THE PROTECTIVE EFFECTS OF PLASMA GELSOVIN AND ALPHA 1-ANTITRYSIN ON ISCHEMIC STROKE OUTCOME IN RATS

By

HUONG LE MOLDTHAN

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY UNIVERSITY OF FLORIDA

2012
To my beloved grandmother, parents and husband, Matthew Michael Moldthan
ACKNOWLEDGMENTS

Obtaining my doctorate has been the most challenging goal of my life so far. Yet, starting is the best decision that I will never regret. During the journey to this dissertation, both figurative as well as literal, I am deeply indebted to the many people who have provided me inspiration and supported my studies.

My greatest gratitude goes to Dr. Jeffrey Hughes, my former mentor who gave me an opportunity to do research in his lab and for his excellent supervision, as well as his great sense of humor. I would like to gratefully thank Dr. Sihong Song for continuing to guide me in a new project when Dr. Hughes left. I also would like to express my thanks to Dr. Michael King and other committee members, Dr. Christopher Batich and Dr. Guenther Hochhaus for their detailed comments and great suggestions. Without their guidance, I would not have had such a great experience in completing this challenging project.

I would like to express my special thanks to Dr. Aaron Hirko, for his constant help, patience and invaluable advice. A sincere thank you goes to Jeffrey Thinschmidt, who was always willing to help me with handling animals, performing laser-Doppler imaging experiments, providing great comments on dissertation as well as my talks, and contributing to a very happy lab environment. I also would like to thank Matthew Isaacson for analyzing the data.

I truly acknowledge the Department of Pharmaceutics for all of their administrative work. I also would like to thank the Department of Pharmacology and Therapeutics, the McKnight Brain Institute, and Department of Veterans Affairs where I performed most of the animal work and whose facilities I used.

I would like to send my many thanks to my professors, teachers, supervisors, lab mates and friends at Utrecht University in the Netherlands and to those whom I was fortunate enough to meet during my studies. Coincidently, I met Dr. Wouter Driessen when I came to the University
of Florida. He was a former graduate student at Utrecht University. Even as he was busy finishing up his doctorate, he was willing to teach, work, and give great advice for my project. I would like to express my sincere thanks for his invaluable support, intelligence and enthusiasm. I am truly grateful for wonderful old friends in Vietnam. I greatly appreciate your kindness and friendship via the internet or personally, when I went home. Although I am not able to name each of you here, I will never forget any of you!

Lastly, I would like to express my deepest thanks for my family members. Most important among them are my parents, who love me unconditionally, support me by all means, and trust me implicitly. They are willing to do anything I need or even what they think is good for me. I would like to express my eternal gratitude to my siblings. My sister, Ha Thu Le, is not only my best friend but also my “soul” mate. She shares my life’s point-of-view and constantly inspires me to reach my goals, especially during my time in the U.S., which is made difficult by the nearly ten thousand miles separating me from my home town and my family. My two brothers, Hoc Van Le and Hien Van Le always wish me the best of luck and deeply sympathize with me simply because I am living on the other side of the world. I would not have easily accomplished this important goal in my life without the constant support, love, inspiration and patient proof-reading, of my husband, Matthew Michael Moldthan. I would like to thank my parents in law, Maxine and Rollan Ross, who are constant encourage me to finish this dissertation. I would like to thank my grandmother for her most loving assistance, as well as insistence on my attending graduate school. She would cook, take care of my apartment and do anything I needed in order for me to continue my efforts to attend graduate school. She is in her 90s now but she is still healthy and vibrant. I love her dearly and it pains me that my studies have taken me so far away from her.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ACKNOWLEDGMENTS</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>9</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>10</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>14</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1  INTRODUCTION</td>
<td>16</td>
</tr>
<tr>
<td>History of Stroke</td>
<td>16</td>
</tr>
<tr>
<td>Ischemic Stroke</td>
<td>17</td>
</tr>
<tr>
<td>Overview of Stroke</td>
<td>17</td>
</tr>
<tr>
<td>Focal Ischemic Stroke</td>
<td>18</td>
</tr>
<tr>
<td>Animal Models for Focal Ischemic Stroke</td>
<td>20</td>
</tr>
<tr>
<td>Intraluminal MCAO model</td>
<td>20</td>
</tr>
<tr>
<td>Embolus models</td>
<td>20</td>
</tr>
<tr>
<td>Endothelin-1-induced MCAO model</td>
<td>21</td>
</tr>
<tr>
<td>Pathophysiology of Focal Ischemic Stroke</td>
<td>21</td>
</tr>
<tr>
<td>Mechanisms of cellular cell death</td>
<td>22</td>
</tr>
<tr>
<td>Sources of free radical generation</td>
<td>25</td>
</tr>
<tr>
<td>Inflammatory response to cerebral ischemia</td>
<td>27</td>
</tr>
<tr>
<td>Blood brain barrier and focal ischemic stroke</td>
<td>31</td>
</tr>
<tr>
<td>Research Approach</td>
<td>36</td>
</tr>
<tr>
<td>The Current Options for Ischemic Stroke Therapy are Limited</td>
<td>36</td>
</tr>
<tr>
<td>Therapeutic Strategies with pGSN and AAT</td>
<td>39</td>
</tr>
<tr>
<td>Plasma GSN and ischemic stroke</td>
<td>40</td>
</tr>
<tr>
<td>Alpha 1-antitrypsin and ischemic stroke</td>
<td>44</td>
</tr>
<tr>
<td>Hypothesis</td>
<td>47</td>
</tr>
<tr>
<td>2  METHODOLOGY</td>
<td>48</td>
</tr>
<tr>
<td>Rats Used as an Animal Model for Focal Ischemic Stroke</td>
<td>48</td>
</tr>
<tr>
<td>Endothelin-1-Induced Middle Cerebral Artery Occlusion Model</td>
<td>51</td>
</tr>
<tr>
<td>Materials</td>
<td>52</td>
</tr>
<tr>
<td>Experimental Procedures</td>
<td>53</td>
</tr>
<tr>
<td>Animal Model and Treatments</td>
<td>53</td>
</tr>
<tr>
<td>Laser Doppler Perfusion Imaging</td>
<td>54</td>
</tr>
<tr>
<td>Behavioral Tests</td>
<td>56</td>
</tr>
<tr>
<td>Cylinder test</td>
<td>56</td>
</tr>
<tr>
<td>Vibrissae test</td>
<td>57</td>
</tr>
<tr>
<td>Histopathology</td>
<td>57</td>
</tr>
</tbody>
</table>
3 THE PROTECTIVE EFFECTS OF PLASMA GELSONIN ON ISCHEMIC STROKE OUTCOME IN RATS .................................................................61

Introduction .............................................................................................................................61
Results .....................................................................................................................................63
Treatment of pGSN did not Interrupt ET-1 Induced Artery Contraction .........................63
Treatment of pGSN Significantly Reduced ET-1 Induced Behavioral Deficits ...............64
Cylinder test ..........................................................................................................................64
Vibrissae test .........................................................................................................................64
Treatment of pGSN Reduced MCAO Induced Brain Damage .....................................65
Discussion ...............................................................................................................................65
Conclusion ..............................................................................................................................67

4 ALPHA 1-ANTITRYPSIN MITIGATED ISCHEMIC STROKE DAMAGE IN RATS.......72

Introduction .............................................................................................................................72
Results .....................................................................................................................................74
Human AAT Did not Affect the ET-1-Induced Ischemia ...................................................74
Local Delivery of hAAT Mitigates ET-1-Induced Stroke Outcome ...................................75
Systemic Delivery of hAAT Mitigated ET-1-Induced Stroke Outcome .........................77
Discussion ...............................................................................................................................79
Conclusions ..........................................................................................................................81

5 GENERAL DISCUSSION, CONCLUSIONS AND FUTURE WORK .........................87

General Discussion .................................................................................................................87
Conclusions ...........................................................................................................................102
Future Work ..........................................................................................................................104

LIST OF REFERENCES ....................................................................................................106

BIOGRAPHICAL SKETCH ...............................................................................................143
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Treatment schedule for laser-Doppler perfusion imaging</td>
<td>60</td>
</tr>
<tr>
<td>2-2</td>
<td>Treatment schedule of for aCSF, ET-1 alone, ET-1+pGSN, and ET-1+hAAT (i.c.) groups</td>
<td>60</td>
</tr>
<tr>
<td>2-3</td>
<td>Treatment schedule for ET-1+saline and ET-1+hAAT (i.v.) groups</td>
<td>60</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1</td>
<td>Perfusion measurements of pGSN study</td>
<td>68</td>
</tr>
<tr>
<td>3-2</td>
<td>Cylinder test of pGSN study</td>
<td>69</td>
</tr>
<tr>
<td>3-3</td>
<td>Vibrissae test of pGSN study</td>
<td>70</td>
</tr>
<tr>
<td>3-4</td>
<td>Infarction area labeled for mitochondrial activity of pGSN study</td>
<td>71</td>
</tr>
<tr>
<td>4-1</td>
<td>Perfusion measurements of AAT study</td>
<td>82</td>
</tr>
<tr>
<td>4-2</td>
<td>Cylinder test of ET-1 alone vs. ET-1 and intracerebral hAAT delivery</td>
<td>83</td>
</tr>
<tr>
<td>4-3</td>
<td>Vibrissae test of ET-1 alone vs. ET-1 and intracerebral hAAT delivery</td>
<td>84</td>
</tr>
<tr>
<td>4-4</td>
<td>Infarction area labeled for mitochondrial activity of AAT study</td>
<td>85</td>
</tr>
<tr>
<td>4-5</td>
<td>Cylinder test of ET-1 alone vs. ET-1 and intravenous hAAT delivery</td>
<td>85</td>
</tr>
<tr>
<td>4-6</td>
<td>Vibrissae test of ET-1 alone vs. ET-1 and intravenous hAAT delivery</td>
<td>86</td>
</tr>
<tr>
<td>4-7</td>
<td>Infarction area calculated from MRI images using Image J v5.0</td>
<td>86</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
<td></td>
</tr>
<tr>
<td>AAT</td>
<td>Alpha 1-antitrypsin</td>
<td></td>
</tr>
<tr>
<td>aCSF</td>
<td>Artificial cerebrospinal fluid</td>
<td></td>
</tr>
<tr>
<td>AGP</td>
<td>$\alpha$-1 acid glycoprotein</td>
<td></td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis-inducing factor</td>
<td></td>
</tr>
<tr>
<td>AMPA</td>
<td>$\alpha$-amino-3-hydroxy-5-methyl-4-propionate</td>
<td></td>
</tr>
<tr>
<td>AMRIS</td>
<td>Advanced magnetic resonance imaging and spectroscopy</td>
<td></td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
<td></td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptotic-protease activating factor-1</td>
<td></td>
</tr>
<tr>
<td>APC</td>
<td>Activated protein C</td>
<td></td>
</tr>
<tr>
<td>APP</td>
<td>Acute phase protein</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
<td></td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
<td></td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
<td></td>
</tr>
<tr>
<td>CBF</td>
<td>Cerebral blood flow</td>
<td></td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
<td></td>
</tr>
<tr>
<td>cGSN</td>
<td>Cytosolic gelsolin</td>
<td></td>
</tr>
<tr>
<td>CINC</td>
<td>Cytokine-induced neutrophil chemoattractant</td>
<td></td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
<td></td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
<td></td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>CVA</td>
<td>Cerebral vascular (or Cerebrovascular) accident</td>
<td></td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
<td></td>
</tr>
<tr>
<td>ECASS</td>
<td>European cooperative acute stroke study</td>
<td></td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
<td></td>
</tr>
<tr>
<td>ESRD</td>
<td>End-stage renal disease</td>
<td></td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
<td></td>
</tr>
<tr>
<td>FAF</td>
<td>Familial amyloidosis of Finnish type</td>
<td></td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
<td></td>
</tr>
<tr>
<td>FIB</td>
<td>Fibrinogen</td>
<td></td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
<td></td>
</tr>
<tr>
<td>GSN</td>
<td>Gelsolin</td>
<td></td>
</tr>
<tr>
<td>hAAT</td>
<td>Human alpha 1-antitrypsin</td>
<td></td>
</tr>
<tr>
<td>HNE</td>
<td>Human neutrophil elastase</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular cell adhesion molecule-1</td>
<td></td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
<td></td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
<td></td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
<td></td>
</tr>
<tr>
<td>JAM</td>
<td>Junction adhesion molecule</td>
<td></td>
</tr>
<tr>
<td>JNK</td>
<td>Jun-N-terminal kinase</td>
<td></td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
<td></td>
</tr>
<tr>
<td>LPA</td>
<td>Lysophosphatidic acid</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
<td></td>
</tr>
<tr>
<td>LRP</td>
<td>Low-density-lipoprotein-receptor-related protein</td>
<td></td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>LTP</td>
<td>Lipoteichoic acid</td>
<td></td>
</tr>
<tr>
<td>MC</td>
<td>Mast cells</td>
<td></td>
</tr>
<tr>
<td>MCA</td>
<td>Middle cerebral artery</td>
<td></td>
</tr>
<tr>
<td>MCAO</td>
<td>Middle cerebral artery occlusion</td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
<td></td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
<td></td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
<td></td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
<td></td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
<td></td>
</tr>
<tr>
<td>NF</td>
<td>Nuclear factor</td>
<td></td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
<td></td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
<td></td>
</tr>
<tr>
<td>NVU</td>
<td>Neurovascular unit</td>
<td></td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet-activating factor</td>
<td></td>
</tr>
<tr>
<td>PAR-1</td>
<td>Protease-activated receptor 1</td>
<td></td>
</tr>
<tr>
<td>PARP-1</td>
<td>Poly(adenosine diphosphate ribose) polymerase 1</td>
<td></td>
</tr>
<tr>
<td>PBF</td>
<td>Phosphate buffered formaldehyde</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
<td></td>
</tr>
<tr>
<td>pGSN</td>
<td>Plasma gelsolin</td>
<td></td>
</tr>
<tr>
<td>PI(4,5)P2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
<td></td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear neutrophil</td>
<td></td>
</tr>
<tr>
<td>PTGS</td>
<td>Prostaglandin-endoperoxide synthase</td>
<td></td>
</tr>
<tr>
<td>RAP</td>
<td>Receptor activated protein</td>
<td></td>
</tr>
</tbody>
</table>
rhu-pGSN  Recombinant human plasma gelsolin
RNS        Reactive nitrogen species
ROS        Reactive oxygen species
rtPA       Recombinant tissue plasminogen activator
s.e.m.     Standard error of the mean
SAINT (I, II)  Stroke-acute-ischemic-NXY-treatment trial (I, II)
STAIR      Stroke treatment academic industry roundtable
TIA        Transient ischemic attack
TJ         Tight junction
tMCAO      Transient middle cerebral artery occlusion
TNF        Tumor necrosis factor
TNF-α      Tumor necrosis factor-alpha
TTC        2,3,5-triphenyltetrazolium chloride
TWEAK      Tumor necrosis factor-like weak inducer of apoptosis
uPA        Urokinase plasminogen activator
VCAM-1     Vascular cell adhesion molecule-1
VEGF       Vascular endothelial growth factor
VSM        Vascular smooth muscle
VSMC       Vascular smooth muscle cell
XO         Xanthine oxidase
XOR        Xanthine oxidoreductase
Plasma gelsolin and alpha 1-antitrypsin, used in this study, are human serum proteins with multiple functions, including anti-cell death pathways and anti-inflammatory effects that can be utilized to develop ischemic stroke therapy.

The effects of the two proteins were tested on middle cerebral artery occlusion (MCAO) induced by the potent vasoconstrictor peptide endothelin-1 (ET-1). In this model, ET-1 is injected adjacent to left middle cerebral artery (MCA) in rats. To test the effects of the candidate therapeutic agents in the MCAO model, rats were post-treated by a discrete injection of pGSN or AAT at the same site of ET-1 injection or by an intravenous injection of AAT at the same time of ET-1 injection. Artificial cerebrospinal fluid (aCSF) and sterile saline were utilized as negative controls. The MCAO was verified using a laser Doppler perfusion imaging system in separate groups of animals. Cylinder and vibrissae tests were performed before and 72 hours after MCAO to assess motor/sensorimotor functional deficits. Infarct volumes were determined via infarct area calculation 72 hours after MCAO using magnetic resonance imaging (MRI) and 2,3,5-Triphenyltetrazolium chloride (TTC) assay. Results from these experiments showed: 1) pGSN or AAT (both local and systemic deliveries) treatment did not interrupt ET-1 induced
MCAO as a disease model; 2) pGSN or AAT treatments significantly mitigated ET-1 induced functional deficits assessed by both cylinder and vibrissae tests; 3) pGSN local delivery, AAT local delivery or AAT systemic delivery markedly reduced brain injury (infarct volumes) by 49%, 83%, and 63%, respectively, compared to controls. Taken together, these findings show that plasma gelsolin and alpha 1-antitrypsin are potential novel therapeutic drugs for the protection against neurodegeneration following ischemic stroke.
CHAPTER 1
INTRODUCTION

This chapter starts with a brief history of stroke followed by an extensive review of stroke animal models, pathophysiology of ischemic stroke, and current ischemic stroke therapies, with a focus on focal ischemic stroke, the main topic of this dissertation. Furthermore, an overview of plasma gelsolin and alpha 1-antitrypsin as therapeutic strategies and the central hypothesis behind using these therapeutic proteins in stroke are discussed.

History of Stroke

The first mention in medical literature of what is called stroke today, was during Hippocrates’ time. It was called apoplexy, which means “struck down by violence” in Greek. This was due to the fact that a person developed sudden paralysis and a change in well-being. Physicians had little knowledge of the anatomy and function of the brain, the cause of stroke, or how to treat it (1). The word “stroke” first appears in recorded English in 1599 (2).

In the mid-17th century, Johann Jacob Wepfer did post-mortem examinations of patients that died with apoplexy and discovered that they had bleeding in the brain (3). Based on these findings, he was the first to hypothesize that death was caused by this bleeding. He also discovered that a blockage of the major arteries that supply blood to the brain could have similar effects.

In the 20th century, the term “cerebral vascular accident”, or CVA, entered the medical terminology following the development of angiography techniques in the early 1900s (4), and apoplexy was divided into categories based on the cause of the blood vessel problem.

Stroke is also known as "brain attack" to represent the fact that it is caused by a lack of blood supply to the brain which is similar to a heart attack caused by a lack of blood supply to the heart. The term brain attack also implies that it requires immediate interventions.
Ischemic Stroke

Overview of Stroke

Stroke is defined as the “rapidly developed clinical signs of focal or global disturbance of cerebral function, with symptoms lasting more than 24 hours or leading to death, with no apparent cause other than of vascular origin” (5) (page 114). Although this definition does not include transient ischemic attack (TIA), which is discussed later in this section, it covers most cases of strokes.

Stroke occurs so quickly, it is also known as a brain attack or a cerebrovascular accident as it is primarily caused within the vasculature. It results from the interruption of the blood supply to the brain, often because a blood vessel bursts or becomes blocked by a clot. This causes insufficient oxygen and nutrients supplied to the brain, ultimately leading to brain damage. Broadly, strokes can be classified into two major categories: hemorrhagic and ischemic.

A hemorrhagic stroke is the bleeding into or around the brain, which causes the compression of the brain tissues, reducing blood supply to those tissues, which subsequently leads to ischemia. This can occur in several ways. One common cause is a bleeding aneurysm, a weak or thin part of an artery wall which can break under arterial pressure and spill blood into surrounding brain tissues. Another cause of hemorrhagic stroke is when an artery breaks open due to plaque-deposited artery walls. Also, an individual with arteriovenous malformation also has a risk of having a hemorrhagic stroke (Stroke: Hope through research, National Institute of Neurological Disorders and Stroke, accessed March 15th, 2012).

Arteries ruptured within the brain result in hemorrhagic stroke called intracerebral hemorrhage. Arteries ruptured outside the brain result in hemorrhagic stroke called subarachnoid hemorrhage in which blood bleeds under the meninges or outer membranes of the brain into the thin fluid-filled space below the arachnoid layer and above the pia mater layer surrounding the
An ischemic stroke results from the blockage of blood flow to a certain area of the brain, leading to ischemia, and eventually causing brain tissue death. Ischemic strokes can be further divided into two subtypes, global and focal. Global strokes are caused by the decrease in blood supply to the entire brain (6) which is primarily involved in cardiac arrest. Focal strokes are often caused by the occlusion of a major cerebral artery, such as the middle cerebral artery (7).

