NUTRITIONAL INTERVENTION AND MODELING
OF ACUTE ISCHEMIC STROKE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Cameron Rink, B.S.

******

The Ohio State University
2008

Dissertation Committee:

Professor Chandan Sen, Advisor
Professor Sashwati Roy, Co-Advisor
Professor Sampath Parthasarathy
Professor Narasimham Parinandi

Approved By:

______________________________
Advisor

Human Nutrition Graduate Program
ABSTRACT

The documented origins of functional-foods and disease intervention date back thousands of years to ancient Egyptian culture where honey was used as a wound dressing. Recent scientific review has explored and affirmed the therapeutic benefits of this olden remedy. While septic wounds were a leading cause of death then, modern man faces very different causes and contributors of morbidity today. Stroke is currently the third leading cause of death in the United States. The purpose of this work was to study potential nutritional interventions for risk factors and outcomes affecting acute ischemic stroke and to develop a robust pre-clinical stroke model to improve the translation of potential therapeutics to the clinic.

**Objective:** The objectives of this dissertation were three-fold: (i) To characterize a basis for nutritional intervention of acute ischemic stroke risk factors by using niacin-bound chromium in the prevention of metabolic syndrome; (ii) to determine the *in vivo* relevance of the natural vitamin E, α-tocotrienol, in neuroprotection following acute ischemic stroke; and (iii) to develop a pre-clinical model of acute ischemic stroke in canines to bridge the translational gap that exists between laboratory and clinical stroke research.
and to improve upon existing stroke models for testing α-tocotrienol efficacy prior to clinical study.

Experimental approach and results: As part of this dissertation, we first described a nutritional intervention for metabolic syndrome, a constellation of risk factors that are known to contribute to incidence and severity of outcomes in stroke. To model metabolic syndrome, we employed mice with a spontaneous mutation that induced obesity, glucose intolerance, and dyslipidemia. Prophylactic supplementation of these mice with a chromium complex significantly improved their lipid profile and modestly improved glucose clearance. Examination of the adipose tissue transcriptome using genome-wide microarray analysis revealed a myogenic response to chromium supplementation.

Next, we assessed the in vivo potential for a direct nutritional intervention of stroke-induced pathology with a natural vitamin E isoform, α-tocotrienol (αT3). A multi-generational supplementation study was conducted to determine αT3 tissue concentration for biological relevance. High pressure liquid chromatography analysis of αT3 concentration in brain tissue was found to be within the nanomolar range relevant to in vitro study neuroprotection results. We followed up these findings by testing the neuroprotective potential of αT3 in
a rodent model of stroke. Spontaneously hypertensive rats, prone to stroke, had significantly reduced infarct volume when supplemented with αT3 as compared to placebo controls. The mechanism of neuroprotection by αT3 in cell culture study was reportedly by two cytosolic targets, c-Src and 12-lipoxygenase (12-Lox). The active form of phosphorylated 12-Lox was reduced in stroke affected tissue of orally administered αT3 rats as compared to placebo controls. Supporting the role of 12-Lox as a key mediator of stroke-induced cell death, 12-Lox deficient mice had significantly smaller infarct volume as compared to wild-type matched controls. 12-Lox metabolizes arachidonic acid from the lipid bilayer into the eicosanoid 12-S-hydroperoxyeicosatetraenoic acid (12-S-HPETE). The rate of respiration in isolated brain mitochondria was inhibited by incubation with 12-S-HPETE. Furthermore, 12-S-HPETE promoted mitochondrial dysfunction by reducing inner membrane potential, and exacerbating permeability transition pore opening (PTP). 12-S-HPETE induction of PTP was inhibited in isolated brain mitochondria by co-treating them with αT3.

The final objective of this thesis was to develop a pre-clinical model of stroke in which potential neuroprotective agents, such as αT3, could be tested prior to clinical trial. We developed a minimally invasive method of transient middle cerebral artery (MCA) occlusion in canines which benefits from a
neuroradiological approach to visualize the stroke event. Using 3T magnetic resonance imaging (MRI), we quantified the infarct volume in canines (n=4) and reported very tight reproducibility. Histological analysis of stroke and contra-lateral cortex confirmed stroke-induced neurodegeneration in the MCA territory.

**Conclusions:** This dissertation describes prospective nutritional interventions for stroke-related risk factors and stroke-induced infarction. These natural dietary factors hold great promise in reducing the incidence and severity of stroke. Development of a minimally invasive pre-clinical model of stroke will further help to bridge the gap between laboratory benchwork and clinical study so that potential therapeutics, such as these, can be identified and translated to the clinical setting.
This dissertation is dedicated to my loving wife, Kristi,

my soon to be born daughter, Campbell,

and to the Glory of my Creator.
ACKNOWLEDGMENTS

There are many to whom I am indebted to and would like to thank for their unconditional love and support throughout my graduate school years. First, to my parents who taught me the value of education and inspired me to always “do my best, and check my work”. Second, to my siblings, whom I consider my closest friends, thank you for being a constant source of encouragement. And last, but by no means least, appreciation is due to my amazing wife Kristi – whose humble sacrifice and unwavering support these past years made this accomplishment achievable – it could not have been done without you.

I am also forever grateful to my mentor Dr. Chandan Sen, whose advice, counsel, and contagious passion for science as well as life I deeply cherish. Genuine appreciation is also due to my co-advisor, Dr. Sashwati Roy, for her mentorship and for always leaving her door open to scientific discussion. Furthermore, sincere thanks to the Sen Lab project leaders - specifically, to Dr. Savita Khanna for her friendship and selfless giving of time and training, and to Dr. Sabyasachi Biswas for technical support and reminding me to laugh time and again. I would also like to thank my graduate committee members, Dr. Chandan Sen, Dr. Sashwati Roy, Dr. Sampath Parthasarathy and Dr.
Narasimham Parinandi for dedicating their time, effort and guidance through the candidacy and doctoral defense process.

A sincere thank you also to my peers and supporters in the lab and in collaboration: Dr. Navdeep Ojha for his MRI expertise, Jared Radtke and Molly McCoy for animal support, Dr. Greg Christoforidis for surgical technique training, and Dr. Valerie Bergdall and Jeanne Greene for veterinary project support.

I greatly appreciate the support provided by the faculty and staff of The Ohio State University Interdisciplinary Ph.D. Program in Nutrition (OSUN); whose assistance in navigating my doctoral program was essential. Appreciation also goes to the DHLRI leadership and staff for project and administrative support. Furthermore, I would like to thank Dr. Christopher Ellison, Chairman of the Department of Surgery, who encouraged and supported this body of work within the Department of Surgery.

Finally, I acknowledge the support of my University sponsored First Year Fellowship, my American Heart Association pre-doctoral fellowship AHA 0615240B, and the project support of NIH grant NS42617 awarded to Dr. Chandan Sen. I would also like to recognize the following organizations for the opportunities to share my research and for the awards and honors received as
VITA

May 25th, 1978 .......... Born – Columbus, OH

1996 – 2000 ............ B.S. Finance, Hillsdale College

2002 – 2004 ............ Research Assistant, The Ohio State University

2005 – 2006 ............ Graduate Research Associate, Laboratory of Molecular Medicine, The Ohio State University

2006 – present ........... Graduate Fellow, Laboratory of Molecular Medicine, The Ohio State University

PUBLICATIONS


FIELDS OF STUDY

Major Field: Human Nutrition

Emphasis: Nutritional Intervention of Cerebrovascular Disease
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>vi</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>vii</td>
</tr>
<tr>
<td>Vita</td>
<td>x</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xvi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xvii</td>
</tr>
<tr>
<td>Doctorate of Philosophy: Roots and Concept</td>
<td>1</td>
</tr>
</tbody>
</table>

## Chapters:

1. Nutritional intervention of risk factors affecting acute ischemic stroke ........................................ 10
   
   1.1 Introduction ......................................................................... 10
   
   1.2 Materials and methods .................................................. 27
   
   1.3 Results and discussion ............................................... 32
   
   1.4 Conclusions ...................................................................... 43
   
   1.5 Tables .............................................................................. 45
   
   1.6 Figures ............................................................................ 48

2. Nutritional intervention of acute ischemic stroke by the natural vitamin E α-tocotrienol .......................... 56
2.1 Bioavailability and tissue distribution of vitamin E isomers in vivo

2.1.1 Introduction

2.1.2 Materials and methods

2.1.3 Results and discussion

2.1.4 Conclusions

2.1.5 Figures

2.2 Unique neuroprotective properties of the natural vitamin E a-tocotrienol

2.2.1 Introduction

2.2.2 Materials and methods

2.2.3 Results and discussion

2.2.4 Conclusions

2.2.5 Figures

3. Minimally invasive, interventional pre-clinical model of acute ischemic stroke in canines

3.1 Introduction

3.2 Materials and methods

3.3 Results and discussion

3.4 Conclusions
3.5 Figures........................................................................162

4. Summary and general discussion........................................174

List of references......................................................................182
# LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Primer sequence used for real-time PCR analysis .................. 45</td>
</tr>
<tr>
<td>1.2</td>
<td>List of genes in subcutaneous fat that were up-regulated in response to NBC supplementation ........................................ 46</td>
</tr>
<tr>
<td>1.3</td>
<td>List of genes in subcutaneous fat that were down-regulated in response to NBC supplementation ........................................ 47</td>
</tr>
<tr>
<td>3.1</td>
<td>Physiologic parameter in canines pre-, during, and post MCA occlusion ......................................................... 152</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1.1</td>
<td>Lepr\textsuperscript{db} KO mouse phenotype</td>
</tr>
<tr>
<td>1.2</td>
<td>Body weight in response to niacin-bound chromium (NBC) supplementation</td>
</tr>
<tr>
<td>1.3</td>
<td>Plasma lipid profile in response to niacin-bound chromium (NBC) supplementation</td>
</tr>
<tr>
<td>1.4</td>
<td>Blood glucose and clearance in response to niacin-bound chromium (NBC) supplementation</td>
</tr>
<tr>
<td>1.5</td>
<td>GeneChip data analysis scheme</td>
</tr>
<tr>
<td>1.6</td>
<td>Cluster images illustrating genes sensitive to NBC in subcutaneous fat tissue</td>
</tr>
<tr>
<td>1.7</td>
<td>Quantitative expression analyses of genes up-regulated in response to NBC supplementation</td>
</tr>
<tr>
<td>1.8</td>
<td>Quantitative expression analyses of genes down-regulated in response to NBC supplementation</td>
</tr>
<tr>
<td>2.1</td>
<td>Eight naturally occurring vitamin E isoforms</td>
</tr>
<tr>
<td>2.2</td>
<td>Schematic representation of the study design aimed at examining the long-term effects of oral vitamin E supplementation in rats</td>
</tr>
<tr>
<td>2.3</td>
<td>(\alpha)-Tocotrienol and (\alpha)-tocopherol levels in the skin of rats</td>
</tr>
<tr>
<td>2.4</td>
<td>(\alpha)-Tocotrienol and (\alpha)-tocopherol levels in abdominal adipose and gonads of rats</td>
</tr>
<tr>
<td>2.5</td>
<td>(\alpha)-Tocotrienol and (\alpha)-tocopherol levels in the heart of rats</td>
</tr>
</tbody>
</table>
2.6 α-Tocotrienol and α-tocopherol levels in the lungs of rats ............. 86
2.7 α-Tocotrienol and α-tocopherol levels in the vastus lateralis skeletal muscle of rats .............................................. 87
2.8 α-Tocotrienol and α-tocopherol levels in the brain and spinal cord of rats ................................................................. 88
2.9 α-Tocotrienol and α-tocopherol levels in the blood of rats ............. 89
2.10 α-Tocotrienol and α-tocopherol levels in the liver of rats .............. 90
2.11 α-Tocotrienol and α-tocopherol levels in tocotrienol supplemented TTP-deficient mice ..................................................... 91
2.12 Glutamate excitotoxicity pathway ........................................... 128
2.13 Cystolic αT3, but not αTOC, protects neurons from glutamate induced death .............................................................. 129
2.14 c-Src and 12-lipoxygenase in glutamate-induced neuronal death .......................................................... 131
2.15 12-Lipoxygenase mediated metabolism of arachidonic acid ........ 132
2.16 AA metabolite, 12-S-HPETE, induces cell death in GSH depleted HT4 neurons ............................................................. 133
2.17 12-Lipoxygenase-deficient mice are resistant to transient focal cerebral ischemia ......................................................... 134
2.18 αT3 and αTOC levels in the brain of spontaneously hypertensive rats .............................................................................. 135
2.19 Oral αT3 supplementation protects against stroke-induced injury and 12-Lox phosphorylation in SHR brain ....................... 136
2.20 Histological analyses of post-stroke infarct zone cortical sections ................................................................................ 137
2.21 Inhibition of mitochondrial ATP synthesis (state 3 respiration) by 12-S-HPETE ........................................ 138
2.22 12-S-HPETE and 12-S-HETE compromise mitochondria inner membrane potential ........................................... 139
2.23 Loss of mitochondrial membrane potential following treatment with 12-S-HPETE and 12-S-HETE ................................. 140
2.24 12-S-HPETE induced loss of mitochondrial membrane potential in neurons .................................................. 141
2.25 α-Tocotrienol protects mitochondria from 12-S-HPETE exacerbated PTP opening .................................................. 142
2.26 Summary diagram of 12-S-HPETE effects on mitochondrial dysfunction ...................................................... 143
3.1 Vertebrobasilar approach to MCA occlusion in canines .................. 162
3.2 Non-invasive fluoroscopic guidance of canine cerebrovascular system ......................................................... 163
3.3 Fluoroscopic guidance of right MCA occlusion ......................... 164
3.4 3T MRI evidence of PCOM and BA affected stroke ...................... 165
3.5 Enhanced contrast angiography of left MCA occlusion ................. 166
3.6 Angiographic assessment of ischemic territory at risk of stroke-induced infarction ............................................. 167
3.7 Coronal slices of MCA territory stroke-induced brain lesion ........... 168
3.8 Stroke-induced infarct volume as determined by MRI .................. 169
3.9 Volumetric reconstruction of stroke-induced infarct lesion ............. 170
3.10 Histological analysis of post-stroke infarct and control tissue .......... 171
3.11 Exploration of alternative MCA occlusion models: 
a canine craniectomy trial..............................................................172

