular capillary endothelial cells of the kidney; however, some production comes from the liver as well, with a larger contribution from the liver during fetal development. Previously, the actions of EPO were thought to be limited to erythroid progenitor cells where it binds to surface receptors (EPO-R) and acts to inhibit apoptosis. The intracellular cascades involved in the effects of EPO on erythroid progenitor cells include Janus kinase 2 (JAK2)/signal transducer and activator of transcription 5 (STAT5), Akt/NFκB, and Ras. The overall effect of EPO is to inhibit caspase-induced apoptosis (Somerville et al., 2001). The EPO/EPO-R system has been shown to be vital to life as EPO-R knockout mice and EPO knockout mice both die in utero due to severe anemia (Lin et al., 1996). The timing of EPO-R expression in erythropoiesis is very specific and decreases as erythroid cells mature. In recent years, the presence of EPO and EPO-R in other organ systems has been explored.

EPO and its receptor in the brain

Evidence is now accumulating for additional actions of EPO within other organ systems including the central nervous system. EPO and EPO-R mRNA have been isolated from the brains of humans, nonhuman primates, and rodents (Digicaylioglu et al., 1995, Marti et al., 1996). Specifically, immunoreactive EPO has been found in the cortex and hippocampus of non-diseased human and mouse brain (Bernaudin et al., 1999, Siren et al., 2001a). The ability of peripheral or exogenous EPO to cross the BBB has been a topic of debate. In the healthy

brain, EPO is thought to cross the BBB at a very slow rate similar to albumin, with brain uptake of EPO measured at 0.05% to 0.1% of the injected dose per gram of brain tissue (Banks et al., 2004). When administered intravenously, the increase in EPO in CSF occurs approximately 3 h after administration. EPO transport across the BBB may occur via a first order transmembrane or non-receptor mediated mechanism as it follows first-order kinetics (Xenocostas et al., 2005). Healthy and schizophrenic patients administered radio-labeled EPO intravenously show definitive evidence of EPO's ability to cross the BBB, with peak EPO levels occurring at 4 hours after administration (Ehrenreich et al., 2004).

There is also accumulating evidence that exogenous and peripheral EPO cross the BBB in even greater amounts in states of CNS injury. In CSF of traumatically brain-injured patients, EPO is increased, and the degree of its increase correlates with severity of BBB disruption. In studies of normal healthy individuals, EPO has also been detected in the CSF of adults and neonates not administered exogenous EPO, suggesting a role for brain-derived EPO in normal brain function and development (Marti et al., 1997). Further evidence of brain-derived EPO comes from patient studies demonstrating EPO levels in CSF that do not correlate with serum EPO levels. Reports suggest that brain-derived EPO comes from multiple cellular sources. Astrocytes and neurons from mice have been found to produce EPO (Masuda et al., 1994, Bernaudin et al., 1999) while cultured oligodendrocytes from embryonic rat brains, but not mature brains, also produce EPO (Sugawa et al., 2002). Together, these studies provide evidence that EPO production in the brain has a specific role in development and in normal brain function. In addition, peripheral or exogenous EPO can and does cross the BBB in states of health and disease. The mechanisms by which EPO regulate neural development and its role in the diseased brain remain unclear, however, specific cell types involved in EPO production and that express EPO-R have been suggested.

EPO-R is a type I cytokine receptor lacking intrinsic tyrosine kinase ability. EPO-R contains a single transmembrane domain and several cytoplasmic domains, which are associated

with JAK2. JAK2 provides tyrosine kinase activity that occurs following EPO binding to an EPO-R homodimer and conformational change of the EPO-R homodimer. Phosphorylation of EPO-R by JAK2 results in signal transduction through STAT5, Akt, MAPK and other pathways. Evidence for the importance of EPO-R in normal development and physiology is abundant. In mice, high levels of EPO-R are expressed in the neural tube, peaking at E10.5. By birth, EPO-R expression is markedly reduced and remains at low levels into adulthood. Human studies show similar results with EPO and EPO-R expression occurring between about 7 and 24 weeks of fetal development (Juul et al., 1998a, Juul et al., 1998b). Structural changes also occur in the brains of EPO and EPO-R knockout mice. Prior to death due to severe anemia, EPO and EPO-R knockout mice exhibit thinner neuroepithelium, reduced brain size, and hypoplasia of the fourth ventricle compared to wild-type littermates (Yu et al., 2002). Neuronal cultures from these knockout mice also demonstrate attenuated proliferation and increased sensitivity to hypoxia (Liu et al., 1997).

Together, these studies demonstrate a role of the EPO/EPO-R system in normal brain development, some details of which have yet to be elucidated. EPO-R expression has been found on multiple cell types within the CNS. Cultured neurons have been found to contain EPO-R mRNA as well as EPO-R protein (Masuda et al., 1993) as well as astrocytes (Bernaudin et al., 1999). Interestingly, immunohistochemical analysis has revealed that astrocytic processes surrounding brain capillaries strongly express the receptor. That same study demonstrated EPO-R immunoreactivity localized within endothelial cells as well (Brines et al., 2000).

Regulation of EPO and EPO-R in hypoxia/ischemia

EPO and EPO-R have been shown to be upregulated in hypoxic conditions *in vitro* and *in vivo* (Tan and Ratcliffe, 1992, Marti et al., 1996). In the brain, EPO mRNA levels have been shown to increase up to 20-fold during hypoxic exposure. Although this is less than the reported 200-fold increase in EPO mRNA increase in the kidney during hypoxia, it is thought to have

physiologic relevance (Marti et al., 1996). In a study of human brain autopsy specimens, data suggests an upregulation of EPO and EPO-R following ischemic infarct or hypoxic damage in the aged human brain as well (Siren et al., 2001b). Cell culture has been used to determine which cell types are responsible for the upregulation of EPO in hypoxia. Primary mouse astrocytes show a 100-fold increase in EPO expression following hypoxia (Marti et al., 1996, Bernaudin et al., 2000). EPO-R mRNA expression also increases in response to hypoxic conditions as demonstrated in cultured hippocampal neurons (Chin et al., 2000, Lewczuk et al., 2000).

Controversy exists regarding the exact mechanism of EPO and EPO-R upregulation in states of hypoxia. Most studies support a mechanism involving hypoxia-inducible factor 1 (HIF-1) transcription of *EPO* gene (Wenger, 2000), although other evidence exists for a HIF-2 mechanism (Rankin et al., 2007). HIF-1 is a highly conserved transcription factor composed of α and β subunits. HIF-1 β is constitutively expressed in the nucleus, while the α -subunit is hydroxylated at proline residues facilitating binding of the von Hippel-Lindau protein, ubiquitination, and subsequent proteasomal degradation in normoxic conditions. During hypoxia, lack of oxygen inhibits the HIF prolyl-hydroxylase due to its use of oxygen as a co-substrate, and ubiquitination and degradation of HIF-1 α does not occur. When released from degradation, HIF-1 α is free to form a heterodimer with HIF-1 β , which then binds to specific DNA sequences located in hypoxia response elements. HIF-1 gene targets include genes involved in angiogenesis, such as VEGF, vasomotor control, apoptosis, and erythropoiesis. *EPO* was the first target gene of HIF-1 to be described, and, as evidence suggests, remains an important gene target during conditions of reduced oxygen (Wang and Semenza, 1993).

Neuroprotection by EPO in animal models

The EPO/EPO-R system has only recently been implicated in the response of the brain to ischemia. With the discovery of the human gene for EPO by Lin et al. in 1985, the doors were opened for production of EPO for research and clinical purposes. In 1989 the FDA approved the

use of EPO (Epogen) for anemia of chronic disease. The first studies describing the neuroprotective affect of EPO in animal models of cerebral ischemia utilized permanent occlusion of the middle cerebral artery in stroke-prone spontaneously hypertensive rats (Sadamoto et al., 1998). The rats were intracerebrally infused with epoietin alpha for 28 days following injury and demonstrated an attenuation of post-ischemic navigational disability as well as a reduction in infarct volume. Since that initial investigation, the research on EPO as a neuroprotective agent has increased greatly. Models of experimental stroke as diverse as devascularization (Kolb et al., 2007) and thromboembolic occlusion (Wang et al., 2004), in both permanent (Faure et al., 2006) and transient (Esneault et al., 2008) ischemia have all reported neuroprotective effects of EPO. Neuroprotection has been measured by both reduction in infarct volume (Belayev et al., 2009) as well as improvement in post-ischemic neurobehavioral assessments (Fletcher et al., 2009). Likewise, the method of administration and the use of EPO analogs have also been explored. Intraperitoneal (Li et al., 2009), intracerebroventricular (Kolb et al., 2007), intravenous (Villa et al., 2007), and intranasal (Fletcher et al., 2009) administration all provide neuroprotection. EPO analogs, including non-hematopoietically active ones, have also been shown to reduce infarct (Erbayraktar et al., 2003) and improve post-ischemic functional recovery (Villa et al., 2007). One study determined the time window for the neuroprotection by EPO by administering EPO 24 h before, simultaneously with, 3, 6, and 9 h after ischemic insult and reported neuroprotection with EPO administration up to 6 h after experimental stroke (Brines et al., 2000).

Despite the abundance of preclinical studies showing neuroprotection by EPO, the mechanism by which it acts is not fully understood. One leading hypothesis is that EPO acts to inhibit apoptosis in the injured brain, much like its role in erythropoiesis (Villa et al., 2003). Immunohistochemical analysis has demonstrated an EPO-mediated attenuation of programmed cell death in rats (Siren et al., 2001a) and gerbils (Sakanaka et al., 1998) following experimental stroke. Specific mechanisms of apoptosis inhibition that have been elucidated include EPO-

mediated expression of anti-apoptotic genes *bcl-2* and *bcl-xL*. Likewise, EPO has been shown to increase expression of *XIAP* and *c-IAP2*, inhibitors of apoptosis genes, in cortical neurons (Digicaylioglu and Lipton, 2001). Upon binding of EPO to EPO-R, JAK2 phosphorylation leads to phosphorylation and activation of PI3K. EPO-mediated activation of the PI3K/Akt pathway serves to inhibit apoptosis by maintaining mitochondrial membrane potential by inhibiting the release of cytochrome C and by regulating caspase activity (Chong et al., 2003). Activation of Akt also serves to maintain inhibitory phosphorylation of the pro-apoptotic fork head transcription factor (FOXO3a), attenuating its ability to activate transcription of apoptotic genes (Mahmud et al., 2002). In addition to its role in inhibiting post-ischemic apoptosis, EPO may also play a role in reducing the excitotoxic damage that results in the aftermath of the initial cell death. *In vitro* studies in which cell death was triggered by glutamate receptor agonists showed that EPO protects neuronal cells from excitotoxicity (Bernaudin et al., 1999, Siren et al., 2001a). Cerebellar granule neurons exposed to hypoxia were also protected by EPO which inhibited calcium-induced glutamate release (Kawakami et al., 2001).

Further evidence of the effects of EPO on calcium regulation suggest a potential role of this mechanism in EPO-mediated neuroprotection. *In vitro* studies suggest that EPO may regulate calcium flux in neurons by plasma membrane T-type voltage dependent calcium channels (Kawakami et al., 2001). In rat striatal slices, EPO has been shown to stimulate dopamine release and enhance potassium-induced acetylcholine release (Yamamoto et al., 2000), while EPO is also associated with increased intracellular concentration of monoamines in a pheochromocytoma cell line (Masuda et al., 1993). Together, these studies suggest that EPO may act to stimulate neuronal function, perhaps in the subacute phase of recovery from injury, by enhancing calcium channel activation and release of neurotransmitters, although more definitive studies, including *in vivo* experiments, are needed.

Clinical trials of EPO for the treatment of ischemic stroke

Due to the success of EPO in preclinical models of stroke, EPO moved into clinical trials for the treatment of acute stroke. The first report to be released came from a clinical trial conducted in Germany and was published in 2002. In that initial proof-of-concept trial, researchers demonstrated that intravenously administered EPO entered the brain of stroke patients and EPO treatment was associated with improvement at 30 days in neurologic scales including the NIH Stroke Scale, the Scandinavian Stroke Scale, the modified Rankin scale, and the Barthel Index (Ehrenreich et al., 2002). Importantly, patients receiving EPO did not show increases in hematocrit, a side effect that would increase risk of re-infarction. A larger clinical trial began in 2003 and enrolled 522 patients by 2008. Results from this clinical trial were published in 2009 and did not demonstrate any beneficial effect of EPO as measured by the 90-day Barthel Index. In addition, the mortality in EPO treated patients was 16.4% compared to 9.0% in placebo-treated patients (CI 1.16 to 3.38, $p=0.01$) (Ehrenreich et al., 2009). The negative result from this clinical trial may be attributed to an unknown interaction between tPA and EPO as a high percentage (63.4%) of study participants received tPA. Before further clinical trials are conducted, a better understanding of EPO's action in the ischemic brain must be elucidated, including potential interactions with current standard of care, namely, tPA.

EPO and estrogen

One possible mechanism by which estrogen worsens stroke outcome is by attenuating the hypoxia-induced increases in *EPO* gene expression. It has long been known that gonadal hormones affect erythropoiesis with estrogen having a dampening effect and testosterone increasing hematocrit (Piliero et al., 1968). Reports as early as 1972 suggested that estrogen regulates both basal and hypoxia-induced increases in erythropoietin (Peschle et al., 1972). More recent studies also demonstrate that estrogen diminishes plasma EPO levels (Wang et al., 1998) as well as the hypoxia-induced elevation of both EPO and HIF-1 α in cell culture (Mukundan et al., 2004). Furthermore, this attenuation is abolished by pre-treatment with the ER inhibi-

tor ICI 182,780 indicating a receptor-mediated mechanism. Western blot analysis of nuclear HIF-1 α suggests that estrogen may decrease hypoxia-induced EPO expression by inhibiting expression and activity of HIF-1 α (Mukundan et al., 2004). In light of recent findings of the deleterious effects of estrogen on cerebral ischemia in postmenopausal women, the estrogenic effects on the EPO/EPO-R system may be a potential mechanism to explain those clinical data, and thus warrant further investigation.

Conclusion

The idea of estrogen as a neuroprotectant first emerged with evidence that premenopausal females experience less damage and greater functional and cognitive recovery from neurologic insult than males, with this disparity disappearing around the age of menopause. In hypoxia/ischemia models of neural injury, many studies have shown estrogen to be neuroprotective (Simpkins et al., 1997, Toung et al., 1998, Culmsee et al., 1999, Alkayed et al., 2000) with an equal number of mechanisms postulated to explain these results. Estrogen attenuates BBB disruption from neurologic insult, reduces edema, lowers levels of inflammatory mediators (Maggi et al., 2004), activates anti-apoptotic pathways (Dziennis et al., 2007), and has antioxidant capabilities (Behl et al., 1997) in young animals and cell culture models. Some of these actions are receptor-mediated while others have nongenomic mechanisms.

However, many questions regarding the mechanisms by which estrogen acts remain unanswered. Specifically, why is estrogen neuroprotective in young animals, but not in postmenopausal women? An estimated 10 million women were taking HRT in 2000 for alleviation of menopausal symptoms. Then, following the release of reports from the WHI and subsequent analyses of that data showing that estrogen increases the incidence and severity of stroke (Wassertheil-Smoller et al., 2003, Anderson et al., 2004, Bath and Gray, 2005, Sare et al., 2008), widespread use of estrogen therapy is no longer recommended. One mechanism worth further exploration is the effects of estrogen on the EPO/EPO-R system in the ischemic brain.

By more fully understanding the mechanisms of estrogenic action in the aged, injured brain, parameters for the safe and effective use of female gonadal hormones may be possible. However, most basic science research in neural injury is conducted on healthy, young animals, despite the fact that there are age-related differences in estrogenic activity in the brain (Table 1.1). Animal studies show age-dependent alterations in ER levels following estrogen treatment (Belisle et al., 1985, Thakur and Ghosh, 2009), while studies from human cadavers demonstrate age-related changes in the specific splice variant of the ER (Ishunina and Swaab, 2008), indicating that aged individuals may have greater levels of a less active form of ER in specific brain regions. This disregard of age-dependent differences in the actions of female gonadal hormones, has led to unnecessary patient morbidity and mortality (Anderson et al., 2004); thus, research models of disease that account for age-related differences must have a place in future investigations of estrogenic activity.

Figure 1.1. Prevalence of stroke increases with age. Data from the NHANES III demonstrates that prevalence of stroke increases with age in both men and women (Simon and Hudes, 1999). Due to the longer lifespan of women, they experience greater stroke burden over their lifetime.

Figure 1.2. Estrogenic effects on gp130 signaling. Gp130 signaling plays an important role in the injured brain. Although quiescent in the healthy brain, upon injury, STAT3 activation leads to transcription of genes that promote cell survival. STAT3 gene deletion studies show enhanced neuronal apoptosis as well as increased infarct size after middle cerebral artery occlusion. Estrogen has been shown to increase levels of activated STAT3 (Dziennis et al., 2007), however, estrogen also increases inhibitors of the pathway, ERK1/2 and SOCS3 (Wong et al., 2003).

Figure 1.3. Estrogenic effects on cerebral vasculature. Although ER α immunoreactivity has been found in the nucleus, mitochondria and on the membrane of endothelial cells from cerebral blood vessels, the role of these receptors in estrogen-mediated vasodilation is unclear. Evidence suggests that estrogenic alteration in myogenic tone occurs via activation of endothelial-derived nitric oxide synthase and cyclooxygenase-1, which result in vasodilation.

Table 1. Reported age-related differences in estrogenic action and receptors in the brain.