Transient ischemic attack, also called a mini-stroke, is defined as “a brief episode of neurological dysfunction caused by focal brain or retinal ischemia, with clinical symptoms typically lasting less than one hour, and without evidence of acute infarction” (8) (page 1715). This definition illustrates the importance of paying attention to TIA, giving a quick evaluation and treatment to avoid cerebral ischemia. Since the symptoms of TIA initially are like a stroke but then resolves with no noticeable effect, TIA used to be considered a benign event. However, it recently has become a critical indicator of impending stroke (9).

Ischemic strokes, in origin, are approximately 87% of all strokes worldwide (10) with the remainder being hemorrhagic strokes. This has led ischemic stroke to be an extensively studied field in the last few decades. Animal models have been used extensively to study ischemic stroke, particularly focal ischemic stroke, which is more common than global ischemic stroke. Global stroke models are generally associated with the models of circulation resultant from cardiac arrest rather than stroke (11, 12). This dissertation focuses on focal ischemic stroke.

**Focal Ischemic Stroke**

Focal cerebral ischemia involves a sufficient enough reduction in regional cerebral blood flow (CBF) in a specific vascular territory to alter cerebral function and usually occurs clinically as an ischemic stroke due to blood clots. Blood clots can result in ischemia and ultimately
infarction in several ways. A clot formed in a part of the body other than the brain can travel through blood vessels and become lodged in a brain artery. This clot is called an embolus, and is often formed in the heart. A blood clot formed in an artery, which stays attached to the arterial wall until it develops large enough to block the artery, is called a thrombus. Cerebral ischemia can also be due to stenosis, a narrowing of the artery resulting from the build-up of plaque along the arterial wall, e.g. atherosclerosis, which is the gradual deposition of cholesterol and other lipids in the innermost layer of arterial walls (National Institutes of Neurological Disorders and Stroke, accessed March 6th, 2012).

In a typical focal ischemic stroke animal model, the middle cerebral artery is occluded, either permanently, or only temporarily, allowing subsequent reperfusion. The latter was chosen for this study because permanent ischemia model results in a region of severe ischemic damage which represents only a minority of human strokes (13).

Transient MCAO models produce both ischemia and reperfusion which mimics the majority of human strokes. Transient ischemic stroke induces varying degrees of ischemic injury depending on the duration (14), location, and intensity of ischemia (15). Therefore, one can manipulate conditions suitable to the study objectives. It is also one of the most relevant models as it correlates with the conditions such as TIA, spontaneous thrombolysis, and treatment-induced thrombolysis, e.g. tissue plasminogen activator, the standard therapy for ischemic stroke patients. This is also one of the models which best mimics the clinical situation in which patients undergo therapeutic recanalization of the cerebral blood vessel following stroke (16). Furthermore, transient ischemic stroke models offer a higher survival-rate in study animals in comparison to permanent ischemia, allowing for long-term effects in drug studies.
Animal Models for Focal Ischemic Stroke

Several transient ischemic stroke models have been developed over the years. Examples include an intraluminal MCAO, embolus models, and endothelin 1-induced MCAO model which are relatively extensively used in the development for stroke therapies. These models can be performed on both mice and rats (17).

Intraluminal MCAO model

This model involves inserting a monofilament suture into the internal carotid artery to block blood flow to the MCA for a certain period of time depending on the design of the study, then withdrawing the suture to allow reperfusion. The detailed techniques and procedures have been described in numerous studies (14, 18-20). The typical induced infarct areas are in the lateral caudatoputamen and frontoparietal cortex (21). The infarct is reproducible, reperfusion is easily obtained when the suture is withdrawn, and animals can survive up to months, making the model beneficial for functional outcome evaluations in testing neuroprotective drugs (22).

Although this model has been popular since in the 1980s, it has several shortcomings which can affect the lesion size. For instance, slight physical differences in sutures (23), insertion position of the suture (24), accidental premature reperfusion (25), and spontaneous hyperthermia due to long duration of surgery (26) can significantly alter the infarct size and may obliterate the testing agent. In addition to this, the technique requires adequately trained and experienced personnel.

Embolus models

Thromboembolic and photochemical MCAO models are commonly used. In the thromboembolic model the blockage of the MCA is induced by injecting a blood clot directly into the common carotid artery or into the carotid artery via a retrograde catheter placed in an external carotid artery (27). Scientists have shown great interest in this model because of its utility and resemblance of human ischemic stroke. Also, it can be used to evaluate thrombotic
therapies (28). However, the main disadvantages of this model are inhomogeneous infarct size, diffuse location of infarcts, and risk of microembolization (27). Photochemical MCAO is induced by systemically injecting a photoactive dye in combination with irradiation of several branches of the distal MCA (29). This model produces consistent infarct and is more reliable in the Sprague-Dawley than in the Wistar rat strain (30). The disadvantage is that the photochemical reaction can cause microvascular injury, vasogenic edema, and BBB disruption which does not allow penumbra formation (31). Therefore, this model may not be used for neuroprotective drugs.

**Endothelin-1-induced MCAO model**

The model is induced by the intracranial injection of endothelin-1 (ET-1), a potent natural vasoconstrictor peptide, proximal to the MCA using a stereotaxic system. After ET-1 application, CBF decreases and induces significant cerebral ischemic injury within the MCA vicinity (32, 33). Although the model has some limitations (discussed in Chapter 5), the consistency in location, size, severity, duration of the infarct, and the avoidance of surgery complications, provide distinct advantages over other models (17). This model may be useful for neuroprotective agent studies and is utilized in this dissertation project.

**Pathophysiology of Focal Ischemic Stroke**

Interruption or decrease (approximately 50% compared to base line) of blood supply to the brain initiates a complex cascade of neuronal events within minutes. Insufficiency of major energy substrates including oxygen and glucose, slows or stops adenosine triphosphate (ATP) synthesis in mitochondria. As energy failure occurs, ion pumps situated in the cell membrane malfunction, leading to massive influx of sodium and calcium; and efflux of potassium, which together cause depolarization. This is accompanied by an inflow of water, resulting in intracellular edema (swelling cells). Once cell membrane is depolarized, neurons release
neurotransmitters, including glutamate and aspartate. Uncontrolled release of glutamate at ischemic sites activates N-methyl-d-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-propionate (AMPA), and metabotropic glutamate receptors causing an increase in intracellular calcium. Depolarization also opens presynaptic voltage-activated calcium channels, releasing even more glutamate. Calcium overload ultimately results in excitotoxicity which leads to cell death. This is known as necrosis, and it occurs rapidly (within 5-10 min) (34). The primary necrotic region resulting from severe ischemia is commonly called the core of the infarct, or umbra of a stroke (16).

In addition to primary cell death due to oxygen and glucose deprivation, the core of the infarct is surrounded by a penumbra of compromised tissues in which programmed cell death or apoptosis and inflammation occur (16). In the penumbral region, the cells can repolarize at the expense of further energy consumption and depolarize again in response to high concentrations of glutamate and potassium ions. Such repetitive depolarizations/repolarizations result in increased release of neurotransmitters (35). This, coupled with the generation of free radicals, release of other biologically active molecules from damaged mitochondria and dead cells, plus infiltration of leukocytes, can continuously exacerbate injury in the penumbra. Following the hours to weeks it takes for the secondary pathology to develop and revolve; necrosis is replaced by other death mechanisms.

**Mechanisms of cellular cell death following cerebral ischemia**

Multiple cellular death pathways, including necrosis and three known types of genetically programmed cell death, appear to be triggered by ischemia and are involved in mitochondrial malfunction. These death pathways probably can concomitantly be present in the same cell (36) following cerebral ischemia; and a cell may switch back and forth between different pathways (37).
**Necrotic cell death pathway.** As mentioned above, necrosis is characterized by the loss of cellular membrane integrity as well as the irreversible swelling of the cytoplasm and its organelles. Typically, the necrotic cells spill their contents into surrounding tissues, resulting in an inflammatory response (discussed later in this section).

**Type I genetically programmed cell death (Type I apoptosis),** also known as caspase mediated cell death, can be initiated by the loss of mitochondrial membrane integrity, cytoplasmic cytochrome C release, and indirectly released cytokines. It has been demonstrated that in cerebral ischemia, mitochondrial cytochrome C, an essential membrane protein of the mitochondrial respiratory chain, is translocated from mitochondria to the cytosolic compartment (38). Upon release to the cytoplasm, cytochrome C binds apoptotic protease activating factor-1 (Apaf-1), and activates caspase-9, resulting in the formation of cytosolic apoptosomes. Caspase-9 then activates caspase-3, a frequently activated death protease, leading to random cleavage of DNA fragments via the activation of endonucleases (39). Caspase-3 and -9 have also been shown to play crucial roles in neuronal death following ischemia (40, 41). Moreover, in caspase-11 knockout animal models, a reduction in apoptosis and a defect in caspase-3 activation, have been demonstrated following cerebral ischemia, suggesting that caspase-11 is a crucial initiator responsible for the activation of caspase-3 (42). Caspase-11 is also responsible for caspase-1 activation which has the ability to enhance apoptosis (43). Caspase-1 is known to produce cytokines, such as IL-1β, IL-1α, and IL-18 (44); thus cytokines in apoptosis can also be considered as effectors during brain ischemia.

**Type II genetically programmed cell death (Autophagy)** is a caspase-independent cell death and involves autophagy. Autophagy is a regulated process of degradation and recycling of cellular constituents, involved in bioenergetic management of starvation (45). It has recently
been shown to be up-regulated in many stress conditions, such as cardiac ischemia/reperfusion (46). It also has been shown that cerebral ischemia enhances autophagy and the autophagic death cells have been detected at different time points post-ischemia in vitro and in vivo (47, 48). Autophagocytosis can occur when the NMDA receptor is activated excessively in neuronal cell culture. The neuronal death in rat organotypic hippocampal slices exposed to NMDA associated with autophagy and endocytosis, which completely prevented by c-Jun-N-terminal kinase (JNK) inhibitor, suggesting that autophagy as well as endocytosis is mediated by the JNK (49).

It has been reported that apoptosis-inducing factor (AIF) rapidly migrates from mitochondria to the nuclei of injured neurons following transient MCAO (tMCAO) (50). When AIF is released into the cytosol, it initiates a caspase-independent cellular suicide involving chromatin condensation and cleavage of DNA into characteristic 50kb fragments, inducing cell death (39). Decrease in AIF expression leads to a reduction in infarct size and cell death in the ischemic penumbra. Also, inhibition of poly(adenosine diphosphate ribose) polymerase 1 (PARP-1), an enzyme responsible for AIF release from mitochondria, lessens AIF migration and reduces neuronal cell death by more than 80% (50). Taken together, activated PARP-1 promotes the release of AIF which is a mediator of caspase-independent apoptosis.

Type II genetically programmed cell death is an important mechanism for dealing with protein aggregation and non-functional organelles (e.g. lysosomes, and especially mitochondria). However, when it becomes excessive it can result in cell death in response to starvation, osmotic stress, and TNF release (51).

**Type III genetically programmed cell death (Parthanatos)**, the last intrinsic cell death mechanism, driven by generation of oxygen and nitrogen species (ROS/RNS), is also known as parthanatos (52). Free radicals, specifically from the mitochondrial membrane, cause DNA
damage via oxidation, methylation, depurination, and deamination (53). DNA damage activates PARP-1, an important nuclear enzyme. Activated PARP-1 promotes the release of AIF from the membrane of mitochondria. These findings imply type III apoptosis is interrelated to type II apoptosis.

Sources of free radical generation contribute to exacerbate brain injury.

Reperfusion injury after transient ischemia is associated with overgeneration of free radicals, e.g. ROS/RNS, resulting in oxidative and nitrosative stress. Three primary sources of free radicals, mitochondrial dysfunction, two enzymes (xanthine oxidase (XO) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase), and activated leukocytes are discussed below.

Mitochondria are a major cellular site of free radical generation in physiological and pathological conditions. It is estimated that 1 to 4% of all oxygen used by mitochondria is converted into ROS. This is caused by “electron leak” at the respiratory chain transporting electrons from reduced pyridine nucleotides to O₂ molecules (54), primarily resulting in O₂⁻ generation. As the reducing state of the respiratory chain increases, the more electrons can leak from the chain, promoting the production of O₂⁻ radicals. This process depends on the concentration and tension of oxygen (55). Superoxide anions then rapidly dismutate to H₂O₂ either spontaneously or enzymatically via manganese superoxide dismutase. The rate of ·O₂⁻ and/or H₂O₂ production increases as mitochondrial Ca²⁺ increases (56). The rate of H₂O₂ production is a function of the oxidation-reduction state of the electron carriers, decreased carriers result in increasing the H₂O₂ production. During ischemic events, the respiratory chain is in a reduced state due to little or no oxygen supplied and overload of Ca²⁺ in mitochondria. This may lead to production of O₂⁻ radicals. Paradoxically, during reperfusion, the quick influx of oxygen to ischemic tissue results in overgeneration of ROS. The mitochondrial burst of ROS

25
production can escape cellular antioxidant defenses and cause damage to DNA, proteins, and membrane lipids. As discussed earlier, an increase in Ca^{2+} concentration can activate nitric oxide synthase (NOS), provoking the formation of ·NO which reacts to O_2^{-} to produce ONOO^{-} (peroxynitrite) radical, a potent oxidizing agent. Following cerebral ischemia, reperfusion or reoxygenation can cause oxidative stress to damage the brain tissues.

**Xanthine oxidase and NADPH oxidase are other important sources of ROS formation in post-ischemic tissues.** Enzyme xanthine oxidase (XO), also known as xanthine oxidoreductase (XOR), plays crucial role in purine nucleotide catabolism in humans. During ischemia, intracellular Ca^{2+} levels rise activating a protease that converts xanthine dehydrogenase (XDH) into XO. In the meantime, purines are catabolized, and XO substrates, hypoxanthine and xanthine, accumulate. In addition, adenosine triphosphate (ATP) breakdown due to hypoxic conditions causes the accumulation of XO substrates. When oxygen is again available (on reperfusion), xanthine oxidase may produce more superoxide (O_2^{-}), consequently, of ·OH (57, 58). Niconinamide adenine dinucleotide phosphate (NADPH) oxidase, a membrane-bound enzyme complex, expressed in neutrophils (59). It has been documented that activated NADPH oxidase contributes to ROS generation (60, 61). The activation of NADPH is triggered by protein kinase C (PKC) or by increase intracellular calcium concentration during cerebral ischemia (62)

**Activated leukocytes contribute to ROS/RNS generation.** Myeloperoxidase (MPO), mainly released by activated neutrophils and monocytes (subtypes of leukocytes), is characterized by powerful pro-oxidative and pro-inflammatory properties (63). MPO catalyzes the conversion of hydrogen peroxide and chloride anion to hypochlorous acid (HOCI), a cytotoxic radical. Thus, leukocyte infiltration promotes inflammation, and secretion of MPO
In summary, formation of reactive oxygen and nitrogen species involves multiple injury mechanisms, such as mitochondrial inhibition, activation of xanthine oxidase and NADPH oxidase enzymes, and infiltrating leukocytes. Excessive amount of free radical leads to oxidative and nitrosative stress, which contributes to the pathology of ischemic stroke.

Oxidative and nitrosative stress resulting from excessive amount of free radicals, debris from death cells induced by various pathways, and blood clots from re-canalization of the occluded blood vessel in the brain all contribute to immune response signaling. The role of the major inflammatory components (microglia, astrocytes, leukocytes, and mast cells) is detailed below.

**Inflammatory response to cerebral ischemia**

**Microglia play a bifunctional role during post-ischemic inflammation.** Microglial cells, the resident macrophages of the brain, are very sensitive to delicate alterations in their neuronal microenvironment. Microglia comprise the innate immunity in the brain and defend against infections as well as toxic substances released from dying cells in the brain by scavenging and engulfing unwanted pathogens and cellular debris. In normal condition, microglia often present in a ramified state, the resting form, which composes of long branching processes projecting out from the small cellular body. When the “sensors” of the microglial membrane bind to pathogen-derived molecules or other microglial activating agents, the cells transform into an activated stage associated with amberoid morphology and expression of inflammatory genes. Although the precise role and the exact time course of microglia in stroke are still debatable, the resident microglia are the first inflammatory cells that rapidly respond to cerebral ischemia. Experimental data have been shown that microglia are activated within minutes of ischemia onset and release
plethora of pro-inflammatory mediators, including IL-1β, tumor necrosis factor-alpha (TNF-α), and chemokines, e.g. monocyte chemoattractant protein-1 (MCP-1), all of which exacerbate tissue damage (64-67). Microglial proliferation typically reaches a maximum at 48-72 hours after focal cerebral ischemia and may continue for several weeks after the initial insult (68, 69). However, microglia can also produce cytoprotective substances, such as brain-derived neurotrophic factor (BDNF), insulin-like growth factor I (IGF-I), and several other growth factors (70). These data imply that microglia activation during ischemia, as an inflammatory indicator; have both deleterious and beneficial effects depending on the balance between pro- and anti-inflammatory secreted molecules.

**Astrocytes secrete inflammatory factors.** Astrocytes, the primary glial cells in the CNS, play a variety of physiological roles in CNS homeostasis. They are also known as astroglia and express glial fibrillary acidic protein (GFAP), a protein required for normal functions such as maintenance of BBB integrity. In ischemic stroke, GFAP expression is up-regulated, leading to astrocyte structural and functional changes (71). Reactive oxygen species produced by transient oxygen-glucose deprivation have been demonstrated to cause astrocyte mitochondrial membrane depolarization. ROS have also been shown to damage astrocytes through activating the PARP-1 cell death pathway (72). The expression of astrocyte inducible nitric oxide (iNO), an ROS, has been found within several hours and is maximal 2 to 3 days after ischemia (73, 74). Astrocytes have the capability to express major histocompatibility complex (MHC) antigens and co-stimulatory molecules that are critical for antigen presentation and T-cell activation in brain inflammation. Astrocytes also participate in promoting Th2 (anti-inflammation) response and suppressing interleukin-12 (IL-12) expression (75). Following cerebral ischemia, activated astrocytes secrete many inflammatory factors, such as interleukins (IL-1, IL-6), interferons (IFN-
α, IFN-β), MMPs, and tumor necrosis factor (TNF-α, TNF-β) (75-77), that can contribute to delayed neuronal death.

Moreover, tumor necrosis factor-like weak inducer of apoptosis (TWEAK), a member of tumor necrosis factor superfamily, has been reported to be produced by neurons, endothelial cells, and astrocytes (78, 79), and can induce the generation of pro-inflammatory mediators, including IL-8 and IL-6, by interaction with its Fn14 receptor found on astrocytes (80). Both TWEAK and Fn14 protein-levels are elevated in the ischemic penumbra and injection of a soluble Fn14 decoy receptor in an animal model of MCAO, can decrease the infarct volume (79). These data indicate ischemia induced astrocyte activation can potentially elevate brain inflammation.

Finally, neurons cannot survive in the brain without close interactions with astrocytes. Therefore, in any region where astrocytes fail to survive, there may well be failure of neuronal survival.

**Leukocytes produce inflammatory mediators.** In the sub-acute phase (hours to days), infiltrating leukocytes release cytokines and chemokines, especially excess amount of ROS and induction of MMP (mainly MMP-9). Infiltration of leukocytes occurs through three stages: rolling on the surface of endothelial cells, adhesion to the endothelial wall, and migration through the endothelium or diapedesis. These processes are mediated by adhesion molecules, including selectins (P-, E- and L-selectins), immunoglobulin superfamily (vascular cell adhesion molecule 1 (VCAM-1), intracellular cell adhesion molecule (ICAM-1)), and integrins (CD11a-c) (81). Leukocytes express cathepsins, a class of globular liposomal proteases that degrade extracellular matrix via MMPs. Leukocytes (lymphocytes) contain granzymes that degrade DNA, hence inducing caspase-independent apoptosis cascade (type II apoptosis). Granzymes,
cathepsins A and G are serine proteases, and they could be targets of serine protease inhibitors (serpins) to mitigate brain inflammation induced by leukocyte activation.