3.12 Minimally invasive endovascular approach to MCA occlusion........173
DOCTORATE OF PHILOSOPHY: ROOTS AND CONCEPT

*Putting the “Ph” into the PhD*

**Introduction**

“There is nothing so stupid as the educated man if you get him off the thing he was educated in.” – Will Rogers (American humorist, radio personality)

The Doctorate of Philosophy degree, or PhD, is the highest earned academic degree in U.S. postsecondary education according to the United States Department of Education. More than 45,000 doctoral degrees are earned in the U.S. every year in a divergent range of fields from Economics to Textiles and Clothing [1]. Common to all fields of doctoral graduate study are the specialization and focus of a research interest that becomes the heart of the doctoral candidate’s study. Often time, and having learned by personal experience, this focal point can so consume and occupy the candidate’s field
of view that the greater context of the work is lost in the minutia of the moment. Sadly, the truest sense of philosophical passion can be lost in a disillusioned desire to attain a particular statistical value rather than a meaningful understanding of what the data truly represents. As an unintended consequence, the “Ph” can be separated from the “D” lending credence to comedian/philosopher Will Roger’s humorous take on the “educated man”. The purpose of this brief introductory chapter, therefore, is to reflect on the origins of the Doctorate of Philosophy degree, the learning process that necessitates a philosophical approach, and the philosophical basis for the conduct of scientific research within this dissertation.

History of the PhD

“The medieval university looked backwards; it professed to be a storehouse of old knowledge. The modern university looks forward, and is a factory of new knowledge.” – Thomas Henry Huxley (English biologist)

The true origins of the Doctorate of Philosophy degree are obscure at best. During the 13th century, a system of degrees seems to have come into existence throughout Europe. The three grades common to all were those of Scholar, Bachelor, and Masters (sometimes called Doctor or Professor), each with a unique academic role [2]. The most junior title was that of Scholar, who
attended lectures and argued on set questions in the schools, successive in rank was the Bachelor whose role was that of student-teacher seeking to obtain a license to teach on his own, and finally the highest in rank were those who obtained mastership, or Masters, who carried an obligation to lecture and mentor others in the university setting. Over time, and as more academic disciplines developed, the title ‘Doctor’ replaced that of ‘Masters’ for chief degrees in faculties outside of Theology and Arts [2].

It was during the mid 1800’s that the modern American PhD took shape. Yale’s Daniel Coit Gilman spent two years attending seminars at German universities following the completion of his bachelor’s degree in 1852. Upon his return he helped develop the nation’s first PhD program. Borrowing heavily from his experience with graduate school study abroad, he helped to propose a plan in conjunction with Yale’s Department of Philosophy and Arts to offer a doctoral degree “in accordance with the usage of German universities” [3]. Students were expected to undertake two years of graduate study, achieve proficiency in languages (Greek and Latin), pass an examination on their studies, and complete a thesis based on results of original chemical or physical investigation to be reviewed by faculty. In 1861, Yale trustees granted the first American Doctorate of Philosophy degrees to three students who achieved the “high standard of attainments” set before them. While the core requirements have remained largely unchanged, Doctorate of Philosophy degrees have grown greatly in number granted and
universities in which they are offered. The Survey of Earned Doctorates by the National Science Foundation reported 45,596 PhD degrees were presented by 417 research doctorate-granting institutions in 2006 alone [1]. The vast amount of research, volume of knowledge generated, and physical resources necessary to produce so many doctoral degrees each year in the U.S. are inestimable. The accomplishments of the modern American university are indebted to Daniel Coit Gilman who not only championed graduate level learning and development of the PhD, but also the concept of the modern research university.

While the establishment of higher education in the United States was ingrained in the founding father’s vision of the country, it took a considerable amount of time to achieve. A little more than a decade after declaring independence from the British, a federal mandate was developed at the 1787 Constitutional Convention calling for the creation of a national university [3]. Despite the fact that this mandate was never fully realized, it eventually spawned the organization of state sponsored universities. In part from the lobbying efforts of former President Thomas Jefferson and Congressman Charles Mercer, the first state university, supported by Virginia, was created in 1825. Many more universities, both state and private, were founded throughout the 1800’s; including The Ohio State University in 1870. But it wasn’t until 1876 with the founding of John Hopkins University that the modern model for the American research university was conceived [4]. Appointed as
university president, Daniel Coit Gilman expressed his transformative proposal for the “new American research university” in his inaugural address. Owing many of his ideas to his time spent studying the German graduate education system, Gilman exclaimed that the university should do more than create “learned pendants, simple artisans, or cunning sophists, or pretentious practitioners.” Rather, “the object of the university was to develop character… not so much to impart knowledge to pupils, as to whet the appetite, exhibit methods, develop powers, strengthen judgment, and invigorate the intellectual and moral forces” [3]. Gilman’s extraordinary vision, with an emphasis on imparting not knowledge alone, but with wisdom, set the cornerstone for the philosophical underpinnings of the American PhD degree and the modern American research university.

Philosophy of the PhD

“Wisdom is the right use of knowledge. To know is not to be wise. Many men know a great deal, and are all the greater fools for it. There is no fool so great a fool as a knowing fool. But to know how to use knowledge is to have wisdom.” – Charles Spurgeon (British pastor, author)

It has been estimated that across all fields of graduate study, the attrition rate for PhD programs is between 40 and 50 percent [5]. This is in stark comparison to the more than 90% completion rate for students in
American medical schools and 98% completion rate for students in the top 20 American law schools [6]. It is my personal belief that a contributing factor to the disparity in graduation rates is the difficulty in fulfilling the challenging and ill-defined philosophical component of a PhD program. Having two siblings who have taken medical school coursework at two different Big Ten universities, I have had the opportunity to compare the philosophical approach of my graduate school education to the more didactic medical (professional) student education. The point is not to insinuate that a medical school education is any less challenging (if anything my siblings’ coursework was more demanding), however, it is to draw upon the observation that the emphasis of an MD education is on the memorization of medical facts and the systematic diagnosis of disease states – and for good reason! The ultimate responsibility of a physician is the care of patients in their specialized field which necessitates a vast array of medical knowledge and the analytical prowess to diagnose in order to provide effective therapy. Conversely, the ultimate responsibility of a Doctorate of Philosophy graduate is to advance the knowledge base of their respective field of study. To do so requires not only an in-depth understanding of their field, but the ability to formulate paradigm shifting questions, implement a course of research and to translate the outcomes into new and appreciable knowledge. This responsibility necessitates the philosophical component of the degree.
At first pass, philosophy and science seem juxtapose. That is, philosophy is defined as the pursuit of wisdom; whereas science is primarily characterized as the pursuit of knowledge [7]. It appears on the surface then contradictory to earn a Doctorate of Philosophy degree in a scientific field such as human nutrition. However, the two are undeniably inseparable as a doctoral student must have a mastery of scientific knowledge along with the wisdom to apply it to their respective field and body of research. Were knowledge enough to earn the degree, the thesis and imminent oral examination, tests of the application of knowledge, would not be necessary.

If “wisdom is the right use of knowledge”, as stated by Spurgeon, then philosophy’s role in the Doctorate of Philosophy degree is in the method of pursuing and applying the scientific knowledge – or more succinctly, the epistemological approach to the science. In the case of earning a PhD in a scientific field, it is therefore equally as important as to how a question is approached as much as the result of the answer to the question. To that end, the approach to asking a scientific question is uniquely individualistic. While doctoral students have the benefit of an advisor to help guide and focus their curiosity, it is ultimately up to the student to implement an approach for their research. Therein lies the challenge of earning a PhD.
Personal Philosophical Approach

“Science is a way of thinking much more than it is a body of knowledge.”
– Carl Sagan (American astronomer)

This thesis is the culminating effort of four years spent in the Ohio State University Nutrition PhD program. During this time I have taken a diverse array of courses ranging from vitamin and mineral metabolism to neuroimmunology. In the laboratory, I took it upon myself to learn and apply an equally divergent range of techniques from general molecular biology applications such as real-time PCR to surgical modeling of acute brain ischemia/reperfusion injury. This diversity in education and training is a core component of my philosophical approach to the research efforts described in this dissertation and to earning my doctoral degree. While one may argue that such a broad background lacks an appropriate degree of focus common to scientific work, I would appeal that by employing an uncommonly divergent perspective my research is uniquely focused to address the topic of nutritional intervention and modeling in cerebrovascular affective disorders.

Whenever possible, I have attempted to draw upon this wide range of techniques and disciplines in approaching my research. By doing so, my goal was to span both basic and applied scientific research. To that end, this dissertation contains both focused and broad analysis of issues affecting cerebrovascular disease. Chapter 1 spotlights a broad category of risk factors
that contribute to ischemic stroke incidence and pathology under the umbrella of metabolic syndrome. The concept of a nutritional intervention for disease is explored in the context of a dietary nutrient, niacin-bound chromium, that improves phenotypic changes in lipid profile as explained by significant genotypic differences between control and treatment groups. Next, chapter 2 looks at the unique neuroprotective properties of the natural vitamin E, α-tocotrienol, in the context of ischemic stroke. This relatively unknown vitamin E isomer is first characterized in terms of tissue distribution in order to ascribe relevance for stroke-related supplementation study. Subsequently, the effects of dietary supplementation on stroke-induced infarct volume and a cellular mechanism for neuroprotection are investigated using in vivo modeling of stroke pathology. The third chapter aims to bridge a gap that exists between laboratory benchwork and clinical study of stroke therapeutics. Chapter 3 highlights the development and characterization of a novel pre-clinical model of stroke that endeavors to improve the translation of potential stroke therapeutic agents, such as α-tocotrienol, to the clinic. The final chapter, 4, provides a summary and general discussion for the entire body of work.
CHAPTER 1

NUTRITIONAL INTERVENTION OF RISK FACTORS AFFECTING ACUTE ISCHEMIC STROKE

1.1 Introduction

1.1.1 Background

While the hypothesis-driven scientific basis for nutritional research is a relatively recent endeavor, the profound effects of food and diet as related to health have long been realized. The Egyptians were known to use honey to prevent infection in wounds [8]. Recent scientific literature has supported the therapeutic effectiveness of this ancient remedy [8, 9]. Garlic has a storied history of use as a panacea for disorders amongst a multitude of cultures. Ancient Mesopotamians prescribed garlic for toothaches while the Chinese associated it with protection of the spleen, kidney and stomach [10]. Perhaps
one of the earliest dietetic experiments on record is found in the Book of Daniel, a chronicle from the Bible’s Old Testament of an adviser to King Nebuchadnezzar, ruler of Babylon from 605-562 BC. Early in the book, Daniel questions the steward’s excessive diet of rich delicacies and wine and prescribes a healthier alternative rich in vegetables and water [11].