Model	Treatment	Injury	Age-related difference	Reference
post mortem human	-	-	increased MB1 splice variant of ER α in hypothalamus of aged women compared to younger women	(Ishunina and Swaab, 2008)
aged mouse	-	-	decreased expression of MTA1 and decreased interaction with ER α transactivation domain compared to young adult female mice	(Thakur and Ghosh, 2009)
aged female mice	60-day time release pellet of 1.7mg 17 β -estradiol	-	decreased astrocytes and microglial cells in dentate gyrus and CA1 compared to young adult female mice	(Lei et al., 2003)
aged female rat	6 μ g/kg 17 β -estradiol (i.v.)	-	decreased ER nuclear concentrations in multiple brain regions compared to young adult female rats	(Wise et al., 1984)
aged female mouse	0.2 μ g 17 β -estradiol (s.c.)	-	decreased ER and decreased binding kinetics of ER in hypothalamus/pituitary compared to young adult female mice	(Belisle et al., 1985)
aged female rat	60-day time release pellet of 1.0mg 17 β -estradiol	forebrain excitotoxic injury	increased IL-1 β expression, decreased nerve growth factor compared to placebo-treated, aged female rats	(Nordell et al., 2003)
aged female gerbils	single, high dose 4mg/kg pre-treatment	global ischemia	improved performance on memory tasks, fewer TUNEL and caspase 3 positive cells in CA1, CA2 compared to placebo-treated, aged female gerbils	(Wappler et al.)
aged female rats	3-week time release pellet of 25 μ g 17 β -estradiol for 7 days	filament occlusion of MCA	reduced cortical and striatal infarct volume, no changes in cerebral blood flow compared to placebo-treated, aged female rats	(Alkayed et al., 2000)

MB1, mamillary body, exon 1; ER, estrogen receptor; MTA1, metastasis associated protein 1; i.v. , intravenous; s.c., subcutaneous; IL-1 β , interleukin-1 β ; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP nick end labeling; CA, cornu ammonis; MCA, middle cerebral artery

Chapter Two

Estrogen worsens outcome from middle cerebral artery occlusion in aged rats

Abstract

BACKGROUND AND PURPOSE – Although estrogen is neuroprotective in young adult animal models of stroke, clinical trials demonstrate that estrogen increases the incidence and severity of stroke in aged women. We have previously shown that experimental stroke pathophysiology differs between young adult and aged rats. The aim of this study is to determine the effects of estrogen following middle cerebral artery occlusion and reperfusion in young adult and aged female rats.

METHODS – Young adult (3 month) and middle-aged (9 month) female rats were treated with subcutaneously implanted pellets containing 17β -estradiol or placebo until they reached 4 months or 18 months of age, respectively. Focal embolic stroke was performed by middle cerebral artery occlusion (2h) with fibrin clot followed by reperfusion with intravenous human recombinant tissue plasminogen activator in treated and untreated young adult and aged rats. Histological and functional outcomes were measured at 24h after middle cerebral artery occlusion.

RESULTS – Aged rats treated with 17β -estradiol had significantly increased infarct volumes compared to placebo-treated aged rats. Young adult rats treated with 17β -estradiol had significantly decreased infarct volumes and functional outcome compared to ovariectomized young adult rats. Aged female rats treated with 17β -estradiol had decreased post-ischemic GFAP-positive astrocytes and altered astrocyte morphology.

CONCLUSIONS – Estrogen acts in an age-dependent manner in the post-ischemic rat brain. In young adult rats, estrogen is neuroprotective; chronic estrogen treatment during aging leads to worsened ischemic brain injury in aged female rats.

Introduction

The Women's Health Initiative (WHI) showed for the first time in a large-scale clinical trial that hormone replacement therapy (HRT) was linked to higher incidence of stroke (Wassertheil-Smoller et al., 2003). Meta-analyses of the WHI and other smaller clinical trials demonstrate that women taking estrogen experience greater stroke severity (Bath and Gray, 2005, Sare et al., 2008). These findings contradict data from prior animal studies indicating a neuroprotective role of estrogen (Culmsee et al., 1999, Merchenthaler et al., 2003, Rau et al., 2003, Hoffman et al., 2006, Connell et al., 2007, Lebesgue et al., 2009) and raise concerns regarding the safe and appropriate use of HRT.

Aging may be a contributing factor accounting for the contrast between animal studies and clinical trial findings. Previous studies from our lab underscore the importance of creating clinically relevant models of disease, which in the case of ischemic stroke includes the use of aged animals. Ischemic stroke in aged rats is characterized by increased ischemia/reperfusion injury, earlier disruptions of the blood-brain barrier, exacerbated neuronal degeneration, higher mortality, and reduced functional outcome (Rosen et al., 2005, Dinapoli et al., 2006, DiNapoli et al., 2008).

In the present study, we compare the effects of estradiol (E2) administration on ischemia/reperfusion injury and functional recovery following experimental stroke between young adult and aged female rats. Using a model of transient ischemic stroke with reperfusion, we confirmed the findings from previous studies showing that E2 acts as a neuroprotectant in young adult female rats. Then, for the first time, we report that chronic E2 administration in aged female rats led to worsened ischemic brain injury and increased mortality following experimental stroke. The results presented in this study parallel the findings of the WHI in a rat model of ischemic stroke and reperfusion and suggest a potential age-related difference in the effects of estrogen on stroke.

We also investigated a potential pathway to which the age-dependent effects of estrogen may be attributed. Previous studies from our lab have shown age-related changes in the Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) pathway, a signaling cascade known to regulate astrocyte reactivity. Specifically, previous reports demonstrated that aged rats have a truncated activation of STAT3 accompanied by enhanced expression of suppressor of cytokine signaling 3 (SOCS3). In the present study, we investigated whether estrogen may also exert its age-dependent effects on post-ischemic response through alterations in JAK2/STAT3. Despite its importance in the age-related differences in post-ischemic outcome of young adult and aged rats, our data do not support a prominent role of the JAK2/STAT3 pathway by estrogen. Instead, we demonstrate that estrogen treatment in aged female rats acts to reduce post-ischemic astrocyte reactivity, has pleiotropic effects on post-ischemic inflammation, and decreases expression of estrogen receptor α (ER α).

Materials and Methods

Chemicals and Animals. Isoflurane was purchased from Halocarbon (River Edge, NJ). Human recombinant tissue plasminogen activator (tPA) was gifted from Genentech (South San Francisco, CA). E2 and placebo pellets (1.5mg; 90 d release) were purchased from Innovative Research of America (Sarasota, FL). Sixty-five female Sprague-Dawley rats (3 months (N=20) and 9 months (N=45)) were purchased from Hilltop Animal Laboratory (Scottsdale, PA) and housed under 12 h:12 h light/dark conditions with food and water provided ad libitum. All procedures were in accordance with guidelines set forth by the West Virginia University Animal Care and Use Committee.

Treatment Protocol. Rats were randomly divided, within age groups, into three treatment groups. Ovariectomy was performed as follows: at 9 months of age, rats were anesthetized with isoflurane (4% induction; 2% maintenance). Once anesthetized, rats were placed on a surgical platform and lateral abdominal regions prepped for surgery using aseptic technique. A 10 mm

dorsal/ventral incision into the lateral abdomen between the rib cage and hind limb was made through the skin. The underlying abdominal muscle was separated with iridectomy scissors to expose the ovarian fat pad. Ovaries were excised, muscle layer sutured, and skin incision closed using 5-0 absorbable sutures. Topical antibiotic (gentamicin) was applied. Intact rats received a sham ovariectomy and were implanted with placebo pellet. OVX rats received bilateral ovariectomy and were implanted with placebo pellet. E2 rats were bilaterally ovariectomized and implanted with E2 pellet. E2 administration to 3 month old rats lasted for 30 d and pellets in 9 month-old rats were replaced every 90 d until rats reached 18 months of age (Figure 2.1). Serum E2 was measured at 4 and 18 months using [I^{125}] radioimmunoassay (U of Virginia Ligand Core, Charlottesville, VA). All rats underwent a transient ischemic stroke by occlusion of the right middle cerebral artery (MCAO) and tPA (5 mg/kg; i.v.) reperfusion under isoflurane anesthesia (4% induction, 2% maintenance), as previously described (Dinapoli et al., 2006). Ischemia was defined by a perfusion drop across the MCA territory of greater than 80% as determined by laser Doppler measurement of cerebral blood flow and successful reperfusion was denoted as a return to greater than 80% of baseline perfusion rate by 30 min after tPA administration. Rats not achieving these standards for depth and duration of ischemia as well as reperfusion were excluded from the study. At 24 h after MCAO, functional assessment was measured by an investigator blinded to the treatment using the modified Neurological Severity Score (mNSS) (Li et al., 2002).

Determination of infarct volume and Neuropathology. At 24 h after MCAO, rats were anesthetized with isoflurane, transcardially perfused with 100mL of 0.9% saline, and brains were removed and sliced coronally in 2 mm intervals. Sections were incubated with 2% 2,3,5-triphenyltetrazolium chloride (TTC) for 15 min at 37°C for determination of infarct volume and cerebral edema using methods previously described (Yang et al., 1998). After TTC staining, brain slices were fixed in 10% neutral-buffered formalin overnight. Formalin-fixed brain sections were embedded in paraffin, cut into 8 μ m sections and mounted onto slides. Mounted sections

were labeled for degenerating neurons with Fluoro-Jade B, reactive astrocytes using anti-glia fibrillary acidic protein (GFAP; 1:1,000; Dako, Glostrup, DK), microglia using anti-ionic calcium binding protein (Iba-1; 1:500; Wako, Richmond, VA), and ER α using anti-ER α (abcam; 1:100; Cambridge, MA). Secondary antibody for GFAP, Iba-1, and ER α was biotinylated anti-rabbit IgG (1:500, Vector Laboratories, Burlingame, CA). Immunolabeling was visualized by HRP Avidin D (1:1000; Vector Laboratories, Burlingame, CA) coupled with 3,3' diaminobenzidine chromagen kit (Vector Laboratories, Burlingame, CA). Microscopic analysis of GFAP and Iba-1 immunostained sections was performed using an Olympus AX70 Provis microscope (Center Valley, PA) and fluorescent confocal microscopy was performed with a Zeiss LSM 510 Confocal (v4.2 SP1).

Real-time PCR amplification. At 12 h after MCAO, rats (n=3 rats/group) were anesthetized with pentobarbital sodium (65 mg/kg, i.p.) and transcardially perfused with 100 ml saline (0.9% NaCl). Brains were removed and the following regions dissected: ipsilateral (to MCAO) cortex, ipsilateral striatum, contralateral cortex, and contralateral striatum and stored in RNAlater. Total RNA was isolated and purified from brain regions using Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). Concentration and purity of RNA was determined using NanoDrop and considered for use only if A260/A280 was between 1.8 and 2.1. Total RNA (1 μ g) was reverse-transcribed to cDNA using SuperScript™ III RNase H- and oligo (dT)12-18 primers (Invitrogen) in a 40 μ l reaction. Real-time PCR analyses of IL-6, OSM, LIF, TNF α , CCL-2, JAK2, STAT3, and BDNF were performed using an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems; Foster City, CA) with TaqMan® chemistry. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control to normalize for differences in amount of cDNA added to reactions. Specific primers and dual labeled internal fluorogenic (FAM/TAMRA) probe sets (TaqMan® Gene Expression Assays) were used according to the manufacturer instructions (Applied Biosystems). All PCR amplifications (40 cycles) were performed in a total volume of 20 μ l, containing 2 μ l cDNA, 1 μ l of the specific Assay on Demand® primer/probe mix, 1 μ l

GAPDH primer/probe mix, and 10 μ l of TaqMan® Universal master mix (Applied Biosystems). Relative quantification of gene expression was performed using the comparative threshold ($\Delta\Delta C_T$) method as described by manufacturer (User Bulletin 2; Applied Biosystems). Changes in mRNA expression level were calculated following normalization to GAPDH and expressed as fold change.

Tissue preparation and immunoblotting. At 24 h following MCAO, rats (n=5 rats/group) were euthanized by focused microwave irradiation (3 kW for 1.5 s) in microwave applicator (Muromachi Kikai, Inc; Tokyo, Japan) to preserve steady-state phosphorylation of proteins (O'Callaghan and Sriram, 2004, Scharf et al., 2008). Brains were removed and the following regions dissected: ipsilateral (to MCAO) cortex, ipsilateral striatum, contralateral cortex, and contralateral striatum and stored at -80° C for later use. Following collection of all tissues, brain regions were weighed, homogenized in 10 volumes of hot (85–95 °C) 1% SDS, and total protein was determined by bicinchoninic acid method (Smith et al., 1985) using bovine serum albumin standard. Alterations in gp130 signaling pathway were assessed by quantifying total and phosphorylated STAT3^{Tyr705} and total and phosphorylated ERK1/2 (42kD and 44kD) using immunoblot analysis with detection of fluorescent signals with infrared fluorescence scanner (Licor Biosciences; Lincoln, NE). Primary antibodies used included rabbit anti-STAT3 (1:500), rabbit anti-phospho-STAT3^{Tyr705} (1:500; Cell Signaling Technology, Inc., Beverly, MA), mouse anti-ERK1/2 (1:500), rabbit anti-phospho-ERK1/2^{Tyr204} (1:500) purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Following incubation with primary antibodies, blots were washed with phosphate buffered saline with 0.1% Tween 20 (1 x 15 min; 2 x 5 min) and incubated with fluorescent-labeled anti-rabbit and anti-mouse IgG antibodies (1:2500) for 1 h.

Statistical analysis. Data are presented as mean $\pm$ S.E. Statistical significance in measured and calculated parameters was determined using two-way ANOVA with Tukey's post-hoc analyses. Changes in weight gain were assessed using one-way ANOVA with repeated measures. Mortality data was assessed with Chi-squared analysis. Level of significance was set at $p < 0.05$.

Results

Confirmation of three distinct treatment groups in 18 month-old rats. Serum E2 levels were measured from blood collected before MCAO (Table 2.1). In aged rats, the three treatment groups could be differentiated by serum E2 levels. E2 rats had elevated serum E2 levels compared to Intact rats and OVX rats. Serum E2 levels in young adult female rats range from 10 to 70pg/mL during their 4 day estrous cycle. Serum levels in young adult rats showed that OVX rats had a significant reduction in serum E2 as compared to both E2 and Intact rats. No difference in serum E2 was measured between young adult E2 and young adult Intact rats.

E2 is neuroprotective in 4 month-old rats following transient ischemia and reperfusion. In young adult rats, E2/placebo administration lasted for 30 d at which time rats underwent MCAO with tPA reperfusion (n=6/group). Consistent with prior studies, our results demonstrate a significant reduction in cortical ($p=0.030$) and striatal (0.018) infarct volumes at 24 h post-MCAO as compared to young adult OVX rats (Figure 2.2A). No difference in cortical ($p=0.728$) or striatal ($p=0.997$) infarct volumes were measured between young adult E2 and young adult Intact rats. Young adult E2 rats demonstrated a significant improvement in functional outcome, as measured by mNSS compared to young adult OVX rats ($p=0.028$) (Figure 2.2B). Of the 20 young adult rats in the study, two rats died during MCAO surgery without confirmed infarction and were excluded from further analysis. All remaining young adult rats met the ischemia/reperfusion criteria and were included in the study. The mortality rate from ischemic stroke in young adult rats was 17% in OVX group and 0% in both Intact and E2 groups.

E2 exacerbates ischemic brain injury in 18 month-old rats following ischemia and reperfusion

In aged rats, we measured a significant increase in total infarct volume in E2 rats as compared to OVX ($p=0.041$) and Intact ($p=0.028$) rats (Figure 2.3A). Most of the increase in E2 infarction volume occurred in the cortex. No difference in striatal infarct volume between the treatment groups was measured. Assessment of functional outcome by mNSS showed no difference in

neurological score between treatment groups at 24h following MCAO (Figure 2.3B). Of the 45 aged rats in the study, 3 died before reaching 18 months of age, 5 were excluded from analysis for not meeting ischemia/reperfusion inclusion criteria. Of the remaining 37 rats, 9 E2, 5 OVX, and 6 Intact rats met the inclusion criteria for ischemia/reperfusion, but died before 24h. A significant increase in mortality rate was observed in E2 rats as compared to Intact rats (Table 2.1). No significant differences in cerebral edema were measured between treatment groups (Figure 2.4).

E2 increases neurodegeneration and reduces astrogliosis following ischemia and reperfusion.

E2 rats demonstrated increased neuronal degeneration in both striatum and cortex compared to that observed in Intact and OVX rats, but no differences were noted in Fluoro-Jade B staining intensity between Intact and OVX rats (Figure 2.5). Reactive astrocytes were labeled using GFAP and were most prevalent in ipsilateral striatum and cortex. Compared to Intact and OVX rats, E2 rats showed reduced astrocyte hyperplasia in the ipsilateral hemisphere (Figure 2.5). High magnification microscopy revealed long, thin astrocytic processes in Intact rats compared to fewer and thicker GFAP-positive astrocytic processes in E2 rats (Figure 2.6). Abundance of microglia was similar in all rats with microglia in the ipsilateral cortex displaying phagocytic phenotype. No morphologic differences in microglia were observed between E2 and Intact groups.

E2 has pleiotropic effects on post-ischemic inflammation in aged rats. Despite its well-known role as an anti-inflammatory agent, estrogen may have different effects on the post-ischemic inflammatory response in the aged rat brain. To test the effects of E2 on post-ischemic inflammation, we determined the mRNA levels of three markers of inflammation: interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), and monocyte chemoattractant protein-1 (MCP-1) (Figure 2.8). Our results indicate that estrogen has pleiotropic effects on post-ischemic inflammation as it significantly decreases post-ischemic mRNA levels of IL-1 β compared to Intact and OVX rats ($p < 0.001$ for both), but has the opposite effect on post-ischemic TNF- α mRNA levels ($p < 0.001$ compared to both Intact and OVX). Interestingly, the effects of E2 on MCP-1 are not directly re-

lated to circulating E2 level as Intact and E2 rats both have significantly higher post-ischemic MCP-1 mRNA levels compared to OVX rats ($p=0.003$ and $p=0.002$, respectively).

Chronic estrogen supplementation leads to decreased ER α expression. Previous studies have suggested that estrogen-mediated neuroprotection in young adult animals is mediated by ER α . To determine if alterations in ER α expression play a role in the deleterious effects of estrogen in the aged female rat brain, we immunolabeled brain sections with ER α antibody (Figure 2.7). In all brain sections, ER α immunoreactivity was restricted to the infarcted region and ER α + cells had neuronal morphology. No ER α immunoreactivity was observed in the contralateral (to ischemia) hemisphere. In ischemic cortex, E2 rats had reduced numbers of ER α + cells compared to both Intact and OVX rats. In the ischemic striatum, no differences in the number of ER α + cells between treatment groups were observed.