**Mast cells are responsible for BBB damage, brain edema, and hemorrhage.** Mast cells (MCs) are present in the brain, particularly inside the BBB (>96%) (82). They contain many granules (i.e. histamine and heparin) which are released upon activation. It is important to note that MCs can respond to activation either via exocytosis of the content of secretory granules, commonly known as degranulation (83), leading to acute release of mediators into the extracellular environment, or under a controlled fashion resulting in differential release of mediators (84, 85). This permits stimulus-specific release of individual mediators, which also may be different among MC types (86). Different MC triggers, such as IL-1, IL-33, and LPS can produce multiple mediators, independent of exocytosis (83). Brain edema, a consequence of ischemia, can be affected by MC activation. In animal models, it has been shown that ischemic brain edema can be reduced by 40% by treatment with the MC-stabilizing agent sodium cromoglycate (87). Moreover, histamine plays a role in brain edema in global ischemic stroke (88, 89), extrapolating that MCs may also be involved in edema of focal ischemia since histamine is a major MC mediator. This evidence indicates that mast cells may contribute to aggravated edema in ischemic stroke. Mast cells play a crucial role in CNS inflammation, particularly by recruiting leukocytes to sites of vascular inflammation by inducing all of the primary mechanisms involved in leukocyte infiltration during acute inflammation via secretion of histamine, adhesion molecules, selectins, and platelet-activating factor (PAF) (90). MCs’ activation in immature animals due to hypoxia-ischemia also results in exacerbating brain damage. It has been demonstrated that a rapid increase in MC count and their activation results in neuronal loss during hypoxic-ischemic brain damage of immature rats, and the damage is
reduced more than 50% by controlling the number of MCs (91). It is possibly caused by the contribution of MC-derived histamine (92). Importantly, inhibition of MCs with cromoglycate can decrease hemorrhage formation during cerebral ischemia-reperfusion following post-ischemic administration of recombinant plasminogen activator (rtPA), a thrombolytic agent (93). The reduction in hemorrhage formation may play a critical role in improving neurological outcomes and reducing mortality.

**Blood brain barrier and focal ischemic stroke**

In addition to the activation of inflammatory processes via stimulation of major components of the immune system, such as microglia, astrocytes, leukocytes, and mast cells, ischemic stroke ultimately leads to disruption of the BBB. The BBB is a semipermeable barrier between the CNS and the systemic circulation. It can be considered as the bodyguard of the CNS serving to maintain the fragile homeostasis of the brain. In addition to endothelial cells, the BBB is constituted of pericytes, astrocytes, neurons, and extracellular-matrix (ECM), which have been commonly known as the neurovascular-unit (NVU) (94). Each component of the NVU operates in harmony to regulate BBB functions, such as microvascular permeability, ion gradients, nutrient uptake, toxic waste removal, and cerebral hemodynamics. Indeed, an alteration of any of the individual constituents may cause BBB impairment. BBB disruption directly contributes to cerebral vasogenic edema, hemorrhagic transformation, and poor outcomes of the disease.

Both distinct stages of ischemic stroke, ischemia and reperfusion, can devastate BBB permeability and tight junctions (TJs) regulation, leading to complex clinical prognosis of the disease. Ischemia results in loss/shortage of oxygen and nutrients in the core and to the surrounding tissues. The endothelium at the core sustains the greatest severity of insult and likelihood for dysregulation and disassembly. The increase in paracellular permeability of BBB TJs has been observed after several hours following ischemia (95). Within minutes of ischemic
onset, the internal capillary diameter shrinks due to endothelial swelling. Ischemic tissues become acidic due to anaerobic respiration, which release lactate (96). Lactacidosis, caused by the lactate (lactic acid), directly contributes to swelling of endothelial cells, neurons, and astrocytes. Moreover, induction of proteases (i.e. tissue plasminogen activator (tPA), MMPs, cathepsins, and heparanases) via activation of inflammatory molecules, contributes to BBB degradation.

Continuous biochemical events following ischemia are strongly linked with downstream endothelial responses. As mentioned earlier, within the penumbra region, human brain expresses of several proteins, including caspases-1,- 3,- 8, -9, death receptors, Apaf-1, and p53, which are capable of activating apoptotic pathways. Pro-inflammatory mediators (e.g. IL-1, TNF-α) are induced, followed by upregulation of chemokines (e.g. MCP-1), and cytokine-induced neutrophil chemoattractant (CINC), involved in the activation of endothelium (97). Moreover, increased expression of intercellular adhesion molecules and cytokines initiates endothelial inflammatory activation, subsequently promoting leukocyte infiltration into the brain. Leukocytes (both circulating and infiltrating) and activated microglia are recruited to the ischemic zone further accelerating inflammation and toxic free radical production. Increased leukocyte migration perturbs the molecular organization of the TJ complex, including breakdown of occludin, a membrane protein found at high concentrations in BBB TJs, and zonula occluden-1 (ZO-1), a cytoplasmic protein involved in BBB TJ formation and regulation (98). The entry of neutrophils in the ischemic brain also causes disorganization of the actin cytoskeleton leading to increased BBB TJ permeability (99).

Reperfusion or compensation through collateral circulation is a process (either spontaneous or via re-canalization of the blood occlusion) which can re-establish blood supply and limit the
brain damage. However, it can contribute to additional physiological complications, and has the potential for hemorrhagic transformation. For instance, blood vessels weakened by ischemic stroke break turning to hemorrhagic stroke form. Increased BBB TJ opening or paracellular permeability may occur upon reperfusion following three stages: hyperemia, phase-1, and -2 (also known as “Biphasic permeability”) (94). The degree of permeability depends on a number of factors, including duration of ischemia, degree of reperfusion, and experimental model (94).

Hyperemia occurs as the result of an acute elevation in regional CBF. It couples with the loss of cerebral auto-regulation and therefore is passively dependent on perfusion. After hyperemia, hypoperfusion of ischemic region initiates, resulting in lacking of nutrients necessary for tissue recovery. Hypoperfusion may also induce neutrophil adhesion, following inflammatory response in the most susceptible tissues, and further induce to the next stage of increased BBB paracellular permeability. Phase-1 of biphasic permeability occurs within 3-8 hours of reperfusion onset. Alteration in permeability in this phase is primarily caused by enzymatic ECM degradation, increased inflammation and oxidative stress (100, 101). A continuous period of ischemia results in increase in edema, and consequently initiates phase-2 of biphasic permeability (102). This final phase initiates 18-96 hours after reperfusion and is coupled with angiogenesis and increased vasogenic edema (103-105).

Neutrophil (leukocyte) extravasation time-span during reperfusion is also dependent on the severity and form of ischemic insult. It has been shown that the highest concentration of neutrophil infiltration occurs at 6 and 48 h following a transient middle cerebral artery occlusion (tMCAO), and 12 and 72 h after a permanent MCAO in rats (106). The association between neutrophil infiltration and paracellular permeability responses of the BBB TJ indicates that the inflammatory mediator is related to BBB dysfunction.
Blood brain barrier dysfunction caused by ischemic stroke appears to be associated with inflammatory response. Initial induction of pro-inflammatory cytokines and adhesion molecules provokes the activation and migration of leukocytes (i.e. neutrophils) across endothelium. Once activated at parenchyma, leukocytes and microglia produce more inflammatory mediators, including cytokines and TNF-α. Interleukin-1β, IL-6, and TNF-α have been found in the CSF of ischemic stroke patients (107) and in ischemic brains of animals (108). Cytokines TNF-α, IL-1β, and IL-6 have been demonstrated to disrupt BBB in vitro study and the activation of cyclooxygenase (COX) could be a key role in this neuroinflammation (109). The BBB permeability is reduced by COX inhibitors in rat models (e.g. indomethacin) (110). Cyclooxygenase enzymes or prostaglandin-endoperoxide synthases (PTGS) catalyze the reaction in metabolism of arachidonic acid (AA), which is released from phospholipid membrane breakdown. This implies that AA cascades in ischemia-reperfusion increases BBB disruption. In addition, it has been documented that TNF-α, upregulated by neurons and astrocytes via activation of MMPs in the ischemic region precedes BBB permeability (111). Cytokines acting upon the vascular endothelium can indirectly stimulate the production and release of MCP-1, a major component involved in leukocyte extravasation into parenchyma and CINC (97), a member of the inflammatory chemokine IL-8 family. Cytokines/chemokines also stimulate induction of endothelial adhesion molecules, such as ICAM-1, P- and E-selectin, and leukocytes (112). Interleukin-1β expression brings about delayed and localized neutrophil recruitment, and breakdown of the BBB. Administration of chronic IL-1 in rat striatum has shown the marked increase in recruitment, vasodilation, and increased BBB breakdown at the highest concentration of IL-1 (days 8 and 14 post-injection); activation of microglia and astrocytes was identified on day 14 post-injection (113). Other mediators, including iNOS, free radicals, and MMPs induced
by neutrophils and mononuclear phagocytes (100) can directly or indirectly signal to accelerate the BBB paracellular permeability.

Regarding TJ protein, it has been demonstrated that junction adhesion molecule (JAM) is directly associated with inflammation. JAM-1 has been shown redistributed following TNF-α up-regulation from the junctions to the endothelial surfaces in vitro (114, 115).

There is abundant evidence showing that mast cells are involved in sequential events during cerebral ischemia via inducing many vasoactive mediators; it is rational to hypothesize that MCs contribute to BBB regulation. Indeed, in the rat model of focal cerebral ischemia, a 50% increase in BBB breakdown following the triggering of MC degranulation has been reported. Interestingly, pharmacological regulation (i.e. cromoglycate) of MCs reduces BBB breakdown by 50% over the control (87, 116). In similar studies with additional administration of tPA, there has been shown a reduction in tPA-mediated hemorrhagic transformation (117). One of the strong possibilities is that MCs’ bioactive mediators perturb BBB regulation and the basal lamina integrity. Some evidence suggests proteases, such as MMPs, plasminogen activators, and cathepsins, in the disruption of the matrix of the basal lamina (116). MMPs, a family of zinc-containing endopeptidases, can degrade most of extracellular matrix components, i.e. collagens, gelatin, elastin, fibronectin, and vitronectin (118). Specifically, MMP-2 and MMP-9 have involvement in the degradation of basal lamina, subsequent BBB breakdown and hemorrhagic transformation (119-121).

Collectively, the pathophysiology of ischemic stroke is extremely complex. It occurs in a series of biochemical cascades involving multiple factors and components with over-lapping and redundant features. Insightful understanding of the disease is necessary for more effective intervention and drug development.
Research Approach

The Current Options for Ischemic Stroke Therapy are Limited

Tissue plasminogen activator (tPA) is a serine protease which catalyzes the conversion of plasminogen into active plasmin. In plasma, plasmin primarily acts to dissolve fibrin filaments in blood clots. This subjects the blood clot to further proteolysis by other enzymes, thus dissolving the clot and restoring the blood flow in the occluded blood vessel. The Food and Drug Administration (FDA) has approved tPA as a therapy for acute ischemic stroke patients since 1996 with limited application, e.g. within 3 h (European Cooperative Acute Stroke Study, ECASS III, extended to 4.5 h in 2008 (122)) time-to-treatment window and with no apparent hemorrhage. Genentech, Inc (San Francisco, CA, U.S.A.) has manufactured recombinant tPA (rtPA; generic name: Alteplase; brand name: Activase). The drug is given intravenously with a dose of 0.9 mg/kg and (123) and in some cases may be given directly into an artery within a relatively narrow time window. Less than 2% of patients qualify for this treatment (124), because most patients do not seek medical assistance quickly enough, since the proper diagnostic procedure is requires time, and the drug itself can increase risks of cerebral hemorrhage and brain injury. The major side effects of the drug possibly occur through MMPs (e.g. MMP-9), and other signaling pathways associated with protease-activated receptor 1 (PAR-1), low-density-lipoprotein-receptor-related protein (LDL receptor-related protein or LRP), and NMDA receptor, and are detailed below.

Administration of tPA can lead to BBB disruption and cerebral hemorrhage via activation of MMP-9. Levels of MMP-9 are increased both in plasma and brain of acute ischemic stroke patients, and further elevated after tPA administration (125-127). It has been well established that MMP-9 is involved in mediating BBB disruption as well as inducing neurotoxicity in thrombolytic therapy (128). In early stages of the ischemic cascade, elevated MMP-9 levels can
lead to degradation of ECM and vascular basement membrane, thereby opening the BBB. Breaking-down of BBB leads to leukocyte infiltration, brain edema, and hemorrhagic stroke transformation (129). Logically, inhibition of MMPs can reduce hemorrhage and improve outcome in experimental embolic stroke model treated with tPA (130). However, tPA-MMP-9 activation could also have beneficial effects on brain tissue recovery after stroke during the later stage by promoting vascular remodeling, angiogenesis, neurogenesis, axonal regeneration (131) as well as synaptic plasticity (132) and glial remodeling by degradation antagonistic molecules within glial scar (133). This implies that combination therapies of MMP inhibitor and other tPA-related pathways may limit the neurotoxic effects and extend the therapeutic time window of tPA in brain ischemia.

Tissue plasminogen activator can cause neurotoxicity and increased BBB permeability through PAR-1. It has been reported that plasminogen and tPA (endogenous and exogenous) can be present in the brain. Therapeutically administered tPA and plasminogen are able to cross the BBB (129, 134) while endogenous tPA is synthesized by neurons and glia, and is particularly abundant in the hippocampus and hypothalamus (135, 136). In the brain, tPA can convert plasminogen into plasmin, which provokes PAR-1. Protease activated receptors (PARs) are G protein-coupled receptors that are activated by proteolytic cleavage of their N-terminus. The upregulation of PAR-1 during experimental ischemia in hippocampal slice culture suggests that PAR-1 may play a role in pathological effects. Indeed, in PAR-1 knockdown mice, the infarct volume was reduced 3.1 times after transient ischemic stroke (137). This provides evidence that the toxic effects of tPA may be mediated by PAR-1 activation.

Tissue plasminogen activator can also exacerbate brain injury via the LRP pathway and oxidative stress. Low-density-lipoprotein-receptor-related protein, LRP, is a multifunctional cell
membrane receptor, expressed in liver and throughout the brain, particularly high in the
cerebellum, cortex, hippocampus, and brain stem (138). During cerebral ischemia, tPA induces
the shedding of the extracellular domain of LRP in neurons and in perivascular astrocytes (139).
The interaction between LRP and urokinase plasminogen activator (uPA), a serine protease used
as a thrombolytic agent, has been shown to regulate vascular contractility (140), and BBB
permeability (141, 142). In tPA-deficient mice, the cerebral infarct is reduced 50% vs. wild type
animal after ischemic stroke, suggesting that tPA can increase stroke-induced injury, and that
increase is independent of tPA’s thrombolytic effects (143). Inhibition of tPA activity using
neuroserpin, a potent endogenous tPA inhibitor, can reduce infarct volume by 64% at 72 hr
(144). Blood brain barrier permeability increases when injecting tPA into CSF of mice without
ischemia, and this is mitigated by an LRP antagonist, a receptor-associated protein (RAP),
implying tPA induces neurotoxicity via LRP receptor. In addition to crossing disrupted BBB,
tPA may be able to cross intact BBB via LRP-mediated transcytosis to enter the parenchyma
under either ischemic or non-ischemic conditions (145, 146). It has been shown that LRP
signaling has an important role in the tPA-induced expression and activation of MMP-3 (147)
and MMP-9 in tissue cell culture as well as animal models of stroke (148). In vitro, MMP-3
expression increases in mouse-brain endothelial cell lines under ischemic stress and following
treatment with tPA. This increase was suppressed by the LRP inhibitor or nuclear factor (NF)-κB
activation (147). Recombinant tPA (rtPA) upregulates MMP-9 expression in cultured human
brain ECs; this response is remarkably inhibited in the ECs treated with small interfering RNA
(siRNA) to suppress LRP (148). Taken together, tPA contributes to BBB damage via activation
of MMP-3 and MMP-9, which is regulated by LRP and NF-κB pathways. Furthermore, the tPA-
LRP interaction has resulted in activation of NF-κB in astrocytes, and induction of iNO
following MCAO in rodent model (149). This might be an additional source of ROS, eventually leading to more BBB disruption in ischemic stroke.

Tissue plasminogen activator interacts with NMDA receptor resulting in excitotoxicity effects. The NMDA receptor (named for the selective glutamate receptor agonist, N-methyl-d-aspartate) consists of variety of NMDA subunits including NR1 subunit (150). Glutamate is the main excitatory neurotransmitter in the brain. In cerebral ischemia, increased glutamate levels may cause BBB opening and induce further brain damage (151). The modulation of the NMDA-induced neuronal death by tPA has demonstrated that tPA forms a stable complex with the NR1 subunit in vitro (152), therefore amplifying the influx of calcium during ischemic excitotoxicity. Blocking the tPA-NR1 interaction reduces excitotoxic necrosis in mice (153, 154). Also, recent findings have shown that the surface trafficking of tPA-NMDA receptor increases by enzymatic activity of MMP-9 (155) which can promote BBB disruption, hemorrhaging, and further induce excitotoxicity.

Taken together, evidence has strongly supported that tPA can induce neurotoxicity, edema, and hemorrhagic transformation through a number of mechanisms, such as activating MMPs (especially MMP-9) and protease activated receptor-1, and interacting with LRP and NMDA receptor. Thus, to improve it, it is of utmost importance to overcome the undesired effects of tPA which may be possible by applying combination therapy with other neuroprotective agent(s).

**Therapeutic Strategies with pGSN and AAT**

As described above, the pathological mechanisms of ischemic stroke are exceptionally complex, involving multiple processes, which can have both beneficial and detrimental effects. Limiting the permanent damage and promoting recovery mechanisms are critical to improve the outcome of stroke. Plasma gelsolin (pGSN) and alpha 1-antitrypsin (AAT) could contribute to limiting cell death, promoting recovery mechanisms, and ultimately improve stroke outcome.
The remainder of this chapter focuses on properties of the two proteins for enhancing stroke therapy strategies.

**Plasma GSN and ischemic stroke**

Gelsolin (GSN) is a ubiquitous protein (156, 157), and is also known as brevin or actin-depolymerizing factor (158). It was first discovered and isolated from rabbit lung macrophages in 1979. It is a calcium-dependent regulatory protein which regulates the reversible gel-sol transformations which occur in cytoplasm (159), hence the name *gel-sol*-in. Originally described as an actin-binding protein, GSN exists in both intracellular (cytoplasmic protein, cGSN) and extracellular (a secreted protein or plasma gelsolin, pGSN) forms, and a single gene on human chromosome 9 encodes both cytoplasmic and secreted variants (160). Alternative transcription initiation sites and selective RNA processing lead to distinct mRNA messages that produce unique protein products. Plasma GSN consists of a single 755-amino acid polypeptide chain (84 kDa) including a 25-amino acid N-terminal extension (160) that distinguishes it from cGSN (82 kDa). Most cells secrete pGSN, however smooth, skeletal, and cardiac muscle cells produce larger amounts of pGSN (156). It has been discovered a second cytoplasmic isoform named gelsolin-3, 11 amino acid longer than cGSN, which is coded by the same gene as the other isoforms and expressed in cytoplasm of oligodendrocytes in white matter tracts of the rat CNS (161). Gelsolin-3 has been shown to involve in myelin formation and CNS development (161). Gelsolin is also expressed throughout the human CNS, particularly in CSF (101, 162), where it mostly comes from neurons (162, 163) and neuronal growth cones (164), suggesting that GSN may have a certain role in the brain. Plasma GSN has a relatively long half-life of 2.3 days (165), and it circulates in serum with concentrations from 190 to 300 mg/mL (2.3 to 3.6 µM) (166).