12 “Please test your servants for ten days, and let them give us vegetables to eat and water to drink. 13 Then let our appearance be examined before you, and the appearance of the young men who eat the portion of the king’s delicacies; and as you see fit, so deal with your servants.” 14 So he consented with them in this matter, and tested them ten days. 15 And at the end of ten days their features appeared better and fatter in flesh than all the young men who ate the portion of the king’s delicacies. 16 Thus the steward took away their portion of delicacies and the wine that they were to drink, and gave them vegetables. – Daniel 1:12-16

Today, the role of food as related to health has taken on a preventative approach with a focus on the prophylactic benefits of balanced nutrition and the maintenance of wellness from diet. This method distinctly contrasts that of modern medicine which emphasizes treatment after diagnosis, frequently with a pharmaceutical solution. Despite such a divergent approach to address the well-being of the individual, the eminent medical historian Henry E. Sigerist
once noted that “there is no sharp borderline between food and drug” [12]. History bears witness to this statement as exemplified by the discovery and elucidation of the chief nutrients in the late 19\textsuperscript{th} and early 20\textsuperscript{th} century.

1.1.2 Deficiency disease and vitamin discovery

In 1897, Dutch scientist Christian Eijkman characterized a nutritional basis for the pathology of beriberi disease, a debilitating illness associated with paralysis due to polyneuritis and congestive heart failure. Early descriptions of the disease have been traced back more than 2000 years to ancient Chinese text that refers to the illness as \textit{kak-ke} (“disease of the legs”) [10]. The relatively modern name of “beriberi” stems from Sinhalese (Sanskrit) origins in which the term means “sheep” and is thought to have connotations of partial paralysis inducing a sheep-like walk in the afflicted. Beriberi reached epidemic status at the turn of the 19\textsuperscript{th} century due to the advent of the steam-powered rice mill. Because of the milling process, commercially milled rice became increasingly inexpensive during this time, making it an attractive staple to a larger populous. As part of mill processing, the outer hulls of the rice grain were removed, leaving behind smooth white kernels, termed “polished rice” [10].

Eijkman, a Dutch military surgeon, was charged with uncovering what was presumed to be an infectious cause of beriberi disease. For many years
most medical authorities, influenced by the work of Pasteur, believed that a bacterium was the instigator [13]. Eijkman’s experiments involved inoculating water fowl with supposedly infected material from beriberi patients [10]. The birds, which were normally fed with crude rice, remained healthy, until by fortunate chance they were switched to a diet of boiled and polished white rice left over by the hospital. Almost immediately, the animals began to show polyneuritis symptoms similar to those found in human beriberi disease. It was only later, when the same animals were returned to the crude rice diet by order of the hospital chief (who had at this point forbidden the use of “luxury” white rice for laboratory animals), and the beriberi symptoms disappeared that Eijkman made the connection that a dietary factor may be at play [10]. In subsequent work, Eijkman noted that by simply restoring the inner hull of the rice to the diet, beriberi patients experienced a rapid recovery. Eijkman’s monumental discovery would eventually lead to his awarding of the 1929 Nobel Prize in Physiology and Medicine.

Another Dutch scientist, Gerrit Grijns, followed Eijkman’s work closely and was the first to describe “that in food there occur in small quantities unknown organic substances essential for health” [14]. Grijns aptly named them “protective substances”. Fifteen years after Eijkman’s initial discovery, Polish biochemist Casimir Funk successfully isolated the water soluble component of the rice hull that prevented beriberi. Because the substance contained an amine group, he appropriately named the compound a “vital
amine” or “vitamine”. Over time, “vitamine” was shortened to “vitamin” as more essential nutrients related to deficiency diseases were discovered that did not contain amine groups [10].

In 1913, Lafayette Benedict Mendel and Thomas Burr Osborne discovered a fat-soluble accessory food substance that was clearly distinct from the water-soluble factor revealed in the beriberi studies [13]. Their finding came from the comparison of two purified diets fed to white rats – one with dried whole milk, the other with dried skim milk. Rats kept on the skim milk diet eventually lost weight and died. However, the substitution of butter for some of the lard in the skim milk diet prevented death and demonstrated that butter contains trace amounts of a fat-soluble organic substance that was essential in nutrition for life [15]. Three weeks prior to Mendel and Osborne’s manuscript submission, a group from the University of Wisconsin submitted a manuscript to the same journal as Osborne and Mendel entitled “The necessity of certain lipins in the diet during growth” [16]. The authors, McCollum and Davis, reported an astonishingly similar finding as Mendel and Osborne with rats fed “the ether extract of egg or of butter”. Despite both articles appearing in the same volume of The Journal of Biological Chemistry, McCollum and Davis were credited with the discovery of “fat-soluble A”; which eventually became known simply as vitamin A [13]. McCollum and Davis’ work eventually paved
the way for the differentiation of fat soluble vitamins A and D in a host of deficiency diseases including xerophthalmia (A), night blindness (A), rickets (D), and osteomalacia (D) [17].

By mid-20th century, a pivotal shift in the consideration of nutritional factors and disease had occurred. No longer were foodstuffs merely pharmacologic interventions for disease, but rather nutritional deficiencies were identified as cause for disease. This new-founded concept appeared foreign and radical to the prevailing mindset of the time that was based heavily on the germ theory of disease. Throughout the early 20th century, vitamins and other essential nutrients continued to be discovered and characterized in cases of deficiency disease. Eventually, Eijkman’s cure for beriberi disease was classified as a member of the vitamin B family and another water-soluble vitamin, C, was recognized for its ability to prevent scurvy [18]. Iron deficiency was found to cause anemia and respiratory failure [19]. During this time, nutritional factors in excess also became associated with pathological conditions. Dietary surfeit of salt (sodium chloride) was linked to primary hypertension in the 1940’s [20].

1.1.3 Modern nutritional disorders: metabolic syndrome

At present, most cases of single nutrient deficiency disease like scurvy and beriberi have been eradicated in the U.S. due to the nutritional fortification
of common foods and beverages such as breakfast cereals and juices [21, 22]. The majority of nutrition-related pathologies affecting Americans today are more related to long-latency diseases such as heart disease, stroke, and type-II diabetes. Today, heart disease and stroke represent the number one and number three causes of death in the United States respectively [23] and more than 14.8 million Americans suffer from type-II diabetes [24]. A component or contributing risk factor for all three of these diseases is metabolic syndrome [25].

Metabolic syndrome encompasses a constellation of cardio and cerebrovascular risk factors. Multiple prospective cohort studies have identified metabolic syndrome as a significant risk factor for ischemic stroke [26, 27]. Another study implicated a three-time greater risk of stroke occurrence in patients diagnosed with metabolic syndrome [28]. According to criteria of the National Cholesterol Education Program Adult Treatment Panel III (ATP III), diagnosis of metabolic syndrome is made when 3 or more of the following risk factors are present: abdominal obesity, elevated triglyceride levels, low levels of high-density lipoprotein cholesterol (HDL), elevated blood pressure, and impaired glucose metabolism [29]. On the basis of the National Health and Nutrition Examination Survey (NHANES) III data, the prevalence of metabolic syndrome in the United States is 23.7% for all adults and 43.5% for adults ≥60 y of age [23]. Contributing to the high incidence of metabolic syndrome in the U.S. are increasingly sedentary lifestyles while food
availability and portion size increase. Results of the recent NHANES survey revealed that more than 61% of adult females and 70% of adult males are classified as overweight or obese in the United States as defined by a Body Mass Index (BMI) of 25 or greater [30]. As the prevalence of obesity has risen in the U.S., so has that of type-II diabetes. Colditz et al reported that the relative risk of diabetes increases by 25% for each additional unit of BMI higher than 22 kg/m² [31]. The transition from obesity to diabetes is made by a progressive defect in insulin secretion coupled with a gradual rise in insulin resistance. Both insulin resistance and defective insulin secretion appear very prematurely in obese patients, and both worsen toward diabetes. Diabetes is itself associated with abnormalities in fatty acid metabolism resulting in an irregular lipoprotein cascade affecting large chylomicrons and small HDL cholesterol [32]. Indeed, the etiological factors of metabolic syndrome are related and contribute to one another.

The first chapter in this dissertation describes a potential nutrient intervention for metabolic syndrome and illustrates the candidate’s training and background in live animal (in vivo) experimentation and modern molecular biology techniques such as high-density DNA microarray gene expression analysis. The significance of the research lies in the prospect for a natural, nutrient-based, intervention of a cluster of stroke-related risk factors, under the umbrella of metabolic syndrome, as an alternative or adjunct to pharmaceutical-based therapeutics.
1.1.4 Chromium’s link to metabolic syndrome

In the mid 1950’s it was discovered that brewer’s yeast contained a “glucose tolerance factor” that prevented diabetes in experimental animals [33]. Eventually, the glucose tolerance factor (GTF) emerged as a biologically active form of trivalent chromium that could substantially lower plasma glucose levels in genetically altered mice meeting the criteria for metabolic syndrome [34]. Studies of low-molecular-weight chromium deficiency have led to the identification of this element as a trace essential element involved in the action of insulin [35, 36]. Clinical interest in chromium supplementation soared in response to the observation that a patient receiving total parenteral nutrition developed severe indications of diabetes that were reversed on chromium supplementation [35, 37]. At present, chromium is added as a standard supplement to total parenteral nutrition [38]. The major impediment to the use of orally administered chromium is poor absorption of trivalent chromium in its inorganic form. Trivalent chromium is more available in yeast, and, more recently, organic-bound chromium has been found to be a bioavailable formulation for oral supplementation [39]. A combination of chromium and niacin has been reported to significantly decrease total cholesterol and total lipid levels in serum [40]. Chromium supplementation holds promise in the management of type-II diabetes [41]. Dietary supplementation of chromium has been postulated to potentially reduce body fat mass and increase lean body mass [42]. Supplemental niacin confers diverse benefits with respect to
both the quantity and quality of lipid and lipoprotein particles [43, 44]. Low doses of niacin are a treatment option for dyslipidemia in patients with type 2 diabetes [45].

We sought to examine the effects of oral niacin-bound chromium, a chromium (III) – niacin complex, supplementation on the plasma lipid and glucose profile of mice with metabolic syndrome. Mice homozygous for the diabetes spontaneous mutation (Lepr<sup>db</sup>) were studied. These mice meet the criteria for metabolic syndrome, which include: dyslipidemia, insulin resistance and obesity [46, 47]. Lepr<sup>db</sup> mice become identifiably obese around 3–4 wk of age. Elevations of plasma insulin begin at 10–14 d and of blood sugar at 4–8 wk. Homozygous mutant mice are polyphagic, polydipsic, and polyuric. Exogenous insulin fails to control blood glucose levels, and gluconeogenic enzyme activity increases. The influence of niacin-bound chromium supplementation on the transcriptome of subcutaneous fat of these mice was examined using high-density, comprehensive, whole mouse genome expression arrays.

1.1.5.1 Introduction to genome expression arrays

Since their inception in the 1990s, DNA microarray technologies have evolved rapidly to become a key high-throughput technology for the simultaneous measurement of the relative expression levels of thousands of
individual genes [48]. The applications for which this technology has been adopted are numerous and include gene discovery, disease diagnosis, drug discovery (pharmacogenomics), and toxicological research (toxicogenomics). Despite the widespread adoption of DNA microarray technology, there remains some skepticism regarding data obtained using this technology. Data originating from these assays can exhibit unexplained variations between experiments and platforms, and the multitude of methods available to analyze the data can result in drastically different interpretations of the same dataset. The complex nature of a microarray experiment introduces many potential sources of variability. These include but are not limited to (A) image acquisition and image processing, (B) data normalization and mining analyses, and finally (C) annotation and interpretation of results. Taken together, these potentially confounding factors must be carefully considered in the design and implementation of a bifactorial gene microarray experiment. The final introductory sections define potential limitations, pitfalls and solutions of microarray experimentation.