Estrogenic effects on JAK2/STAT3 signaling. Prior studies have shown that aged animals have an altered response of the Janus kinase 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3) pathway following MCAO. To test the effects of estrogen on this pathway following MCAO, we measured mRNA levels of cytokine activators of glycoprotein 130 (gp130) in the ischemic cortex (Figure 2.9). We found significant increases in interleukin-6 (IL-6) based on MCAO in both Intact and OVX treatment groups at 12 hours after MCAO, but not for E2 rats ($p<0.001$ for Intact and OVX, $p=0.954$ for E2). Compared to Intact rats, E2 rats had a significant attenuation in post-ischemic upregulation of both IL-6 ($p=0.003$) and OSM ($p<0.001$) mRNA at 12 hours after MCAO. We also measured JAK2 and STAT3 mRNA levels at 12 hours after MCAO and found no significant differences in JAK2 between treatment groups in sham-operated rats ($p=0.267$) or following MCAO ($p=0.255$) (Figure 2.10, panels A, B). Post-ischemic STAT3 mRNA was significantly affected by MCAO ($p<0.001$), but not treatment ($p=0.068$). No significant differences of treatment or MCAO were measured in mRNA levels of BDNF, a gene target of STAT3 implicated in post-ischemic recovery (Figure 2.10, panel C). Total STAT3 protein and phosphorylated (activated) STAT3 protein were measured at 24 hours following MCAO

(Figure 2.11). No significant differences were found in total or phosphorylated STAT3 in the ischemic cortex. In the ischemic striatum, phospho-STAT3 was significantly increased in E2 rats. Extracellular-signal-regulated kinase1/2 (ERK1/2) is an important modulator of the JAK2/STAT3, and, like STAT3 is phosphorylated to be activated. We measured total and activated protein levels of ERK1/2 and found no significant differences between treatment groups in ischemic cortex or ischemic striatum from naïve or treated rats (Figure 2.12).

Discussion

The present study showed, for the first time, that aged female rats treated with E2 have worsened stroke injury and increased mortality following MCAO. Previous reports have shown that E2 administered prior to experimental stroke is neuroprotective in young adult rats. Using subcutaneously implanted time-release E2 pellets, we confirmed neuroprotection by E2 in young adult rats. In contrast, female rats ovariectomized at 9 months of age and administered similar E2 pellets until 18 months of age, experienced higher mortality, increased infarct volume, and exacerbated neurodegeneration (most pronounced in the cortex) following MCAO. These findings support the concept that estrogenic effects on the post-ischemic brain differ over the lifespan and demonstrate for the first time, an animal model that produced results that parallel findings of the WHI (Wassertheil-Smoller et al., 2003, Anderson et al., 2004).

The importance of animal models of disease that closely mimic the clinical presentation cannot be overstated. This study employed a number of approaches to improve our understanding of estrogenic effects on focal ischemic stroke. First, E2 rats received treatment starting at 9 months of age and physiologic E2 levels were maintained until 18 months of age, thereby avoiding any prolonged period of hypoestrogenicity. Next, we accounted for the effects of age on stroke outcome. Our lab has repeatedly demonstrated that ischemic stroke injury and recovery in aged rats (18 to 20 months old) is markedly different from that in younger animal models of ischemic injury (Dinapoli et al. 2006). Finally, a method of focal cerebral ischemia implementing

embolic occlusion of the middle cerebral artery (MCAO) with tissue plasminogen activator (tPA) reperfusion was used to more accurately model the clinical manifestation of thromboembolic stroke treated with tPA. Together, these factors support the potential for translating the findings from our model into human populations.

Our results do not support what has become known as the “timing of initiation hypothesis” which claims that a prolonged period of hypoestrogenicity following menopause leads to irreversible vascular changes, that are not mimicked in ovariectomized animals immediately supplemented with E2 (Wise et al., 2005, Suzuki et al., 2007). A period of hypoestrogenicity is thought to contribute to the increased stroke incidence and worsened stroke outcome found in women taking HRT. According to proponents of this hypothesis, HRT could be used safely if treatment begins close to the time of menopause onset. However, our results show that E2 administration initiated at 9 months of age offered no neuroprotection and, in fact, led to higher mortality and increased infarct size following MCAO. Likewise, clinical data show that the timing of initiation hypothesis is not substantiated in analysis of a subset of WHI participants who began taking HRT within 5 years of menopause (Prentice et al., 2009).

The mechanism by which E2 acts following ischemic injury remains unknown. Due to the known anti-inflammatory actions of estrogen in young adult animals, we investigated whether chronic estrogen supplementation altered the post-ischemic inflammatory response in aged female rats. We measured mRNA levels of three inflammatory mediators, IL-6, TNF α , and MCP-1 in the ischemic cortex of rats at 12 h after MCAO. We found that E2 supplementation has pleiotropic effects on inflammation in the post-ischemic brain with post-ischemic E2 rats demonstrating increases in both TNF α and MCP-1 compared to sham-operated counterparts. However, IL-6 levels in E2 rats did not increase following MCAO compared to approximately 20-fold increases in Intact and OVX rats following MCAO. TNF α , however, was greatly increased in E2 rats following MCAO and were significantly greater than the post-ischemic increases in both Intact and OVX rats. Intact and E2 rats had comparable levels of MCP-1 following MCAO, while

OVX rats showed an attenuated post-ischemic increase in MCP-1 mRNA levels. Alongside the post-ischemic inflammatory response, cerebral edema commonly complicates the post-ischemic recovery. Female gonadal hormones, particularly progesterone, but to some degree estrogen as well, are thought to decrease edema resulting from brain injury (O'Donnell et al., 2006). However, in aged female rats we did not see any effect of treatment on cerebral edema as measured by hemisphere volume comparisons using TTC-stained brains. A more accurate method of calculating cerebral edema, such as dry tissue weight, may find subtle differences between groups. Taken together, these findings suggest that estrogen has little effect on the post-ischemic inflammatory response as measured by inflammatory cytokine mRNA levels within the ischemic tissue and cerebral edema.

Another potential mechanism of estrogenic action is through alterations or reductions in ischemia-induced astrocyte activation. Estrogenic effects on post-ischemic astrocyte response have been studied using both traditional immunohistochemistry (Barreto et al., 2007) as well as biophotonic/bioluminescent imaging (Cordeau et al., 2008) and demonstrate marked reductions in GFAP-positive cells in animals treated with E2 (Barreto et al., 2009). In the present study, we assessed presence of astrocyte reactivation using GFAP immunostaining in aged rats and found significantly fewer GFAP-positive cells in the ischemic hemisphere of aged E2 rats compared to Intact counterparts. Although the role of reactive astrocytes in the injured brain remains unclear, the absence of GFAP protein in knockout mice leads to higher mortality and altered astrocyte response to penetrating brain lesion (Pekny et al., 1999). Increased ischemic brain injury in E2 rats, and subsequent reduction in GFAP-positive astrocytes, suggest a potentially beneficial role of reactive astrocytes in ischemic stroke pathophysiology. Using these findings as a foundation, future studies will investigate the effects of E2 on post-ischemic astrocyte response.

Pathways responsible for astrocyte activation following ischemic injury were a likely target of estrogenic activity and the focus of a subset of experiments in this study. The

JAK2/STAT3 signaling cascade has been shown to regulate astrocyte reactivity and has also been implicated in age-related differences in post-ischemic response (Dinapoli et al. 2006). We assessed both total and phosphorylated STAT3 levels in our aged rat groups at 24 h after MCAO and found no differences in protein levels of STAT3 or phospho-STAT3 between groups. We also found no differences in protein levels of a key regulator of that pathway, ERK1/2 or the activated form, phospho-ERK1/2 between treatment groups. Our Western blot analysis data was supported by mRNA results demonstrating both increased and decreased levels of activators of the g-coupled receptor linked to the JAK2/STAT3 pathway, known as glycoprotein 130 (gp130). E2 significantly decreased IL-6 mRNA but had the opposite effect on another gp130 ligand, OSM. Messenger RNA levels of a third gp130 activator, LIF, were not significantly different between groups. Taken together, these results do not support the hypothesis that estrogen worsens stroke outcome in aged female rats by acting on the JAK2/STAT3 pathway.

Chronic estrogen supplementation may result in reductions in estrogen receptors that, in ischemic conditions, may have deleterious effects. Specifically, ER α has been implicated in the neuroprotective effects of estrogen in young adult rats (Merchenthaler et al., 2003, Dubal et al., 2006). ER α knock-out mice administered E2 show a complete reversal of its neuroprotective effects, while no differences in E2-mediated neuroprotection are noted in ER β knock-out mice, indicating a prominent role of ER α in the neuroprotective action of E2 (Sampei et al., 2000, Dubal et al., 2001). To determine whether chronic E2 administration altered the expression levels of ER α in aged rats, we immunostained brain sections using ER α antibody. We discovered that ER α expression is decreased in E2-treated rats, suggesting that decreases in ER α in the aged, chronically E2-treated brain may account for the loss of E2-mediated neuroprotection. This decrease in ER α was most pronounced in the cortex of E2 rats, coinciding with the area of increased infarct volume. Interestingly, we also observed that ER α expression was limited to the infarct region, indicating that ischemic stimulus is a likely activator of ER α expression. Furthermore, the expression of ER α appeared to be limited to cells with neuronal morphology, contra-

dictory to previous reports demonstrating ER α in astrocytes of both injured and non-injured brains (Spence et al., , Jung-Testas et al., 1992). Together, these results offer further support for ER α as mediator of E2-induced neuroprotection, and are a beginning towards determining the mechanism of estrogenic action in the aged, post-ischemic brain.

Despite the clinical relevance of our model, there are several caveats to be considered in the present study. Aged OVX rats had significantly higher weight at time of MCAO compared to both Intact and E2 rats (Table 2.1). This difference in weight may have resulted in alterations in circulating levels of estrone and E2 due to conversion of androstenedione and testosterone in adipose tissue (Lukanova et al., 2004). However, levels of E2 measured from blood collected at time of sacrifice demonstrated E2 levels below the detectable range in OVX rats. Local conversion of steroid hormones to E2 by the enzyme aromatase may also play a role in stroke pathophysiology (McCullough et al., 2003), but was not measured in this study. Local aromatase expression has been shown increase following neural injury, thus future experiments may involve post-ischemic quantification of this enzyme (McCullough et al., 2003, Peterson et al., 2004). Despite differences in infarct size, aged rats showed no significant differences in functional outcome following MCAO as measured by the mNSS. This result may be due to limitations of that assessment in aged rats. Although useful in young adult rats, the mNSS requires significant motor coordination to perform many of the subtests in the assessment. Furthermore, due to the high mortality in the aged E2 rats, both infarct volumes and mNSS scores may be underestimated by a survivor bias.

An additional consideration arises from the use of time-release pellets for hormone administration. Upon implantation, serum E2 levels increase rapidly followed by a slow decline in hormone level, which differs from the more constant hormone levels achieved by oral supplementation in women. Likewise, the E2 used in the laboratory setting differs from commonly prescribed E2. Clinically prescribed conjugated equine estrogen is a mixture of several estrogens, with the most common preparation containing estrone sulfate, equilin sulfate, and equilenin sul-

fate. Further complicating the study of estrogenic effects are reports demonstrating variable levels of the hormone based on patient adiposity (Lukanova et al., 2004), a factor that was not accounted for in the WHI studies.

Summary

In conclusion, the results of this study are a beginning towards understanding age-related effects of estrogen in the post-ischemic brain. In the year 2000, an estimated 10 million women were taking HRT for the treatment of menopausal symptoms. Since the release of reports from the WHI, FDA guidelines for the use of HRT have been altered to recommend that patients take the lowest effective dose for the shortest amount of time. The perceived cardioprotective and neuroprotective actions of estrogen were not substantiated in those clinical trials, despite years of animal research to the contrary (Culmsee et al., 1999, Merchenthaler et al., 2003, Rau et al., 2003, Hoffman et al., 2006, Connell et al., 2007, Lebesgue et al., 2009). Age-related differences in estrogenic activity may account for those discrepancies. Here, we demonstrated for the first time deleterious effects of E2 treatment in an aged animal model of ischemic stroke, which parallel the findings of the WHI (Wassertheil-Smoller et al., 2003). We also demonstrate that estrogenic action in the aged brain is not likely mediated through the JAK2/STAT3 pathway. However, we did observe that worsened stroke injury in aged female rats was accompanied by downregulation of the ER α , suggesting a role for this receptor in estrogen-mediated neuroprotection. Although further investigation is warranted to gain greater understanding of the mechanism(s) of estrogenic actions on the aged brain following ischemic injury, development and use of a model that replicates the clinical findings is an essential component for progress in the field.

	Weight (g)				Serum (pg/mL)	E2	Mortality (%)
	Pre	12 mo.	15 mo.	18 mo.			
Intact	342 ± 14	361 ± 7	398 ± 13	400 ± 13	16.4 ± 3.0		40
OVX	347 ± 12	474 ± 16	500 ± 16	540 ± 14	5.2 ± 0.2		33
E2	352 ± 11	377 ± 9	396 ± 9	423 ± 27	36.6 ± 3.2		60

Table 2.1. Weight gain, serum E2, and mortality of aged female rats.

Figure 2.1. Schematic of serum E2 levels of young adult (A) and aged (B) treatment groups.

Figure 2.2. Lack of endogenous or supplemented estrogen leads to increased infarct volume in young adult rats. (A) Young adult OVX rats demonstrated increased infarct size following MCAO with tPA reperfusion compared to both Intact and E2 counterparts. Cortical, striatal, and total infarct size were all significantly increased in young adult OVX rats compared to both young adult Intact and young adult E2 rats. (B) Lack of endogenous or supplemented estrogen led to worsened functional outcome in young adult rats. Young adult OVX rats showed higher scores on the modified Neurological Severity Score, indicative of worsened functional outcome. There was no significant difference in mNSS scores between Intact and E2 rats. * $p < 0.05$ as compared to Intact; # $p < 0.05$ as compared to E2.

Figure 2.3. Estrogen treatment leads to increased cortical and total infarct volume in aged rats. (A) E2 rats demonstrate increased cortical and total infarct following MCAO with tPA reperfusion. There was no significant difference in striatal infarct volume between treatment groups. (B) E2 treatment did not significantly alter functional outcome. Aged E2 rats did not show worsened functional outcome despite having increased cortical and total infarct volume. (C) Representative brain slices from Intact and E2 treated rats demonstrating a significant increase in infarcted tissue in E2 rats. * $p < 0.05$ as compared to Intact; † $p < 0.05$ as compared to OVX.

Figure 2.4. Estrogen administration does not alter cerebral edema following MCAO. There were no significant differences between treatment groups in cerebral edema formation as measured at 24 h after MCAO with tPA reperfusion.

Figure 2.5. Exacerbated neurodegeneration and reduced astrogliosis in ischemic hemisphere of aged E2 rats. Fluoro-Jade B staining was used to assess neuronal degeneration at 24 h after MCAO in ischemic cortex of aged rats. E2 rats (G) had increased neuronal degeneration compared to both Intact (A) and OVX (D) rats. Post-ischemic reactive astrogliosis was observed using anti-glial fibrillary acidic protein (GFAP) and revealed reduced astrogliosis in aged E2 rats (H) compared to both Intact (B) and OVX counterparts (E). Inflammatory response was assessed with immunostaining of microglia (C, F, I). No significant effect of treatment was observed in microglia immunostaining by Iba-1. Bar = 100µm.

Figure 2.6. Reduced GFAP-positive astrocytes and altered astrocyte morphology in aged E2 rats. High magnification microscopy of GFAP immunostaining in aged Intact (A) and E2 (B) rats revealed fewer GFAP positive cells in ischemic cortex of E2 rats. Individual astrocytes were observed to have fewer and thicker GFAP positive processes in aged E2 rats (D) compared to Intact rats (C). No differences in microglia abundance or morphology were observed in aged rats. (E) Intact rats had comparable abundance of Iba-1 positive cells in the ischemic cortex as E2 counterparts (F). Individual cells had similar phagocytic morphology in both Intact (G) and E2 (H) rats. Bar (A,B,E,F) = 25 μ m, (C,D,G,H) = 10 μ m.

Figure 2.7. Estrogen supplementation leads to reduced estrogen receptor- α (ER α) in aged female rats. ER α immunolabeled cells in the ischemic cortex and ischemic striatum have structural morphology consistent with neurons. ER α -positive cells appear more abundant in the ischemic cortex of Intact and OVX rats compared to E2 rats (A, C, E). No observable differ-

ences were noted in abundance of ER α -labeled cells in the ischemic striatum (B, D, F). Scale bar = 50 μ m.

Figure 2.8. Estrogen has pleiotropic effects on post-ischemic inflammation. (A) E2 rats do not exhibit ischemia-induced increase in interleukin-1 β mRNA in the ischemic cortex compared to rats lacking endogenous or supplemented estrogen. (B) However, tumor necrosis factor- α (TNF α) shows the opposite effect with E2 treatment. Intact and OVX rats have TNF α mRNA levels increased compared to sham-operated rats, but E2 rats have significantly increased post-ischemic elevation of TNF α . (C) Monocyte chemoattractant protein (MCP-1) is increased in all rats following MCAO compared to sham-operated rats. Intact and E2 rats have significantly greater increases in MCP-1 mRNA following ischemia compared to OVX rats. ** $p < 0.001$ compared to E2; $\natural$ indicates $p < 0.01$, $\natural\natural$ $p < 0.001$ compared to sham-operated; $\natural$ $p < 0.01$ compared to OVX; $\natural\natural$ $p < 0.001$ compared to OVX.

Figure 2.9. Estrogenic effects on mRNA levels of activators of gp130 signaling in the ischemia cortex. E2 rats do not exhibit ischemia-induced upregulation of interleukin-6 (IL-6) mRNA following MCAO (A), but do demonstrate post-ischemic increases in leukemia inhibitory factor (B) and oncostatin M (C) mRNA compared to sham-operated rats. Post-ischemic upregulation of OSM mRNA is attenuated in E2 rats compared to Intact and OVX groups. * $p < 0.01$, ** $p < 0.001$ compared to E2; †† $p < 0.001$ compared to sham-operated.