**Plasma GSN plays a vital role in primary tissue injury via actin-binding activity.**

Actin, the most abundant protein in mammalian cells, has fundamental roles in cell integrity,
structure, and mobility. Monomeric globular actin (G-actin), 42 kDa, can noncovalently (and reversibly) polymerize into filamentous actin (F-actin) under physiological conditions (167). When G-actin is released from damaged or dying cells, its polymerization is thermodynamically liberated in the extracellular space leading to toxic F-actin formation. Rats receiving an intravenous injection of large quantities of G-actin die quickly with symptoms of pulmonary venous obstruction by actin filaments, pulmonary microthrombi, and endothelial injury (168). Human actin-containing sera directly induce toxicity to endothelial cells (169). Long actin filaments increase the blood viscosity, interfere with microcirculation, and cause the development of secondary tissue injury. GSN, a part of an actin-scavenger system, has three distinct actin-biding sites (170-172), and may prevent these toxic effects. Similar to cGSN, pGSN can actively bind (association constants, \( K_a = 10^9/\text{mol/L} \)) to the barbed end of F-actin, capping it to prevent the addition of monomers (171), and bind to the sides of F-actin and sever actin filaments. These interactions are regulated by \( \text{Ca}^{2+} \) ion (at micromolar levels), pH (<6.5), and polyphosphoinositides (173). Consequently, pGSN can depolymerize F-actin and remove it from circulation, thus preventing actin-exposure toxicity, an increase in blood viscosity, and possible blood flow disturbance (168). Therefore, massive actin released from damaged tissues can result in pGSN depletion. Indeed, there is abundant evidence demonstrating that depletion of pGSN is associated with various clinical conditions. There has been consistent observation of lowered levels of pGSN in hepatic failure (174, 175), acute lung injury (176, 177), malaria (178), cardiac injury (175), sepsis (179, 180), and major trauma (181). Moreover, previous studies have implicated that pGSN in CSF is a marker for a number of neuropathologies. For example, gelsolin levels in Alzheimer patients’ CSF were significantly reduced compared to healthy age-matched controls (145). Another example is GSN-c68, the 68-kDa carboxyl-terminal fragment of
gelsolin identified as a source of the insoluble peptide in familial amyloidosis of the Finnish type (FAF), was found in the CSF of a subject diagnosed with FAF (146). It has been reported that CSF gelsolin concentration is varied in certain neurological conditions, i.e. under normal conditions CSF gelsolin concentration is approximately 5 µg/mL (182); is 2.1 ± 0.7 µg/mL in patients with multiple sclerosis and is 1.95 ± 2.9 µg/mL in patients recovering from a subarachnoid hemorrhagic stroke (183). The variation of CSF gelsolin concentrations indicates that it may play an important role in managing inflammation and/or other biological processes in the CNS. A recent study has shown that pGSN levels in stroke patients are significantly reduced compared to controls, and it can be a biomarker for prognosis after ischemic stroke (184).

Gelsolin has also been shown to be involved in actin reorganization in microglia, promoting neuronal regenerative processes following brain inflammation. These processes include the production of anti-inflammatory cytokines, synaptic stripping, and recruitment of neurons and astrocytes to the damages area (185). Moreover, it has been demonstrated that gelsolin-mediated actin depolymerization results in reduced calcium influx through NMDA receptors and voltage-dependent calcium channels, and serves a neuroprotective role following excitotoxic and ischemic insults (186, 187).

**Plasma GSN has anti-inflammation properties by interacting with potent inflammatory mediators.** Catastrophic actin leaked from cellular injury may induce pro-inflammatory cytokine production (e.g. TNF-α), impair the microcirculation as well as damage multiple organs (168, 188, 189). Actin binds to pGSN causing local and systemic pGSN depletion that allows localized mediators to be released and initiate systemic inflammatory responses. Recombinant pGSN has been shown to be protective against acute inflammatory response associated with tissue injury (190). Besides binding to actin, pGSN also binds to a
number of bioactive lipids including lysophosphatidic acid (LPA), lipoteichoic acid (LTA), and lipopolysaccharide (LPS) (87, 191-193). Lysophosphatidic acid has multiple biological functions, such as a lipid mediator as well as a precursor in phospholipid biosynthesis. Indeed, pGSN binds LPA with an affinity $K_d = 6$ nM (194), and LPA inhibits the F-actin severing activity of pGSN (87).

It has been reported that pGSN inhibits PAF-mediated P-selectin expression by 77% (195). Plasma GSN also strikingly inhibited PAF-induced superoxide anion production of human peripheral neutrophils (polymorphonuclear neutrophils, PMN) in a concentration-dependent manner (195). Platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine), is a potent, pro-inflammatory phospholipid with diverse physiological and pathological effects (126). PAF does not circulate in the blood under normal conditions, but it is up-regulated in septic and trauma patients, and PAF concentration may increase further at the site of local inflammation (141). The ability to inhibit LPA and PAF bioactivity suggests that pGSN has anti-inflammatory activity during acute inflammation.

Plasma GSN is a substrate for MMPs (196). Matrix metalloproteinases, zinc-dependent endopeptidases, are up-regulated during the inflammatory response (197), and in stroke (198). It has been demonstrated that, in the fluids of a burn wound, there was an inverse correlation between pGSN and metalloproteinase levels; gelsolin proteolytic fragments of 49 kDa were also detected (197), probably because pGSN is cleaved by MMPs and gelsolin fragments also play a certain role in the process.

**Plasma GSN has anti-apoptotic activity.** It has been documented that changes in dynamics of actin cytoskeleton result in the release of ROS and subsequent programmed cell death (199). It is also well-known that GSN is a cytoskeletal regulator and has roles in apoptosis.
GSN is a substrate for caspase-3, a key mediator of apoptosis, and N-terminal GSN fragments have been shown to promote morphological changes in numerous cell types (200), and it is implicated that the GSN fragments are effectors of apoptosis. However, the full length of GSN suppresses apoptosis by forming a complex with phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) which inhibits caspase-3 and -9 activity (201). It has also been shown that GSN inhibits apoptosis by blocking the loss of mitochondrial membrane potential and preventing caspase-3 activation (202, 203). In several models of neuronal cell death, GSN has demonstrated protection against excitotoxicity-induced apoptosis by altering the actin cytoskeleton in response to Ca$^{2+}$ influx (203). GSN-knockout neurons have augmented cell death and are susceptible to glucose/oxygen deprivation. In support of the protective effect of GSN in vivo, GSN-knockout mice have shown to be more vulnerable to brain ischemia (187). Therefore, increasing GSN expression may ameliorate neurodegeneration during ischemic stroke.

Most studies indicate that the restoration and/or increase in the levels of pGSN in injured animals have beneficial effects. Further, Critical Biologics Corporation has investigated recombinant human pGSN (rhu-pGSN) as a replacement therapy for the treatment of hypogelsolinemia in high-risk patients presenting with end-stage renal disease (ESRD), cystic fibrosis patients, and other critical care complications.

(http://www.lehigh.edu/~inbios21/PDF/Fall2008/Palmer_11032008.pdf)

**Alpha 1-antitrypsin and ischemic stroke**

Alpha 1-antitrypsin (AAT or A1AT or α$_1$-antitrypsin), also known as alpha-1 proteinase inhibitor (A1PI), was first isolated in 1955 and the name antitrypsin came from its ability to inhibit pancreatic trypsin (204). The “α-1” part of the name comes from the alpha band of globulins formed during protein electrophoresis and is part of sub-region 1 of that band. AAT, a serpin (serine proteinase inhibitor), is a globular, single-chain 394-amino acid glycoprotein of 52
kDa that is about 15% carbohydrate (205). AAT is produced primarily in hepatocytes and released into blood circulation through the liver (206). It is also produced by other tissues, such as Paneth cells of the gastrointestinal tract, kidney, and lung (207, 208). In normal conditions, concentrations of AAT in serum are between 1.0 to 2.5 mg/mL (20-50 µM) (206), in CSF is approximately 7.6µg/mL (209), and it is fairly stable with a half-life of 4-5 days in humans (206). It can cross the plasma membrane of several types of cells and exert distinct extracellular and intracellular functions. For mice, serum AAT peaks within 10 min., and the half-life is similar to humans after intraperitoneal (i.p.) or subcutaneous (s.c.) injection (210).

**AAT has anti-inflammatory properties.** AAT is a primary serpin in the blood and is a major protector against proteolytic digestion by human neutrophil elastase (HNE) through inhibiting HNE at the associate rate constant (Ka) of about 10^7 M^-1 s^-1 (211). The resultant complex renders the proteinase inactive and the complex is then removed from circulation (212).

Serine proteinases and their inhibitors play important roles in several inflammatory processes including blood coagulation, fibrinolysis, and complement activation. AAT is a primary acute protein with potent anti-inflammatory activities, such as the efficient inhibition of neutrophil elastase and proteinases, as well as activity against cathepsin G, thrombin, trypsin, and chymotrypsin (213). Many of these proteinases target receptor proteins related to pro-inflammatory cytokine expression and cell signaling (214). Human AAT (hAAT) appears to be an inhibitor of both extracellular matrix degradation and PMN influx in the lungs of the mice exposed-to the-cigarette model (215). Similar matrix degradation and infiltration occurs in inflammation following stroke, and AAT may be able to interfere with matrix degradation and leukocyte infiltration that promotes edema, hemorrhage, and neurotoxicity. AAT is one of many acute phase proteins (APP) (e.g. C-reactive protein (CRP), acidic α-1 glycoprotein (AGP), and
fibrinogen (FIB)) elevated in human blood in response to transient brain ischemia (216). The inflammatory reaction following stroke may exacerbate the clinical outcome of stroke patients, and these patients require treatments for inflammation (216). It is not known whether these elevated AAT levels correlate with functional outcome, but AAT serves as a major anti-inflammatory serum protein. It inhibits the production of pro-inflammatory cytokines interleukin (IL)-1β, IL-6, IL-8 and TNF-α, and enhances anti-inflammatory cytokine IL-10 production through increasing cellular cyclic adenosine monophosphate (cAMP) levels (217, 218). This subsequently inhibits the leukocyte recruitment in inflammation, and it is independent from AAT protease inhibitor activity. AAT-mediated interruption of IL-8 binding to its receptors limits neutrophil infiltration in lungs (219). AAT has also been shown to decrease neutrophil influx and TNF-α production in kidneys during ischemia followed by reperfusion (220). Therefore, AAT may similarly act to reduce the recruitments of inflammatory mediators to inflammatory sites in the brain, preventing the organ damage.

**AAT has anti-apoptotic and cytoprotective effects.** In addition to inhibiting serine proteases, AAT also inhibits cysteine proteinase, e.g. caspase-3 that plays a central role in apoptosis (221, 222). For instance, AAT has been demonstrated to restrict apoptosis by internalizing into alveolar cells (lung cells) and interacting with caspase-3 (223). It has been shown that AAT can protect pancreatic β-cells against apoptosis in vitro and in vivo via caspase-3 inactivation and is a potential therapeutic for diabetics (222, 224), in animal models of ischemia in the kidney (220), as well as in liver cells in a model of hepatic ischemia/reperfusion injury (225). It has recently been documented that caspase-8 and caspase-3/7 are involved in activation of microglia and subsequent inflammatory-mediated neurotoxicity (226). This suggests that AAT anti-caspase activity may be highly significant in limiting stroke pathology.
that results from intrinsic inflammatory processes. Furthermore, cathepsin and calpain are hypothesized to play key roles in autophagic cell death (227), and AAT, a member of the serpin family, can inhibit calpain and cathepsin therefore reduce apoptosis.

Cytoprotection is critical in protection of healthy tissues immediately following an initial ischemic insult, preservation of organ functions, and prevention of organ failure. Therefore, AAT could be a good candidate for ischemic stroke.

Taken together, with its anti-inflammatory, anti-apoptotic, anti-hemorrhagic, and potential antioxidant properties (228, 229), and by inhibiting enzymatic activity of granzymes, calpains, and caspases AAT appears as a promising therapeutic agent for ischemic stroke.

AAT is approved by the FDA for use in treating diseases associated with insufficient AAT activity usually due to genetic variants. It has an excellent safety profile based on growing use in chronic obstructive pulmonary disease and genetic insufficiencies, with no apparent cytotoxicity (218, 230). Three commercial formulations available, Prolastin, Zemaira, and Aralast (231) are purified extracts from human plasma and delivered intravenously.

Hypothesis

The central hypothesis of this dissertation is that outcomes following ischemic stroke will be improved by delivery of pGSN and hAAT. This will be tested in a rat model using both localized and systemic delivery.
Ischemic stroke occurs most commonly in the vicinity of the middle cerebral artery (MCA) in humans. Data coming from experimental animal models delineate important mechanisms and the development of novel therapeutic strategies because of the difficulty in obtaining information from humans in terms of expense, time, and ethics in certain circumstances. An endothelin-1-induced middle cerebral artery occlusion model has been shown to produce well-defined areas of infarct, is reproducible, a minimally invasive procedure, and has the ability to achieve reperfusion in rats. This method can be utilized with the management of a laser-Doppler imaging system to ascertain perfusion/reperfusion in cerebral ischemia. The outcome parameters are defined by neurological status (motor and sensorimotor functions) and ischemic brain lesion quantification (either in vivo with magnetic resonance imaging (MRI) or post-mortem with brain tissue staining).

**Rats Used as an Animal Model for Focal Ischemic Stroke**

Studies in human stroke are extremely limited due to the difficulty in collecting postmortem tissues at different time points after stroke onset where neuronal death occurs. Therefore, ischemic stroke research concentrates mainly on experimental ischemic stroke in animal models. There are many animal models to study mechanisms and neuroprotective strategies. Using the appropriate animal models is very important to predict the efficacy of drugs that may benefit humans. However, each animal model has its own advantages and disadvantages. Rats were selected as animal model for the specific objectives of this project because the following reasons.

Rat models have been shown to approximate human conditions well, such as cerebral anatomy and physiology, ability of analyzing physiology and brain tissues, genetic homogeneity within strains, low cost, and public and institutional acceptability in term of ethical aspect
compared to large animals (97, 232, 233). Large animals (higher species) are often used in later studies once positive results are achieved in small animal models prior to clinical trials (recommendations of Stroke Therapy Academic Industry Roundtable, STAIR, published in 1999).

Stroke outcome measurements include testing motor and sensorimotor functions corresponding to brain damage, as well as evaluating infarct size. The rat has a moderate size which permits easy monitoring of physiologic parameters and evaluation of brain specimens (234). Somatosensory deficits are often present in humans following stroke (235); this has commonly been reported in rats as well (236, 237). The movements of a human required to reach for a target object are similar to rats (238, 239). Tests have been established which measure motor and sensorimotor deficits acquired following focal brain damage, including the cylinder and vibrissae-stimulated forelimb placing tests (240, 241).

There are also several issues regarding the use of rats in animal models for stroke disease in humans. These include the infarct size, reperfusion, and functional recovery.

Stroke can occur when a small or large artery is occluded which is associated with small or large infarct volume which the arteries supply, respectively. Human strokes are often relatively small (range 28-80 mm$^3$) (13), while animal models usually induce bigger infarct volumes (the infarct size of in Sprague-Dawley rat MCAO ranges from 60-400 mm$^3$) (242-244). Therefore, the findings from animal models may introduce a potential bias for large clinical trials including stroke patients with less severe, and hence smaller infarct volumes, particularly in the first-ever stroke group (245, 246). In addition, the ratio of the volume of gray matter to white matter in the cerebral hemisphere in humans is smaller than that of rats and preclinical studies have extensively examined neuroprotective gray matter in rats (247). Neuroprotective agents which
work on gray matter do not necessarily have the same effects on white matter, which makes up a large proportion in the human brain.

Reperfusion occurring in stroke patients comes primarily from three sources. First, approximately 15-18.8% of all strokes have early spontaneously recanalization as early as 6-8 hours from stroke onset (248, 249). Second, collateral blood flow via the circle of Willis and leptomeningeal vessels has been observed using a regional angiographic system (250, 251). Last, the thrombolytic agent, tPA (intravenous injection within 3-4.5 hours from stroke onset) or the intravascular clot retrieval devices, Ancrod (252) can promote reperfusion. Moreover, leptomeningeal vessels have been shown to provide blood flow in the penumbra and improve stroke outcome (253, 254). Recovery after stroke includes at least three processes: resolution of acute tissue damage, behavioral compensation, and neuroplasticity (255). Cellular reparation within the penumbra involves axonal sprouting to establish new patterns of cortical connections (255), newly generated neurons, possibly to replace losses in the penumbra (256, 257), and reorganization of cortical maps (258). In fact, connection between animal models to these areas is not well-established as animal models often produce a wide-spread damage to many different brain regions, such as rostral and medial frontal cortex, lateral temporal cortex, and occipital cortex (259-261), therefore it is difficult to study for the patterns of cellular reparation following stroke.

Functional recovery of stroke involves behavioral deficits. However, most experimental studies focus on reducing damage areas in rodent models. As mentioned above human functional deficits after stroke occur with damage to specific circuits, including cortical maps. The recovery of motor, sensory (262), and language (263) involves a progressive reparation and recovery of the penumbra, and behavioral compensation following stroke (264, 265).
Endothelin-1-Induced Middle Cerebral Artery Occlusion Model

Human ischemic stroke in origin is more prominent than hemorrhagic stroke and mainly caused by occlusion of MCA. Therefore MCAO models are the most widely used in stroke research, with the goal of evaluating of new treatments (266, 267). Rat models have been shown to produce well-defined areas of infarct within the neocortex and caudate nucleus which are feasible for quantification (268). MCAO results in a reduction of CBF in both the cortex and striatum, however, the degree and the reduction of blood flow depend on the duration of MCAO, the site of occlusion along the MCA, and the amount of the collateral blood into MCA territory. MCAO models can be permanent, or temporary to allow reperfusion, depending on the study objectives. Permanent ischemia models allow the study of cerebral ischemia without interference of reperfusion effects, temporal/transient ischemia models allow investigating reperfusion injury which often occurs in human (due to recanalization or thrombus disintegration) (269, 270).

It is understandable that there is no “ideal” ischemic stroke model due to the diversity of human stroke itself. The best model selected for investigating therapeutic agents should satisfy the following criteria: (A) simulating the physiological conditions in human stroke; (B) creating re-producible lesions; (C) employing relatively simple techniques and minimal invasiveness; (D) showing the capability of monitoring physiological parameters; (E) allowing outcome measurements, such as behavior evaluations and brain analysis; and (F) low cost (271). The dissertation studies employ MCAO induced by endothelin-1 (ET-1), a potent vasoconstrictor peptide, in rats as a model to evaluate the effects of two proteins, pGSN and AAT. Microinjection of ET-1 adjacent to the rat MCA has shown a reproducible pattern of focal cerebral infarction (272). The technique is relatively easy to perform (i.e., intracerebral injection follows stereotaxic coordinates) and minimizes the surgery complications (33). The data that have been collected from the present project include behavior tests and brain damage
measurements using histology and MRI. Further discussion about advantages and disadvantages of the model are detailed in Chapter 5.

ET-1, a small peptide, belongs to an endothelin (ET) peptide family. The ET family includes three 21-amino acid peptides ET-1, -2, and -3 (273). ET-1 is the predominant isoform in vasculature and has the most potent endogenous vasoconstrictor agent (the order of potency is ET-1 > ET-2 >> ET-3). ET-1 acts in both paracrine and autocrine fashion by interacting with ET receptors in vascular smooth muscle (VSM) and endothelial cells, thereby modifying vascular function (274). There are three known ET receptors, ETA, ETB, and ETc. ETA and ETB receptors are widely expressed in VSM cells (VSMCs), however, the ETA receptor appears to be the predominant subtype in medial VSMCs. The mechanism of vasoconstriction of ET-1 is its binding to the specific cell surface ETA receptor in VSMC, which induces G-protein-dependent stimulation of phospholipase C, leading to the formation of inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) by hydrolysis of phosphatidyl inositol. IP3 up-regulates the intracellular concentration of calcium (Ca^{2+}), which in turn results in vasoconstriction (275, 276). In healthy adults, ET-1 concentrations in basal plasma are 0.7-5 pg/mL (277, 278). Studies of the kinetics of ET-1 clearance in a three-compartment model showed an approximate terminal half-life of 455 minutes. It has been shown that ET-1 presents long-lasting action in many in vitro vascular tissue preparations, e.g. cerebral, coronary, and mesenteric arteries (279-283). Furthermore, ET-1 injection in a dose-dependent manner, in vivo directly into the rat brain parenchyma with induces local CBF to ischemic levels (33, 284-286).