1.1.5.2 Array and probe design, limitations

All DNA microarrays are based on the hybridization of experimental nucleic acid molecules to a synthesized array of oligonucleotide probes that correspond to particular gene targets. Probes are deposited onto a suitable
substrate by either direct contact-spotting, ink-jet deposition, or in situ synthesis. For the sake of focus and brevity, this discourse will focus on the Affymetrix GeneChip® products used by our laboratory and discussed in the subsequent study. In the case of GeneChip microarrays, multiple 25-mer oligonucleotide probes are synthesized in situ on quartz wafers using a photolithographic process. Wafers are then divided to form up to hundreds of GeneChip arrays that can accommodate more than 40,000 distinct probe sets each. For example, in the case of the latest mouse genome array from Affymetrix, some 45,000 probe sets corresponding to more than 34,000 different genes are synthesized on each chip. Divergent probe sets corresponding to the same gene product are commonly used to improve accuracy and reduce the likelihood that results are due to non-specific hybridization. Technologies associated with in situ synthesis (i.e. Affymetrix) give high feature shape uniformity; however the synthesis of the probes is not 100% efficient, which results in the presence of truncated sequences [48]. Consequently, the features are comprised of a heterogeneous population of truncated synthesis products that do not share ideal hybridization characteristics. Less than ideal hybridization with truncated probes may partially contribute to the observed trend for underestimated fold changes in differential expression of genes in microarray analysis as compared to real-time PCR analysis. Other microarray platforms, such as robotic printing of purified oligonucleotides, do not share this shortcoming; however they have other undesirable outcomes such as irregular probe distribution [49].
1.1.5.3 Array imaging, pitfalls and solutions

Beyond the inherent limitations of synthesizing microarray chips, other considerations must be accounted for when designing a GeneChip experiment related to image acquisition and image processing. To acquire and analyze the data of a GeneChip microarray experiment, a high resolution scanner is used to measure the fluorescent light intensity emitted by the labeled cRNA bound to the probe arrays. Upon successful hybridization and subsequent fluorescent excitation, the array will emit a pattern of light that correlates to their respective hybridized probes and consequently their associated gene products. The current software used by our laboratory for acquisition and analysis of the image produced by hybridization is GeneChip Operating Software (GCOS). This software is an updated, all inclusive, version of the software described for use in the study, Affymetrix Microarray Suite 5.0 (MAS) and Data Mining Tool 2.0 (DMT). The GCOS, and previously MAS, software incorporates an alignment algorithm that uses the checkerboard image of control probes, located at the corners of the probe array, to superimpose a grid on the scan image. The software algorithm aligns the grid so that each square in the grid delineates a probe cell. Furthermore, probe intensities are calculated on the basis of the grid alignment; thus making it critical that the grid is properly arranged. The software algorithm will occasionally misalign the grid; thereby affecting image analysis and the miscalculation of probe
intensities. Fortunately, it is possible to correct for this deficit using the software to manually arrange the grid such that the checkerboard control probes are correctly aligned in each of the four corners of the chip. In addition to proper grid alignment, it is also important to check the raw image files (DAT files) for scratches, dust or debris on the surface of the chip. While Affymetrix maintains strictly controlled environments in the synthesis of their chips, some artifacts may already be present or introduced after handling the chips that upon image analysis interferes or significantly alters the probe intensity values. If artifacts are found, the affected grids can be masked to prevent skewing of other data on the chip, but little can be done to recover the affected probe data [47].

1.1.5.4 Raw data normalization

Once image acquisition and analysis of raw feature intensities has been successfully achieved, the resulting data must be normalized in order to compare gene expression data across multiple chips. Since the following research employs a bifactorial experimental design and each chip is representative of one experimental subject, all chips (generally 4/group: ie. 4 control and 4 treatment) must be normalized so that gene expression patterns can be compared between each subject and across each group. Prior to normalization, background noise correction is essential for determining
intensities that accurately reflect the amount of RNA present for each gene on an array. Normally, GeneChip software corrects for background variation by segmenting an image into 16 (by default) squares that cover the entire image. For each block, the lower 2% (by default) of the feature intensities for that block are averaged, and this average is subtracted from each feature in the block. One assumption implicit in this method is that feature-to-feature background variation is not significantly different. There are other methods to background correct and normalize data based on internal controls built into the array. In the Affymetrix GeneChip system, each gene is represented by 11-20 perfect match and mismatch pairs of probes, each probing a different region of the mRNA transcript, typically within 600 base pairs of the 3' end. The purpose of the mismatch pairs of probes is to detect the degree of non-specific hybridization in the determination of background noise. The robust multi-array analysis (RMA) method of Irizarry et al. [50] does not account for mismatch probes in the array. It employs perfect match probe intensity distributions for signal and background intensities respectively, and uses quantile normalization and a log-scale expression plus probe effect model that is fit to define the RMA expression estimate for each gene [51]. The more rigorous and widely accepted GC-RMA method describes an algorithm similar to RMA, but also incorporates the mismatch probes using a model based on GC content (GC-RMA) that accounts for higher binding affinities of GC rich probes to determine background noise correction and normalization.
1.1.5.5 Data mining and gene annotation

Following background correction and normalization across all chips, data mining to determine differential gene expression between experimental groups can be performed. Under a bifactorial experiment design, one can employ Student’s $t$-test to determine if the mean gene expression value between control and treatment groups is statistically significant, assuming two normally distributed populations about their respective means and equal variances. To increase the statistical rigor of the test, one may also employ Bonferonni correction. The Bonferroni correction is a safeguard against multiple tests of statistical significance on the same data, where 1 out of every 20 hypothesis-tests will appear be significant at the alpha = 0.05 level purely due to chance. It states that if an experimenter is testing $n$ independent hypotheses on a set of data, then the statistical significance level that should be used for each hypothesis separately is $1/n$ times what it would be if only one hypothesis were tested. For example, when testing two hypotheses, instead of a $p$ value of 0.05, one would use a stricter $p$ value of 0.025. Finally, another manner in which a more selective set of differentially expressed genes may be selected for is by using a fold change cut-off value. For example, while statistically significant, a gene that is differentially expressed only 1.1 times greater in a treated group as compared to controls may not have significant biological relevance. Often times, a fold-change of greater than 1.1 (either up- or down-regulated) is used to attain a more biologically relevant
group of differentially expressed gene candidates. This latter approach is employed in our experimental design to improve upon the rigor of statistical analysis.

After differential gene candidates have been statistically determined, a final consideration one must undertake is that of gene annotation. While GeneChip microarrays offer the opportunity to simultaneously examine the expression levels of a large number of genes, the technology actually measures the relative abundance of comparatively short strands of nucleic acid sequence, which act as reporters for the gene under investigation. Therefore, the correct association of probe sequence with the sequence of the gene of interest is paramount to meaningful data generation. Unfortunately, the same gene sequence can be submitted to public databases from different sources, which may result in it being assigned different names or descriptions [52]. An array is therefore only as good as its probe annotation status, and in reality many arrays of both academic and commercial origin have been shown to have high proportions of incorrect probe assignment [53]. Fortunately, commercial probe annotation is frequently updated to address this potential problem though it is always in the best interest of the researcher to check for the latest annotation status.
1.2 Materials and methods

1.2.1 Animals and supplementation protocol

Mice homozygous for the diabetes spontaneous mutation ($Lepr^{db}$) become identifiably obese around 3–4 wk of age (Fig. 1.1). Elevations of plasma insulin begin at 10–14 d age and of blood sugar at 4–8 wk. We therefore chose to start our study at 10 wk of age. Homozygous mutant mice are polyphagic, polydipsic, and polyuric. A number of diabetes-related features are observed on the C57BLKS background, including an uncontrolled rise in blood sugar, severe depletion of the insulin producing -cells of the pancreatic islets, and death by 10 mo of age. Exogenous insulin fails to control blood glucose levels, and gluconeogenic enzyme activity increases [54, 55]. Male obese $Lepr^{db}$ diabetic mice ($n = 14$, BKS.Cg-m +/+ $Lepr^{db}$/J, stock 000642; Jackson Laboratories, Bar Harbor, ME) were received at 8 weeks of age and randomly divided into the following groups: supplemented with niacin-bound chromium ($n = 7$; NBC) and placebo control ($n = 7$; PBO). Mice were maintained under standard housing conditions at 22 ± 2°C with 12:12-h light-dark cycles. With an allowance of 2 wk for environmental and trainer handling acclimation, mice began a supplementation regimen of either NBC suspended in water at a concentration of 10 mg/kg bw or a matching volume of water that served as PBO at 10 wk of age. NBC used for the supplementation in this study was identical to the preparation examined in human studies [39]. NBC containing 10% elemental chromium in a fine-mesh powder (CM-100M) was
obtained from InterHealth Nutraceuticals (Benicia, CA). The dose of NBC to supplement was determined on the basis of two factors: 1) a small pilot study with a smaller dose of 2 mg·kg$^{-1}$·d$^{-1}$ and shorter supplementation time of 4 wk revealed just less than statistically significant changes in phenotypic outcomes, and 2) a review of literature indicating that human chromium consumption that exceeds the adequate daily dietary intake is safe [56-59]. Consistently, we saw no signs of overt toxicity in this *in vivo* study.

Throughout the course of treatment, all mice had access to standard mouse chow (Harlan) and water *ad libitum*, with the exception of 12 h before blood glucose and lipid profiling and 6 hours before the oral glucose tolerance test. Oral gavaging was implemented using a 22G feeding needle from Popper and Sons (New Hyde Park, NY). Mice were gavaged 5 days/wk for a period of 10 wk. The body weight of mice, by which the dose of NBC was determined, was obtained at baseline (i.e., start of study) and every 2 wk throughout the course of study. Total volume of the aqueous supplement throughout the study averaged 146 ± 20μl. Following 10 wk of supplementation, mice were euthanized, and subcutaneous fat was isolated for RNA extraction and microarray analysis.
1.2.2 Blood glucose and lipid profiling

Blood glucose and lipid panel profiling was performed at baseline before any supplementation (wk 0) and at wk 6. Following a 12 h fast, mice were briefly anesthetized with isoflurane to acquire 100 μl of blood via the retroorbital vein. The blood samples were subjected to glucose and lipid profile analyses using a clinical CardioChek analyzer (Polymer Technology Systems, Indianapolis, IN). Parameters analyzed include blood glucose, total cholesterol, HDL cholesterol, triglycerides, LDL, and the total cholesterol-to-HDL cholesterol ratio.

1.2.3 Oral glucose tolerance test

On the eighth week of supplementation, all mice were subjected to an oral glucose tolerance test (OGTT). As a part of this test, blood was drawn after 6 hours of chow withdrawal. Baseline (t=0) blood glucose measurements were recorded using the CardioChek analyzer. Mice were then challenged with 1.5 mg/g body wt of D-glucose (Sigma) dissolved in water, and blood glucose levels were measured at 30, 60, and 120 min after glucose challenge.
1.2.4 GeneChip probe array analysis

Total RNA was isolated from subcutaneous fat by grinding the tissue under liquid nitrogen as previously described [60, 61]. RNA was purified using the RNeasy kit (Qiagen), and quality was checked using a Bio-Rad Experion RNA HighSens chip. Targets for microarray hybridization were prepared according to a previously described protocol [62]. Briefly, samples were hybridized for 16 h at 45°C to GeneChip Test-2 arrays to assess preparation quality. On verification of sample quality, the targets were hybridized to Affymetrix mouse genome arrays (430 v2.0) under the conditions listed above for the Test-2 arrays. The arrays were washed, stained with streptavidin-phycoerythrin, and then were scanned with the GeneArray scanner.

1.2.5 Microarray data analysis

Raw data were collected and analyzed using the Affymetrix Microarray Suite 5.0 (MAS) and Data Mining Tool 2.0 (DMT) software as described previously [60]. Additional processing of data was performed using the dChip software. A detailed analysis scheme has been illustrated (see Fig. 1.5). Absolute analysis was utilized to identify differentially expressed genes[60]. The t-test was performed using DMT on absolute files generated from MAS. Genes that significantly ($P<0.05$) changed (increased or decreased) in the supplemented group compared with the control group were selected. Next, the
dChip (v 1.3, Harvard University) software was used to further filter genes using the following criteria: 1) fold change>1.2; 2) $t$-test, $P<0.05$; and 3) present call in all experimental (NBC) samples for upregulated genes, and present call in all baseline (PBO) samples for downregulated genes. For data visualization, genes filtered using the statistical ($t$-test) approach were subjected to hierarchical clustering using the dChip (v1.3) software [60].

1.2.6 Real-time RT-PCR analysis

Expression levels of candidate genes (calsequestrin-1, CASQ1; tropomyosin-1, TPM1; enolase-3, ENO3; glucose phosphate isomerase, GPI1; uncoupling protein-1, UCP1; cell death-inducing DNA fragmentation factor, CIDEA; tocopherol transfer protein, TTP) and GAPDH mRNA were independently determined using real-time RT-PCR. In brief, total RNA (5μg) was reverse transcribed into cDNA using oligo-dT primer and Superscript II. RT-generated DNA was quantified by real-time PCR assay using double-stranded DNA binding dye SYBER Green-I as described previously [60]. Individual gene primer sequences are listed in Table 1.1.
1.2.7 Data presentation and analysis

Data are shown as means ± SD. Differences between means were tested using Student’s t-test or ANOVA, as appropriate. Difference between means was considered significant at \( P < 0.05 \).

1.3 Results and discussion

1.3.1 Effect of NBC supplementation on body weight

Oral supplementation of NBC to the obese diabetic mice for 10 weeks had no significant effect on bw compared with PBO fed controls (Fig. 1.2). While subjected to gavaging, animals were observed for early removal criteria such as mutilation, extreme lethargy, guarding, and weight loss of greater than an average of 10% bw/wk. On the basis of these early removal criteria, no animals had to be pulled from the study. Furthermore, chow and water consumption were monitored daily during gavaging, and no discernable difference was observed in the consumption between experimental and control groups. There were no overt differences in phenotypic body composition observed between NBC-supplemented and PBO mice following the 10 wk of oral gavaging.