Figure 2.10. Estrogen may not act through JAK2/STAT3 following MCAO. Post-ischemic alterations in JAK2 (A) and STAT3 (B) mRNA do not show significant differences based on treatment group, although there is a significant increase in STAT3 mRNA with MCAO. (C) Brain-derived neurotrophic factor, a key target of STAT3, does not show alterations dependent on estrogen status or MCAO. $^{***}p < 0.001$ compared to sham-operated rats.

Figure 2.11. Estrogen level does not affect activation of STAT3 following MCAO. Total and phosphorylated STAT3 proteins were not altered by E2 supplementation at 24 h after MCAO in ischemic cortex or ischemic striatum of aged female rats.

Figure 2.12. Post-ischemic total and activated ERK1/2 protein are not affected by estrogen. Protein levels of neither isoform of ERK1/2 nor their activated forms were altered by estrogen status at 24 h after MCAO in ischemic cortex (A) or ischemic striatum (B) of aged female rats.

Chapter Three

Estrogen down-regulates erythropoietin receptor following middle cerebral artery occlusion in aged female rats

Abstract

Previous reports have demonstrated that the neuroprotective actions of estrogen in young adult animal models of stroke do not translate into aged female rats chronically supplemented with estrogen. In the present study we investigate a potential mechanism involved in the deleterious effects of estrogen in the aged female rat brain. In rats chronically supplemented with estrogen or placebo, we measure a significant reduction in erythropoietin (EPO) receptor mRNA in the ischemic cortex following transient occlusion of the middle cerebral artery. We also observe decreased EPO-receptor (EPO-R) protein expression in the penumbral cortex and striatum using immunohistochemical techniques. In a second group of rats, also treated with estrogen, we attempt to rescue estrogen treated rats from exacerbated stroke injury through administration of erythropoietin. Results confirm that EPO administration attenuates estrogen-induced increase in infarct volume, and is accompanied by decreased expression of ER α in the ischemic penumbra. Together, the results presented here support the hypothesis that chronic estrogen supplementation leads to reduced brain ER α expression, reduction in post-ischemic EPO-R response, and worsened stroke outcome.

Introduction

Previously, combined estrogen and progestin hormone replacement therapy (HRT) was widely prescribed to women for the treatment of menopausal symptoms. HRT was thought to perpetuate the neuroprotective and cardioprotective actions of endogenous estrogen, since the hormone had been repeatedly shown to do so in animal studies (Simpkins et al., 1997, Toung et al., 1998, Culmsee et al., 1999, Wang et al., 1999, Alkayed et al., 2000, Dubal and Wise, 2001). This clinical practice came into question when the Women's Health Initiative (WHI) released reports on the effects of HRT (2003) and estrogen supplementation alone (2004) on stroke prevalence and outcome (Wassertheil-Smoller et al., 2003, Anderson et al., 2004) that conflicted with data from animal studies. In randomized controlled trials of women aged 50–79 years, researchers found an increased risk of ischemic stroke with HRT or estrogen supplementation alone, warranting the premature termination of the trials. Since then, meta-analyses of data from the WHI and other smaller clinical trials have also shown increased stroke severity with HRT (Sare et al., 2008).

Estrogen is a known modulator of erythropoietin (EPO) and its receptor (EPO-R). Estrogen and testosterone have opposite effects on erythropoiesis with estrogen acting to decrease erythropoiesis and testosterone acting to increase it (Piliero et al., 1968). Both in vitro and in vivo studies indicate that EPO and EPO-R are upregulated in the brain following hypoxic exposure (Chin et al., 2000, Siren et al., 2001b). Yet, estrogen has been shown to decrease hypoxia-induced upregulation of EPO in cell culture (Mukundan et al., 2004). These effects are likely mediated through hypoxia-inducible factor-1 (HIF-1), the HIF-1 α subunit of which is released from proteasomal degradation in low oxygen conditions (Wang and Semenza, 1993). The specific mechanism of EPO/EPO-R action in hypoxia is unknown, but experiments in which EPO-R is inhibited following ischemia show worsened stroke outcome, suggesting that the EPO/EPO-R system is beneficial (Chen et al.). In addition, numerous studies have demonstrated neuroprotection by exogenous EPO treatment in acute stroke (Digicaylioglu and Lipton, 2001, Genc et

al., 2004, Zhang et al., 2006a, Gonzalez et al., 2007, Ehrenreich et al., 2009). The effects of estrogen on the EPO/EPO-R system in the post-ischemic female rat brain have not been explored to date, and are the focus of this study. We hypothesized that estrogen may alter the post-ischemic activation of the EPO/EPO-R system in the aged female rat brain. To test our hypothesis, we administered estradiol (E2) to female rats as they aged and studied the effects of acute EPO treatment following transient occlusion of the middle cerebral artery.

Materials and Methods

Chemicals and Animals. Isoflurane was purchased from Halocarbon (River Edge, NJ). Human recombinant tissue plasminogen activator (tPA) was gifted from Genentech (South San Francisco, CA). E2 and placebo pellets (1.5mg; 90 d release) were purchased from Innovative Research of America (Sarasota, FL). Sixty-nine female Sprague-Dawley rats (9 months (N=45) and 15 months (N=24)) were purchased from Hilltop Animal Laboratory (Scottsdale, PA) and housed under 12h:12h light/dark conditions with food and water provided ad libitum. All procedures were in accordance with guidelines set forth by the West Virginia University Animal Care and Use Committee.

Treatment Protocol. In the first cohort of animals, nine month-old female rats were randomly divided into three treatment groups: Intact, OVX, and E2. Ovariectomy/sham was performed as follows: rats were anesthetized with isoflurane (4% induction; 2% maintenance). Once anesthetized, rats were placed on a surgical platform and lateral abdominal regions prepped for surgery using aseptic technique. A 10 mm dorsal/ventral incision into the lateral abdomen between the rib cage and hind limb was made through the skin. The underlying abdominal muscle was separated with iridectomy scissors to expose the ovarian fat pad. Ovaries were excised, muscle layer sutured, and skin incision closed using 5-0 absorbable sutures. Topical antibiotic (gentamicin) was applied. Intact rats received a sham ovariectomy and were implanted with placebo pellet. OVX rats received bilateral ovariectomy and were implanted with placebo pellet. E2 rats were

bilaterally ovariectomized and implanted with E2 pellet. E2 pellets in 9 month-old rats were replaced every 90 d until rats reached 18 months of age. In the second cohort of animals, fifteen month-old rats were randomly divided into two treatment groups, Intact and E2. At time of treatment randomization, they were also randomized to receive EPO or saline following MCAO. Intact rats were implanted with placebo pellets, E2 rats received pellets containing 17 β -estradiol, described for the first cohort. Rats in the second cohort also received treatment until they reached 18 months of age. Serum E2 was measured at 18 months in all rats using [I^{125}] radioimmunoassay (U of Virginia Ligand Core, Charlottesville, VA).

Middle cerebral artery occlusion. The first cohort of rats underwent a transient ischemic stroke by occlusion of the right middle cerebral artery (MCAO) and tPA (5 mg/kg; i.v.) reperfusion under isoflurane anesthesia (4% induction, 2% maintenance), as previously described (Dinapoli et al., 2006). Ischemia was defined by a perfusion drop across the MCA territory of greater than 80% as determined by laser Doppler measurement of cerebral blood flow and successful reperfusion was denoted as a return to greater than 80% of baseline perfusion rate by 30min after tPA administration. Rats not achieving these standards for depth and duration of ischemia as well as reperfusion were excluded from the study. Intact and E2 rats of the second cohort underwent a transient ischemic stroke by occlusion of the right middle cerebral artery (MCAO) with modified suture (Doccol, Redlands, CA) under isoflurane anesthesia (4% induction, 2% maintenance). Ischemia was defined by the same criterion as for rats receiving MCAO with fibrin clot. Immediately preceding suture removal, a subset of Intact and E2 rats received recombinant human erythropoietin (i.p., 5000U/kg). Rats not receiving EPO were administered a similar injection of vehicle. At 24 h after MCAO, functional assessment was measured by an investigator blinded to the treatment using the modified Neurological Severity Score (mNSS) (Li et al., 2002).

Determination of infarct volume and Neuropathology. At 24 h after MCAO, rats were anesthetized with isoflurane, transcardially perfused with 100 mL of 0.9% saline, and brains were re-

moved and sliced coronally in 2 mm intervals. Sections were incubated with 2% 2,3,5-triphenyltetrazolium chloride (TTC) for 15 min at 37°C for determination of infarct volume using methods previously described (Yang et al., 1998). After TTC staining, one 2 mm brain slice, corresponding to the territory of the MCA (0.7 – 2.7 mm from bregma) was fixed in 10% neutral-buffered formalin overnight. Formalin-fixed brain sections were embedded in paraffin, cut into 8 µm sections and mounted onto slides.

Immunohistochemistry. Mounted sections were treated with 0.3% H₂O₂-30% methanol in PBS to suppress endogenous peroxidases. Nonspecific binding was blocked using 10% horse serum. Sections were incubated in substrates overnight at 4°C. Brain sections were incubated with biotinylated goat anti-rabbit or horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) followed by an avidin-biotin complex process (Vector Laboratories). Finally, the brain sections were exposed to stable 3,3'-diaminobenzidine tetrahydrochloride (DAB). EPO-receptor was labeled with anti-EPO-R (1:100, Novus Biologicals, Littleton, CO). Microscopic analysis of sections was performed using an Olympus AX70 Provis microscope (Center Valley, PA) and confocal microscopy was performed with a Zeiss LSM 510 Confocal (v4.2 SP1).

Real time PCR. Total RNA was isolated from brain homogenates using Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). Only RNA with 260/280 between 1.80 and 2.10 and 260/230. Real time PCR analyses of the JAK/STAT array (Table 3.1; SA Biosciences, Frederick, MD) was performed using an ABI PRISM 7700 sequence detection system (Applied Biosystems; Foster City, CA) according to manufacturers instructions.

Enzyme-linked Immunosorbant Assays. Prior to sacrifice, CSF was collected under isoflurane anesthesia (4% induction, 4% maintenance) from the cisterna magna. If blood contamination occurred during needle withdraw, CSF was centrifuged at 1000 x g for 20 minutes and clear CSF was removed and stored at -80° C for later use. Blood was collected from rats transcardially prior to perfusion. For serum analysis, whole blood was allowed to clot at room temperature for 2 hours followed by centrifugation at 1000 x g for 20 minutes. Serum layer was removed and

stored at -80° C for later use. EPO, IL-6, and TNF- α levels were determined by ELISA per manufacturer recommendations (R&D Systems, Minneapolis, MN).

Statistical analysis. Data are presented as mean $\pm$ S.E. Statistical significance in measured and calculated parameters was determined using two-way ANOVA with Tukey's post-hoc analyses. Level of significance was set at $p < 0.05$.

Results

Post-ischemic EPO-R downregulation by E2. An array specific to the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway was used to determine the effects of chronic E2 supplementation on the post-ischemic alterations in mRNA of effectors in the pathway in the first cohort of rats, consisting of Intact, E2, and OVX groups. Rats in the first cohort received E2 or placebo supplementation from 9 months until 18 months of age. Confirmation of differences in serum E2 levels from these rats have been reported on previously (Table 2.1). Compared to Intact rats, E2 treatment led to upregulation of prolactin (PRL; 5.0 fold) and prolactin-receptor (PRL-R; 33.1 fold) mRNA in the ischemic cortex. E2 supplementation also caused 4.9 fold downregulation of EPO-R in the ischemic cortex (Figure 3.1).

EPO-R expression decreased in ischemic penumbra of E2 rats. Immunohistochemical analysis of EPO-R expression in the first cohort of rats indicated a reduced EPO-R expression in rats receiving E2 (Figure 3.2). Few EPO-R+ cells were observed within the ischemic core, but differences in EPO-R expression were noted in the penumbral region, particularly in the penumbral cortex. Cells staining for EPO-R had astrocyte and microglia morphology in all treatment groups, although a cellular marker was not used to verify cell types. Differences in number of EPO-R immunolabeled cells were much less pronounced in the penumbral striatum.

Confirmation of distinct treatment groups. Serum E2 levels were measured from blood collected prior to pellet implantation and after 75 days in the second cohort of rats which were treated with E2 or placebo from 15 months until 18 months of age (Figure 3.3). At baseline, prior to treat-

ment group randomization, no differences were measured in serum E2 levels. Following 75 days of E2 treatment, however, two distinct treatment groups could be differentiated by serum E2 level, where rats treated with E2 had significantly elevated serum E2 levels compared to Intact rats.

Enzyme-linked immunosorbent assay for EPO in cerebrospinal fluid and serum. Results from ELISA for EPO in CSF and serum of Intact and E2 rats revealed significantly higher levels of EPO in the CSF of Intact rats compared to their respective serum EPO level (Figure 3.4). However, despite EPO administration in a subset of each treatment group, no significant differences were measured in serum or CSF levels of EPO between EPO and saline treated rats, potentially indicating unreliable results.

Effects of chronic E2 and acute EPO in post-ischemic markers of systemic inflammatory. Post-ischemic serum tumor necrosis factor α (TNF α) and interleukin-6 (IL-6) were measured by enzyme-linked immunosorbent assay to determine systemic inflammatory profile (Figure 3.5). Serum TNF α was significantly increased by E2 treatment ($p=0.003$), but not by EPO administration ($p=0.081$). TNF α levels were increased by EPO treatment in E2 rats from 8.5 ± 1.9 pg/mL to 16.5 ± 2.1 pg/mL, but this increase was not statistically significant. IL-6 levels in Intact and E2 rats treated with EPO were increased compared to saline treated rats (346 ± 87 pg/mL compared to 218 ± 98 pg/mL in Intact rats, and 392 ± 87 pg/mL compared to 177 ± 65 pg/mL in E2 rats), but this difference also did not reach significance ($p=0.076$).

Acute treatment with EPO reduces infarct volume in estrogen-treated rats. At 2 h following initiation of cerebral ischemia by suture occlusion of the middle cerebral artery, a subset of Intact and E2 rats ($n=4$ /group) received 5,000 U/kg of EPO (i.p.). Infarct volume was measured at 24 h after initiation of MCAO (Figure 3.6, panel A). Cortical infarct volume of E2 rats was higher than that of EPO and saline treated Intact rats as well as E2 + EPO rats, but it did not reach significance ($p=0.182$). There were no differences based on E2 or EPO treatment in striatal infarct volume. Total infarct volume showed a significant effect of EPO treatment ($p=0.044$), although

no differences in functional impairment following MCAO were measured between groups using the modified Neurological Severity Score (Figure 3.6, panel B).

E2 supplementation is associated with greater post-ischemic disruption of tissue architecture and presence of petechial hemorrhage, which is reduced by acute EPO treatment. Brain sections from the middle cerebral artery territory from rats in each treatment group were stained with hematoxylin and eosin at 24 h after MCAO and studied for cellular and structural alterations (Figure 3.7). E2 supplementation was associated with enhanced disruption of tissue architecture and accompanied by petechial hemorrhage, most pronounced in the ischemic cortex. This disruption of tissue architecture was attenuated in E2 rats receiving acute EPO treatment. In Intact rats, EPO treatment enhanced tissue vacuolation in the cortex, but did not affect the ischemic striatum.

Both EPO-R and ER α expression are attenuated in the post-ischemic, estrogen treated rat brain. At 24 h after MCAO, brain sections from Intact and E2 rats receiving saline or EPO were immunolabeled with EPO-R or ER α . Similar to results from the first cohort of rats, ER α + cells were only present in the ischemic core with some extent into the penumbra. No ER α + cells were observed in the contralateral hemisphere or outside the territory of the MCA. Cellular morphology of ER α + cells was consistent with that of neurons, although no cellular marker was used. No pronounced differences in ER α expression were observed based on EPO treatment. A prominent decrease was observed in the number of ER α + cells in the penumbral regions of E2 rats treated with saline compared to both EPO and saline treated Intact rats. In contrast, EPO-R immunolabeling was present only in the penumbral region and was not observed in the ischemic core in any treatment group (Figure 3.9). EPO-R+ cells extended into the contralateral hemisphere and were abundant in the corpus callosum of all rat brains studied. EPO-R+ cells had morphology consistent with that of astrocytes or microglia. No EPO-R+ cells with neuronal morphology were observed. No differences in number of EPO-R+ cells were noted based on EPO treatment, however E2 rats were observed to have reduced EPO-R+ cells both in the penum-

bral cortex as well as the penumbral striatum, similar to results observed in the first cohort of rats.

Discussion

E2 has been repeatedly shown to be neuroprotective in young adult animal models of stroke. However, we have previously reported that female rats treated with E2 during aging experience exacerbated infarct volume compared to age-matched, placebo treated rats. In the present study, we examined a potential mechanism by which estrogen worsens cerebral injury following experimental stroke in aged female rats. Our data suggest that deleterious effects of estrogen in the aged female rat brain may be linked to the post-ischemic EPO/EPO-R system. In the first cohort of female rats, treated with E2 from 9 months until 18 months, we demonstrate an almost 5-fold downregulation of EPO-R mRNA in the ischemic cortex. Analysis of EPO-R protein expression by immunohistochemistry revealed significantly fewer EPO-R+ cells in rats receiving E2, as well. In contrast, previous studies reported in the literature both in vitro and in vivo demonstrate upregulation of EPO-R in neurons and glia cells following hypoxic/ischemic insult (Chen et al.). Administration of EPO has been demonstrated to have neuroprotective capacity in preclinical models of stroke. These reports, together with our findings, support the notion that effects of estrogen to worsen stroke injury involve downregulation of EPO-R.

Based upon our initial findings, we designed a second experiment in which E2 and placebo treated rats received EPO treatment (5,000 U/kg, i.p.) at 2 h after the initiation of ischemia. If EPO-R reduction were responsible for increased infarct size in E2-treated rats, we hypothesized that treatment with EPO would rescue E2 rats from worsened stroke injury. Furthermore, based upon previous preclinical reports, we expected EPO to have neuroprotective qualities, leading to reduced infarct volume in placebo treated aged female rats. EPO has been studied extensively in young adult animal models and, based upon those preclinical studies, moved to clinical stroke trials (Ehrenreich et al., 2002). In Phase II trials, EPO failed to improve stroke

outcome and was associated with an increased mortality, leading to the termination of its development as a stroke therapeutic (Ehrenreich et al., 2009). The failure of EPO in clinical trials may be attributable to the lack of thorough investigation into its mechanism of action in a clinically relevant animal model. In addition, the majority of patients receiving EPO that subsequently died were reported to have also received tPA. Clinical researchers speculate as to whether an unrecognized tPA-EPO interaction could have occurred, leading to the detrimental outcome. For this reason, we chose to use the suture occlusion model of MCAO in the second cohort of E2 and placebo treated rats in order to permit reperfusion without the use of tPA.