**Materials**

Rat ET-1, MW = 2492.0, purchased from American Peptide Company, Inc, (CA, U.S.A.), was dissolved in sterile phosphate buffered saline (PBS) to make a stock concentration of 80 µM (80 pmol/µl). The stock solution was stored at -20°C. ET-1 was thawed, centrifuged, and placed
on ice until ready to inject. Human pGSN, 1 mg/mL in sterile saline stock solution, was a generous gift from Critical Biologics Corporation (MA, U.S.A.) Artificial cerebrospinal fluid (aCSF) was obtained from Fisher Scientific, Inc. (PA, U.S.A.) Human AAT (alpha 1-protein inhibitor, Prolastin C), dissolved in sterile water, was from Grifols, Inc. (NC, U.S.A.) Other materials, such as phosphate buffer saline (PBS), saline, formalin, and isoflurane were purchased from Fisher Scientific (NH, U.S.A.)

Experimental Procedures

Animal Model and Treatments

All procedures were performed with prior approval from the University of Florida Institutional Animal Care and Use Committee (IACUC). Male Sprague-Dawley rats, 7-8 weeks in age, (weight 220–250 g) were purchased from Charles River Laboratories International, Inc. (MA, U.S.A.) Pair-housed rats were maintained in controlled environments on a 12/12 hour light/dark cycle in plastic cages, received ad libitum food and water. The rats were acclimated for three or more days before the start of any experiments during the light phase. Animals with incomplete data sets due to death, and non-response to behavior tests are eliminated from the study data.

A model of transient/reversible middle cerebral artery occlusion (MCAO) using ET-1 was performed as previously described (287). Briefly, animals were anesthetized (induced with 5% isoflurane in 1 L/min oxygen and maintained at 1.5-2.0% and 0.5 L/min), placed prone in a stereotaxic frame (Braintree Scientific, Inc., MA, U.S.A.) and secured in the flat skull position. Under aseptic conditions, the scalp was then retracted and a midline incision was made in the skin from the point above bregma until the lambda point, using a stainless steel sterile blade. A small hole (3 mm i.d.) was drilled in the cranium adjacent to the left MCA at 0.2 mm anterior, 5.2 mm lateral (33), and 1.0 mm from the skull bottom (3 µL of methylene blue was injected in
other animals with the same coordinates to verify the injection site, then the brain was sectioned to expose the MCA and the injection site was visualized, data not shown). A 27-gauge needle was used to inject ET-1 or pGSN or hAAT at a rate of 1 µL/min. After ET-1 injection, the needle was left in place for an additional 3 min before being slowly withdrawn. The injection system was flushed by distilled water and loaded with subsequent treatment agents. The detailed schedule for treatments is described in tables 2-1, 2-2 and 2-3.

Body temperature was maintained between 35ºC and 37ºC throughout surgery, using a circulating water blanket (YSI telethermometer and Gaymar T-pump system). Respiratory rate and general skin color were observed as an indirect assessment of heart rate. The scalp was then closed with suture wound clips, or thread wound sutures for the group subjected to MRI later. The animals were kept at 37ºC until totally recovered. After full recovery, animals were returned to their home cages conditions for outcome evaluation 72 h after surgery.

During the acute post-operative period and anytime that there is any acute concern, any animal displays the signs of distress or a loss > 15% body weight, animals will be treated or euthanized following the approved protocol.

Laser Doppler Perfusion Imaging

Laser-Doppler imaging system (MOORLDI system at the Department of Pharmacology and Therapeutics, University of Florida) was used to measure the relative flux/perfusion of the brain which is strongly associated with the spatial and temporal characteristics of the changes in cerebral blood flow (CBF) response (288-290). The principle of the technique is that the dynamic blood flow in the vasculature results in a Doppler frequency shift of the scattered laser light, which is photodetected and then processed to build a color-coded map of blood flow. The technique allows for repeatedly assessing perfusion changes over a wide brain by scanning a low-power laser beam across the skull (290).
Imaging studies measuring sequential perfusion changes after ischemia often require the animals to be immobilized for long periods of time (at least 2.5 h). Isoflurane, a volatile anesthetic, has been shown to reduce early neuronal death in animal models of focal cerebral ischemia (291). Therefore, animals used in these study, exposed to isoflurane for approximately 3 h, were not used for testing the effects of the treatment agents to avoid the influence of isoflurane, and were sacrificed following the last measurement (survival time was about 4 h).

The scalp was retracted from animals’ head and two holes (~3 mm i.d.) were made in each hemisphere. The treatment details were exactly the same as the treatments in the “Animal Model and Treatments” above, except that an aCSF group was not included, there were three animals per group, one animal died during imaging due to isoflurane sensitivity (overdosed), and was replaced. The imaging was performed at four different time points: pre-injection, 10-20 min, 30-35 min, and 55-65 min after ET-1 injection. The pseudo-coloring of relative flux (number of red blood cells multiplied by speed) illustrates “cool” colors for relative low flux and “warm” colors for relative high flux. The 16-bit color scans were made with an arbitrarily assigned unit from 0 (lower limit) to 1,000 or more (upper limit). The scan speed was 10 ms/pixel and the total scan (scan area was about 1.8 cm x 2.3 cm) duration per animal was approximately 10 min. Actual blood flow is highly correlated to flux except at supranormal pressures (292). Indeed, percentage reductions of perfusion on the injected side of the brain were calculated using the following equation 2-1.

\[
\text{Flux (\%) = } \frac{F_i}{F_c} \times 100
\]  

(Equation 2-1)

where Fi is the flux value of the ipsilateral side (injected side) and Fc is the flux value of the contralateral side (opposite or unaffected side).
Behavioral Tests

In brain models of focal ischemic stroke, the sensorimotor cortex or striatum in one hemisphere is frequently damaged with severe injury compared to other area of the brain. Unilateral injury to the forelimb region of the rat brain motor and sensorimotor cortex causes immediate deficits in the somatosensory functions. The deficit in the use of the contralateral forelimb can be measured (293). The behavioral tests, such as cylinder and vibrissae tests, allow assessing motor and sensorimotor asymmetries. These tests have been useful in studies of recovery of function following central nervous system (CNS) injury, as well as for evaluation of pharmacological interventions (294-299).

Cylinder test

Cylinder test is also known as forelimb use for vertical-lateral exploration test. The test examines the levels of preference for using the non-impaired forelimb for weight shifting movements during spontaneous vertical exploration of the walls of a cylinder after unilateral cerebral ischemia.

The test encourages the rodent (rats in this study) inside a specially designed cylinder (40 cm height, 20 cm i.d.), to use the walls for upright support and vertical exploration. The cylinder walls reveal forelimb asymmetries that have resulted from different forms of brain injury, including cortical damage (300) and nigrostriatal neurodegeneration (240). A video camera with slow-motion playback capability records (3 min each animal) the number of times the animal uses the ipsilateral (affected) or contralateral (less affected) forelimb alone or uses both simultaneously for upright support. Intact animals typically use both limbs equally for upright support. But after damage to the motor system, animals show an asymmetric reliance on the less-affected (ipsilateral) limb. An experimenter blinded to treatment conditions calculates the
percentage of two forelimbs used from the number of total attempts for upright support and vertical exploration.

**Vibrissae test**

Vibrissae test is also called vibrissae-evoked forelimb placing (unskilled reaching for a stable surface) test. The test studies the sensorimotor/proprrioception deficits.

To determine whether an animal has asymmetrical sensorimotor perception, the test is designed to hold the animal by the torso with its forelimbs hanging freely, and then slowly move the animal laterally toward the edge of the table or countertop until the vibrissae of one side make contact with the edge. Intact animals typically quickly place the ipsilateral forelimb on the edge of the surface when the ipsilateral vibrissae brush the table edge. In contrast, animals with damage to the motor system often respond slowly (or do not respond) to vibrissae stimulation on the ipsilateral side compared to contralateral side (240). A camera records ten iterations of vibrissae stimulation for each side and the corresponding responses. An experimenter blinded to treatment conditions calculates the time between vibrissae stimulation and "placing response" for each side using Windows Movie Maker 2007.

**Histopathology**

Among a number of histological methods, the 2,3,5-triphenyltetrazolium chloride (TTC) staining assay is one of the most commonly used to measure ischemic lesions. The TTC assay has been shown to be reliable and consistent compared to other traditional histologic markers, e.g. hematoxylin and eosin (H&E) assay (301), thionin staining (302), and cresyl violet staining technique (303). TTC is a white crystalline powder and soluble in water. It is enzymatically reduced to red lipid-soluble compound, 1,3,5-triphenylformazan (TPF) in living tissues due to the activity of various dehydrogenases (important enzymes in oxidation of organic compounds,
abundant in the inner membrane of mitochondria (304)). Thus, pale or white TTC tissues indicate dead tissues, since these enzymes have been denatured or degraded.

Animals in model and treatment groups were allowed to survive for 3 days. The brains were quickly isolated, placed in cold PBS (0-4°C) for 30 min and then sectioned into 2-mm-thick coronal slices using a brain slice matrix (Leica Microsystem, IL, U.S.A.) The tissue sections were held in cold PBS for 3 min before they were incubated in TTC solution (0.05% TTC in PBS) for 30 min at 37°C. The sections were washed three times (one minute each) with PBS and fixed in 0.1 M phosphate buffered formaldehyde (PBF). Calibrated digital images of tissue sections were made at 600-dpi scanner resolution with 48-bit color and saved as TIFF files. The infarct areas were quantified by visual thresholding of TTC-labeled (normal) and unlabeled (infarct) tissue, and the measurement of each area was completed using Image J version 5.0 (NIH). Infarct volume was determined as the sum of the infarct areas of all sections of each brain. The ratio of average infarct volumes of treated rats to untreated rats was used as a dependent measure for evaluating pGSN or hAAT effects.

Magnetic Resonance Imaging

A magnetic resonance imaging (MRI) scanner uses information about the movement of the water molecules to determine the infarct after ischemic stroke in living subjects. Measurement of infarct volume in ischemic stroke patients using MRI have shown that the technique is an accurate predictor of clinical outcome in humans and it is a relevant measure to use to assess the outcome of therapy both in human and animal studies (305).

Three days following ET-1 induced ischemic stroke, damage was visualized in live animals from the groups ET-1 alone (N = 8) and ET-1+hAAT (i.v.) (N = 14) maintained under 1-2% isoflurane anesthesia using Advanced Magnetic Resonance Imaging and Spectroscopy (AMRIS) facility in the McKnight Brain Institute of the University of Florida. An 11T/470 MHz
MRI Spectrometer (Magnex Instruments, U.K.) using a Bruker Avance Console and Paravision software (Bruker BioSpin MRI, Inc, MA, U.S.A.) were used to generate a standard T2-weighted spin-echo sequence (rapid acquisition with relaxation enhancement) through continuous 1-mm-thick sections covering the entire brain region. Body temperature was monitored and maintained at 35-37°C for the entire scan time with heated ventilation. Total acquisition time for MR imaging was about 35 minutes. Image analysis and infarct area quantification are done by Jim version 5.0 and Image J. An experimenter blinded to treatment conditions calculated the total infarct areas of animals.

**Image Analysis**

Infarct areas of brain slices from all experiments, including from TTC assay and MRI imaging were analyzed in a blinded manner using the NIH Image J version 5.0. Regions of interest (ROI) were created encompassing infarct areas of each sections (in mm²), and total infarct volume were the estimated using the sum of infarct area of all slices of each animals.

**Statistical Analysis**

Unless specified, all data are expressed as arithmetic means ± s.e.m. Two-way ANOVA (time and treatment) was followed by Bonferroni *post hoc* test to compare behavioral dependent variables between groups. Comparisons of infarct area were made by two-tailed Student’s t-test; and p-values less than 0.05 were considered to be significant.
Table 2-1. Treatment schedule for laser-Doppler perfusion imaging

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Number of animals</th>
<th>Survival rate</th>
<th>Date collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1 alone</td>
<td>3 µL of ET-1 (i.c.)</td>
<td>3</td>
<td>3/3</td>
<td>3</td>
</tr>
<tr>
<td>ET-1+pGSN</td>
<td>3 µL of ET-1 (i.c.)</td>
<td>3</td>
<td>3/3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3 µL of pGSN (i.c.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ET-1+hAAT(i.c.)</td>
<td>3 µL of ET-1 (i.c.)</td>
<td>3</td>
<td>3/3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3 µL of hAAT (i.c.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ET-1+hAAT(i.v.)</td>
<td>3 µL of ET-1 (i.c.)</td>
<td>3</td>
<td>3/3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1 mL of hAAT (i.v.)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2-2. Treatment schedule of for aCSF, ET-1 alone, ET-1+pGSN, and ET-1+hAAT (i.c.) groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Number of animals</th>
<th>Survival rate</th>
<th>Date collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1 alone</td>
<td>3 µL of ET-1 (i.c.)</td>
<td>3</td>
<td>3/3</td>
<td>3</td>
</tr>
<tr>
<td>ET-1+pGSN</td>
<td>3 µL of ET-1 (i.c.)</td>
<td>3</td>
<td>3/3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3 µL of pGSN (i.c.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ET-1+hAAT(i.c.)</td>
<td>3 µL of ET-1 (i.c.)</td>
<td>3</td>
<td>3/3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3 µL of hAAT (i.c.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ET-1+hAAT(i.v.)</td>
<td>3 µL of ET-1 (i.c.)</td>
<td>3</td>
<td>3/3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1 mL of hAAT (i.v.)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Two animals in ET-1 alone group were excluded because of death and incomplete data sets. One animal in ET-1+pGSN group was excluded due to difficulty of defining the infarct area.

Table 2-3. Treatment schedule for ET-1+saline and ET-1+hAAT (i.v.) groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Number of animals</th>
<th>Survival rate</th>
<th>Date collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1+saline</td>
<td>3 µL of ET-1 (i.c.)</td>
<td>8</td>
<td>8/8</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>1 mL of saline (i.v.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ET-1+hAAT</td>
<td>3 µL of ET-1 (i.c.)</td>
<td>12</td>
<td>12/12</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>1 mL of hAAT (i.v.)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 3
THE PROTECTIVE EFFECTS OF PLASMA GELSONIN ON ISCHEMIC STROKE OUTCOME IN RATS\(^1\)

Introduction

Stroke or brain attack occurs when the blood supply to the brain is interrupted, usually because a blood vessel is blocked by a clot or loses structural integrity permitting hemorrhage. The disease is not subject to a particular race or ethnic group (306). In 2009, 795,000 strokes occurred in the United States, i.e. a stroke occurs once every 40 seconds and a death occurs every 4 minutes (10). According to the Centers for Disease Control and Prevention (CDC), the total cost of stroke was $68.9 billion and the number is expected to rise. Of all strokes, 87% are ischemic (10). Currently, recombinant tissue plasminogen activator (rtPA) is the only FDA-approved therapeutic agent for ischemic stroke. rtPA is effective only if intravenously administered within 3 to 4.5 h of stroke onset, and can have adverse neurotoxic effects even with proper use (122). The drug can only be used within a narrow time window after a stroke begins and only about two percent of stroke patients are able to access rtPA therapy. Therefore, development of new agents for stroke is essential.

The mechanisms involved in stroke injury and repair are extremely complex, involving excitotoxicity and necrotic cell death occurring within minutes of stroke onset (307). As well, there is increasing evidence showing that genetically programmed cell death during post-ischemic tissue inflammation (that can last days to weeks) has a detrimental effect (308, 309). Therefore, therapeutic strategies targeting that delay or dampening inflammatory responses could inhibit the progression of the tissue damage and improve the overall outcome of stroke.

\(^1\) This part is mainly a manuscript that was published in Experimental & Translational Stroke Medicine, 2011, 3-13
Gelsolin (GSN) is a ubiquitous (156, 157) actin filament-severing, capping and actin nucleation protein of eukaryotes. Originally described as an actin-binding protein, GSN exists in both intracellular (cytoplasmic protein, cGSN) and extracellular (a secreted protein or plasma gelsolin, pGSN) forms. pGSN, also known as brevin and actin-depolymerizing factor, consists of a single 755-amino acid polypeptide chain (84 kDa) including a 25- amino acid N-terminal extension (160) that distinguishes it from cGSN (82 kDa). Most cells secrete pGSN, however smooth, skeletal and cardiac muscle cells produce larger amounts of pGSN (156). The plasma concentration of pGSN is 200-300 mg/L (159, 310, 311) and isolated human and rabbit pGSN have a plasma half-life of 2.3 days (165). Because pGSN derives from muscle tissue, it must pass through interstitial fluid of the extracellular matrix to localize in the blood. pGSN also exists in human cerebrospinal fluid (CSF) (162). Although certain functions for the intracellular isoforms have been described, the function(s) of the plasma isoforms remain unclear. The high affinity of pGSN for filamentous actin (F-actin) (K_a > 10^9/mol/L) (312) suggests that its physiological function is likely related to its actin-binding properties. pGSN may scavenge actin leaked from injured tissue and limit subsequent damage instigated by extracellular filamentous actin (166). Studies have shown that large amounts of F-actin could potentially increase the blood viscosity and perturb blood flow through the microvasculature (168).

It is also well established that pGSN levels decrease in blood in acute inflammation conditions that involve tissue damage (176, 313-315). Consistent with the idea that pGSN is not only a biomarker for inflammation but also an important protective factor, repletion of pGSN in a mouse model of endotoxemic sepsis led to solubilization of circulating actin aggregates and significantly reduced mortality in mice (179). GSN knockout mice neurons are vulnerable to glucose/oxygen deprivation, and pharmacological brain actin depolymerization restored
resistance to ischemic stroke in knockout mice (187). The knockout mice results could not
determine which endogenous form of gelsolin is responsible, or whether gelsolin in or near the
infarct mediates neuroprotective effects. Gelsolin-overexpressing transgenic mice demonstrate
neuroprotection against experimental stroke (187), but it is not known whether these effects are
mediated by pGSN or cGSN, or whether it is GSN near the infarct that mediates the protection.

To test the hypothesis that proximal administration of pGSN can antagonize stroke
pathology, we induced transient middle cerebral artery occlusion (tMCAO) in male rats via
intracranial injection of ET-1, a potent vasoconstrictor, and post-treated with discrete brain
injection of pGSN. Cylinder and vibrissae tests were used to examine sensorimotor function
before and 72 h after MCAO to assess functional deficits. Whole brain laser Doppler perfusion
imaging was performed through the skull to verify MCAO effectiveness. Infarct volumes were
examined 72 h after MCAO using 2,3,5-triphenyltetrazolium chloride (TTC) assay.

Results

Treatment of pGSN did not Interrupt ET-1 Induced Artery Contraction

In order to test the effect of pGSN and ET-1 on MCAO, cohorts (n = 3) of rats were
injected with ET-1 to induce transient middle cerebral artery occlusion (MCAO).

Approximately 5-10 min after ET-1 injection, pGSN or saline was injected at the same
location. The time points of scanning were based on ET-1 injection time. The relative perfusion
unit (PU) or blood flow values of animals before injection were in the range of 800-1,600 PU
and the difference between two hemispheres of the brain was not statistically significant. After
20 min following ET-1 injection, the flux values on the injection side dropped to the range of
300-600 PU. The calculations were made using equation 2-1 described in the methods. Relative
flow values showed a rapid decrease to ~ 50% of baseline in all animals and flow remained
maximally decreased for 20- 30 min after ET-1 injection (Figure 3-1). ET-1 injection resulted in
a drop in perfusion immediately after injection regardless of pGSN injection at the same
location. Reperfusion was observed in both groups after ~ 60 min. These results indicate that
pGSN injection did not interrupt the induction of artery contraction by ET-1.