Metabolic syndrome is associated with numerous cerebrovascular risk factors including obesity, diabetes mellitus and dyslipidemia. Both insulin
resistance and defective insulin secretion appear very prematurely in obese patients, and both worsen similarly toward diabetes [63]. There is scant evidence suggesting that supplemental chromium may cause weight loss [42]. Recently, a meta-analysis was performed to assess the evidence of chromium supplements for reducing bw. The results indicated a relatively small effect of chromium picolinate compared with placebo for reducing bw [64]. This outcome is not surprising, because it is well accepted that effective strategies of weight loss require management approaches in a combined approach of dietary therapy and physical activity by using behavioral interventions [65]. Thus controlled studies testing the effect of chromium alone may not generate overt changes in body weight outcomes. Interestingly, NBC supplemented daily over 2 months by African American women undergoing a modest dietary and exercise regimen influenced weight loss and body composition [39]. Such encouraging effects may be attributed to the combinatorial therapeutic approach adopted including supplementation, diet, and exercise. Although the current experimental design did not affect bw, it was powerful for examining the specific effects of NBC by itself in vivo.

1.3.2 Effect of NBC supplementation on plasma lipid profile

Metabolic syndrome is associated with hypertension and a greater risk for cerebrovascular disease as a result of elevated levels of circulating total
cholesterol, low-density lipoproteins (LDL), and triglycerides, which are known to induce atherosclerosis. While NBC supplementation had no significant effect on age-dependent weight gain in the Lepr\textsuperscript{db} obese diabetic mice, there were significant differences in the blood lipid profiles between NBC- and PBO supplemented groups (Fig. 1.3). Lepr\textsuperscript{db} mice supplemented with NBC had significantly (20%) lower total cholesterol, higher (25%) high-density lipoprotein (HDL) cholesterol, lower (54%) LDL cholesterol, and lower (43%) triglyceride levels compared with PBO-gavaged mice following 6 wk of supplementation. The total cholesterol (TC)-to-HDL ratio (TC:HDL) is frequently used to assess the risk of heart disease [66]. Following 6 wk of oral supplementation, mice treated with NBC had a significantly lower (37%) TC:HDL ratio compared with mice in the PBO group orally gavaged with a matching volume of water.

Obese diabetic mice are known to have significantly higher levels of TC, LDL, and triglycerides [67]. Metabolic syndrome as defined by the American Heart Association is characterized by a group of metabolic risk factors in any one individual that include abdominal obesity, high triglycerides, low HDL cholesterol, high LDL cholesterol, and insulin resistance or glucose intolerance [68]. The mice used in the current study represent a powerful tool to model the metabolic syndrome [46, 47]. NBC supplementation clearly improved the lipid profile of the blood plasma. Lower TC, higher HDL cholesterol, lower LDL cholesterol, and lower triglyceride levels in response to
NBC were highly significant findings. These findings are consistent with the literature on humans and experimental rodents. Twelve weeks of chromium supplementation has been observed to lower levels of blood plasma LDL cholesterol and lower levels of TC as well as lower HDL cholesterol and triglyceride in rats [69]. Chromium has been implicated as a cofactor in the maintenance of normal lipid and carbohydrate metabolism. In humans, high dietary intake of chromium over a long period lowered TC, triglyceride, and hemoglobin A(1C) in blood [70]. Two mo of chromium supplementation resulted in a clinically useful increase in HDL cholesterol levels in men taking beta-blockers [71]. In a double-blind crossover study with humans, it was noted that levels of TC, LDL cholesterol, and apolipoprotein B (the principal protein of LDL) decreased significantly while the subjects were supplemented with chromium. The concentration of apolipoprotein A-I, the principal protein of HDL fraction, increased during the treatment. The HDL cholesterol level was elevated slightly, but not significantly, during chromium supplementation. In contrast, only apolipoprotein B was altered significantly during supplementation with the placebo. In a double-blind, placebo controlled trial with obese diabetic patients, it was reported that, following 6 mo of NBC supplementation, significant improvements were found in LDL levels, total-to-HDL cholesterol ratios, and TC levels [72]. These observations support the assertion that dietary chromium supplementation is efficacious in lowering blood lipids in humans.
1.3.3  Effect of NBC supplementation on glucose clearance

In the present study, no difference was observed in fasting blood glucose levels of supplemented and control Lepr\textsuperscript{db} mice following 6 weeks of NBC supplementation (Fig. 1.4A). Eight wk into the study, an OGTT was implemented to elucidate the effects of NBC supplementation on carbohydrate metabolism in obese diabetic mice. After a glucose challenge of 1.5 mg/g bw, mice that were supplemented with NBC had no significant difference in blood glucose challenge response (0 min) or the rate of clearance at 30 and 60 min compared with PBO-supplemented mice (Fig. 1.4B). From 60 to 120 min, however, there was a decline in the rate of blood glucose clearance, as NBC-supplemented mice had a significantly greater reduction in the percent change of blood glucose compared with PBO-gavaged control mice.

Although NBC supplementation had a significant influence on lipid profile of the blood plasma, blood glucose was not influenced. The Lepr\textsuperscript{db} mouse model is characterized by an uncontrolled rise in blood sugar, severe depletion of the insulin-producing β-cells of the pancreatic islets, and death by 10 mo of age. Exogenous insulin fails to control blood glucose levels, and gluconeogenic enzyme activity increases. Under such severe genetic conditions, it is understandable that the dietary supplement NBC was not effective in changing blood glucose levels. The marginal effect of NBC on OGTT suggests improved clearance of glucose in treated mice. This indication is consistent with the current literature demonstrating that supplemental
chromium may ameliorate hyperglycemia after a glucose load [73]. Results of a human study show that dietary chromium supplementation raised HDL cholesterol and improved insulin sensitivity in those with evidence of insulin resistance but normal glucose tolerance [74].

1.3.4 Response of adipose tissue transcriptome to NBC supplementation

This work represents the first to investigate the genome-wide effects of chromium supplementation in a rodent model of obesity and diabetes. To achieve a comprehensive analysis of adipose tissue gene expression profiling after 10 weeks of either NBC or PBO supplementation, high-density DNA microarray analysis was performed. The overall objective of the data mining approach was the elucidation of candidate genes within the adipose tissue that were sensitive to oral NBC supplementation. Figure 1.5 illustrates the data analysis scheme adopted. This design is consistent with previous studies by our laboratory [60, 61]. Of the 45,101 probe sets analyzed, only a very small and consistent subset was up- or down-regulated in response to NBC supplementation. This reflects a specific effect of oral NBC and argues against a genome-wide perturbation caused by the nutritional supplement. The dChip software enabled graphic visualization of the tight consistency of the NBC-sensitive genes in the subcutaneous adipose tissue (Fig. 1.6). A sample of
NBC-sensitive up-regulated and down-regulated genes are listed in table 1.2 and 1.3 respectively.

Validation of DNA microarray data was carried out by real-time PCR analysis of select candidate genes. Genes of interest were chosen on the basis of a significant fold change >1.2. While accurately predicting the direction of the fold change, we have previously reported that DNA microarray analyses tend to underestimate the degree of gene induction compared with real-time PCR analysis [60, 61]. This property is evident in the present study’s validation of both up-regulated and down-regulated genes by real-time PCR (Figs. 1.7 and 1.8). Interestingly, a large number of genes up-regulated by a 1.6-fold change or greater in adipose tissue were myogenic in nature. NBC supplementation up-regulated the expression of the following genes: CASQ1, a Ca^{2+} mediator for muscle contraction; TPM1, a myosin regulator; and both ENO3 and GPI1, key enzymes necessary for glycolysis. Findings from quantitative real-time PCR assay were consistent with the results of the DNA microarray analysis (Fig. 1.7). Real-time PCR validation of the down-regulated genes CIDEA, UCP1, and TTP also was consistent with the DNA microarray results (Fig. 1.8).

Adipose cells and skeletal myoblasts are derived from a common mesodermal stem cell, indicating that both cells have a closer relationship in the developmental lineage than the other somatic cells. Recently, it has been demonstrated that cells from adipose tissue exhibit mesenchymal plasticity
such that they display skeletal myogenic potential [75]. This is consistent with the observation that preadipocytes are capable of myogenic differentiation [76]. Adipose tissue is known to contain pluripotent mesenchymal cells that are capable of differentiating along the myogenic lineage [77]. Adipose tissue, like bone marrow, is derived from the embryonic mesenchyme and contains a stroma. Stem cell population within the adipose stromal compartment, also known as processed lipoaspirate cells, may differentiate toward a myogenic lineage [78]. Indeed, a myogenic fate of adipose stromal cell-derived common pluripotent stem cells has been reported [79]. MyoD exerts a master transcriptional control over the myogenic differentiation cascade. Studies of organotypic cultures of fat tissue and a long-term culture of in vitro differentiated adipocytes demonstrate that MyoD provokes morphological changes in mature adipocytes that can be summarized as loss of fat content, acquisition of a fusiform shape, and eventual fusion with committed neighbor cells. In vivo, MyoD gene transfer into rat fat pads demonstrated that, while structural proteins of muscle lineage were expressed, they coexisted with specific adipocyte proteins [80]. Unbiased genome-wide interrogation of the effect of dietary NBC on the transcriptome of the subcutaneous adipose tissue posits that the supplement resulted in the upregulation of muscle-specific gene expression. Whether NBC triggered a myogenic response in the fat tissue is an interesting, testable hypothesis that resulted from our microarray studies. NBC supplementation had an overall positive impact on the fat genome, where it induced many more genes than it repressed. Genes encoding proteins
involved in glycolysis, muscle contraction, muscle metabolism, and muscle development were specifically up-regulated in response to NBC supplementation. Expression of muscle-specific genes in the fat tissue is known, over time, to diminish the fat content of the tissue. This transdifferentiation process is well tolerated by the fully differentiated and mature adipocytes [80].

Enolase, or ENO3, was the most sensitive among all genes induced in the fat tissue in response to NBC supplementation. Enolase is a dimeric glycolytic enzyme exhibiting tissue-specific isoforms. The enzyme catalyzes the interconversion of 2-phosphoglycerate and phosphoenolpyruvate. In adult human muscle, >90% of enolase activity is accounted for by the β-enolase subunit, the protein product of the ENO3 gene [81]. The transcription of ENO3 is regulated by an intronic muscle-specific enhancer that binds myocyte-specific enhancer factor-2 proteins and ubiquitous G-rich box-binding factors [82]. Deficiency in β-enolase leads to myopathy [81]. In the order of the magnitude of change, the next NBC-sensitive gene in the fat tissue was calsequestrin. Calsequestrin is by far the most abundant Ca\(^{2+}\)-binding protein in the sarcoplasmic reticulum of skeletal and cardiac muscle. It allows the Ca\(^{2+}\) required for contraction to be stored at total concentrations of up to 20 mM, while the free Ca\(^{2+}\) concentration remains at ~1 mM. This storage capacity confers on muscle the ability to contract frequently with minimal run-down in tension [83]. Chromium is known to induce the plasmalemmal Ca\(^{2+}\)-ATPase in
smooth muscle cells, raising the possibility that bioactive chromium modulates calcium metabolism [84]. Indeed, chromium has been shown to reduce intracellular Ca\(^{2+}\) concentration loads in vascular smooth muscle cells and thereby reduce peripheral vascular resistance in insulin-resistant states [85]. Some of the other most-affected genes that were induced in response to NBC supplementation in the fat tissue include actin and tropomyosin-1. Actin plays a dynamic role in muscle contraction and many cellular motility events that occur when the motor domain of myosin uses the energy of ATP hydrolysis to move along the actin filament [86]. Tropomyosin, in association with the troponin complex, plays a central role in the Ca\(^{2+}\)-dependent regulation of striated muscle contraction. Skeletal isoforms are composed of two types of subunits, α and β [87]. TPM1 encodes tropomyosin-1 or α-tropomyosin [88]. Finally, glucose phosphate isomerase (GPI) is another glycolytic enzyme that is responsible for the conversion of glucose-6-phosphate to fructose-6-phosphate. It has been reported that glycolytic genes, such as GPI and ENO3, are down-regulated in the visceral adipose tissue of morbidly obese patients compared with non-obese counterparts [89]. Increased GPI expression in subcutaneous fat in response to NBC supplementation is consistent with a myogenic induction of adipose tissue, as GPI is highly expressed in skeletal muscle [90]. Genes in the adipose tissue that were down-regulated in response to NBC supplementation included CIDEA and UCP1, which represent key components involved in the thermogenic role of brown adipose tissue [91, 92]. The UCP1 gene is uniquely expressed in brown adipose tissue
and uncouples ATP generation and dissipates the energy as heat [93]. White adipose tissue contributes to UCP1-independent thermogenesis [94]. Brown adipose tissue expresses the thermogenic UCP1. UCP1 is positively regulated by peroxisome proliferator-activated receptor (PPAR) agonists and retinoids through the activation of the heterodimers PPAR/retinoid X receptor (RXR) and retinoic acid receptor (RAR)/RXR and binding to specific elements in the UCP1 enhancer [95]. UCP1 and adipocyte-specific genes are down-regulated by extracellular-regulated kinases and p38 mitogen-activated protein kinase-dependent pathways [96]. The expression of UCP1 seems to be tightly related to adipocyte growth [97]. Cell death-inducing DNA fragmentation factor-α (DFFA)-like effector A (CIDEA) belongs to a family of pro-apoptotic proteins that has five known members in humans and mice. CIDEA regulates energy balance and adiposity. CIDEA-null mice are resistant to obesity and diabetes [92]. CIDEA mRNA is expressed in white human fat cells and in brown mouse adipocytes. Lowering of CIDEA gene expression by the RNA interference approach stimulates lipolysis [98]. Both UCP1 and CIDEA are regulated by similar pathways because both genes are down-regulated by TNFα [96, 98]. The pathways involved in NBC-dependent down-regulation of UCP1 and CIDEA remain unknown. Tocopherol transfer protein (TTP) is the primary carrier of α-tocopherol from the liver to peripheral organs including adipose tissue [99]. Expression of TTP in subcutaneous fat is high, as adipose tissue serves as a reservoir for >90% of the lipid-soluble vitamin stored in the body [100]. α-Tocopherol is a potent lipid-phase antioxidant. Down-regulation of
TTP expression is expected to lower the levels of α-tocopherol in adipocytes compromising their lipid-phase antioxidant defense mechanism.