At 24 h after MCAO by suture occlusion, we calculated a significant reduction in total infarct volume in rats receiving EPO, supporting our hypothesis that EPO administration to E2 rats could return their infarct size to baseline levels for aged rats. No statistically significant differences were measured in cortical or striatal infarct volumes, however, this may be due to the small sample size used in our second study ($n=4/\text{group}$). Particularly in the cortex, there was a trend toward decreased cortical infarct volume with EPO treatment in E2 rats ($p=0.182$). We did not, however, find an improvement in infarct volume with EPO treatment in Intact rats, as has been reported in the past using young adult animals. Histologic evaluation of ischemic sections revealed greater disruption of tissue architecture with E2 treatment, which was attenuated in E2 rats receiving EPO. Functional assessment with the mNSS, however, did not support the notion of worsened stroke injury in E2 rats or attenuation by EPO treatment. Previous reports from our lab have demonstrated that aged rats provide unique challenges to post-ischemic functional testing due to reduced basal locomotor activity. Therefore, data showing null differences in functional outcome between groups as measured by the mNSS does not provide a complete profile of post-ischemic functional impairment.

Although this data does not prove causality, namely that E2-mediated downregulation of EPO-R leads to exacerbated stroke injury which is corrected by acute EPO treatment, this study provides supporting evidence for that hypothesis and an avenue for further exploration into the

mechanisms of estrogenic action in the ischemic brain. To better understand how estrogen regulated EPO-R expression in the post-ischemic brain, we performed immunohistochemistry for ER α , the estrogen receptor implicated in estrogen-mediated neuroprotection in young adult animals (Sampei et al., 2000, Dubal et al., 2001, Dubal et al., 2006). We observed that aged rats treated with E2 have reduced ER α expression, which is most pronounced in the penumbral cortex and striatum. ER α expression was not affected by EPO treatment, as expected. Taken together, our findings support a proposed mechanism of estrogenic action in the post-ischemic brain where chronic estrogen supplementation leads to reduced ER α expression which, in turn, reduces EPO-R expression and exacerbates ischemic injury. In this proposed mechanism, and supported by our data, it is the EPO/EPO-R system, rather than estrogen directly that confers neuroprotection and is altered by chronic E2 treatment. However, there are many additional details left unexplained in this proposed model, thus warranting further study. Most importantly, perhaps, is the question of age-related differences in estrogenic action.

In addition, the present study has some limitations and leaves some questions unanswered. First and foremost are the unexpected results from the EPO ELISA which do not show elevated serum EPO in rats receiving EPO and show significantly higher levels of EPO in the CSF compared to the serum in all treatment groups. The most plausible conclusion to draw from this experiment is that the ELISA did not accurately measure EPO levels. There are two main reasons why this may be true: 1) EPO levels from samples were calculated from the equation of the best fit line of the standard curve and were outside the range of the standards, thus limiting the accuracy of the assay, and 2) samples containing lysed red blood cells can alter results from colorimetric ELISAs, and some samples did have hemolysis present. The second caveat involves the type of estrogen supplementation used, which is unlike commonly prescribed estrogen therapy. Clinically prescribed conjugated equine estrogen is a mixture of several estrogens, with the most common preparation containing estrone sulfate, equilin sulfate, and equilenin sulfate. In contrast, laboratory studies, like the one presented here, use the most active form of es-

trogen, 17 β -estradiol in a time-release matrix or silastic capsule. This time-release matrix is also imperfect, and upon implantation, serum E2 levels increase rapidly followed by a slow decline in hormone level; this dosage profile differs from the more constant hormone levels achieved by oral supplementation in women.

Finally, a potentially clinically relevant result from our studies that is likely unrelated to exacerbation of stroke injury, warrants discussion. In the debate surrounding HRT, stroke severity is not the major concern; rather, the increased risk of stroke in women taking HRT or estrogen alone is the primary focus of discussion. Based on the data from our JAK/STAT array, we propose that estrogenic effects on the PRL/PRL-R system may account for increased stroke risk in women taking HRT or estrogen alone. In our first cohort of rats, E2 supplementation led to alterations in PRL and PRL-R in the post-ischemic brain, specifically, a 5-fold upregulation of PRL mRNA and a 33-fold upregulation of PRL-R mRNA at 12 hours after MCAO. Research has indicated that PRL-R is present on hematopoietic tissue and PRL/PRL-R activity is implicated in the promotion of clot formation, specifically affecting platelets (Dardenne et al., 1994).

Observational support of the role of PRL and PRL-R comes from pregnant and puerperium women, states in which stroke and venous thromboembolism (VTE) risk is the highest in women under age 50 and which coincides with the time of increased PRL (Wallaschofski et al., 2001). Likewise, females taking hormonal birth control or hormone replacement therapy are also at increased risk of VTE and stroke (Pymar and Creinin, 2001), and, as demonstrated in an animal model here, have elevated PRL and PRL-R mRNA in the brain. The mechanism by which PRL enhances clotting is thought to involve upregulation of P-selectin, an adhesion molecule on the surface of platelets that enhances platelet aggregation (Raaz et al., 2006). It is unknown if PRL acts within the brain to enhance clotting or if PRL/PRL-R are involved in stroke risk or outcome. However, considering the supposed platelet-activating actions of PRL and the effects of E2 to increase PRL and PRL-R in the brain, further investigation is warranted.

Summary

Preclinical studies have demonstrated that estrogen is neuroprotective in young adult animal models of stroke. We have previously demonstrated that estrogen does not confer neuroprotection in the aged female rat model of stroke, and, in fact, increases mortality and exacerbates ischemic brain injury. In the present study, we show that in the same estrogen-treated rats that exhibited increased mortality and worsened stroke injury, there was downregulation of EPO-R mRNA in the ischemic cortex accompanied by reduced EPO-R protein expression, as observed by immunostaining. In a second cohort of aged female rats treated for 90 days with estrogen or placebo, we demonstrate attenuation of the estrogenic effects on infarct size with administration of EPO at 2 h after initiation of MCAO. EPO treatment also reduced disruption of tissue architecture and pettichae formation within the ischemic parenchyma. Lastly, we observe reduced ER α protein expression in the same aged female rats. Together, these data support the hypothesis that chronic estrogen supplementation reduces ER α expression and subsequently inhibits post-ischemic EPO-R expression resulting in worsened stroke injury. There remain significant questions to be answered in order to validate and gain a better understanding of this proposed model of estrogenic action in the post-ischemic aged rat brain.

Unigene	GeneBank	Symbol	Description	Gene Name
Rn.198688	NM_012488	A2m	Alpha-2-macroglobulin	A2MAC1, A2m1, A2maa, MGC114358, Mam, RATA2MAC1
Rn.163202	NM_053669	Sh2b2	SH2B adaptor protein 2	Aps
Rn.10323	NM_031535	Bcl2l1	Bcl2-like 1	Bcl-xl, Bcl2l, Bclx, bcl-X
Rn.22279	NM_171992	Cond1	Cyclin D1	-
Rn.10089	NM_080782	Cdkn1a	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	Cip1, Waf1
Rn.6479	NM_024125	Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	Il6dbp, LAP, NF-IL6, TCF5
Rn.163154	NM_019302	Crk	V-crk sarcoma virus CT10 oncogene homolog (avian)	Crko
Rn.16463	NM_017096	Crp	C-reactive protein, pentraxin-related	Aa1249, Ab1-341, Ab2-196, Ac1-114, Ac1262, Ac2-069, Ba2-693
Rn.72599	NM_001029901	Csf1r	Colony stimulating factor 1 receptor	CSE-1-R, MGC125014, c-fms
Rn.42930	NM_133555	Csf2rb	Colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)	Cs2rb1
Rn.7391	NM_145672	Cxcl9	Chemokine (C-X-C motif) ligand 9	MGC105312, Mig, Scyb9
Rn.37227	NM_031507	Egfr	Epidermal growth factor receptor	ERBB1, ErbB-1, Erip
Rn.22394	NM_017002	Epor	Erythropoietin receptor	MGC108723
Rn.54498	NM_022924	F2	Coagulation factor II (thrombin)	-
Rn.2609	NM_012950	F2r	Coagulation factor II (thrombin) receptor	MGC93622, Par1, TRGPC
Rn.9677	NM_012724	Fcgr1a	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	Iger01, RATIGERO1
Rn.16643	XM_215643	Fcgr1a	Fc fragment of IgG, high affinity Ia, receptor (CD64)	FcgammaRI, Fcgr1, Fcgr1b
Rn.198318	XM_216605	G1p2	Interferon, alpha-inducible protein (clone IFI-15K)	-
Rn.92350	NM_133293	Gata3	GATA binding protein 3	-
N/A	XM_221882	Gbp1	Guanlylate binding protein 1, interferon-inducible, 67kDa	-
Rn.2178	NM_017094	Ghr	Growth hormone receptor	GHR, MGC124963, MGC156665
Rn.83614	NM_139327	Hmga1	High mobility group AT-hook 1	Hmgl, Hmgly
Rn.105738	XM_213649	Ilfnr1	Interferon (alpha, beta and omega) receptor 1	-
Rn.10795	NM_138880	Ilfg	Interferon gamma	IFNG2
Rn.19927	NM_053783	Ilfng1	Interferon gamma receptor 1	Ilfng1
Rn.64632	NM_057193	Il10ra	Interleukin 10 receptor, alpha	-
Rn.9872	NM_013163	Il2ra	Interleukin 2 receptor, alpha	IL2RAC
Rn.14508	NM_080889	Il2rg	Interleukin 2 receptor, gamma	Ab2-183, Cd132
Rn.108255	NM_201270	Il4	Interleukin 4	Il4e12
Rn.10471	NM_133380	Il4ra	Interleukin 4 receptor, alpha	Il4r
Rn.12138	NM_001008725	Il6at	Interleukin 6 signal transducer	Ac1055
Rn.6396	NM_012591	Irf1	Interferon regulatory factor 1	-
Rn.163277	NM_001012041	Irf9	Interferon regulatory factor 9	Isgf3g
Rn.90191	XM_342872	Jak1	Janus kinase 1	-
Rn.18909	NM_031514	Jak2	Janus kinase 2	-
Rn.11159	NM_012855	Jak3	Janus kinase 3	RATJAK3
Rn.93714	NM_021835	Jun	Jun oncogene	-
Rn.15806	NM_021836	Junb	Jun B proto-oncogene	-
Rn.47894	NM_001024344	Slt1	Signaling threshold regulating transmembrane adaptor 1	-
Rn.222008	XM_001060362	Il20	Interleukin 20	-
Rn.32086	NM_133523	Mmp3	Matrix metalloproteinase 3	-
Rn.198731	XM_345572	Mpl	Myeloproliferative leukemia virus oncogene	-
Rn.12072	NM_012603	Myc	Myelocytomatosis oncogene	MGC105490, RNCMYC, c-myc, nMyc
Rn.2411	XM_342346	Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NF-kB
Rn.10400	NM_012611	Nos2	Nitric oxide synthase 2, inducible	Nos2a, iNos

Unigene	GeneBank	Symbol	Description	Gene Name
Rn.90070	NM_012576	Nr3c1	Nuclear receptor subfamily 3, group C, member 1	GR, Gcr, Grl
Rn.127158	NM_001006961	Osm	Oncostatin M	-
Rn.55127	XM_214030	Pdgfra	Platelet derived growth factor receptor, alpha polypeptide	APDGFR, PDGFACE
Rn.24369	XM_217188	Pias1	Protein inhibitor of activated STAT, 1	-
Rn.9759	NM_012629	Prl	Prolactin	PRLB, PRLSD1, Pr1a1, Prol, RATPRLSD1, RNPRL
Rn.9757	NM_012630	Prlr	Prolactin receptor	MGC105486, RATPRLR
Rn.11317	NM_012637	Pltn1	Protein tyrosine phosphatase, non-receptor type 1	MGC93562, Ptp
Rn.53971	NM_013016	Sirpa	Signal-regulatory protein alpha	Bit, Ptgs1, SHPS-1
Rn.90166	XM_001062978	Ptprc	Protein tyrosine phosphatase, receptor type, C	CD45, L-CA, Lca, RT7, T200
Rn.11295	NM_145779	Pzp	Pregnancy-zone protein	-
Rn.22261	XM_221655	Il10rb	Interleukin 10 receptor, beta	RGD1560373
Rn.145609	XM_001067317	RGD1562071	Similar to Src-like adaptor protein-2	-
Rn.162251	XM_001067909	Stam	Signal transducing adaptor molecule (SH3 domain and ITAM motif) 1	RGD1564499
Rn.47568	XM_575987	Socs5	Suppressor of cytokine signaling 5	RGD1564914
Rn.212982	NM_001005892	Stp1	SFFV proviral integration 1	Pu.1
Rn.11069	NM_134456	Sh2bpm1	SH2-B PH domain containing signaling mediator 1	Sh2-b, Sh2b
Rn.10635	NM_013130	Smad1	SMAD family member 1	Madh1
Rn.2755	NM_019191	Smad2	SMAD family member 2	MGC156656, Madh2
Rn.10636	NM_013095	Smad3	SMAD family member 3	Madh3
Rn.9774	NM_019275	Smad4	SMAD family member 4	Madh4
Rn.146857	NM_021692	Smad5	SMAD family member 5	Madh5
Rn.82754	NM_145879	Socs1	Suppressor of cytokine signaling 1	Cish1, Socs-1
Rn.205056	NM_058208	Socs2	Suppressor of cytokine signaling 2	Cish2, Socs-2
Rn.127801	NM_053565	Socs3	Suppressor of cytokine signaling 3	Cish3, Socs-3, Sai-3
Rn.62929	XM_223934	Socs4	Suppressor of cytokine signaling 4	-
Rn.44609	NM_012655	Sp1	Sp1 transcription factor	-
Rn.112600	NM_031977	Src	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	-
Rn.33229	NM_032612	Stat1	Signal transducer and activator of transcription 1	-
Rn.24237	NM_001011905	Stat2	Signal transducer and activator of transcription 2	-
Rn.10247	NM_012747	Stat3	Signal transducer and activator of transcription 3	MGC93551
Rn.137580	NM_001012226	Stat4	Signal transducer and activator of transcription 4	-
Rn.154399	NM_017064	Stat5a	Signal transducer and activator of transcription 5A	Stat5
Rn.54486	NM_022380	Stat5b	Signal transducer and activator of transcription 5B	-
Rn.6880	XM_343223	Stat6	Signal transducer and activator of transcription 6	-
Rn.6504	NM_001025625	Stub1	STIP1 homology and U-Box containing protein 1	MGC116422
Rn.162521	NM_139194	Fas	Fas (TNF receptor superfamily, member 6)	Tnfrsf6
Rn.140313	XM_233741	Tyk2	Tyrosine kinase 2	-
Rn.10845	NM_031777	Usf1	Upstream transcription factor 1	-
Rn.162877	NM_173290	Yy1	YY1 transcription factor	NF-E1, UCRBP
Rn.973	NM_001007604	Rplp1	Ribosomal protein, large, P1	MGC72935
Rn.47	NM_012583	Hprt1	Hypoxanthine phosphoribosyltransferase 1	Hgprtase, Hprt, MGC112554
Rn.92211	NM_173340	Rpl13a	Ribosomal protein L13A	-
Rn.107896	NM_017025	Ldha	Lactate dehydrogenase A	Ldh1
Rn.94978	NM_031144	Actb	Actin, beta	Actx

Table 3.1. Probes tested in JAK/STAT array

Figure 3.1. Estrogen supplementation leads to downregulation of EPO-R mRNA following MCAO. Post-ischemic mRNA levels of modulators of the JAK/STAT signaling pathway were measured at 12 h following MCAO in the first cohort of rats. Compared to Intact rats, E2 rats had increased mRNA levels of prolactin (PRL) and prolactin-receptor (PRL-R) and nearly a 5-fold decrease in EPO-R mRNA. Level of significance set at $p < 0.05$, indicated by lines surrounding center line.

Figure 3.2. Estrogen treated rats have reduced EPO-R immunostaining in the ischemic penumbra. Immunostaining for EPO-R in the first cohort of rats was most prominent in all samples in the penumbral region and was not observed in the ischemic core. Compared to both Intact (A, B) and OVX (C, D) rats, E2 rats had reduced post-ischemic EPO-R immunolabeling (E, F), most pronounced in the penumbral cortex (E). EPO-R immunostaining was observed to be most prominent in the penumbral cortex of OVX rats (C). Scale bars = 100 μ m.

Figure 3.3. Significant difference in serum E2 levels between E2 and placebo treated rats.

Serum E2 levels from the second cohort of rats prior to randomization at 15 months of age (Pre) and two weeks before MCAO, at 18 months of age (Post). Rats treated with pellets containing 17β -estradiol had significantly higher serum E2 levels at 18 months compared to rats administered placebo pellets. * $p < 0.05$ compared to placebo treated rats using one-way ANOVA with Tukey's post hoc analysis.

Figure 3.4. Serum and CSF levels of EPO. Serum and CSF were collected at time of sacrifice at 24 h after MCAO in the second cohort of rats and EPO levels were measured by enzyme-linked immunosorbent assay. (A) There were no significant differences in serum EPO levels between treatment groups. (B) Statistical analysis could not be performed on CSF levels from E2 groups due to a sample size of one in E2 + EPO group.

Figure 3.5. Serum tumor necrosis factor- α is increased by estrogen. (A) TNF- α levels were measured in serum by enzyme-linked immunosorbant assay in the second cohort of rats. A subset of Intact and E2 rats received EPO (i.p., 5000U/kg). In Intact rats, EPO had little effect on TNF- α levels, while E2 treatment led to a significant increase in TNF- α that was further increased by EPO treatment. (B) E2 supplementation did not have significant effects on serum interleukin-6 levels. EPO treatment tended to increase serum IL-6 levels in rats at 24 h after MCAO, but data did not reach significance ($p=0.07$). * $p<0.05$ compared to Intact rats using two-way ANOVA with Tukey's post hoc analysis.