**Treatment of pGSN Significantly Reduced ET-1 Induced Behavioral Deficits**

To test the protective effect of pGSN, behavioral studies were conducted. Initially, five rats
were randomly assigned into ET-1 injection alone or ET-1 plus pGSN group. In the ET-1 only
group, one rat died and one rat was paralyzed after ET-1 injection and was euthanized
immediately. In ET-1 plus pGSN treated group, one rat did not show any infarction by TTC
staining and was excluded. Therefore, three animals in ET-1 group and four animals in ET-1 plus
pGSN group were examined for behavioral deficits and brain damage.

**Cylinder test**

As shown in Figure 3-2, ET-1 induced MCAO resulted in profound impairment of
contralateral forelimb function three days following injection. ET-1-treated animals showed
significant reduction of symmetrical forelimb use during wall exploration (from approximately
80% to 20%), while artificial cerebrospinal fluid (aCSF) injection had no effect. In the pGSN
treatment group, the percentage of symmetrical forelimb use was significantly higher than the
control group (20% vs. 70%, p-value < 0.01). The number of attempts to explore the wall was
also lower than before surgery (data is not shown). These results indicate that pGSN treatment
significantly prevented MCAO-induced damage to the motor system.

**Vibrissae test**

To test the effect of pGSN on sensorimotor system, we also performed vibrissae testing.
As expected, vibrissae-stimulated placing of the ipsilateral forelimb was not affected 3 days after
experimental ischemic stroke (Figure 3-3A). Contralateral forelimb placing was significantly
slowed (from 1 s to 18 s) in the control (ET-1 alone) group (Figure 3-3B). Intriguingly, pGSN
treatment significantly reduced the time of contralateral forelimb placing compared to control group (18 s vs. 9 s, p-value < 0.01). These results indicated that pGSN treatment significantly prevented the loss of sensorimotor function induced by MCAO.

**Treatment of pGSN Reduced MCAO Induced Brain Damage**

In the control rats, ET-1 produced large and reproducible unilateral infarcts that involved the rostro-central dorsolateral cortex and basal ganglia, corresponding to the full extent of the MCA territory. The infarction volume in the pGSN-treatment group was reduced by 49% compared with the control group (Figure 3-4). Sparing was observed in both cortical and subcortical structures.

**Discussion**

The current study reports for the first time the use of pGSN as a protein drug to reduce injury after transient local ischemic stroke. We demonstrate that pGSN can improve sensorimotor recovery in conjunction with substantial reduction in infarct volume present three days after stroke.

Transient middle cerebral artery occlusion (tMCAO) induced by ET-1, a potent vasoconstriction peptide, decreased relative cerebral blood flow in brain tissue served by the MCA by 50% in all groups. In previous studies, injection of ET-1 adjacent to MCA has been shown to reduce blood flow 30-75% in the region supplied by the artery including cortex and basal ganglia, and to produce subsequent ischemic neuropathology in these regions of the brain (32, 286, 316).

Our results demonstrate that after 10-20 min following ET-1 intracranial injection, the ipsilateral cerebral relative blood flow decreased approximately 50% in all groups of animals (Figure 3-1), indicating that all animals initially have comparable ischemic strokes and that pGSN did not reduce the magnitude of the original ischemic event, but limited the subsequent
degeneration and associated loss of function. This suggests that pGSN does not interfere with ET-1 action on its receptors, and corresponds to studies in which GSN knockout mice had larger infarct volume at 22 h even though reductions in CBF during MCAO were not altered (187).

Although this study shows that pGSN in or near an infarct can reduce neuropathology and functional loss due to ischemic stroke, the mechanism by which pGSN mediates protection are not yet clear. One likely mechanism involves actin depolymerization. Upon tissue injury due to glucose/oxygen deprivation (CBF significantly reduced), large amounts of actin can be released from damaged cells into the extracellular space. Since the ionic conditions in the extracellular fluid favor actin polymerization, high amounts of F-actin could be released to potentially increase the viscosity of blood and perturb blood flow through the microvasculature. The actin severing protein gelsolin has a secreted plasma isoform (called plasma gelsolin), which is constitutively active in the high extracellular calcium concentrations of plasma. Plasma gelsolin severs extracellular F-actin to short filaments, and by capping barbed ends, prevents polymerization and favors monomer release. Therefore, pGSN acts as “debris cleaner” limiting inflammation and possibly decrease blood clogging [22]. Another possible mechanism is through anti-apoptotic activity of pGNS. In Jurkat cells, overexpression of gelsolin inhibits cytokine induced apoptosis (317). It has been reported that gelsolin can form complex with phosphatidylinositol 4,5-bisphosphate and inhibit caspase-3 and -9 activities (201). In addition, pGSN may also play an important role in regulating inflammation.

Histone deacetylase inhibitor-mediated neuroprotection against MCAO has been associated with GSN upregulation and reductions in filamentous actin, neither of which was shown to occur in GSN-knockout mice in which the treatment was ineffective (318). Also, GSN
can modulate the actin cytoskeleton regulation of numerous ion channels responsible for elevated cytotoxic intracellular calcium and glutamate excitotoxicity (186, 319, 320).

Gelsolin is regulated by phosphatidylinositol 4,5-bisphosphate (PIP₂), and contains a lipid signaling binding domain. This domain has been shown to bind to a number of bioactive lipids including lysophosphatidic acid (LPA), lipoteichoic acid (LTA), and lipopolysaccharide (LPS) (87, 191-193). LPA levels have been shown to be increased in patients suffering ischemic stroke (321). LPA signaling has also been shown to regulate a number of pro-inflammatory genes (322). Increasing gelsolin levels during stroke may serve to modulate the inflammatory response thereby offering protection against the inflammation related neurodegeneration following stroke.

Further emphasis of the potential importance of GSN in stroke comes from recent reports that circulating pGSN levels are reduced in ischemic stroke sufferers and is highly predictive for first-year mortality from ischemic stroke (184). Matrix metalloproteinases (MMPs), zinc-containing endopeptidases that participate in both normal and pathological processes, are upregulated during inflammatory conditions (197), including stroke (198). Plasma gelsolin is cleaved in vitro by MMP-3, MMP-2, MMP-1, MMP-14 and MMP-9 (323) which may be the cause of the severe depletion of pGSN observed in patients who suffer ischemic stroke. Replacing lost pGSN may interrupt pro-inflammatory cascade and result in decreased brain damage.

Conclusion

The current study offers a proof of principle that delivery of pGSN following ischemic stroke results in neuroprotection and can reduce both sensory and motor deficits that arise following stroke. Future research aimed at characterizing improved delivery, dose response, temporal, safety, pharmacokinetic issues, and physiological mechanisms for further preclinical development of this promising strategy are called for.
Figure 3-1. Perfusion measurements of pGSN study. (A) Color photographs of an animal brain recorded at different time points (pre-injection, 10-20 min, 30-35 min, and 60-65 min from ET-1 injection time) by a digital camera of laser Doppler system, corresponding closely with the blood flow image. Look-up table shows arbitrarily assigned perfusion unit (PU) from the lower limit 0 to upper limit 1,000 and above. The skulls demonstrate the holes and injection sites. (B) Percentage perfusion reduction as a function of time calculated using equation 2-1. All rats were injected with ET-1 (240 pmol in 3 µL PBS) proximal to the left middle cerebral artery. About 5-10 min after ET-1 injection, pGSN (35.71 pmol in 3 µL saline) was intracranially injected (i.c.) at the same site in a group and no injection in the other group. The dotted line represents the average levels in pGSN treated group (N = 3). The solid line represent levels in control group (N = 3). The differences at all-time points were not statically significant.
Figure 3-2. Cylinder test of pGSN study. Rats were placed in a transparent cylinder for 3 min. Animal forelimbs used during exploration were scored. Each bar represents the average percentages ± s.e.m. of using both forelimbs in the indicated group. ET-1 group, N = 3, ET-1+pGSN, N = 4, *: p-value < 0.05; **, p-value < 0.01, ***, p-value < 0.001.
Figure 3-3. Vibrissae test of pGSN study. (A) Time in seconds to ipsilateral forelimb placement on the countertop. (B) Time in seconds to contralateral forelimb placement on the countertop. ET-1 group, N = 3, ET-1 + pGSN, N = 4, **: p-value < 0.01, ***: p-value < 0.001. Data are means ± s.e.m.
Figure 3-4. Infarction area labeled for mitochondrial activity of pGSN study (2,3,5-triphenyltetrazolium chloride, TTC). ET-1-alone group N = 3, pGSN treatment group, N = 4. *, p-value < 0.05. Four TTC-staining slices from two animals, two slices from each animal, one animal from each group, at the area receiving the most blood from the MCA. Off-white color areas show dead tissues while red areas indicate vital tissues. Data are means ± s.e.m.
CHAPTER 4
ALPHA 1-ANTITRYPSIN MITIGATED ISCHEMIC STROKE DAMAGE IN RATS

Introduction

World-wide, there are more than 50 million survivors of stroke and TIA (324), producing an immense burden on the healthcare infrastructure as well as national economies (124). At present, recombinant tissue plasminogen activator (rtPA) is the only FDA-approved therapeutic agent for ischemic stroke and is effective only if administered intravenously within 3 to 4.5 h of ischemic stroke onset. The major functions of rtPA are dissolving blood clots and promoting reperfusion. Paradoxically, it can cause neurotoxicity through the N-methyl d-aspartate (NMDA) receptors and disruption of the neurovascular matrix through matrix metalloproteinase (MMP) dysregulation (325). Also, in the United States, less than 2% of stroke patients are able to access the tPA treatment due to a narrow time window after stroke begins and the diagnostic procedure (124). Therefore, more effective therapies are needed in order to improve the outcome of stroke.

Ischemic stroke, the dominant type of all strokes, initiates a series of events, including cellular bioenergetic failure, excitotoxicity, oxidative stress, BBB disruption, microvascular injury, homeostatic activation, and inflammation (326). The inflammation starts within a few hours of stroke onset and characterizes the secondary or delayed response to ischemia (327). It involves activation of microglia and astrocytes, as well as influx of hematogenous cells recruited by cytokines, adhesion molecules and chemokines. These pro-inflammatory mediators penetrate activated blood vessel walls and invade into the parenchyma (81, 210). Once activated, leukocytes and microglia produce more inflammatory cytokines and chemokines, nitric oxide (NO) via inducible nitric oxide synthases (iNOSs), reactive oxygen species (ROS), and matrix metalloproteinases (MMPs). Pro-inflammatory mediators and toxic molecules contribute to inflammation response and further potentiate brain injury, leading to apoptosis and necrotic cell
death of the potentially viable tissue. Increasing evidence shows that genetically programmed cell death during post-ischemic tissue inflammation (which can last days to weeks) has a detrimental effect (308, 309). Therefore, therapeutic strategies which target that delay or mitigate inflammatory responses, could inhibit the progression of the tissue damage and improve the overall outcome of stroke.

Human alpha 1-antitrypsin (hAAT) is a serum proteinase inhibitor which has anti-inflammatory and apoptotic, and cytoprotective properties. AAT is primarily synthesized in the liver (205), secreted into a serum level of 1.0-2.5 mg/mL (20-50 μM), CSF level of 7.6 μg/mL at normal condition, (209, 230, 328, 329), and is relatively stable with a half-life of 4-5 days in human (330, 331), but as an acute phase reactant, the hAAT serum level can rise two to four fold in response to tissue injury (332, 333), AAT synthesis mediated by LPS can be raised to eight fold (334), and even 100 fold in A549 cells when stimulated with cytokines and transforming growth factor b (335). Previous studies have demonstrated that AAT inhibits the production of pro-inflammatory cytokines interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor-alpha (TNF-α), and enhances anti-inflammatory cytokine IL-10 production by increasing cellular cyclic adenosine monophosphate (cAMP) levels (217, 218). AAT has shown anti-apoptotic and anti-inflammatory effects as early as 2 h and as late as 24 h following ischemia/reperfusion in an animal model by inhibiting neutrophil superoxide production, TNF-α production, and decreasing caspase-1 and -3-like activities (220, 336). AAT-mediated interruption of IL-8 binding to its receptors limits neutrophil infiltration in the lungs (337). In addition to inhibiting serine proteases, AAT also inhibits cysteine proteinase (e.g., caspase-3) activity. Coupled with the ability to enter cells, this has been shown to protect cells (e.g., pancreatic β-cells, lung, kidney and liver cells) against apoptosis (222).
Important note that the total protein concentration in CSF is about 200 times lower than in the blood plasma (338), and 80% CSF protein is serum-derived and 20% is produced intrathecally (339), therefore AAT levels increase in neurodegenerative diseases such as, Alzheimer disease or dementia (209) may come from peripheral source by crossing BBB.

Moreover, recent studies have shown that neuroserpin, a serin protease inhibitor (related to AAT) expressed in neurons (340), exerts a neuroprotective effects after ischemic stroke due to its ability to complex with serine proteases tPA, uPA, and plasmin which are similar to AAT. The negative correlation between the decreased neuroserpin serum levels and levels of molecular markers (e.g. TNF-α, IL-1β, and ICAM-1) of brain damage, suggesting that neuroprotective properties of neuroserpin might be associated with the inhibition of excitotoxicity, inflammation, and BBB compromise following ischemic stroke (341).

Collective evidence implicates that AAT may hold therapeutic potential in limiting stroke pathology that results from intrinsic inflammatory processes. In the present study, we tested the protective effects of AAT in ET-1-induced transient middle cerebral artery occlusion (tMCAO) in rats.

**Results**

**Human AAT Did not Affect the ET-1-Induced Ischemia**

In order to test the effects of hAAT on middle cerebral artery occlusion (MCAO) induced by ET-1, cohorts (nine rats were divided randomly into three equal groups) of rats were stereotaxically injected with ET-1 adjacent to the left MCA of the brain. About 5-10 min after ET-1 injection, hAAT or saline was intracranially injected at the same site in two groups ET-1+hAAT (i.c.) group or ET-1+saline group, and the third group was intravenously injected, ET-1+hAAT (i.v.) group. The relative reduction flux in rat brain was measured using a laser Doppler scanner. As shown in Figure 4-1A, approximately 20 min following the ET-1 injection,
the flux statistic at the injection site dropped markedly from ~ 1,600 PU to less than 900 PU in all groups (i.e. ET-1+saline group, ET-1+hAAT (i.c.), and ET-1+hAAT (i.v.) groups). The calculations were made using equation 2-1, (METHODOLOGY). The average relative flux values in all groups were drastically reduced by 40-50% compared to the base line. The maximally reduced flux was maintained for up to 35 min, followed by the initiation of reperfusion (Figure 4-1B). No significant difference among the three groups was detected, illustrating that hAAT both locally and systemically delivery did not alter the ischemic induction of ET-1.

**Local Delivery of hAAT Mitigates ET-1-Induced Stroke Outcome**

In a separate experiment, five rats were intracranially injected with ET-1 and hAAT as previously described. Data from the control groups, receiving aCSF or ET-1 alone, were retrieved from our previous study (287) as the set of experiments were performed during the same time period and using identical methodology. Three days after ET-1 injection, behavioral tests and brain damage evaluation were performed.

Cylinder test results showed that animal behavior was normal before treatment, i.e. percentages of both forelimbs used for postural support in aCSF, ET-1 alone, and ET-1+AAT (i.c.) groups were 80.0±7.0%, 79.20±5.16%, and 82.50±4.15%, respectively; and no difference among the three groups (Figure 4-2). Three days after ET-1 injection, the use of both forelimbs in ET-1 alone group was significantly reduced (22.6±6.3%), indicating profound impairment of contralateral forelimb function whereas no impairment was observed in the group which received the aCSF injection (76.67±4.08%). In the hAAT treatment group, ET-1+AAT (i.c.), the use of forelimb was significantly higher than that in ET-1 alone group (60.0±6.1%, p-value < 0.05). These results clearly demonstrate that local administration of hAAT mitigated the deficit of motor function caused by ischemic stroke in rats.
Vibrissae tests were performed to test the effect of hAAT on sensorimotor function. Data in Figure 4-3A shows the ipsilateral forelimb placing response to vibrissae stimulation before and 72 h post-MCAO measured in seconds. Before ET-1-induced ischemic stroke, the response time periods in aCSF, ET-1 alone, and ET-1+hAAT (i.v.) group were 1.54±0.04s, 1.76±0.18s, and 1.07±0.21s, respectively. Three days after ischemic stroke they were 1.92±0.71s, 2.15±0.19s, and 1.34±0.37s, respectively; and there is no statistically significant difference among the three groups. This indicates ipsilateral forelimb placing was not affected by the middle cerebral artery occlusion in rats after three days. As shown in Figure 4-3B, contralateral forelimb placing time in ET-1+hAAT (i.c.) group was significantly shorter than that in ET-1 alone group (5.79±1.17s, 18.92±4.14s, respectively, and p-value < 0.001), suggesting that hAAT treatment is able to limit the loss of sensorimotor function induced by MCAO in rat model. The contralateral forelimb placing time period in aCSF group remained the same (1.10±0.08s) after surgery vs. before surgery, and significantly shorter compared to ET-1 alone model group. This implies that and ET-1 injection induced profound sensorimotor deficits which were not affected by the surgery procedure. After the above behavioral tests, all animals were sacrificed and subjected to brain damage evaluation.

Brains were sectioned into 2-mm-thick sections and stained using TTC assay. Images of the brain sections were obtained from a scanner and the infarct areas were quantified using Image J v5.0. Total infarct volume of each animal was defined by the sum of the infarct area of all sections. Results are presented in Figure 4-4, the average of total infarct areas of the model group (receiving ET-1 alone) was significantly larger than that of the hAAT treatment group (198.9±13.3 mm² and 33.7±2.8 mm², respectively, p-value < 0.001). Typically, the infarct in the
model group involves the rostro-central dorsolateral cortex, subcortex, and basal ganglia while in the treatment group the infarct area was limited to the cortex region.

**Systemic Delivery of hAAT Mitigated ET-1-Induced Stroke Outcome**

Although direct local injection of hAAT clearly prevented brain damage from ET-1-induced MCAO, the procedure is invasive and may not be practically translated into clinical application. In order to test the effect of another route of hAAT delivery on ischemic stroke, we performed experiments using separate groups of animals. Since we found no effects from aCSF in the previous studies, only control and hAAT treatment groups were utilized. Eight rats in the control group received ET-1 (i.c.) and 1 mL of sterile saline (i.v.) Fourteen rats in AAT treatment group were intracranially (i.c.) injected with ET-1 and intravenously (i.v.) injected with hAAT (40 mg/kg) immediately after ET-1 injection.

The rationale for choosing this dose is based on the normal concentration of AAT in humans. From our laboratory experience, at a dose of 50 mg/kg injected intraperitoneally in mice, hAAT can prevent streptozotocin-induced diabetes in mice via disruption of β-cell apoptosis (222); a dose of 25 mg/kg used in mouse model for autoimmune arthritis has shown hAAT is able to reduce inflammation (342). Another study used a dose of 60 mg/kg injected intraperitoneally in mouse model for myocardial ischemia/reperfusion, demonstrated the anti-inflammatory and tissue-protective properties of hAAT (343). Therefore, 40 mg/kg was selected for treatment in rats in this study as it was in the experimental range.

Cylinder test results showed that animal behavior was normal before treatment, i.e. percentages of both forelimbs used for postural support against cylinder walls of ET-1+saline and ET-1+hAAT (i.v.) groups were 84.5±1.2% and 87.9±3.2%, respectively; and no statistical difference between the two groups (Figure 4-5). Three days after the injections, both groups revealed significant deficit compared to pre-treatment. However, the hAAT treatment group
showed significant deficit reduction in using two forelimbs for cylinder exploration compared to the controls (72.6±6.2% and 35.4±5.9%, respectively, p-value < 0.001), suggesting intravenous delivery of hAAT can ameliorate motor function deficit induced by ET-1-induced cerebral ischemia.