1.4 CONCLUSIONS

In summary, this research presents direct evidence establishing that dietary supplementation of NBC is effective in improving the plasma lipid profile in mice demonstrating metabolic syndrome (Lepr<sup>db</sup> mutation). NBC supplementation did not influence age-dependent gain in body weight of the Lepr<sup>db</sup> mice, but had a modest effect on glucose clearance following challenge. Unbiased genome-wide interrogation of the transcriptome of the subcutaneous adipose tissue led to the hypothesis that NBC triggered a myogenic response in the fat tissue. Genes encoding proteins involved in glycolysis, muscle contraction, muscle metabolism, and muscle development were specifically up-regulated in response to NBC supplementation. Genes in adipose tissue that were down-regulated in response to NBC supplementation were fewer than those that were induced. Genes in this category included CIDEA and UCP1, which are known to be regulated by common pathways. The observation that CIDEA-null mice are resistant to obesity and diabetes indicates that the inhibitory role of NBC on CIDEA expression is favorable. Future studies testing the molecular basis of NBC function and long-term outcomes in the treatment of metabolic syndrome are warranted. Furthermore,
the positive effect of chromium supplementation on the plasma lipid profile deserves additional examination in the context of metabolic syndrome comorbidities, such as stroke. In a broader context, this research demonstrates the effectiveness for a nutritional intervention of metabolic syndrome. While NBC supplementation did not appear to have a direct effect on obesity as a risk factor, the positive effects of supplementation on other risk factors of metabolic syndrome, such as plasma lipid profile, indicate that it has therapeutic potential.
1.5 Tables

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primer Sequence 5’ to 3’</th>
</tr>
</thead>
</table>
| CASQ1 | CTTGACAAGGTGGCAA  
         | CAGTTTCCTCAGGGTTGATCTCCT                                      |
| TPM1  | ACCGGAGCAAGCAGCTGGAA  
         | GCACGATCCAACTCCTCCTCAA                                         |
| ENO3  | CCTGTGCCTGCCCTTTAATGTGA  
         | CGTCCTTCCCTACCTGGCCTT                                         |
| UCP1  | CCGCTACACGCGGACCTACAAT  
         | CCGGCAACAAGAGCTGACAGTAA                                       |
| CIDEA | GTCATCACAACTGGCCTGGTTACG  
         | GCCCTGTATAGGTCGAAGGTGACTCT                                     |

CASQ1, cassequestrin 1; TPM1, tropomyosin 1; ENO3, enolase 3; UCP1, uncoupling protein 1; CIDEA, cell death-inducing DNA fragmentation factor

Table 1.1 | Primer sequences for real-time PCR analysis
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>FC Average</th>
<th>FC SD</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enolase 3</td>
<td>7.6</td>
<td>3.6</td>
<td>glycolysis</td>
</tr>
<tr>
<td>Calsequestrin 1</td>
<td>4.6</td>
<td>2.0</td>
<td>regulation of muscle contraction</td>
</tr>
<tr>
<td>Actin</td>
<td>4.4</td>
<td>1.7</td>
<td>muscle contraction</td>
</tr>
<tr>
<td>Tropomyosin 1</td>
<td>3.5</td>
<td>1.3</td>
<td>muscle development</td>
</tr>
<tr>
<td>GTPase-activating RANGAP domain-like 1</td>
<td>3.3</td>
<td>1.0</td>
<td>GTPase activator activity</td>
</tr>
<tr>
<td>Protease inhibitor 16</td>
<td>3.1</td>
<td>1.1</td>
<td>peptidase activity</td>
</tr>
<tr>
<td>Aspartate beta hydroxylase</td>
<td>2.9</td>
<td>0.6</td>
<td>macromolecule metabolism</td>
</tr>
<tr>
<td>Endothelin receptor type B</td>
<td>2.8</td>
<td>0.8</td>
<td>G-coupled protein receptor activity</td>
</tr>
<tr>
<td>Talin 1</td>
<td>2.8</td>
<td>0.4</td>
<td>actin binding</td>
</tr>
<tr>
<td>Sarcalumenin</td>
<td>2.4</td>
<td>0.7</td>
<td>calcium ion binding</td>
</tr>
<tr>
<td>Thromobospondin, type 1, domain 2</td>
<td>2.3</td>
<td>0.5</td>
<td>protein amino acid phosphorylation</td>
</tr>
<tr>
<td>Nebulin-related anchoring protein</td>
<td>2.1</td>
<td>0.3</td>
<td>ion transport</td>
</tr>
<tr>
<td>Glucose phosphate isomerase 1</td>
<td>1.6</td>
<td>0.3</td>
<td>glycolysis</td>
</tr>
</tbody>
</table>

FC, fold change; SD, standard deviation.

**Table 1.2 | List of genes in subcutaneous fat tissue that were up-regulated in response to oral supplementation of niacin-bound chromium**
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>FC Average</th>
<th>FC SD</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDHD domain containing 1</td>
<td>2.7</td>
<td>0.30</td>
<td>lipid catabolism</td>
</tr>
<tr>
<td>Uncoupling protein 1</td>
<td>2.7</td>
<td>1.00</td>
<td>mitochondrial transport</td>
</tr>
<tr>
<td>Neuroepithelial cell transforming gene 1</td>
<td>2.2</td>
<td>0.70</td>
<td>guanyl-nucleotide exchange factor activity</td>
</tr>
<tr>
<td>Cell death-inducing DNA fragmentation factor</td>
<td>2.1</td>
<td>0.02</td>
<td>lipid metabolism</td>
</tr>
<tr>
<td>Betacellulin</td>
<td>1.9</td>
<td>0.49</td>
<td>growth factor activity</td>
</tr>
<tr>
<td>Phosphoenolpyruvate carboxykinase 1</td>
<td>1.8</td>
<td>0.04</td>
<td>gluconeogenesis</td>
</tr>
<tr>
<td>Tocopherol transfer protein</td>
<td>1.4</td>
<td>0.13</td>
<td>vitamin E transport</td>
</tr>
</tbody>
</table>

FC, fold change; SD, standard deviation.

**Table 1.3** | List of genes in subcutaneous fat tissue that were down-regulated in response to oral supplementation of niacin-bound chromium
1.6 Figures

Figure 1.1 | Lepr\textsuperscript{db} KO mouse phenotype. Male Lepr\textsuperscript{db} +/- and +/- mice were imaged at 7 wk of age to compare the noticeable phenotypic difference in girth. Lepr\textsuperscript{db} +/- mice are noticeably obese as compared to their heterozygous counterparts and develop insulin resistance similar to humans with type II diabetes.
Figure 1.2 | Body weight in response to niacin-bound chromium (NBC) supplementation. Two groups of obese diabetic mice (n=7) were orally gavaged 5 d/wk with either NBC (10mg/kg body wt, □) or a matched volume of water (♦). Individual mouse weight was recorded every 2 wk. Data represent mean weight of mice over time (A), mean weekly weight gained (B), and mean cumulative weight gained for duration of supplementation (C) ± SD.
Figure 1.3 | Plasma lipid profile. Lipid profile of the blood plasma was analyzed at baseline (wk 0) and following 6 weeks of oral gavaging with either NBC (10 mg/kg, solid bars) or a matching volume of placebo water (PBO; open bars). Blood (50 μl) collected from the retroorbital vein was used to quantify the following parameters: cholesterol (A), HDL cholesterol (B), LDL cholesterol (C), total cholesterol (TC)-to-HDL ratio (D), and triglycerides (E). *P<0.00005.
Figure 1.4 | Blood glucose and glucose clearance. (A) Blood glucose was determined at baseline (week 0) and following 6 wk of oral gavaging with either NBC (10 mg/kg; solid bars) or a matching volume of placebo water (PBO; open bars). (B) After 8 wk of supplementation, mice administered PBO and NBC were fasted for 6 h, after which they were orally gavaged with an aqueous solution of glucose (1.5 mg/g body wt). Blood samples were collected at administration of glucose challenge and 30, 60, and 120 min thereafter to compare the blood glucose clearance rate between NBC-supplemented (□) and PBO controls (♦). Data represent mean % change of blood glucose between time points. Blood glucose value before fasting was used for baseline reference point. *P<0.005: significant difference between PBO and NBC-supplemented groups.
Figure 1.5 | GeneChip data analysis scheme. Data processing was primarily performed using Microarray Suite 5.0 (MAS) and Data Mining Tool v2.0 (DMT) softwares. Additional data filtration was performed using dChip with the following criteria: 1) fold change > 1.2; 2) t-test, P<0.05; and 3) present call in all experimental (NBC supplemented) samples for upregulated genes. ↑ Increases; ↓ decreases in response to NBC supplementation (10 mg/kg body wt).
Figure 1.6 | Cluster images illustrating genes sensitive to NBC in the subcutaneous fat tissue. Student’s t-test was performed on data from NBC – and PBO-supplemented groups in subcutaneous fat. The genes that significantly (P<0.05) changed between the two groups compared were selected and subjected to hierarchical clustering using dChip software as described in Fig. 1.5. Up-regulated (A) and down-regulated genes (B) in NBC-supplemented compared with PBO (baseline) group.
Figure 1.7 | Quantitative expression analyses of genes up-regulated in response to NBC supplementation. CASQ1, calsequestrin-1; TPM1, tropomyosin-1; ENO3, enolase-3; GPI1, glucose phosphate isomerase-1. *P<0.05.
Figure 1.8 | Quantitative expression analyses of genes down-regulated in response to NBC supplementation. CIDEA, cell death-inducing DNA fragmentation factor; UCP1, uncoupling protein-1; TTP, tocopherol transfer protein. *P<0.05.
CHAPTER 2

NUTRITIONAL INTERVENTION OF ACUTE ISCHEMIC STROKE BY THE
NATURAL VITAMIN E \( \alpha \)-TOCOTRIENOL

2.1.1 Bioavailability and tissue distribution of vitamin E isoforms

2.1.1.1 Background

Chapter 1 introduced a dietary factor, niacin-bound chromium, which improved the lipid profile of mice with metabolic syndrome. Metabolic syndrome is defined by a host of cardio and cerebrovascular risk factors that contribute to stroke. It has been reported that the risk of ischemic stroke is 3-fold higher in patients with metabolic syndrome [101]. Chapter 2 focuses on the remarkable finding by our laboratory that a naturally occurring vitamin E isoform, \( \alpha \)-tocotrienol, reduces the infarct volume caused by acute ischemic stroke. Vitamin E is a generic term for a family of compounds most cited for their antioxidant capacity. Research by this candidate on the natural vitamin E isoform \( \alpha \)-tocotrienol (\( \alpha \)T3) has revealed neuroprotective properties in models
of acute ischemic stroke independent of antioxidant function and not shared by other naturally occurring family members [102, 103]. Tissue distribution of the vitamin E analogs is isoform specific, as are the physiologically relevant tissue concentrations of αT3 necessary to confer neuroprotection. These factors and more must be taken into consideration for the development of a dietary vitamin E supplementation strategy for the prevention of stroke. Chapter 2.1 represents the candidate’s research efforts to elucidate the unique tissue distribution profiles of the naturally occurring vitamin E analogs in order to support the relevance of αT3 supplementation as a prophylactic stroke therapeutic.