Figure 3.6. Acute treatment with EPO reduces infarct volume in estrogen treated rats.

Modified Neurologic Severity Score and infarct volume were measured at 24 h after MCAO in the second cohort of Intact and E2 rats. (A) EPO treatment at 2 h after initiation of MCAO did not significantly alter cortical, striatal, or total infarct volume in Intact rats. In E2 rats, EPO treatment resulted in significantly decreased total infarct volume ($p=0.04$). EPO also decreased cortical infarct volume in E2 rats, but not significantly ($p=0.07$). (B) No significant differences between groups were measured in composite mNSS score. * $p<0.05$ compared to EPO treated rats using two-way ANOVA with Tukey's post hoc analysis.

Figure 3.7. Estrogen leads to greater disruption of tissue architecture and petechial hemorrhage following MCAO. Coronal tissue sections from the infarcted region of rats from the second cohort were stained with hematoxylin and eosin. Overall, saline treated Intact rats (A, B) showed less necrotic damage in the ischemic hemisphere than E2 rats (E, F). Post-ischemic disruption of tissue architecture was observed in the ischemic hemisphere in all specimens, but

was most pronounced in the ischemic cortex of E2 rats not receiving EPO (E, F). Acute treatment with EPO reduced post-ischemic disruption of tissue architecture in E2 rats (G, H) but led to increased tissue vacuolation in both Intact (C, D) and E2 rats (G, H). Scale bars = 100 μ m.

Figure 3.8. E2 rats show reduced ER α expression following MCAO. ER α immunoreactivity was observed in the ischemic core and to a lesser extent in the penumbral region in all treatment groups of rats from the second cohort. Cellular morphology of ER α cells was consistent with that of neurons, although no cellular markers were used. No ER α expression was noted in the contralateral hemisphere. Both saline and EPO treated Intact rats had similar levels of ER α expression in all regions studied (A – H). Compared to Intact rats, both saline and EPO treated E2 rats had fewer ER α + cells, which was most pronounced in the penumbral cortex and striatum (I, L). No significant difference in number of ER α + cells based on EPO treatment was observed. Scale bars = 50 μ m.

Figure 3.9. E2 rats show reduced EPO-R immunoreactivity in the penumbral cortex and striatum. At 24 h after MCAO, brain sections were immunolabeled with EPO-R. Similar to results from the first cohort of E2 rats, rats from the second cohort treated with E2 beginning at 15 months (E, F, G, H) also showed reduced EPO-R+ cells compared to Intact rats (A, B, C, D). This reduction in EPO-R expression was observed in both penumbral cortex as well as penum-

bral striatum. Morphology of EPO-R⁺ cells was consistent with activated astrocytes and microglia. Scale bars = 50µm.

Chapter Four

Estrogen supplementation leads to dry eye syndrome in aged female rats

Abstract

Keratoconjunctivitis sicca (KCS) is a leading cause of patient visits to eye care providers. Hormone replacement therapy is associated with an increased incidence of dry eye symptoms and KCS, but the mechanism by which this occurs is not known. The current study investigates the effects of estrogen supplementation on structure and function of lacrimal and Meibomian glands in a clinically relevant animal model. Following 90 days of estrogen supplementation in aged female rats, tear volume by a modified Schirmer's I test and fluorescein tear break up time (TBUT) were assessed followed by lacrimal and Meibomian gland excision and histologic evaluation. Hematoxylin and eosin staining as well as immunohistochemistry were performed to observe structural changes and expression levels of estrogen receptors α and β in the lacrimal and Meibomian glands. Our principle findings include a reduction in tear volume and TBUT in estrogen treated rats accompanied by increased connective tissue fibrosis in both extraorbital and intraorbital lacrimal glands and reduced acinar lumen in the intraorbital lacrimal glands. These functional and structural changes are likely mediated by $ER\alpha$, as estrogen treatment led to upregulation of $ER\alpha$ expression in lacrimal and Meibomian glands.

Introduction

Dry eye syndrome, or keratoconjunctivitis sicca (KCS), affects nearly 25 million people in the U.S. and is a leading cause of patient visits to both ophthalmologists and optometrists (Schaumberg et al., 2001). KCS is associated with an increased risk of corneal infection and in severe cases has led to permanent vision loss (Gilbard, 2005). The symptoms associated with KCS include ocular dryness, foreign-body sensation or grittiness, pain, burning, photophobia, and blurry vision. The discomfort of KCS has been correlated to reduced work capacity (Lemp, 1998) and increased anxiety and depression (Li et al.). The pathophysiology of KCS is not well studied.

Etiology of keratoconjunctivitis sicca

A normal tear film is essential for the health of the eye. Enzymes and antibodies in tears, namely lysozyme, lactoferrin, B lysin, and immunoglobins A and G serve to protect against ocular infection (Girgis et al., 2003). Growth factors in tears maintain the corneal integrity. Abnormal or insufficient tear film is the result of pathology of one or more structures that produce the layers of the tear film, of which there are three: a mucin layer produced by goblet cells of the conjunctival epithelium, an aqueous layer secreted by the lacrimal glands, and a lipid layer produced by the eyelid Meibomian glands. The mucin layer serves to spread the aqueous layer evenly over the ocular surface and represents a very small percentage of the thickness of the tear film. The aqueous tear layer, produced by the lacrimal glands, makes up over 90% of the thickness of the tear film (Zoukhri, 2006). The lacrimal glands are tubulo-acinar glands and in humans are located in the lacrimal fossa of the frontal bone above and toward the temporal aspect of the orbit. Meibomian glands are holocrine, tubulo-acinar glands that secrete the most anterior tear layer, known as meibum, which contains lipids that serve to prevent evaporation of the underlying aqueous layer. The Meibomian glands are located in a single row along both the superior and inferior lid margins.

Dry eye syndrome is most commonly caused by an inadequate production of the aqueous layer by the lacrimal glands (Kecik et al., 1994). Lacrimal gland dysfunction can result from infiltration of lymphocytes and overall inflammation, which in some cases occurs as a comorbid condition along with another autoimmune disease such as rheumatoid arthritis and/or systemic lupus erythematosus (McCartney-Francis et al., 1997, Zoukhri, 2006). When lacrimal gland inflammation is accompanied by inflammation and/or enlargement of the salivary glands, the clinical diagnosis is Sjogren's Syndrome. However, lacrimal gland dysfunction appearing as part of Sjogren Syndrome is not as common as lacrimal gland dysfunction without comorbid conditions (Chomette et al., 1986, Jabs et al., 1991, Tsubota et al., 2001). Meibomian gland dysfunction can also lead to symptoms of KCS. Genetic analysis of human eyelids from normal controls and patients with KCS reveal upregulation of genes encoding small proline-rich proteins and S100 calcium binding proteins, suggesting that keratinization may play a role in Meibomian gland dysfunction and subsequent KCS (Liu et al.). In addition, Meibomian gland dysfunction can be caused by altered meibum composition (Mathers et al., 1991) or obstruction of the glands (Driver and Lemp, 1996). Without the presence and proper function of each of the tear film layers, dry eye syndrome can result.

Clinical Diagnosis and Management of KCS

Clinical diagnosis of KCS can be determined by the symptoms, but specific tests aide in the determination of the severity of the disease. One common test to measure the abundance of tears is the Schirmer's I Test, in which a graded strip of filter paper is placed in the upper palpebral fissure for 5 minutes (Gontier and Fisch, 1976). Tear volume reaching 5 mm or less on the filter paper is indicative of dry eye syndrome. In the Schirmer's test II the nasal mucosa is irritated by rubbing it with a cotton swab and tear volume is measured. In this test, a tear volume equal or less than 15 mm indicates dry eye syndrome. A third test that is commonly used to assess KCS is tear break up time (TBUT) in which fluorescein dye is added to the ocular surface to allow visualization of the tear film. The tear film is observed with slit lamp microscopy and the

time until the tear film becomes disrupted is measured. Normal TBUT is greater than 10 seconds, while TBUT in KCS is commonly less than 5 seconds. TBUT is most useful in the diagnosis of evaporative KCS in which tear volume may be normal or increased, but tears evaporate quickly due to dysfunction of the lipid layer.

Clinical management of KCS can be costly and includes topical lubricants, immunosuppressive agents, punctal occlusion, and surgery. For mild to moderate cases of KCS, the first line of treatment consists of over-the-counter (OTC) remedies such as artificial tears, humidifiers, and warm compresses. More severe cases of KCS require more aggressive treatment. Prescription strength ophthalmic lubricants can provide a more long-lasting effect than OTC artificial tears (O'Brien and Collum, 2004). In addition, KCS may be treated with ophthalmic anti-inflammatory medications such as cyclosporine to reduce the inflammation and irritation of the conjunctiva. In patients with a definitive autoimmune etiology of KCS, immunosuppressive agents, such as corticosteroid drops, can help alleviate symptoms. These drops also promote healing in patients with corneal abrasion due to KCS. Finally, if other therapies fail to provide relief, surgical treatment is considered. Punctal occlusion is a minor surgical procedure in which the punctae are blocked to reduce the tear outflow to the nose, thus allowing tears to remain in the eye for a longer duration. Punctal occlusion can be performed by inserting a specially designed ocular device, allowing it be reversible; conversely, the punctae can be occluded permanently with cauterization. Depending on the cause of KCS, more invasive surgeries can be performed to correct the underlying cause. For example, in some stroke patients, the ocular muscles are affected creating lag ophthalmos. This allows increased evaporation of tears and can be an underlying cause of KCS. Lag ophthalmos can be corrected by implanting a small gold plate in the eyelid that will facilitate the eyelid in closing. KCS caused by evaporative tear loss due to eyes that open too widely can be corrected by lateral tarsorrhaphy, in which the lateral aspect of the eyelids are sutured together such that the eyes cannot open as widely and close more easily (Bodian, 1965).

Animal Models of KCS

Animal models of KCS have revealed some insights into the multifactorial etiology of the disease, but many questions remain. Current models of dry eye syndrome typically involve single or multiple doses of drugs such as atropine sulphate (Burgalassi et al., 1999), the preservative benzalkonium chloride (Lin et al.), T-cell mitogen, concanavalin A (Seo et al.), or botulinum toxin B (Hongyok et al., 2009). Yet, despite development of these models, no cure for KCS has been developed. To date, no models of KCS have been reported involving the local or systemic administration of female gonadal hormones to the aging female rat, despite the fact that the tear film is known to be affected by hormone replacement therapy (HRT) (Mathers et al., 1998). In the present study, we report a novel model of KCS following chronic systemic administration of estradiol (E2) to aging female Sprague-Dawley rats. Although this model could be used to study KCS therapeutics, it also provides specific clinical relevance related to the reported increase in KCS in women taking HRT. A 2001 report from the Women's Health Study demonstrated a nearly 30% increase of KCS in women taking hormone replacement therapy (HRT), and women with prior hysterectomy taking estrogen alone experienced an almost 70% increase in KCS compared to non-treated women of the same age (Schaumberg et al., 2001). Moreover, there are two reports of estrogen therapy inducing Sjogren's syndrome, an autoimmune disease that targets salivary glands, and the lacrimal gland, leading to KCS (Nagler and Pollack, 2000). These studies show that estrogen is linked to altered tear production. Due to the effects of estrogen on the lacrimal gland in women who develop Sjogren's syndrome, a potential target of estrogenic action is the aqueous tear layer produced by the lacrimal gland. In the present study, we assessed the effects of E2 on tear production and tear break up time in aged female rats. In an effort to clarify the mechanism by which estrogen affects tear production, we also examined lacrimal and Meibomian glands histologically.

Materials and Methods

Chemicals and Animals. Twenty-four female Sprague-Dawley rats, age 15 months, were purchased from Hilltop Animal Laboratory (Scottsdale, PA) and housed under 12 h:12 h light/dark conditions with food and water provided ad libitum. All chemicals, except where noted, were purchased from Sigma-Aldrich (Saint Louis, MO). Isoflurane was purchased from Halocarbon (River Edge, NJ). E2 and placebo pellets (1.5mg; 90 d release) were purchased from Innovative Research of America (Sarasota, FL). All procedures were in accordance with guidelines set forth by the West Virginia University Animal Care and Use Committee.

Treatment Protocol. Intact rats were subcutaneously implanted with placebo pellets and E2 rats received pellets containing 1.5 mg of 17 β -estradiol in a 90-day time-release matrix. Both groups received treatment until they reached 18 months of age. Serum E2 was measured prior to treatment and at 18 months using [I^{125}] radioimmunoassay (U of Virginia Ligand Core, Charlottesville, VA).

Tear Volume Assessment. Tear volume was assessed in rats under isoflurane anesthesia (3 min, 4% induction, 2% maintenance). Modification of Schirmer's filter paper strips was as follows: strips were cut lengthwise in half to reduce width to fit into the rat palpebral fissure and allow for more precise measurements of the rat tear volume. The end before graded markings began was also removed. Only the halves of the strips containing the graded markings were used. Modified Schirmer's strips were bent at the 2 mm mark and placed with forceps into the upper palpebral fissure of the rat for 5 min. After 5 min, test strips were removed and the line on the strip where tears reached was recorded.

Tear Break up Time. Tear break up time (TBUT) was assessed with portable slit lamp with rats under isoflurane anesthesia, simultaneous with measurement of tear volume. Following placement of the modified Schirmer's strip for tear volume assessment, while remaining under isoflurane anesthesia (2% maintenance), rats were positioned laterally to determine TBUT in the contralateral eye. Fluorescein (1.0 μ l, 0.25%, OcuSOFT, Richmond, TX) was administered to the rat eye followed by three manual blinks. Following the third blink, TBUT was measured with porta-

ble slit lamp and time to first break in the tear film recorded. This procedure was repeated three times with the same eye, and averaged.

Histology. At 18 months, following functional assessments of tear volume and TBUT, rats were transcardially perfused and intraorbital and exorbital lacrimal glands were excised. Eyelids were removed for analysis of Meibomian glands. All glands were fixed in 10% buffered formalin overnight followed by processing for paraffin embedding. After embedding, glands were sectioned at 8 μ m and mounted onto slides. One set of slides each was stained with hematoxylin and eosin, immunolabeled with estrogen receptor α and estrogen receptor β (ER α , ER β ; abcam, Cambridge, MA). For immunolabeling, mounted sections were treated with 0.3% H₂O₂-30% methanol in PBS to suppress endogenous peroxidases. Nonspecific binding was blocked using 10% horse serum. Sections were incubated in ER α antibody (1:100) and ER β (1:500) overnight at 4°C. Sections were incubated with biotinylated goat anti-rabbit or horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) followed by an avidin-biotin complex process (Vector Laboratories). Finally, sections were exposed to stable 3,3'-diaminobenzidine tetrahydrochloride (DAB). Microscopic analysis of hematoxylin and eosin, ER α , and ER β stained sections was performed using an Olympus AX70 Provis microscope (Center Valley, PA).

Statistical analysis. Data are presented as mean $\pm$ S.E. Statistical significance in measured and calculated parameters was determined using one-way ANOVA with Tukey's post-hoc analyses. Level of significance was set at $p < 0.05$.

Results

Confirmation of distinct treatment groups. Serum E2 levels were measured from blood collected prior to randomization to treatment groups and again at time of euthanasia. At 18 months, two distinct treatment groups could be differentiated by serum E2 levels with E2 rats having significantly elevated serum E2 levels compared to Intact rats (Figure 4.1)

Aged rats treated with E2 have reduced tear volume and shorter TBUT. To measure functionality of tear glands, we studied the tear volume produced by rats using a modified Schirmer's I test. Tear break up time was measured by fluorescein visualization with slit lamp microscopy (Figure 4.2). Aged rats receiving E2 had significantly less tear volume compared to Intact rats (7.4 ± 0.58 mm and 15.8 ± 2.1 mm, respectively; $p < 0.001$). TBUT was also altered by estrogen treatment with E2 rats having a significant reduction in time to first tear disruption compared to Intact rats (16.4 ± 1.1 sec and 33.7 ± 1.9 sec, respectively; $p < 0.001$) (Figure 4.3).

E2 alters tissue architecture of lacrimal glands in the aged female rat. In both intraorbital and extraorbital lacrimal glands, E2 rats demonstrated increased amounts of connective tissue compared to Intact rats, consistent with fibrosis (Figures 4.3, panels A, B and Figure 4.4, panel B). Fibrosis led to disruption of tissue architecture in the intraorbital lacrimal glands of E2 rats, but was mostly observed in the interlobular space in the extraorbital lacrimal glands. Extraorbital lacrimal glands were also observed to have widened interlobular and intralobular ducts in rats receiving E2. No differences between treatment groups were noted in the structure or morphology of Meibomian glands of the lower lid.

Lack of supplemented estrogen was associated with larger acinar lumen and dacryolith formation in the intraorbital lacrimal gland. Hematoxylin and eosin staining revealed stone-like structures with lamellar rings within the acinar lumen in some regions of the intraorbital lacrimal glands. These lacrimal stones, or dacryoliths also stained darkly with 3,3-diaminobenzidine (Figure 4.6, panel C). Intraorbital lacrimal glands from Intact rats showed numerous dacryoliths compared to very few and smaller ones in E2 rats (Figure 4.4, panel A). In addition, acinar cells of E2 rats appeared to contain more cytoplasm and were more rounded, thereby reducing the acinar lumen size compared to less rounded acinar cells and larger lumen size in Intact rats (Figure 4.4, panels C,D).

Estrogen treatment associated with increased ER α expression in tear producing glands. To begin to examine the mechanism by which E2 acts in the tear producing glands to reduce tear

volume and TBUT, we immunostained glands with ER α antibody. In all glands, ER α immunolabeling of cell nuclei was increased in rats receiving E2 compared to Intact rats. In the intraorbital lacrimal gland, ER α immunostaining was limited to the gland margin in E2 rats.

Discussion

Estrogen has been shown to have deleterious effects on tear production in previous studies (Nagler and Pollack, 2000). In the present study, we assessed the effects of E2 supplementation on tear production and composition in a clinically relevant animal model. By using aged female rats treated with estrogen for 3 months, we created a model of KCS arising from HRT. In this model, we assessed both functional and structural/molecular aspects of tear production including tear volume, TBUT, gland structure, and ER expression. This study showed for the first time that chronic systemic E2 supplementation alters lacrimal and Meibomian gland structure and function in aged female rats, and these changes are associated with increased ER α expression.