Vibrissae test results were presented in Figure 4-6. Relative response time represented time to place forelimb as a percentage of pre-treatment tests, using equation 4-1. The results show the ipsilateral on the left and the contralateral on the right. As expected, the unaffected side limb (ipsilateral to the injection side) was not affected by the MCA occlusion, the relative response time of forelimbs of animals in ET-1+saline and ET-1+hAAT (i.v.) groups were approximately 100% (127.3±23.5% and 99.2±11.5%, respectively). The relative response time of the affected side forelimb (contralateral to the injection side) of both groups were significantly increased (Figure 4-6); however the hAAT treatment group showed significantly less delay in response to vibrissae stimulation compared to control ET-1+saline group (238.7±43.4% and 400.9±87.4%, respectively). This indicates intravenous delivery of hAAT mitigates sensorimotor function deficit by ischemic stroke.

\[
\text{Relative response time} = \frac{\text{Seconds to place (after surgery)}}{\text{Seconds to place (before surgery)}} \times 100
\]  
(Equation 4-1)

Brain damage was evaluated following ischemic insult 72 h using MRI. Infarct size calculation is based on the T2 relaxation maps of each image (1-mm thickness) using Image J v5.0. Total infarct volume was defined by the sum of the infarct area of all sections. Results are shown in Figure 4-7, the infarct size of ET-1+saline and ET-1+hAAT (i.v.) were
141.8±37.6 mm² and 53.0±17.7 mm², respectively, p-value < 0.05, indicating that systemic hAAT administered significantly reduced by ~63%.

Discussion

Human alpha 1-antitrypsin has been used for more than two decades as augmentation therapy for chronic obstructive pulmonary disease (COPD) that is related to AAT deficiency. Our laboratory has been investigating its functions and has shown its beneficial effects in several different experimental models of diseases, including rheumatoid arthritis (344), diabetes (172, 222, 345), and bone loss (346). However, the effect of AAT on brain injury induced by stroke has never been reported. In the present study, we showed that hAAT therapy protected against brain tissue injury and improved stroke outcome. Considering that hAAT treatment has been proven to be safe, these results imply a new clinical application of hAAT for the treatment of stroke, and perhaps other brain diseases associated with inflammation and programmed cell death.

Pathogenesis of stroke is complex and involves inflammation and cell death. Within seconds to minutes after loss of blood flow to a region of the brain, the ischemic cascade is rapidly initiated. Cerebral ischemic excitotoxic mechanism is activated in the territory which leads to apoptotic cell death due to depletion of cellular energy stores. These injured neurons and microglia in both the core and periphery of the lesion are producing pre-inflammatory mediators, such as cytokines and reactive oxygen species (ROS), which activate microglial cells. Therefore, anti-inflammatory and cytoprotective approaches hold great potential for the treatment of stroke. The increasing body of literature shows that hAAT has anti-inflammatory and cytoprotective properties (347). In the present study, we showed that local delivery of hAAT protected ET-1 induced brain injury and mitigated behavioral deficits. Although the mechanism of this protection remains to be further investigated, it is possible that hAAT inhibits ischemia- and
reperfusion-induced neuron cell death by inhibiting caspase-1 and -3 (343). Human AAT may also inhibit local expression of ischemia- and reperfusion-induced inflammatory cytokines and activated astrocytes or microglia. Therefore, administration of hAAT to the site of ischemic stroke insult may mitigate cell death and inflammatory responses.

It is intriguing that systemic delivery of hAAT significantly improves stroke outcome. Since AAT is a 52 kd glycoprotein and may not be able to pass through an intact BBB (348), the following possible mechanisms may contribute to its protective effects. First, ET-1 is a known mediator that increases BBB permeability (349). Also, stroke increases BBB permeability to macromolecules, e.g. Immunoglobulin M (IgM, 450 kd) (350). Second, ET-1 induced ischemia-reperfusion can lead to dysfunction of endothelial cells and temporary leaking of the BBB (351). Third, the injection of ET-1 mechanically, possibly leads to local damage of the BBB. In all cases, circulating hAAT may pass through the BBB, reaching the impaired area of the brain and displaying local protective functions. A fourth possibility is that hAAT may indirectly reduce brain tissue damage by inhibiting the infiltration and migration of lymphocytes to the penumbra of the ischemic region of the brain (219). Considering that ischemic stroke often leads to local BBB damage in human patients, systemic administration of hAAT may be clinically beneficial for patients with early onset of stroke. Although the beneficial effects of systemic administration hAAT may be limited after the recovery of BBB, such treatment may protect the brain from the recurrence of stroke, which occurs commonly in humans. In addition, since BBB damage in stroke is primarily induced by the inflammatory response and results in vasogenic edema and brain damage, hAAT, as an anti-inflammatory protein, can also contribute to recovery of the BBB.
In the present study, approximately 20 min after ET-1 injection relative cerebral blood flow rapidly decreased to 40-50% of baseline in all animals and remained maximally decreased for at least 20-30 min. The result is consistent with the previous study that showed micro-injection of ET-1 near MCA induced a blood flow reduction of 30-75% in the region supplied by the artery (33, 316). There was no significant difference among the three groups in CBF reduction, indicating that hAAT had no direct influence on ET-1 induced MCAO. Therefore, our results indicate that the protective effects of hAAT are mediated not through blocking the activity of ET-1, but rather by blocking the subsequent biochemical/inflammatory responses and limiting damage.

Conclusions

Present study demonstrated that both local and systemic deliveries of hAAT resulted in neuroprotection and mitigation of behavioral deficits induced by ischemic stroke in rats. Results from this study indicate a promising new application of hAAT as a therapy for ischemic stroke.
Figure 4-1. Perfusion measurements of AAT study. (A). Color photographs of an animal brain recorded at different time points (pre-injection, 10-20 min, 30-35 min, and 60-65 min following ET-1 injection) by a digital camera of a laser Doppler system, corresponding closely with the blood flow image. Look-up table shows arbitrarily assigned perfusion unit (PU) from the lower limit of 0 to an upper limit of 1,000 and above. The skulls illustrate the holes and injection sites. The top, middle, and bottom panels are images of ET-1+saline, ET-1+hAAT (i.c.), and ET-1+hAAT (i.v.) groups, respectively. (B). Percentage perfusion reduction as a function of time calculated using equation 2-1. All rats were injected with ET-1 (240 pmol in 3 µL PBS) proximal to the left MCA. About 5-10 min after ET-1 injection, hAAT (1.35 nmol in 3 µL saline for i.c.) or (192 nmol in 1 mL saline for i.v.) was administered at the same site in the two groups. The dotted, closed diamond solid and closed triangle solid lines represent the average levels in ET-1 alone, hAAT (i.c.), and hAAT (i.v.) groups, respectively. N = 3 for all groups. The differences at all time points of all groups were not statistically significant. Data are means ± s.e.m.
Figure 4-2. Cylinder test of ET-1 alone vs. ET-1 and intracerebral hAAT delivery. Rats were placed in a transparent cylinder for 3 min. Forelimb use during exploration by the rats was scored. Each bar represents the average percentages ± s.e.m. of using both forelimbs in the indicated group. ET-1 group, N = 3, ET-1 + hAAT group, N = 5,*,**,***: p-value < 0.05; 0.01; 0.001, respectively.
Figure 4-3. Vibrissae test of ET-1 alone vs. ET-1 and intracerebral hAAT delivery. (A). Time in seconds to ipsilateral forelimb placement on the countertop. (B). Time in seconds to contralateral forelimb placement on the countertop, ET-1 group, N = 3, ET-1 + hAAT, N = 5, ***: p-value < 0.001. Data are means ± s.e.m.
Figure 4-4. Infarction area labeled for mitochondrial activity of AAT study (2,3,5-triphenyltetrazolium chloride, TTC). Two TTC-staining slices from two animals representing each group, the left one for group receiving ET-1 alone, and the right one for groups receiving ET-1 and intracranial hAAT administration. Off-white color areas show dead tissues while red areas indicate vital tissues. ET-1-alone group N = 3, ET-1 and hAAT treatment group, N = 5. ***: p-value < 0.001. Data are means ± s.e.m.

Figure 4-5. Cylinder test of ET-1 alone vs. ET-1 and intravenous hAAT delivery. Rats were placed in a transparent cylinder for 3 min. Forelimb use during exploration by the rats was scored. Each bar represents the average percentages ± s.e.m. of using both forelimbs in the indicated group. ET-1 alone group, N = 8, ET-1 + hAAT (i.v.), N = 14, *: p-value < 0.05, ***: p-value < 0.001.
Figure 4-6. Vibrissae test of ET-1 alone vs. ET-1 and intravenous hAAT delivery. The percentage of pre-MCAO response time calculated using equation 4-1. Data are means ± s.e.m.

Figure 4-7. Infarction area calculated from MRI images using Image J v5.0. *: p-value < 0.05. Data are means ± s.e.m.
CHAPTER 5
GENERAL DISCUSSION, CONCLUSIONS AND FUTURE WORK

General Discussion

Despite the fact that stroke or cerebrovascular accident (CVA) has been known for more than two thousand years and has been intensively researched in the last several decades, it still is the third leading cause of death (following ischemic heart disease and cancer); and the most frequent cause of permanent disability worldwide (352, 353). Stroke poses a massive health burden and becomes a huge financial burden for society. In the United States alone, as many as 800 000 people suffer from the disease annually, i.e. stroke occurs every 40 seconds (10). Stroke is well associated with age and other aged-related diseases, so the absolute numbers of stroke patients are likely to rise with an aging population. Yet, it has limited effective therapeutic options, e.g. only one FDA-approved drug for acute ischemic stroke exists, however, it is only available to less than 5% of all patients (124, 354), because of strict time-restraints on administration post insult. Therefore, innovative and effective therapies are greatly needed. Because the events leading to neuronal damage are heterogeneous and involve many factors, it is challenging to translate data from animal studies to clinical trials. However, in order to rationally decide on the use of novel treatment regimens it is important to review pre-clinical and clinical results of therapeutic strategies undertaken to date.

Ischemic cascade following an ischemic stroke first affects brain cells (mostly neurons) exposed to extreme reductions in blood flow (the ischemic core) which leads to the loss of their membrane potential, cellular structural integrity, and eventually irreversible damage. In surrounding regions (the ischemic penumbra), the reduction in blood flow is sufficient to compromise neuronal function but does not immediately cause neuronal death. Inhibition of such delayed effects offers a promising window for neuroprotective strategies. Over the past ten years
there have been more than 1000 experimental papers and over 400 clinical trials focusing on neuroprotective strategies while there were hardly any publications on this topic prior to the 1990s (355).

Major triggers of ischemic neuronal death are overloaded intracellular calcium concentrations, accumulation of free radicals resulting in oxidative and nitrosative stress, various cell death signaling pathways, and inflammatory reactions. Below is a summary of some of the findings and promising approaches for ischemic stroke therapies based on recent insight of stroke mechanisms.

**Calcium antagonists (Nimodipine).** Following cerebral ischemia raised cytosolic calcium concentrations affect many processes associated with neurotoxicity. That is, influx of calcium into the cells (e.g. through voltage-gated calcium channel and NMDA channels) and increased cycling across ischemia-damaged membranes lead to a sustained rise in cytoplasmic calcium, leading to cell depolarization and neurotransmitters being released from neurons; and calcium overload in mitochondria, thereby resulting in cellular death (356). Cytoplasmic calcium activates enzymes (particularly proteases, lipases, and endonucleases) causing membrane damage (357). Cellular membrane damage and calcium-iNOS activation provide high concentrations of nitric oxide and fatty acid substrates for free radical generation which damages many important biomolecules, e.g. proteins and DNA (358). Glutamate release is stimulated by calcium-dependent exocytosis and has toxic effects on surrounding cells (359, 360).

Based on this rationale, nearly 3400 ischemic stroke patients were enrolled in a clinical trial to test the therapeutic benefit of Nimodipine, a well-known calcium channel antagonist (355). The drug showed positive results in animal models of acute focal ischemic stroke, then was started in ischemic stroke patients and showed beneficial effects (30 mg every six hours
begun within 24 hours of the onset the symptoms), however, the effects were limited to men (361). Based on these positive results, more clinical trials of the drug were conducted at multiple centers. Unfortunately, the results from these studies were inconsistent.

Three considerations for why Nimodipine failed follow. First, intracellular translocations of calcium, resultant from the derangement of mitochondrial sequestration during severe ischemia, might not be susceptible to calcium blockers. Second, Nimodipine is possibly ineffective enough to saturate the Ca\(^{2+}\) binding sites as another calcium antagonist may. Third, the postischemic infusion may need to be longer for a more sustained improvement in the delayed hypoperfusion, which may contribute to ischemic neuronal injury. Consequently, both pre-clinical and clinical studies of this drug showed to be ineffective (355, 362-365).

However, intracellular calcium influx can be attributable to activation of glutamate receptor; inhibition of these receptors may bring other potential therapeutics to stroke therapy in the future.

**Glutamate antagonists.** Under ischemic conditions, intracellular calcium influx is attributable to stimulation of glutamate receptors, thus inhibition of these receptors offer more targets in stroke therapy. Excessive amounts of glutamate, a major excitatory CNS neurotransmitter, are released and capable of inducing excitotoxicity (366-368). Glutamate and related excitatory amino acids activate several receptors, such as NMDA and AMPA, which are relevant to neuroprotection. There have been several compounds in pre-clinical and clinical trials which have tested the neuroprotective efficacy of glutamate antagonists following cerebral ischemia.

MK-801 (Dizocilpine), a non-competitive NMDA antagonist, is the most extensively studied compound. MK-801 binds to NMDA receptors with high affinity and showed infarct
reduction in several experimental models within 1-2 h of ischemia (369, 370). Dextromethorphan (a centrally acting dextrorotatory analog of codeine) and its derivatives, non-competitive NMDA-channel blockers, also demonstrated neuroprotective effects in animal studies of focal ischemic stroke (371, 372). Nevertheless, both MK-801 and dextromethorphan were terminated in clinical trials due to side effects (the phencyclidine-like effects, such as hallucination and agitation) lack of efficacy (373, 374).

Aptiganel (Cerestat, CNS1102) is also noncompetitive NMDA antagonist which decreases infarct volume and behavioral deficits up to 2 h after focal ischemia (375). Studies of Aptiganel safety and tolerability in patients at a low dose (4.5-mg intravenous bolus by infusion, non-weight-adjusted doses) was shown to be a tolerable and to have neuroprotective effects in animal models, however, it causes hypertension and CNS excitation (376). The drug was suspended in phase III because of both a lack of efficacy and safety (377).

CGS 19755 (Selfotel), a competitive NMDA antagonist, has shown ~50% infarct reduction in animal models delivered at 75 min following ischemic insult (378). However, it was discontinued in phase III trials due to lack of efficacy and a trend toward higher mortality (379, 380).

With regard to AMPA receptor antagonism, the AMPA antagonist ZK200755, in phase II trials at multiple centers, transiently worsened the neurological condition in patients with acute ischemic stroke (381). The trial was terminated after 61 patients for safety reasons and no further studies have been reported.

Another AMPA antagonist YM872 (Zonampanel) has potential neuroprotective effects (reduces infarct volume 30-40%) when administered within 2 h of permanent and transient stroke onset in rats (382, 383). YM872 also lessened stroke damage and augmented tPA efficacy.
in rat model of thromboembolic stroke (384). Unfortunately, no reports have been made after clinical trials began in 2001 in human (355).

**Antioxidants/radical scavengers.** Imbalance between oxidants and antioxidants results in oxidative stress. Oxidative and nitrosative stress involves formation of oxygen and nitrogen species through various mechanisms, including mitochondrial inhibition, intracellular calcium overload, reperfusion injury, and inflammation (385). During ischemia and reperfusion, cytosolic levels of free radicals (ROS/RNS) accumulate in the brain tissues and accelerate neuronal damage through membrane lipid peroxidation, DNA damage, and protein dysfunction (386, 387). Hence, free radicals are considered to be an important therapeutic target for improving stroke outcome.

Several compounds have shown neuroprotective effects and are undergoing clinical studies. For instance, Ebselen (2-phenyl-1,2-benzisothiazol-3(2H)-one), a selenium compound with glutathione peroxidase-like activity, has reduced ischemic damage when administered prior to ischemia in mice (388). It has also shown brain injury reduction and neurological improvement in MCAO model in rats (389). A phase III study of 394 patients with maximum time from onset of 24 h in multiple centers are ongoing (Internet Stroke Center, last updated on 19 February, 2009; accessed date 27 February, 2012).

Another antioxidant candidate is Edaravone (MCI-186; 3-methyl-1-phenyl-2-pyrazolin-5-one). Edaravone is an oxygen radical scavenger and blocker of lipid peroxidation. A phase III trial of 252 ischemic stroke patients with a 72-h window of treatment reported that Edaravone improved outcome in patients at 3 months with no serious safety issues (390). In April 2001, Edaravone was approved by the Japanese Ministry of Health, Laboratory and Welfare for
treatment of acute stroke patients, and a post-marketing study has shown that the drug is well-tolerated in all patients (391).

In addition, the novel radical scavenger NXY-059 protected brain tissues in various animal ischemia models, e.g. reducing infarct volume by 77% in MCAO model in rats (392), produced a 51% brain damage reduction in permanent MCAO models in a primates (marmosets) (393), and reduced hemorrhage when combined with tPA in an embolus model in rabbits (394, 395). The drug passed the first clinical trial, Stroke-Acute-Ischemic-NXY-Treatment trial I (SAINT I) involving 1722 patients with acute ischemic stroke and showed significant improvement of primary stroke outcome (396). However, in a larger number of patients (3306, SAINT II) NXY-059 appeared to be ineffective (397) and a depletion of potassium was reported as a side effect (398).

**Anti-inflammatory therapy.** Inflammation has detrimental effects on brain tissues after stroke. It occurs within hours of the initial stroke and can last for several months. Suppression of inflammation using a variety of agents has shown reduction in brain damage and clinical outcome improvement in animal models of stroke. One benefit may be extending the therapeutic window of tPA and decreasing the risk of hemorrhage. Although anti-inflammatory candidates have not yet proved their therapeutic effects in clinical studies, it is important to understand the mechanisms of inflammation in stroke as it would offer potential opportunities for improvement of stroke outcome.

Inflammatory responses following cerebral ischemia initiates with the up-regulation of proinflammatory pathways in neurons, astrocytes, microglia, pericytes, mast cells, and endothelial cells due to ROS, activated proteases, and intracellular components from necrotic cells in the ischemic core. Chemokines and cytokines are secreted, microglia are activated, and
adhesion molecules are up-regulated in vascular endothelial cells. Increased expression of adhesion molecules has been found in thromboembolic stroke, and in permanent and transient focal ischemic stroke in rat (399) and baboon (400) models. Adhesion molecules and chemokines mediate the recruitment of circulating leukocytes to the vessel wall. Leukocytes, especially neutrophils migrate and infiltrate into parenchyma within several hours. Infiltrating leukocytes and activated microglia produce proinflammatory mediators which accelerate the inflammatory reactions, as well as various mediators, including cytokines, NO via inducible nitric synthase (iNOS), ROS, and MMPs. Cytokines can directly lead to cell death. MMPs and other inflammatory effectors (e.g. plasminogen activators and cathepsin) damage the endothelium and other components of BBB resulting in vasogenic edema, microvascular ischemia, and increased susceptibility to hemorrhagic transformation of the infarct. Reperfusion (as discussed in detail earlier) can contribute to ROS generation and promote inflammation. Migrating leukocytes and other hematogenous inflammatory cells can also form aggregates that perturb cerebrovascular microvessels, worsening microvascular perfusion or preventing effective reperfusion (401, 402).

Moreover, other chronic inflammatory states can be deleterious and effectors of stroke outcome. It has been documented that approximately 30% of ischemic stroke patients have a previous infection, and 30% develop an infection while they are in the hospital (403). For instance, atherosclerosis is a chronic inflammatory response in blood vessel walls associated with poor outcome in experimental stroke. It is mediated partly by activation of CD36 (404), which is a multi-ligand scavenger receptor involved in the pathogenesis of artherosclerosis (405). Subacute physiological stress is unfavorable for stroke outcome partially by initiating toll-like receptor 4 (TLR-4)-mediated inflammation in brain ischemic mice (406). Moreover, chronic
hypertension, obesity, and diabetes which are associated with inflammation, exacerbate stroke pathology in animal models of cerebral ischemia (407-409). Recently, modeling peripheral inflammation by injecting lipopolysaccharide (LPS) in rats showed delayed infection 24 h after MCAO and increased the infarct size by 85%. This is associated with microglia/macrophage and infiltrating leukocyte up-regulation and greater functional deficits (403).