2.1.1.2 History of vitamin E

The discovery of vitamin E, as compared to the accounts of vitamins A, B, C, and D described in Chapter 1, was uniquely different in that deficiency in animal models varied from species to species and a specific relevance to human deficiency took several decades to realize. Herbert M. Evans is credited with the discovery of vitamin E in 1922, when he found that a particular “antisterility factor x” was necessary for reproduction in rats [104]. Pregnant female rodents kept on a rancid lard diet would resorb their embryos, but by supplementing their diet with the chlorophyll-rich oil of lettuce leaves, Evans reported that he could restore fertility. Knowing that the antisterility
factor $x$ was a lipid soluble compound, he quickly resolved that his supplement was uniquely different from the other two known lipid soluble vitamins of the time: vitamin A and vitamin D. Two years later, a chemistry professor from the University of Arkansas by the name of Barnett Sure named the fertility factor “vitamin E”, suggesting Evans’ antisterility factor was also essential to humans despite no evidence yet to support such a claim. In 1936 Evans published the chemical formula of vitamin E in the Journal of Biological Chemistry [105].

Ascribing the chemical name of the compound was given to a colleague of Evan’s at The University of California at Berkeley, professor of Greek, George M. Calhoun. Evan’s personal account of the name’s origin from Dr. Calhoun gives humorous insight to what would otherwise be considered a prodigious effort:

“Most scientists, medical men especially,” said Calhoun, “have been guilty of coining Greek-Latin terms, bastards, of course, and we might have to do this.” “What does the substance do?” he asked. “It permits an animal to bear off-spring,” I replied. “Well, childbirth in Greek is tocos,” he said, “and if it confers or brings childbirth, we will next employ the Greek verb phero. You have also said that the term must have an ending consonant with its chemical – ‘ol’, it being an alcohol; your substance is ‘tocopherol,’ and the pleasant task assigned me quickly solved and not worth the delightful four-course dinner you have arranged.” [106]
The relevance of vitamin E to humans proved to be a difficult problem to solve until the late 1950’s with the advent of the free-radical theory of lipid peroxidation [107]. According to this free-radical theory, membrane lipids in all membranes of the body should be susceptible to oxidative damage, and the exact location of damage observed in different animal species would depend upon variables such as specific membrane lipid composition, vitamin E content, and cellular metabolic rate. While it had already been known that vitamin E played an antioxidant role in protecting plant oils from oxidative damage and rancidity, this theory pioneered the concept of oxidative stress and the importance of antioxidants such as vitamin E as related to lipids in mammalian systems. It also provided reasoning for the variable effects of vitamin E deficiency across species, such as infertility in rodents and muscular dystrophy in ducks, as each species has different lipid compositions and requirements. Decades later, the human relevance of vitamin E deficiency became recognized in the medical field in cases of fat mal-absorption or the absence of low-density lipoprotein (LDL), a lipid rich transporter of vitamin E in the blood. Progressive neuropathy and retinopathy leading to crippling ataxia and impaired vision were prevented by high-dose vitamin E supplementation in patients with the genetic absence of LDL [108].
2.1.1.3 The vitamin E family

As nutrition based research continued to refine the characterization of vitamin E, the discovery of additional isoforms and distinctly specific functions broadened the scope of its biological relevance. Today, vitamin E is a generic term used to describe a class of eight naturally occurring, lipid soluble antioxidants that have been broken down into two primary categories based on structure: tocopherols and tocotrienols. Tocopherols consist of a chromanol ring and a 15-carbon tail derived from homogentisate (HGA) and phytyl diphosphate, respectively. Condensation of HGA and phytyl diphosphate, the committed step in tocopherol biosynthesis, is catalyzed by HGA phytoltransferase (HPT) [109]. Conversely, homogentisic acid transferase (HGGT) catalyzes the committed step in tocotrienol biosynthesis. Tocotrienol structure differs only in the degree of saturation that occurs in the phytyl side chain of the molecule – with tocopherols having a saturated phytyl side chain and tocotrienols having a partially unsaturated (3 double bonds) phytyl side chain (Fig 2.1). Because of the unsaturations in the hydrocarbon tail, tocotrienols are thought to assume a unique confirmation with increased flexibility and the ability to induce a greater curvature stress on phospholipid membranes [110]. Within the two larger classes of vitamin E, four different isoforms exist: alpha (α), beta (β), gamma (γ) and delta (δ). These isoforms differ in the position and degree of methylation found on the chromanol ring.
Tocotrienols are the primary form of vitamin E in the seed endosperm of most monocots, including agronomically important cereal grains such as wheat, rice, and barley. Palm oil contains a significant quantity of tocotrienols, with up to 800mg/kg reportedly extractable from the fruits of *Elaeis guineensis* [111]. There are also noted examples of tocotrienol presence in a limited number of dicots, such as members of the *Apiaceae* family which includes cumin, parsley, carrot, cilantro, dill and fennel. Compared to tocopherols, however, tocotrienols are considerably less widespread in the plant kingdom with one report that in 80 different plant species studied, only 24 contained significant amounts of tocotrienol [112]. An analysis of tocopherol and tocotrienol compartmentalization revealed the presence of tocotrienols in non-photosynthetic tissues and organs, such as seeds, fruit and latex. No tocotrienols were detected in photosynthetic tissues. Conversely, tocopherols occur ubiquitously in plant tissue and are the exclusive form of vitamin E in leaves of plants and seeds of most dicots [112].

2.1.1.4 Dietary vitamin E and tissue distribution

The majority of tocopherols in Western (United States) diets are found in plant oils such as corn, soybean and peanut (4). Tocotrienols, in contrast, are predominantly found in Eastern diets, which are richer in palm oil and rice bran as compared to Western diets (8). Palm oil and rice bran contain the highest concentrations of tocotrienols, although both are still heavily enriched
with alpha-tocopherol as well. After consumption, all vitamin E isoforms are equally absorbed in the small intestine passively in association with dietary lipids (6). However, despite the fact that γ-tocopherol constitutes the majority of vitamin E tocopherols found in the Western diet oils, α-tocopherol (αTOC) is still preferentially absorbed and distributed to body tissue via tocopherol transfer protein (TTP), which has favored selectivity for αTOC over all other isoforms in the diet. The affinity of TTP for αTOC is 100%, compared to 12% and 9% for αT3 and γ-tocopherol respectively as determined by a study of competition for distribution from liposomes to membranes in-vitro (7). In this particular study, Hosomi et al, radiolabeled αTOC in the presence of other vitamin E forms in liposomes. The degree of competition between each alternative vitamin E isoform and radiolabeled αTOC for TTP tissue distribution was then calculated as the amount of radiolabeled αTOC transported in the presence of other isoforms relative to a no-competition control. The lack of relative specific affinity of TTP for αT3 led to the notion that availability of dietary αT3 to vital organs is negligible. Indeed it was reported that αT3, supplemented to laboratory chow, does not reach the brain [113].

Our laboratory’s striking observation that αT3, but not αTOC, is potently neuroprotective at nanomolar concentration in vitro led us to revisit tissue uptake of orally supplemented αT3 [114, 115]. In humans subjected to oral supplementation, plasma αT3 rises to a micromolar concentrations, 10 times
in excess of the concentration required for complete neuroprotection [114-
116]. The standard laboratory chow contains excessive amounts of αTOC but
negligible amounts of αT3 [117]. Long-term lack of αT3 in the diet may repress
any putative α3-transport mechanism in vivo. We sought to conduct a long-
term study examining the effects of αT3 or αTOC supplementation, either
alone or in combination, on tissue levels. To evaluate the significance of TTP
in αT3 delivery to tissues, we studied the tissue levels of αT3 in TTP-deficient
mice orally supplemented with αT3 on a long-term basis.

2.1.2 Materials and methods

2.1.2.1 Animals and supplementation protocol

Sprague-Dawley female rats (Harlan, Indianapolis, IN) were maintained
on vitamin E-deficient diet (TD 88163, Harlan) and divided into the following
four groups supplemented (5 days/week) with: (i) αT3 (5 mg/kg bw) (ii) αTOC
(5 mg/kg bw), (iii) αT3 + αTOC (2.5 + 2.5 mg/kg bw), and (iv) placebo vitamin
E-stripped corn oil (volume matched). These rats were identified as first
generation breeders. The female breeders received supplementation through
pregnancy. Supplementation, however, was suspended for a period of 1 week
after the birth of second-generation litter. During this time, handling of mother
rats for supplementation often resulted in killing of the pups by the mother.
Offspring from all groups nursed from their mother until 4 weeks of age. On
the fifth week of age, the offspring were weaned and supplemented with their respective isoform of vitamin E for 1 week. This was followed by tissue harvest for vitamin E analysis. αT3 (90%; free of tocopherol; residual 10% made up of β-, γ-, and δ-tocotrienol) and αTOC (100%) were provided by Carotech Sdn Bhd, Perak, Malaysia. Vitamin E was suspended in E-deficient corn oil (Harlan) for feeding. Second-generation females from each group were bred. All rats received their designated supplementation through pregnancy. As in the case of first-generation rats, supplementation was suspended for 1 week after the birth of third generation rats. After 4 wk of age, the offspring were supplemented with their respective isoform(s) of vitamin E for 1 wk. This was followed by tissue harvest of the third generation rats and vitamin E analyses. The protocol described above was utilized to generate fourth-generation rats. The placebo group, fed with E-deficient corn oil alone, lost fertility and did not breed (Fig. 2.2). The remaining three groups of fourth-generation females were bred with a supplementation protocol similar to that used for the previous generations. On the fifth wk of age, the offspring were weaned and supplemented with their respective isoform(s) of vitamin E for a period of 4 wk. On the eighth wk of age, tissues were harvested for vitamin E analyses. Access to diet was denied to the rats 12 h before harvest. Rats were not supplemented on the day of harvest. The last supplementation was performed 24h before tissue harvest. Whole blood was drawn from the hepatic vein.
TTP knockout mice were provided by Chugai Research Institute for Medical Science, Japan. At the quarantine facility, breeder pairs were not available for daily gavaging. The mice were fed with standard laboratory chow enriched with αT3 (1g Tocomin 50% per kg diet). As reported previously, 1 g of Tocomin 50% (Carotech Sdn Bhd, Perak, Malaysia) contains a mixture of 110 mg αTOC and 119 mg of αT3 [118]. After 3 wk of supplementation on Tocomin-enriched powder diet, two of the three females received from Japan were pregnant. Healthy pups were born 3 wk after pregnancy. The pups and the mother passed quarantine check and were moved to the laboratory animal facility. At this facility, the pups were gavaged five times a week with Tocomin in vitamin E-stripped corn oil (Harlan) at a dose of 250 mg/kg body weight for 7 mo. These mice were then bred to obtain next (second) generation pups. The pups (5 males and 3 females) were weaned on the fourth week of age and gavaged with Tocomin 50% (250 mg/kg body weight) for 1 week. On wk 5 of age, the mice were killed to harvest tissues for vitamin E analysis. Access to diet was denied to the mice 12 h before harvest.

2.1.2.2 Vitamin E extraction and analyses

Excised tissues were cut into small pieces, rinsed in phosphate-buffered saline to remove blood, and stored in liquid nitrogen until analyses. Vitamin E extraction was performed as described previously [119]. Vitamin E analysis was performed using a HPLC-coulometric electrode array detector
(CoulArray Detector Model 5600 with 12 channels; ESA Inc., Chelmsford, MA). This system uses multiple channels with different redox-potentials. αTOC was detected on a channel set at 200mV. αT3 was detected on a channel set at 600 and 700mV as described previously [119].

2.1.2.3 Data presentation

Results are illustrated as means ± SD. Analysis of variance (ANOVA) was used to compare differences between groups in Sprague-Dawley rats. For data collected from TTP-deficient mice, the significance of difference between αTOC and αT3 values in the same tissue was examined by Student's t-test. P < 0.05 was considered to indicate statistically significant difference between means.

2.1.3 Results and discussion

2.1.3.1 Vitamin E supplementation considerations

This work represents a first of its kind effort to investigate the tissue availability of αT3 in response to long-term oral supplementation. A fundamental consideration that influenced the design of this study was that the standard laboratory chow contains excessive amounts of αTOC [117]. In light of the knowledge that natural analogs of vitamin E may compete for specific
transporting mechanisms, we chose to use vitamin E-deficient standardized laboratory chow for this study [120]. Animals maintained on such diet were gavaged with known amounts of specific forms of vitamin E (Fig. 2.2). Another consideration that influenced the study design was our own previous observation that although incorporation of orally supplemented vitamin E into tissues is a slow and progressive process, rapid incorporation of the supplement into tissues of newborn may occur in response to gavaging of pregnant mother rats [118]. To generate proof of principle testing whether dietary αT3 is capable of being transported to vital organs in vivo, we combined long-term oral supplementation with breeding (Fig. 2.2). While second-generation rats in the vitamin E-deficient group lost fertility and failed to reproduce when bred for over 4 mo, rats on tocotrienol supplementation maintained fertility and continued to reproduce comparable to the reference group supplemented with tocopherols (Fig. 2.2).