Specifically, using a modification of the Schirmer's I test, we measured tear volume in Intact and E2 treated aged female rats and found that chronic E2 supplementation leads to reduced tear volume. TBUT was measured with slit lamp microscopy and revealed that tear film undergoes faster evaporative loss in rats receiving E2. This reduced TBUT could result from either inadequate lipid layer production, thus allowing evaporative loss, or an overall reduction in aqueous tear component. Future studies will focus on specific effects of estrogen on tear composition.

To more fully evaluate estrogenic effects on tear producing glands, we removed glands from rats and processed them for histology. Upon examination, lacrimal gland sections revealed several important differences between Intact and E2 rats: 1) increased connective tissue fibrosis in E2 rat lacrimal glands accompanied by widened interlobular and intralobular spaces in the extraorbital glands 2) more rounded acinar cells and reduced acinar lumen size in intraorbital

lacrimal glands of E2 rats, and 3) numerous lacrimal dacryoliths in intraorbital glands of rats lacking supplemented E2. Connective tissue fibrosis in both intraorbital and extraorbital lacrimal glands of E2 rats was also associated with disruption of the normal tissue architecture and may be a contributing factor to the reduced functionality of the glands. In addition, the smaller lumen size of acinar structures in E2 rats may increase likelihood of obstruction and/or reduce the outflow of secretions, thereby contributing to reduced tear volume. In addition to lumen size, acina in both Intact and E2 rats contained protein-rich material that stained more darkly in E2 rats. Darkly stained material within the acinar lumen may indicate greater protein content, but further analysis of tear composition would need to be conducted to verify this hypothesis. Lastly, the presence of numerous dacryoliths in the intraorbital lacrimal glands of Intact rats suggests an estrogen-related inhibition of dacryolith formation. Previous studies suggest patients with symptomatic dacryolithiasis are more likely to be female (59.1%) and over age 70 years, therefore postmenopausal (Repp et al., 2009), thus supporting the notion that female gonadal hormones play a role in inhibiting dacryolith formation. However, dacryolith presence was not associated with reduced function of tears in the present study as Intact rats had significantly higher tear volume and TBUT compared to E2 rats.

Importantly, no significant inflammatory cell infiltrates were observed in glands from E2 rats. Due to the reported cases of HRT leading to the development of Sjogren's syndrome, one hypothesis for the deleterious actions of estrogen in the lacrimal and Meibomian glands is through inflammatory disruption of normal cell function and tissue structure (Toda et al., 1999, Niederkorn et al., 2006, Zoukhri, 2006). Our results do not support this hypothesis of estrogenic action in lacrimal and Meibomian glands of our aged female rat model; however, additional studies, such as mRNA quantification of inflammatory cytokines, would be useful to rule it out.

In order to determine how estrogen mediates its actions on the tear producing glands, we performed immunohistochemistry on sections from lacrimal and Meibomian glands of E2 and Intact rats. Previous report have demonstrated the presence of ER α in lacrimal and Meibo-

mian glands of young adult rats (Spelsberg et al., 2004, Richards and Sullivan, 2009). In the present study, we found ER α immunoreactivity in the lacrimal and Meibomian glands of aged female rats and we show that ER α expression is upregulated by chronic E2 supplementation. We also confirmed a lack of immunostaining of ER β in lacrimal and Meibomian glands (data not shown), as has been reported previously. Together with tear volume and TBUT data, these results support the notion that E2 actions in the tear producing glands are mediated through ER α . The functional consequences of ER α signaling in the tear glands are unknown as estrogen response elements are found on many genes that may affect tear gland structure and/or tear production. Specifically, in the mammary gland, estrogen has been shown to regulate expression of extracellular matrix (ECM) proteins and integrins (Haslam and Woodward, 2003), but estrogenic effects on ECM of lacrimal and Meibomian glands have not been studied to date. Considering our findings of increased connective tissue fibrosis in E2 rats, estrogen-mediated changes in ECM proteins may play a role in those structural changes observed in our model.

The findings presented here are an initial attempt to understand lacrimal and Meibomian gland structure and function in an aged, female, estrogen treated rat model of KCS. However, there are several caveats to the present study. A potentially confounding variable in the tear volume and TBUT assessments is time spent under anesthesia. We attempted to control for rats not closing their eyes during anesthetic induction by standardizing the induction time to 3 min, nevertheless, differences in evaporative tear loss prior to the assessments may have affected our results. Also, the form of E2 supplementation used in this study is unlike commonly used hormone replacement therapy in women. Clinically prescribed conjugated equine estrogen is a mixture of several estrogens, with the most common preparation containing estrone sulfate, equilin sulfate, and equilenin sulfate. We administered the most active form of estrogen, 17 β -estradiol in time-release pellets. However, time-release pellets do not provide a constant dose of E2. Upon subcutaneous implantation of E2 pellets, serum E2 levels increase rapidly followed by a slow decline in hormone level, which differs from the more constant hormone levels

achieved by oral supplementation in women. Despite these caveats, the results presented here offer a beginning towards a more full understanding of the role of E2 in the lacrimal and Meibomian glands.

Summary

In the year 2000, the prevalence of dry eye symptoms in women was estimated to be 16.7% in the Beaver Dam Eye Study Cohort (Moss et al., 2000). Data from the Women's Health Study implicates HRT, particularly estrogen therapy alone, in the development of dry eye syndrome in postmenopausal women (Schaumberg et al., 2001). The effects of estrogen on the lacrimal and Meibomian glands are largely unknown and unstudied. Due to the health and economic burden of dry eye syndrome, a more full understanding of this disease and its etiology should be the focus of ophthalmic research, particularly in aged animal models that mimic KCS arising from HRT.

In the present study, we showed for the first time that aged rats treated chronically with E2 have reduced tear volume and shorter TBUT compared to normally aging female rats. We also demonstrated specific structural changes in the lacrimal glands of rats receiving E2 including increased fibrosis (which disrupted normal tissue architecture in some areas) larger acinar cells, and reduced acinar lumen size. We also noted the presence of numerous dacryoliths in Intact animals which were either not present or much smaller in size in E2 rats. Finally, we performed immunostaining for both ER α and ER β in tear producing glands of both aged Intact and aged E2 treated rats. ER β immunostaining did not reveal ER β expression in any of the tear glands. Conversely, we observed ER α expression in all tear glands which was upregulated in all tear producing glands of rats receiving E2, suggesting that E2-mediated effects on the tear glands are mediated through ER α . Future studies on the effects of estrogen in KCS will focus on the mechanism of estrogenic action - identification of specific proteins that are altered by E2 and that lead to the structural and functional changes we observed in the tear glands.

Figure 4.1. Significant difference in serum E2 levels between E2 and placebo treated rats.

Serum E2 levels of rats prior to randomization at 15 months of age (Pre) and two weeks before gland excision, at 18 months of age (Post). Rats treated with pellets containing 17β -estradiol had significantly higher serum E2 levels at 18 months compared to rats administered placebo pellets. * $p < 0.05$ compared to placebo treated rats using one-way ANOVA with Tukey's post hoc analysis.

Figure 4.2. Modified Schirmer's I test and tear breakup time in the anesthetized rat. (A) A modified Schirmer's filter paper strip fits more easily in the upper palpebral fissure of the Sprague-Dawley rat and allows for improved precision in quantifying tear volume in the rat. (B) A portable slit lamp was used to determine tear break up time in the anesthetized animal. Following administration of fluorescein sodium (0.25%), one researcher manually blinks the eye three times and then aides in holding it open while tear break up time is measured by a second researcher.

Figure 4.3. Estrogen treatment leads to reduced tear volume and lower tear break up time in aged female rats. (A) Tear volume was measured in 18 month old female rats during 3 months of E2 or placebo supplementation. Intact rats had significantly higher tear volume as measured by the modified Schirmer's I test compared to E2 rats. (B) Tear break up time was also measured in rats receiving placebo or E2 treatment using fluorescein sodium (0.25%). Time to first break up of tear film, as observed with slit lamp microscopy, was significantly higher in Intact rats compared to E2 rats. # $p < 0.001$ compared to E2 rats using one-way ANOVA with Tukey's post hoc analysis.

Figure 4.4. Chronic estrogen supplementation leads to alterations in tissue architecture of extraorbital lacrimal glands in aged female rats. At 18 months, following 3 months of E2 or placebo supplementation, extraorbital lacrimal glands were excised and prepared for hematoxylin and eosin staining. (A, B) Compared to Intact rats, E2 rats had increased peri-vascular connective tissue, indicating fibrosis (*). (C, D) E2 rats also demonstrated widened interlobular (*arrowheads*) and interlobular (*arrows*) spaces in the extraorbital lacrimal gland. Scale bars = 100µm.

Figure 4.5. Intraorbital lacrimal glands of aged rats without estrogen supplementation contain dacryoliths and exhibit larger acinar lumen. Intraorbital lacrimal glands from 18 month old female rats were examined following 3 months of E2 or placebo treatment. All rats exhibited protein-rich material within the acinar lumen, which stained more darkly in glands from E2 rats (B, D). Intact rats (A) exhibited larger and more abundant intra-luminal dacryoliths (*d*), as well as larger acinar lumen (C) compared to E2 rats (B, D). Similar to findings in the extraorbital lacrimal glands, E2 rats exhibited greater degree of connective tissue fibrosis and disruption of the acinar structures in the intraorbital lacrimal glands (B) compared to Intact rats (A). Scale bars A, B = 100 μ m; scale bars C, D = 50 μ m.

Figure 4.6. Hematoxylin and eosin staining of lower lid margin showing Meibomian glands in Intact and E2 rats. No structural or morphological differences between Intact and E2 groups were observed in Meibomian glands of the lower lid margin. Scale bars = 100μm.

Figure 4.7. Estrogen receptor α expression is increased in the eye glands of aged female rats receiving E2 supplementation. ER α expression was measured in extraorbital lacrimal glands, intraorbital lacrimal glands, and Meibomian glands of aged female rats following 3 months of E2 or placebo treatment. ER α immunostaining was observed in the nuclei of some cells in all glands of both Intact (A, C, E) and E2 (B, D, F) rats (*arrows*). However, in E2 rats, the number of cells staining for ER α was increased in all glands studied. In the intraorbital lacrimal glands of E2 rats, ER α expression was limited to the gland margin (D). Intraorbital lacrimal

glands of Intact rats contained darkly staining dacryoliths, which were fewer and smaller in glands from E2 rats (C). Scale bars A – D = 100 μ m; scale bars E, F = 50 μ m.

Chapter Five

Hippocampal-dependent memory in aged rats following middle cerebral artery occlusion

Abstract

Current experimental models of stroke commonly utilize young adult rats and involve suture occlusion of the middle cerebral artery. More clinically relevant models have recently been developed that involve aged rats and a fibrin blood clot occlusion of the middle cerebral artery (MCAO). Cognitive and behavioral outcome from experimental stroke are poorly understood, particularly in the recently developed models. In the present study, we assessed hippocampal-dependent memory using the 8-arm radial maze in young adult and aged female rats following middle cerebral artery occlusion with fibrin clot and tissue plasminogen activator reperfusion. Aged rats required greater number of sessions to become trained and had increased trial duration, suggesting reduced locomotor activity. Although baseline differences in reference and working memory errors were observed, they did not reach significance. Following MCAO, no significant differences were found in reference or working memory errors between young adult and aged rats.

Introduction

Although the middle cerebral artery does not supply the hippocampus, cells of the hippocampus can undergo apoptosis following ischemia/reperfusion injury due to excitotoxic damage from the necrotic death and release of glutamate from surrounding cells (Yang et al., 2001, Xu et al., 2007). Damage to the hippocampus is of significant concern following stroke due to its relevance to the behavioral outcome from stroke. Animal models of stroke have undergone significant development and improvement since research began in the field (Kudo et al., 1982, Kaneko et al., 1985, Bederson et al., 1986, Dinapoli et al., 2006). The most common models used today involve insertion of a modified suture or fibrin-rich blood clot into the proximal middle cerebral artery, and are referred to as transient or permanent middle cerebral artery occlusion (MCAO). In both suture and fibrin clot models, reperfusion can be achieved by suture removal or administration of a thrombolytic agent. These models have been shown to produce consistent infarct volumes and functional impairment. However, it is unknown if these newer models of MCAO have effects on the hippocampus. Of particular interest are experimental stroke models that utilize aged animals, which typically have larger infarcts, thus increasing the chance of hippocampal damage (Dinapoli et al., 2006).

The hippocampus is involved in short term memory, memory consolidation, and spatial navigation. Functional assessment of the hippocampus in animal models of brain injury typically relies on hippocampal-dependent navigation, including both working and reference memory. Various types of mazes are often used and depending on their design and use, can test either working or reference memory or both. One commonly used task is the 8-arm radial maze in which rats can be trained to search the arms for food rewards (Toumane et al., 1988). Visual cues in the maze room allow for the assessment of reference memory. With some arms of the maze baited and others not baited, food-restricted rats rely on reference memory to navigate to arms containing food rewards. Working memory is tested by counting the times rats re-enter an arm where a food reward was previously eaten during the same trial.

The focus of the present study was to determine the effects of experimental stroke on hippocampal-dependent memory using the 8-arm radial maze in young adult and aged female rats. We used the 8-arm radial maze to evaluate both reference and working memory errors one week after reversible occlusion of the middle cerebral artery with fibrin clot. Results demonstrate that aged animals require more training sessions to become trained and aged rats take longer to complete the maze task. Despite these training differences, young adult and aged female rats do not exhibit hippocampal-dependent memory losses following MCAO.

Materials and Methods

Chemicals and Animals. Ketamine and xylazine were purchased from Sigma (St. Louis, MO). Human recombinant tissue plasminogen activator (tPA) was gifted from Genentech (South San Francisco, CA). Thirty-six female Sprague-Dawley rats (3 months (N=24) and 18 months (N=12)) were purchased from Hilltop Animal Laboratory (Scottsdale, PA) and housed under 12h:12h light/dark conditions with water provided ad libitum and food restricted to maintain rats at 85% of their ad libitum weight. All procedures were in accordance with guidelines set forth by the West Virginia University Animal Care and Use Committee.

Food restriction and radial maze training. During a one-week acclimation to the training room, rats were fed ad libitum and at the end of the week their ad libitum weight was recorded. The following day food was restricted to 10 g/day including several food rewards (Froot Loops cereal, Kellogg's, Battle Creek, MI), and continued as such until rats reached 85% of their ad libitum feeding weight. Throughout training and testing, rats were maintained at 85% of their ad libitum feeding weight. Rats were acclimated to the 8-arm radial maze (60x10x12 cm high, elevated 70 cm above the floor) in three 10 min trials held on consecutive days. Acclimation trials consisted of rats being placed in the center of the maze under plexiglass cover for 30 sec in groups of 2 or 3 rats. Cover was lifted and rats were given 10 min to freely explore the maze and eat food rewards placed throughout the maze. Training began on the day following the last acclimation trial

and consisted of two trials per day. Training trials used the same four baited arms each time and trials ended when all food rewards were eaten or after 5 min. Total arm entries and total trial duration were recorded along with reference and working memory errors. Training was considered complete when rats achieved zero working memory errors and an average of less than one reference memory error in five consecutive sessions. Rats that failed to become trained within 50 training sessions were excluded from further analysis. Testing trials were performed following one week of recovery from MCAO and occurred once daily for five consecutive days.

Middle cerebry artery occlusion. Upon training completion, rats underwent a transient ischemic stroke by occlusion of the right middle cerebral artery with tissue plasminogen activator (tPA; 5mg/kg, i.v.) reperfusion under ketamine and xylazine anesthesia (5mg/kg and 10 mg/kg, respectively), as previously described (Dinapoli et al., 2006). Ischemia was defined by a perfusion drop across the MCA territory of greater than 80% as determined by laser Doppler measurement of cerebral blood flow and successful reperfusion was denoted as a return to greater than 80% of baseline perfusion rate by 30min after tPA administration. Rats not achieving these standards for depth and duration of ischemia as well as reperfusion were excluded from the study. Sham operation involved similar procedures except that no clot was inserted. At 24h after MCAO, functional assessment was measured by an investigator blinded to the treatment using the modified Neurological Severity Score (mNSS) (Li et al., 2002). Following one week of recovery, rats were tested in the 8-arm radial maze once daily for five consecutive days.

Functional assessment. At 24 h after MCAO, rats were assessed for functional impairment using the modified Neurological Severity Score. This assessment consisted of five subtests to measure voluntary motor function, balance, and motor reflexes. Scores range from 1 to 17 with higher scores indicating more severe functional impairment (Li et al., 2000).

Results

Aged rats had longer trial duration and required more sessions to become trained. Despite the lack of functional impairment of aged rats before undergoing MCAO, the per-trial duration of aged rats was significantly higher compared to young adult rats. By the 25th session, the average per trial duration of young adult rats was 75 sec compared to 126 sec in the aged rat group. The number of training sessions for rats to achieve zero working memory errors and an average of less than one reference memory error in five consecutive sessions was also increased in the aged rat group, but not significantly. By the end of the 25th training session, the majority of young adult rats (92%) had achieved this level of training; however, many more aged rats were not yet trained by the 25th session and 16% of aged rats had not reached trained status by the 50th training session, and were excluded from further analysis.

Aged rats had greater functional impairment following MCAO. Previous reports from our lab and others have demonstrated that aged rats experience worsened functional impairment following MCAO compared to young adult counterparts. In the present study, aged rats had an average composite mNSS of 7 at 24 h after MCAO, while young adult rats had an average composite score of 4, indicating significantly greater degree of functional disability in aged rats following MCAO ($p=0.017$).

Frequency of reference and working memory errors not significantly altered in aged rats. Following one week of recovery from MCAO, rats were tested in the 8-arm radial maze in one session per day for five consecutive days. Reference and working memory errors were recorded. Data demonstrate that there is no significant difference between frequency of reference memory errors in young adult or aged rats subjected to MCAO compared to age-matched or sham-operated rats. Although a trend towards higher reference memory errors was observed in aged rats following MCAO compared to young adult rats following MCAO, data did not reach significance. Likewise, no significant effect of age was measured in frequency of working memory errors following MCAO.