Several agents have been proven to ameliorate inflammation in animal models as well as stroke patients. They focus mainly on leukocyte trafficking (e.g. anti-adhesion molecule therapy), effector molecules (e.g. MMP inhibition), and inflammatory mediators (e.g. albumin therapy).

**Leukocyte trafficking.** Anti-selectin antibody administration before and after transient MCAO in rat model reduced brain damage and hemorrhage (410). Blockade of P-selectin using a monoclonal antibody also decreased infarct volume and improved reperfusion in mice (411). A clinical trial of a murine monoclonal anti-human intercellular adhesion molecule- (ICAM-1) antibody Enlimomab was not effective, and potentially worsened stroke outcome (412). To examine several potential mechanisms for the negative outcome in clinical stroke trial of Elimomab, the murine anti-rat ICAM-1 antibody, 1A29, was injected in rats after focal brain ischemia. The 1A29-treated group did not reduce infarct volume, rather generated rat anti-mouse antibodies and exacerbated the infarct size. Thus, the possible reason why Elimomab failed in the clinical trial is that it activates the immune system in response to foreign protein (413).

**Effector molecules.** MMPs, the largest class of human proteases, comprise of more than 25 different secreted- and cell-surface bound molecules (414). It is well documented that dysregulation of MMP activity may cause the degradation of extracellular matrix and basal lamina proteins which promote brain injury following stroke (415). MMP activity during the
delayed neuroinflammatory response may be beneficial for stroke recovery (128). The standard hypothesis postulates that some MMPs play a central role in the pathology of ischemic stroke.

Many MMPs are significantly increased in brain tissues and plasma following ischemic stroke, both in humans and experimental animals (416, 417). MMP-mediated brain injury occurs through a variety of mechanisms, including breakdown of components of the ECM or via activation of other bioactive compounds (e.g. chemokines and cytokines). In experimental animal studies of stroke, MMP levels are associated with BBB disruption, edema formation, and hemorrhagic transformation events (119). It was demonstrated that inhibition of MMP-9 or treatment with an MMP-9 neutralizing antibody ameliorated brain injury after permanent MCAO in rats (418). MMP-9 knockout mice had significant smaller ischemic lesions compared to wild type mice after permanent focal ischemia, implicating the deleterious role of MMP-9 in the development of brain injury after focal ischemia (419). In addition, MMP-9 has been shown to mediate the hypoxia-edema formation via vascular endothelial growth factor (VEGF) (420).

MMPs induce hemorrhagic transformation following ischemic stroke via degradation of basal lamina and subsequent BBB disruption. It was reported that MMP-2 levels increased in early stages after MCAO in a primate species (421). MMP-2 degrades a protein, claudin-5 which is one of the proteins in TJs, resulting in increased BBB degradation (422). In addition to MMP-2 and -9, MMP-3 and -13 have also recently been shown to be activated after ischemia-reperfusion contributing to BBB dysfunction and worsening stroke outcome in murine models (423-427). Importantly, MMP-9 and -2 are also elevated in brain tissues and plasma in the ischemic human brain (428).

To date, 12 pharmacological inhibitors of MMPs have been in experimental studies demonstrating reduction of infarct size, BBB opening, brain edema, decreased in delayed
neurovascular remodeling, rescuing neurons from apoptosis, and reduction of tPA-induced hemorrhage (414).

Although MMP inhibitors have not yet been approved for stroke therapy, they appear as promising potential therapeutics. A number of studies, both in animals and humans, illustrate the possibilities to improve stroke outcome using several types of MMP inhibitors, including anti-inflammatory agents. Interestingly, they can be combined with the current acute stroke therapy, tPA with protagonist/supportive effects. Perhaps, successful targeting of MMPs requires optimizing inhibition of MMPs’ deleterious activity without interfering with the beneficial effects of brain reparation during stroke recovery.

Learning from failures of the past, blocking calcium channels and receptors associated with calcium overload, antioxidants, and even some promising anti-inflammatory agents have failed or shown only mild efficacy to prove their neuroprotective effects. Because stroke is complex having heterogeneous mechanisms involving multiple factors, it is unlikely that in any approach targeting a single mechanism may provide an effective treatment for stroke patients, not to mention the translation from animals to humans.

**Plasma gelsolin and alpha 1-antitrypsin as novel treatment regimens in stroke.**

We investigated two new potential therapeutic proteins, plasma gelsolin and alpha 1-antitrypsin, for their ability to improve stroke outcome in animal models. Because of the complexity of events leading to neuronal damage following stroke, we hypothesized that it is unlikely that any “single mechanism approach” would provide an effective treatment for stroke patients. Both pGSN and AAT have multiple effects that should be beneficial to ischemic stroke outcome. They possess a myriad of properties which may interrupt or inhibit multiple cell death pathways to prevent secondary injury, and promote regenerative mechanisms. Both are naturally
present in humans, have excellent safety profiles, and one of them (AAT) is has been an FDA-approved drug since 1987 with a dose of 60 mg/kg weekly intravenous infusion for augmentation therapy for AAT deficiency patients (230). Plasma GSN and hAAT have distinct mechanisms that enable them useful for stroke therapy. However, there are overlapping mechanisms of action also including anti-apoptosis and anti-inflammation. Combination of pGSN and AAT may not provide additive effects due to competive mode of action and/or interaction between the two proteins and it requires elaborate study design to evaluate efficacy of the proteins at different stages of the disease.

Within minutes, the lack of blood supply to the brain can cause cells to die in the ischemic core of the infarct. Plasma GSN can prevent actin released from dead cells from polymerization (F-actin) which is toxic to the surrounding tissues in the penumbra. It has been well-established that the penumbra can regain its ability to survive by interrupting the process of genetically programmed cell death (apoptosis) (429). Both pGSN and AAT can contribute to delayed programmed cell death in the penumbra by suppressing apoptotic caspase activation; pGSN can inhibit type I apoptosis while AAT can inhibit all types.

Plasma GSN and AAT both can reduce inflammatory response through different mechanisms. Plasma GSN suppresses inflammation by binding to several bioactive molecules, including LPA which is unregulated during cerebral ischemia. Plasma GSN also interacts with MMPs which are effector molecules in inflammatory process. Alpha 1-antitrypsin, by other mechanisms, suppresses inflammatory reactions through inhibition of neutrophil elastase and proteinases, cathepsins, thrombin, and trypsin; inhibition of pro-inflammatory cytokines and enhancement of anti-inflammatory cytokine IL-10 production via increasing cAMP; anti-neutrophilic inflammation by inhibition of calpain activity (430).
It is interesting that at the site of injury pGSN levels drop due to actin binding while AAT concentrations can raise up to 3-4 times in response to without inducing toxicity (431). It was reported that AAT levels were elevated in the blood of ischemic stroke patients, but there is no evidence of these increased AAT levels correlated with stroke outcome. Increased APP levels are correlated with inflammatory reactions, so AAT, an acute phase reactant, is in response to tissue injury and inhibits inflammation. Both proteins are commonly considered as markers of inflammation (216, 432).

Importantly, pGSN and AAT cannot only be effective therapy for acute stroke therapy but may also be utilized for prevention therapy. Indeed, approximately 25-35% of the 795,000 Americans who have stroke each year will have another stroke within their life time, there is a 40% chance of having another stroke after the first stroke (a transient ischemic attack or mini-stoke) within 5 years, and recurrent strokes often have a higher rate of mortality and disability due to damage to the brain already injured by the original one (Recurrent Stroke Prevention Resources, National Stroke Association, accessed 3 March 2012). It has been shown that as many as 30% of stroke patients have pre-inflammation, the anti-inflammatory proteins can lower the high risk in individuals with systemic inflammation (433), and those with clinical conditions such as cardiovascular disease, hypertension, high cholesterol levels, diabetes, obesity, heavy alcohol consumption, and stress. Having been approved as a long-term treatment therapy with a good safety profile, AAT coupled with a lasting inhibition of inflammation, is likely beneficial for prolonged rehabilitation therapy.

Plasma GSN and AAT have multiple properties that target various pathophysiological stroke pathways including biochemical and cellular pathways of post-ischemic cell death,
thereby they provide neuroprotection, or they indirectly reduce the progression of brain injury and improve the functional outcome.

Although the recombinant tissue plasminogen activator is currently the standard treatment for ischemic stroke, it can only be effective within 3h (extended to 4.5 h in Europe) of symptom onset, and it can cause neurotoxicity, edema, and hemorrhagic transformation (433). The primary beneficial action of tPA is restoring blood flow and promoting reperfusion, which is associated with the induction of inflammatory mechanisms. Either pGSN or AAT can possibly mitigate the inflammation in the brain by limiting brain cell death or injury from pro-inflammatory responses, which may extend the utility of tPA as well as minimize its undesired effects and consequently improve stroke outcomes.

Some limitations and strengths of the study

This study inevitably has some limitations, including ET-1-induced middle cerebral artery occlusion model, behavioral tests, comparison of cortical motor function in rats and humans, time course, dose response and the pharmacokinetics of pGSN and AAT in cerebral spinal fluid, and comparison of pGSN and AAT in rats and human.

The study utilized ET-1, a potent vasoconstrictor peptide, to induce middle cerebral occlusion. It has been reported that the reperfusion process is ET-1-dose dependent (33), in our experiments utilizing laser-Doppler perfusion imaging, reperfusion began as early as 55 min to as long as more than two hours following ET-1 application, implicating the variability of the experiment. Another issue is that ET-1 can induce astrocytosis and facilitates axonal sprouting that may interfere with the production and interpretation of neural repair experiments (13). ET-1 injection to induce a vasospasm model does not induce blood clot which is typical in thrombotic
and embolic strokes in humans. Lastly, brain ischemia cannot be performed in conscious rats, so it requires anesthesia (isoflurane in this study) which may influence testing agent effects.

On the other hand, ET-1-induced MCAO model has some important advantages. The model produces a transient middle cerebral artery occlusion which mimics stroke in human and provides highly reproducible ischemic damage and significant motor deficits (434). Reperfusion occurs in a range of hours (435) which may be more representative of human stroke than the immediate reduction and reperfusion that occurs with intraluminal suture models, if the ET-1 dose is adjusted appropriately to avoid variability. Also, ET-1-induced MCAO model with intracranial injection of ET-1, dose-dependent, and minimal edema (436), makes it possible to target the infarct to specific regions, particularly subcortical areas, which are more difficult to induce in other models, such as intraluminal suture or direct surgical MCAO which requires the use of a clip or silk suture to ligate an artery (437). The model can induce penumbra so it is suitable for studies of neuroprotective agents as well as examining behavioral alterations during postischemic stroke. Microinjection of ET-1 to MCA vicinity is a minimally invasive and simple technique; it provides a high animal survival rate (17), and in our study it was 96% (two dead out of 52 animals studied) which is important for long-term studies of neuroprotective drugs.

The study purposely selected male, young (7-8 week old) Sprague-Dawley rats for testing the neuroprotective effects of the two proteins. Male rats were selected to avoid variability due to the known effects estrogen has on ischemic outcome (438, 439). Similarly, animal age also affects ischemic outcome; e.g. older rats have been shown to develop a larger infarct compared to younger animals (440, 441). The animal strains may also affect cerebral ischemia (30). Therefore, gender, age, and strain of the animal should be taken into consideration.
The study utilized only a single dose of both testing proteins. Thus, dose/response experiments are necessary to provide important information for optimizing the maximal effective doses as well as predicting the side effects which may occur. Although not studied in this project, the availability of the proteins throughout the time course of ischemic stroke is beneficial for maximizing the effective treatment. Importantly, pharmacokinetics (PK) data on both pGSN and AAT in CSF and serum in ischemic conditions are absent in this study, optimized doses of these proteins cannot be determined until their serum levels are investigated.

A single dose of 3 µg of pGSN (local injection), or 70 µg of hAAT (local injection), or 10mg (40 mg/kg) of hAAT (intravenous injection) has shown significant reduction of 49%, 83%, and 63% in infarct size, respectively. The dose/response experiments can be established based on these findings, for instance 1/3 and 3X of the dose could be tested. Also, the infarct measurements were made 72 h post-ischemia and found the effects of both proteins, therefore the time course can also be established to find if multiple doses are need to maximize the outcome.

Two behavioral tests, cylinder and vibrissae, were performed in this study. The cylinder test is to examine the animal forelimb use for postural support. The test encourages the animal, inside a specially designed cylinder, to use the walls for upright support and vertical exploration. The test results reveal only forelimb sensitivity, thus another test to demonstrate the best prediction of the degree of injury to a specific area of the brain linked to the test should be done to assess the hindlimb function. Another potential disadvantage of the cylinder test is that it may not be repeated too often as animals may lose interest in exploring a novel environment and will not perform at all. In fact, in our study, some rats did not move when the test was performed again after three days, indicating the test may be not sensitive enough (442). Besides, the cylinder test has several notable advantages, including its simplicity and relative ease to perform,
it does not require a particular expertise or training, it does not require pre-training of the animals, the brief duration of the procedure (in this study, 3 min/animal) and the data are rapidly obtained (443). In addition, the test provides a true measure of spontaneous forelimb use as the movements that rats exhibit in the home cage. The absence of comparison between rodent cortical motor and human cortical motor can be a caveat drawback of this study.

Vibrissae or vibrissae-stimulated forelimb placing test is a method for revealing sensorimotor/proprioception. The animal is held by the torso with its forelimbs hanging freely, and moved slowly toward a table or countertop so that the vibrissae on the one side contact with the table or countertop, the limb on the same side readily to move forward to gain weight support. The test requires training and highly experienced personnel (444). Animal struggle and abrupt movements may influence on results, so care must be taken (444). However, the test is evaluate an important role that vibrissae play in rat’s sensory environment as rats are thought to use vibrissae (whiskers) as the primary tools for explore their natural environment. Also, the test can be modified for studying the neural events in sensorimotor system that occurs across the midline (445), the anatomical reorganization between two hemispheres following brain damage (299, 446).

The neuroprotective effects of the proteins have been shown 72 h post-ischemia, while the secondary stroke injury can last weeks to months. It cannot conclude the efficacy of the testing proteins for long-term outcome. Also, the pGSN and AAT used in this study come from human serum, the examination of the immune response as well as the functional differences between rat and human pGSN and AAT, which were unavailable in this study.

Conclusions

The major impetus in stroke research is that stroke may be preventable, and once developed it may be manageable by therapeutic interventions. The delayed onset of neuronal
death following cerebral ischemia occurs via a cascade of events that offers numerous opportunities to intervene with neuroprotective agents. Excitotoxicity, genetically programmed cell death, and inflammation are all involved in the delayed neuronal death.

Plasma gelsolin and alpha 1-antitrypsin possess multiple functions, including anti-apoptotic and anti-inflammatory effects, which may improve stroke outcome through multiple mechanisms of action, and which are different from several neuroprotective agents targeting individual pathways (e.g. only anti-inflammation, anti-apoptosis, or MMP inhibitors). The data demonstrate significant reduction in brain damage and improvement in motor and sensorimotor functions in animal model of middle cerebral artery occlusion.

A model of reversible focal ischemia induced by endothelin-1 (ET-1) showed substantial reduction (~50% compared to base line) in cerebral perfusion, an estimation of cerebral ischemia, in all groups, including ET-1 alone, ET-1+pGSN, ET-1+hAAT (i.c.) and ET-1+hAAT (i.v.), using a laser Doppler perfusion imaging system. This suggested that all groups initially receiving MCAO showed no statistical difference between treatments, and neither pGSN nor hAAT interfered with the vasoconstrictive activity of ET-1. Reperfusion was attained after approximately 1 hour of ET-1 application.

Intracranial delivery of a single dose, 3 µg or 35 pmol, of pGSN significantly prevented both motor and sensorimotor deficits three days following ischemic insult. In the cylinder test, examining animal forelimb use for postural support, animals with pGSN treatment performed better than animals in ET-1 alone control group, 70% vs. 20% (p-value < 0.01), respectively. In the vibrissae test (vibrissae-stimulated forelimb placing test), which tests the sensorimotor response, the pGSN treatment group showed less delay in response to vibrissae stimulation compared to the control group (9 s vs. 19 s, p-value < 0.01), respectively. Plasma gelsolin also
markedly reduced total infarct volume, cortical and subcortical regions, 49% compared to control group (p-value < 0.05).

Intracranial and intravenous administrations of human AAT at doses of 70 µg or 1.35 nmol and 40 mg/kg (10 mg/animal), showed significant reduction in infarct size, 83% and 63%, respectively. As expected, the motor and sensorimotor functions were diminished, in cylinder test though, animals performed much better in hAAT (i.c.) treatment group than in the control (60% vs. 23%, p-value < 0.05), and in vibrissae test hAAT (i.c.) treatment group showed less delay in response to vibrissae stimulation (6 s vs. 19s, p-value < 0.001). The study of the effects of intravenously delivered hAAT was performed in two separate groups of animals, ET-1 alone (N = 8) and ET-1+hAAT (i.v.) (N = 12). Human AAT (i.v.) treated group also illustrated significant improvement of motor function (73% in hAAT treatment vs.35% in control group, p-value < 0.001), and sensorimotor function (~50% decrease in delayed time in hAAT treatment group compared to ET-1 alone group, p-value < 0.05).

The dissertation studies have shown that two proteins, plasma gelsolin and alpha 1-antitrypsin, perform multiple functions that may provide multiple beneficial effects for ischemic therapy. These novel therapies may give a promise for ischemic stroke patients, including preventing stroke recurrence.

**Future Work**

These two endogenous proteins offer several avenues of research toward neuroprotection following cerebral ischemia. Further research to validate the modes of action of plasma gelsolin and alpha 1-antitrypsin, including the inhibition of cell death pathways and anti-inflammatory properties of the both proteins are essential for further pre-clinical development. The dose/response and time course need to be determined in order to optimize the protective effects of the agents. Although pGSN and AAT are natural proteins, the safety/toxicity and study of
their long-term effects are important for the treatment of this disease. Also, pharmacokinetic studies should be performed to determine levels of the proteins in both serum and cerebral spinal fluid during cerebral ischemia; this data is essential for dosing adjustment, multiple dose regimens as well as to know whether transient ischemia has any effects on the testing agents. Studying the effects of pGSN and AAT in combination with tissue plasminogen activator could be useful to see whether the proteins can be beneficial for extending the effective time window or necessary dose for tPA thrombolysis. Inclusion of more behavioral tests that assess motor/sensorimotor function sensibility and cognitive function can strengthen the translational applications.
LIST OF REFERENCES


357. P. Nicotera, P. Hartzell, G. Davis, and S. Orrenius. The formation of plasma membrane blebs in hepatocytes exposed to agents that increase cytosolic Ca2+ is mediated by the activation of a non-lysosomal proteolytic system. FEBS Lett. 209:139-144 (1986).


BIOGRAPHICAL SKETCH

Huong Thu Le (Lê Thu Hương, in her native language) was born in Vinh Phúc, Vietnam on October 1975. Huong grew up in a large family with other three siblings. At a very young age, Huong showed herself as a dynamic girl. She likes helping people, which showed in the fact that at the age of 14, she started running errands to help her family. She chose to attend the Hanoi College of Pharmacy among the several schools to which she received admission in 1993. She got her Pharmacist Degree in 1998, and worked for a Japanese pharmaceutical company immediately following graduation. After working seven years in Hanoi, Huong decided to trade Vietnam and the pharmaceutical industry for the Netherlands and academia, starting a master degree in Drug Innovation at Utrecht University. During this master program, she joined Dr. Jeffrey Hughes’ Lab at the University of Florida as an intern. She got her master of science in early August 2007 and returned to Florida where later that month she was admitted into their Ph.D. program in the Department of Pharmaceutics. She has enthusiastically continued to work under the supervision of Dr. Jeffrey Hughes, and completed the project she began working on during her internship. She was assigned to work in Dr. Sihong Song’s Lab on another project when Dr. Hughes left for industrial work. Despite life's many obstacles and hardships, she received her Ph.D. from the University of Florida in the spring 2012 eagerly sought work in her field following graduation. In the last year of her Ph.D. program she met her fiancé, Matthew Michael Moldthan whom she married in December of 2011.