Discussion

This study examined the hippocampal-dependent memory in young adult and aged female rats prior to and following one week of recovery from MCAO or sham surgery. Our results show that aged rats take longer to acquire stable performance in the 8-arm radial maze and a significantly greater number of aged rats do not achieve trained status within 50 training sessions, compared to young adult rats. The present study also demonstrated for the first time that current fibrin clot models of transient MCAO produce no measureable differences in reference or working memory errors in young adult and aged female rats compared to age-matched sham-operated rats. Similarly, no significant differences between young adult and aged rats undergoing MCAO were found. Although histologic confirmation of an absence of hippocampal damage was not performed, behavioral analysis with the 8-arm radial maze demonstrated no significant differences in post-ischemic performance based on age or MCAO.

There are several important caveats to consider regarding these results. Although necessary to ensure motivation in training, calorie restriction and decreased body weight undoubtedly affect the neurologic sequelae of experimental stroke. Reports indicate that calorie-restricted rats perform better on cognitive and motor tasks (Means et al., 1993), although the effects of calorie restriction in aged rats following stroke has not been studied to date. An additional source of neuroprotection in the present study was the anesthetic agent used, which was ketamine. At the time the present study was conducted, few reports existed regarding the neuroprotective effects of ketamine. However, more recently accumulating evidence suggests that ketamine acts through several mechanisms to reduce neuronal injury following insult (Schifilliti et al., , Zhang et al., 2006b). Specifically, studies have demonstrated that ketamine can inhibit N-methyl-D-aspartate (NMDA) receptor, which reduces excitotoxic cell death, an important component of the secondary phase of injury following acute ischemic stroke (Himmelseher et al., 1996). Ketamine can also reduce apoptotic cell death through signaling pathways that result in attenuated levels of pro-apoptotic proteins such as *Bax* (Engelhard et al., 2003). Lastly, neu-

roprotection by ketamine is thought to involve a reduction in post-injury inflammation as measured by cytokines tumor necrosis factor α , interleukin-6, and interleukin-8 (Kawasaki et al., 1999). For these reasons, ketamine is no longer used in studies involving neural injury.

Summary

The present study offers interesting data regarding the hippocampal-dependent memory in young adult and aged rats prior to and following MCAO. Future studies could improve on this data set by including additional behavioral assessments to more fully profile the cognitive differences between young adult and aged rats before and after experimental stroke. Of particular interest would be passive avoidance test and Morris water maze, both of which require hippocampal-dependent memory. To more fully understand basal and post-ischemic locomotor differences between young adult and aged rats, the open-field test would be informative. In addition, histologic examination of the brain would provide invaluable information as to whether hippocampal neurons are affected by experimental stroke. Specific stains, such as Fluoro-Jade B, which labels degenerating neurons would allow visualization of hippocampal damage. Although the fibrin clot model is a clinically relevant model of stroke, a greater understanding of experimental stroke models would come from studies involving multiple models, both transient and permanent occlusion with both suture and fibrin clot. In summary, information regarding the cognitive and behavioral assessment of rats following experimental stroke is lacking in the literature, but has significant clinical relevance. In order to obtain meaningful results from experiments involving stroke, a clear understanding of all facets of the model that is used must be acquired.

Figure 5.1. Aged rats require longer trial duration. Trial duration was measured as time to collect all food rewards with 5 min as the maximum time allowed. During 50 training sessions, aged rats required significantly more time to complete trials compared to young adult rats. * $p < 0.05$ compared to young adult rats using one-way repeated measures ANOVA.

Figure 5.2. No significant differences between young adult and aged rats in number of reference or working memory errors during training. Although aged rats began training with similar numbers of reference (A) and working (B) memory errors, young adult rats had reduced number of both error types by the tenth training session, although the difference did not reach significance. By the 50th training session significantly more aged rats (16%) than young adult rats (4%) had not reached the required level of training to continue in the experiment.

Figure 5.3. Aged rats have greater functional impairment following MCAO. Functional impairment was assessed at 24 h after MCAO using the modified Neurological Severity Score (mNSS), a commonly used battery of motor, balance, and reflexes in stroke research. Consistent with previous reports, aged rats in this study had higher composite mNSS score indicating more severe functional impairment following MCAO compared to young adult rats. * $p < 0.05$ compared to young adult rats using one-way ANOVA with Tukey's post hoc analysis.

Figure 5.4. No difference based on age or MCAO in frequency of reference or working memory errors. Following MCAO, young adult and aged rats showed no significant differences in frequency of reference (A) or working (B) memory errors compared to sham-operated rats. Also, no significant differences were measured between young adult and aged rats following MCAO, although a trend towards higher reference memory errors was observed in aged rats following MCAO.

Chapter Six

Conclusions and Future Directions

Introduction

The dissertation research presented here encompasses several components of the effects of estrogen in an aged female rat model. This research has clinical relevance and importance, as demonstrated by the fact that in the year 2000, an estimated 10 million women were taking HRT for the management of menopausal symptoms. HRT is known to be an effective and cost efficient therapy for menopausal symptoms which can include hot flashes, fatigue, hair loss, sleep disturbance, vaginal dryness, weight gain, cognitive disturbance, loss of libido, headaches, joint pain, depression, and anxiety, among others. Since the release of reports from the WHI, FDA guidelines for the use of HRT have been altered to recommend that patients take the lowest effective dose for the shortest amount of time, potentially leading to undertreatment of menopausal symptoms (Bren, 2003). Clinical studies have demonstrated that not all effects of HRT or estrogen alone are beneficial. Estrogen has been shown to increase the incidence of stroke in postmenopausal women (Wassertheil-Smoller et al., 2003, Anderson et al., 2004), although a recent randomized controlled clinical trial demonstrates that increased stroke incidence does not persist following termination of HRT (LaCroix et al.). In addition, HRT is associated with tear gland dysfunction resulting in dry eye syndrome, a painful condition that is often undertreated. These effects of estrogen therapy were the focus of the dissertation research presented here.

Estrogen and Ischemic Brain Injury

In our first study, we examined the role of chronic estrogen supplementation on experimental stroke injury in young adult and aged female rats. We hypothesized that differences in estrogenic action in ischemic brain injury between young adult animal models and postmenopausal women resulted from age-related alterations in estrogenic action. Evidence from numerous studies indicates that estrogenic actions and receptors in the brain are affected by age. Specifically, there are changes in splice variants of ER α with age (Ishunina and Swaab, 2008),

differential effects of estrogen on abundance of specific cell types with age, and altered effects of estrogen on post-ischemic cognitive performance with age. Using a clinically relevant animal model of E2 supplementation and experimental stroke, we demonstrated for the first time worsened stroke injury and increased mortality with E2 treatment in aged animals following MCAO, which parallel the findings of the WHI (Wassertheil-Smoller et al., 2003). The importance of an animal model that accurately represents the clinical problem cannot be overstated, and the model described here provides an important first step in addressing that issue.

The next step in this research is to identify a mechanism by which estrogen acts in the aged, injured brain. To that end, we explored a number of potential candidates, and revealed important new information that contributed to the development of an evidence-based hypothesis for the mechanism of estrogenic action. One pathway that we explored has been previously implicated in age-related differences in stroke injury. Previous investigations of the JAK2/STAT3 pathway in our lab demonstrate that aged rats experience a truncated activation of STAT3 compared to young adult counterparts (Dinapoli et al. 2010). STAT3 is an important transcription factor that targets several genes important to recovery from stroke, and was a plausible candidate for age-related differences in estrogenic activity in the post-ischemic brain. In addition, suppressor of cytokine signaling 3, an inhibitor of the pathway and attenuator of reactive astrogliosis was previously shown to be upregulated in aged animals (Dinapoli et al. 2010). Based on this foundation of age-related alterations in the JAK2/STAT3 pathway, we hypothesized that estrogen may also exert its age-related effects through JAK2/STAT3. To test this hypothesis, we measured protein levels of total and phosphorylated STAT3 in the ischemic cortex and striatum of our E2 rats in comparison to Intact and OVX rats. We did not find any significant differences based on E2 treatment. We also looked at mRNA levels of JAK2 and STAT3 and found no estrogen-related effect. Finally, we used a JAK/STAT array to more fully evaluate estrogenic effects on post-ischemic JAK/STAT pathways.

Data from the JAK/STAT array used with our first cohort of animals showed that E2-mediated exacerbation of stroke injury was accompanied by downregulation of EPO-R mRNA. We confirmed that E2 rats also had reduced expression of EPO-R protein in the penumbral regions of the post-ischemic brain. Using immunohistochemical techniques we also observed reduced expression of ER α in the ischemic hemisphere of E2-treated rats. These findings led to the design of a second study in which we attempted to rescue E2-mediated exacerbation of stroke injury with acute treatment with EPO, a known neuroprotectant. This study required the use of a different model of MCAO as EPO-tPA interactions have been suggested to lead to increased post-ischemic mortality and are poorly understood. Using the suture occlusion model of MCAO, we hypothesized that if estrogenic effects on EPO-R were responsible for increased infarct size in E2 treated rats, acute treatment with EPO would compensate for reduced EPO-R expression and attenuate the extent of estrogen-mediated injury. Our hypothesis was supported by the data, which showed a reduction in total infarct volume in rats receiving EPO. EPO treatment also reduced disruption of tissue architecture and petechial formation within the ischemic parenchyma. In this second cohort, we also confirmed the estrogen-mediated reduction in EPO-R protein expression in the penumbral region as well as estrogen-mediated attenuation of ER α in the ischemic hemisphere that had been observed in the first cohort of rats.

Taken together, our findings support a proposed mechanism of estrogenic action in the post-ischemic brain where chronic estrogen supplementation leads to reduced ER α expression which, in turn, reduces EPO-R expression and results in exacerbated ischemic injury. In this proposed mechanism, and supported by our data, it is the EPO/EPO-R system, rather than estrogen directly that confers neuroprotection and is altered by chronic E2 treatment. However, this proposed model of estrogenic action in the post-ischemic brain raises additional questions. Specifically, is the EPO/EPO-R system altered prior to ischemic injury or do estrogen-mediated changes only affect the post-ischemic EPO/EPO-R response? Experiments looking at ER α , EPO, and EPO-R in sham-operated and naïve animals as well as in cultured cell lines would

provide valuable information regarding basal effects of estrogen on the EPO/EPO-R system in the brain and in specific cell types.

Next, how exactly does estrogen regulate basal or post-ischemic EPO-R expression? If estrogen only affects post-ischemic EPO-R expression, rather than basal levels, the likely answer involves hypoxia-inducible factor 1 (HIF-1), the transcription factor responsible for hypoxia-mediated upregulation in EPO-R. HIF-1 has two subunits, both constitutively expressed in the cell. However, one subunit, HIF-1 α , is ubiquitin tagged for proteasomal degradation by the enzyme HIF prolyl-hydroxylase, which requires oxygen as a co-substrate. When oxygen becomes scarce, HIF prolyl-hydroxylase cannot ubiquitinate HIF-1 α , thus it is not degraded and can then associate with HIF-1 β to form an activated transcription factor that targets genes important to survival in hypoxic conditions, such as EPO-R. Experiments to test whether E2 acts through HIF-1 would involve protein and mRNA quantification of that pathway in cells or animals treated with estrogen. If inhibitors of HIF-1 transcriptional regulation of EPO-R eliminate the estrogenic effects on EPO-R, that would provide further support of the involvement of HIF-1 in estrogenic downregulation of EPO-R.

Lastly, if estrogen acts through ER α to alter EPO-R in aged female rats leading to increases in post-ischemic infarct size, how does estrogen provide neuroprotection in young adult animals? This question poses the greatest quandary since no known age-related differences in ER α or the EPO/EPO-R system in the brain have been reported. Future investigations of the mechanisms of estrogenic actions should involve young adult counterparts in order to gain insight into the age-dependent activity of estrogen in the brain.

Estrogenic Effects on Tear Producing Glands

The effects of estrogen on the lacrimal and Meibomian glands are poorly understood. Data from the Women's Health Study implicates HRT, particularly estrogen therapy alone, in the development of dry eye syndrome in postmenopausal women (Schaumberg et al., 2001). Ob-

servational data support a role for female gonadal hormones in the tear producing glands. In the year 2000, the prevalence of dry eye symptoms in women was estimated to be 16.7% compared to 11.4% in men (Moss et al., 2000). Dry eye syndrome, or keratoconjunctivitis sicca (KCS), leads to significant distress and can result in severe consequences such as corneal abrasion or visual impairment. Due to the lack of understanding of the etiology and pathophysiology of dry eye syndrome, this disease should be the focus of continuing ophthalmic research. Of specific concern are the effects of estrogen on the structure and function of tear glands, which has been shown to be deleterious and lead to dysfunction and the development of KCS.

In the third study described here, we investigated the lacrimal and Meibomian glands of rats from our second cohort of estrogen treated rats. Two weeks prior to MCAO, tear gland function of E2 and Intact rats was assessed using two commonly used clinical tests: the Schirmer's I test and fluorescein tear break-up time (TBUT). We describe a modification of the Schirmer's I test to adapt it to the smaller tear volumes and eye structure of the rat. Using the modified Schirmer's I test, we found that estrogen treated aged rats had reduced tear volume compared to Intact aged rats. TBUT was also different between the groups with E2 rats showing reduced time to first break in the tear film.

In the second part of this study, we examined the tear glands histologically. Unlike humans, rats have both intraorbital and extraorbital lacrimal glands. Following sacrifice at the end of our experiments involving estrogenic effects on stroke, we excised both lacrimal glands and Meibomian glands on the side contralateral to the MCAO surgery. The lacrimal glands in humans receive blood supply from the ophthalmic artery, a branch off the proximal middle cerebral artery. In order to avoid erroneous conclusions based on restricted blood flow, we did not use glands from the side ipsilateral to MCAO surgery. From histologic evaluation of extraorbital and intraorbital lacrimal glands as well as Meibomian glands of the lower lid, we demonstrated specific structural changes in the lacrimal glands of rats receiving E2. Changes in lacrimal glands included increased fibrosis, which disrupted normal tissue architecture in some areas. We noted

changes in the structure of acini of the intraorbital lacrimal glands with E2 rats displaying larger acinar cells and reduced acinar lumen size. We also noted the presence of numerous dacryoliths in Intact animals which were either not present or much smaller in size in E2 rats.

The last experiment of this study involved immunostaining for both ER α and ER β in tear producing glands of both aged Intact and aged E2 treated rats. ER β immunostaining did not reveal ER β expression in any of the tear glands, which was consistent with previous reports in healthy young adult rats not receiving hormonal therapy (Spelsberg et al., 2004). We did, however, observe ER α expression in all tear glands, which was upregulated in glands of rats receiving E2. This data suggests that E2-mediated effects on the tear glands are mediated through ER α , rather than ER β . However, the exact mechanism by which estrogen alters tear gland structure and function remains unclear. Specific pathways and proteins that are altered by E2 and that lead to the structural and functional changes must be identified. Based on our finding of increased connective tissue fibrosis in glands from estrogen-treated rats, proteins involved in extracellular matrix remodeling are likely candidates for influence by estrogen. Questions remain regarding age-related differences in the effects of estrogen on tear producing glands. Future studies to investigate the mechanism should include young adult counterparts in order to elucidate differences between young adult and aged rats.

Hippocampal-dependent memory in an aged animal model of stroke

The final study presented in this dissertation research sought to characterize the behavioral outcomes from our model of experimental stroke. Behavioral evaluation of current models of stroke is poorly defined. Functional consequences of stroke, however, are of utmost importance and should be a main outcome measure in all stroke studies, as outlined in the STAIR recommendations (Fisher et al., 2009). Our lab has been on the forefront of developing a clinically relevant model of transient ischemic stroke. Specifically, our model utilizes fibrin clot collected from a blood donor rat and monitors depth of ischemia throughout the procedure. By us-

ing a fibrin clot, we permit reperfusion of the MCA by administering tPA, similar to the clinical situation. Coupled with the use of aged animals, this model of transient embolic occlusion of the middle cerebral artery parallels the clinical scenario of stroke unlike any other model used in the field (Dinapoli et al., 2006). However, the behavioral profile resulting from this model and all other experimental stroke models are not well characterized and was the focus of the fourth study described here.

In this study, both young adult and aged female rats were trained on a hippocampal dependent memory task that has been well described and validated, namely, the 8-arm radial maze (Toumane et al., 1988). We found that aged female rats take longer to train and a higher percentage of them do not become trained in 50 sessions, compared to young adult rats. Following MCAO, however, no differences are found in working or reference memory between young adult and aged rats. We also measured no differences in sham-operated rats compared to age-matched MCAO rats, suggesting that our model of stroke does not affect hippocampal-dependent memory. Despite our negative results, future studies to characterize the behavioral outcome from various models of experimental stroke would still hold an important relevance in the stroke field. As mentioned previously, behavioral outcome from stroke is far more important in the clinical setting than tissue volume lost.

Future studies could improve on our data set by including additional behavioral assessments, both those involving hippocampal-dependent memory as well as other cognitive tasks in order to more fully profile behavioral outcomes from experimental stroke in both young adult and aged rats. One specific study of interest, likely to demonstrate differences between young adult and aged rats, is the open-field test, which simply measures locomotor activity. Another potential candidate is gait analysis, which can be measured in great detail with new high-tech devices. To more fully assess hippocampal-dependent memory, studies using the passive avoidance test and Morris water maze would be useful. In addition, histologic examination of the brain would provide invaluable information as to whether hippocampal neurons are affected by exper-

imental stroke. Specific stains, such as Fluoro-Jade B, which labels degenerating neurons would allow visualization of hippocampal damage.

Although the fibrin clot model is a clinically relevant model of stroke, a greater understanding of experimental stroke models would come from studies involving multiple models, both transient and permanent occlusion with both suture and fibrin clot. Also, an enhanced battery of behavioral tests should be explored in the acute and chronic phases of recovery as functional outcome from stroke has the most clinical significance. In conclusion, accurate and meaningful behavioral assessments of animals following experimental stroke are lacking, particularly ones that quantify observed differences between young adult and aged animal. Clinical relevance of stroke research would be greatly enhanced by characterizing more fully the unique cognitive and behavioral outcomes in the various stroke models and utilizing those measures of most relevance in conjunction with infarct volume analysis.

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