2.1.3.2 αTOC and αT3 levels in skin

Baseline levels of αT3 in the skin of tocopherol-fed rats that never received any tocotrienol supplementation were negligible. Orally supplemented tocotrienol was rapidly taken up by the skin. Already in second-generation rats, αT3 levels in the skin of tocotrienol supplemented rats exceeded twice the αTOC levels in that organ. Of note, the αT3 level in the skin matched the αTOC level in the skin of rats fed with a comparable amount
of tocopherol. When tocotrienol and tocopherol were co-supplemented, the uptake of αT3 by the skin was clearly blunted. In this group, αT3 levels were lower than αTOC levels in the skin, suggesting a direct competition between orally taken tocotrienol and tocopherol for delivery to the skin (Fig. 2.3). Longer supplementation resulted in a marked increase in the αT3 levels in the skin of tocotrienol fed rats, indicating a buildup of αT3 over time. Interestingly, the levels of αT3 in the skin of these rats were folds higher than the αTOC level in the skin of tocopherol fed rats. This observation suggests the presence of an effective transport mechanisms delivering αT3 to the skin and efficient retention of αT3 in the skin over time. In the case of tocotrienol as well as of tocopherol feeding, results from third and fifth-generation rats indicate higher levels of vitamin E in the skin of female compared to that of male rats. Co-supplementation of tocotrienol and tocopherol demonstrated favorable uptake of αTOC than αT3. Adipose tissue serves as a storage organ for vitamin E [121]. Analysis of adipose tissue vitamin E content of fifth-generation rats revealed substantially more accumulation of αT3 in that tissue than αTOC (Fig. 2.4). In the co-supplemented group, tissue levels of αT3 and αTOC were comparable (Fig. 2.4). Consistent with the observation in the skin, levels of both forms of vitamin E were higher in the adipose tissue of females compared to that in the males (Fig. 2.4). This observation led us to examine the vitamin E level in the gonads of available rats. Indeed, the levels of both forms of vitamin E studied were significantly higher in ovaries than in the testes (Fig. 2.4). This
gender-dependent effect was more striking for \( \alpha T3 \) than for \( \alpha TOC \). In rats co-supplemented with tocotrienol and tocopherol, \( \alpha TOC \) outcompeted \( \alpha T3 \) for delivery to the gonads (Fig. 2.4).

2.1.3.3 \( \alpha TOC \) and \( \alpha T3 \) levels in heart, lungs, and skeletal muscle

The \( \alpha T3 \) level in the heart, lungs, and skeletal muscle of tocopherol-supplemented rats may be considered as baseline for \( \alpha T3 \) in the heart of rats not supplemented with tocotrienol (Figs. 2.5 – 2.7). Compared to that baseline, the level of \( \alpha T3 \) in the heart, lungs, and skeletal muscle of second-generation tocotrienol-fed rats was substantially higher establishing that oral tocotrienol does get delivered to these organs. The level of \( \alpha T3 \) in these organs of tocotrienol-fed rats was lower than the level of \( \alpha TOC \) in tocopherol-fed rats, indicating that in these rats, the efficiency to deliver oral tocotrienol to the respective organs was lower than that for oral tocopherol delivery. Co-supplementation of tocotrienol and tocopherol clearly compromised delivery of \( \alpha T3 \) to all three organs (Figs. 2.5 – 2.7). The level of \( \alpha T3 \) in the heart, lungs, and skeletal muscle of tocotrienol-fed female rats increased in response to a longer supplementation. Third and fifth-generation females had significantly higher levels of \( \alpha T3 \) in the heart of tocotrienol-fed group animals (Fig. 2.5). Co-supplementation of tocotrienol and tocopherol compromised delivery of \( \alpha T3 \) to the lung and skin (Figs. 2.3 and 2.6). The extent of compromise was most in the second-generation rats. In the third and fifth generations, rats co-
supplemented with tocotrienol and tocopherol had higher levels of αT3 in the lung compared to the corresponding rats in the second generation (Fig. 2.6). In third- and fifth-generation rats, the levels of αT3 were significantly higher in females than in males. Of note, in the third- and fifth-generation rats the αT3 levels in the tocotrienol-fed group were comparable to the αTOC levels in the tocopherol-fed group, indicating comparable delivery and retention of the two forms of vitamin E in the tissue (Figs. 2.3 – 2.10).

2.1.3.4 αTOC and αT3 levels in the central nervous system

In tissues of the central nervous system, brain, and spinal cord, tocotrienol feeding increased the levels of αT3 compared to baseline levels detected in rats never supplemented with any tocotrienol (Fig. 2.8). In second-generation rats, αT3 was appreciably detected. However, the level was folds lower than the αTOC level in the brain of tocopherol-fed rats. This observation indicates that oral αT3 is delivered to the central nervous system but the delivery system is much weaker than the system to deliver α-tocopherol. Co-supplementation of rats with tocotrienol and tocopherol resulted in lower αT3 delivery to the brain as well as to the spinal cord compared to αTOC levels. Longer term supplementation resulted in higher levels of brain αT3 in the third- and fifth-generation rats compared to animals of the second generation. In
both third- and fifth-generation rats fed with tocotrienol, females had higher αT3 in the brain than males. This was also evident in the spinal cord of fifth-generation rats (Fig. 2.8).

2.1.3.5 αTOC and αT3 levels in the blood

In the blood, baseline αT3 levels in rats never fed with tocotrienol (i.e., tocopherol group) were negligible (Fig. 2.9). Tocotrienol supplementation increased the levels of circulatory αT3 even 12h after the last supplementation. The level of αT3 detected in the circulation of tocotrienol-fed rats under comparable conditions for the second, third, and fifth generation were similar. αT3 levels in the blood of tocotrienol-fed rats were roughly a magnitude lower than the levels of αTOC in tocopherol-fed rats. Co-supplementation of tocotrienol and tocopherol resulted in higher levels of blood αTOC than αT3, indicating more efficient uptake and retention of oral αTOC in the circulation than that for αT3. In third- and fifth-generation tocotrienol-fed rats, females had higher blood αT3 levels than males (Fig. 2.9).

2.1.3.6 αTOC and αT3 levels in the liver

In rats never supplemented with tocotrienol, baseline αT3 levels in the liver were negligible (Fig. 2.10). Tocotrienol supplementation increased hepatic αT3 concentration. However, in tocotrienol-fed rats hepatic αT3 content was only half of the αTOC levels in the liver of tocopherol-fed second-
generation rats. Co-supplementation of tocotrienol and tocopherol resulted in preferential uptake of αTOC by the liver. In the third- and fifth-generation tocotrienol-fed rats, hepatic αT3 concentration was higher in the females than in the males. While long-term tocotrienol-supplementation had little effect on the αT3 levels in male rats, in fifth-generation female rats significantly elevated levels of αT3 were noted (Fig. 2.10).

2.1.3.7 Oral αT3 supplementation in TTP-deficient mice

TTP-deficient mice are known to be embryonic lethal because of their inability to deliver tocopherol to tissues [122, 123]. Oral tocotrienol supplementation, but not tocopherol, restored reproductive capability in these mice (Fig. 2.11). Next, we tested whether delivery of oral αT3 to vital organs is dependent on TTP. Orally supplemented αT3 was effectively delivered to several vital organs in TTP-deficient mice. While these results do not rule out the possibility that TTP may contribute to tocotrienol transport in the body, it was clear that TTP does not represent a major or sole mechanism of αT3 transport in the body. In TTP-deficient mice, the adipose tissue represented a major destination for orally consumed αT3. Long-term tocotrienol supplementation to mice resulted in adipose tissue αT3 levels that were folds higher than αTOC levels. Consistent with the observation in our rat study, the skin and skeletal muscle were observed to be efficient in accumulating dietary αT3. In these organs, αT3 levels were folds higher than that of αTOC. In TTP-
deficient mice supplemented with tocotrienol, αT3 levels were significantly higher in the heart than αTOC levels. In the lung and brain of these mice, however, αTOC and αT3 levels were not significantly different. It was clear that oral tocotrienol was indeed delivered to both lung and brain. Consistent with the observation in rats, mice never supplemented with tocotrienol show negligible αT3 levels in all organs. In the spinal cord, αTOC levels were comparable to those in the brain. Tocotrienol supplementation was more effective in raising the αT3 level in the spinal cord than that of the brain. αT3 levels in the spinal cord were multifold higher than the levels of αTOC. Although the levels of αT3 were remarkably high in tissues such as the fat, skin, and muscle, hepatic αT3 concentration in tocotrienol-supplemented TTP-deficient mice was folds lower than the corresponding tocopherol levels. In TTP deficient mice with compromised ability to traffic αTOC from the liver to the peripheral tissues, hepatic αTOC content is known to accumulate. In the blood, both αTOC and αT3 were detected even 12 h after the last supplementation. αT3 levels in the blood were significantly lower than circulating αTOC levels (Fig. 2.11).

2.1.3.8 αT3 tissue delivery in spite of TTP affinity for αTOC

Delivery of orally taken vitamin E to vital organs is a key determinant of the overall efficacy of vitamin E in those tissues. Thus, mechanisms responsible for the transfer of absorbed vitamin E to the tissues have been the
subject of active investigation [124]. TTP has emerged as the major intracellular transport protein for vitamin E, mediating αTOC secretion into the plasma via a non-Golgi-dependent pathway [125]. It has been estimated that TTP has 8.5-fold lower affinity to transport αT3 than αTOC [120]. Inefficiency to transport αT3 to vital organs represents one of the key concerns that have limited enthusiasm for this form of natural vitamin E. Recently, we have reported the first evidence demonstrating the biological effects of trace concentrations of vitamin E [114, 115, 126]. Nanomolar concentration αT3, but not αTOC, was potently neuroprotective. Results of the current study represent the first evidence addressing the effect of long-term tocotrienol supplementation on tissue αT3 levels. Dietary αT3 was effectively delivered to several vital organs among which the skin, adipose, ovaries, and the heart seemed to be preferred destinations within the body. Oral αT3 was also delivered, albeit to a lesser extent, to vital organs such as the brain, lung, testes, and skeletal muscle.

Gender-based differences in the transport of dietary vitamins are known to exist in specific cases [127]. Although the effect of several physiological factors on vitamin E transport has been studied, the gender factor remains to be specifically addressed [128]. Recently it has been demonstrated that γTOC is more rapidly metabolized in women than in men [129]. In all organs tested, we consistently observed higher tissue levels of αT3 in females than in males. This effect was most prominent in response to long term supplementation. Of
interest, gonads of the fifth-generation rats exhibited the most striking
difference. The level of αT3 in the ovary was over five-fold higher than that in
the testes from the corresponding males rats. In the ovary, tocopherol is
known to accumulate via a lipoprotein receptor-dependent mechanism [130].
Whether tocotrienol share that mechanism remain to be tested.

TTP is a soluble 32-kDa protein expressed in liver that selectively binds
αTOC. TTP maintains the concentration of serum αTOC by facilitating αTOC
export from the liver. TTP is required to maintain normal αTOC concentrations
in plasma and extrahepatic tissues [124]. Although TTP is known to bind to
αT3 with a 8.5-fold lower affinity than that for αTOC, it is not clear whether, or
to what extent, the delivery of orally supplemented αT3 to vital organs is
dependent on TTP [120]. Previously it has been reported that TTP-deficient
females are infertile presumably because of vitamin E deficiency [123]. This
important observation was confirmed in another lineage of TTP-deficient mice,
the one used in the current study. Placentas of pregnant TTP-deficient
females were severely impaired with marked reduction of labyrinthine
trophoblasts, and the embryos died at mid-gestation even when fertilized eggs
of TTP-containing wild-type mice were transferred into TTP-deficient recipients
[122]. In our study, TTP-deficient mice fed a standard laboratory chow
containing tocopherol were infertile, consistent with previous observations.
Even in the presence of dietary tocopherol, TTP knockout mice are known to
suffer from tocopherol deficiency [122, 123]. Oral supplementation of the
female mice with αT3 restored fertility, suggesting that tocotrienol was successfully delivered to the relevant tissues and that αT3 supported reproductive function under conditions of αTOC deficiency. This observation was consistent with our observation in the rats where αT3 supplementation spared loss of fertility caused by long-term vitamin E deficiency in the diet.

Accumulation of αT3 in several vital organs of the TTP-deficient mice indicates that the delivery of oral αT3 to these tissues occurs independent of TTP. Heritable mutations in the TTP gene are incident in humans and display low plasma vitamin E levels and pathological conditions such as autosomal recessive Friedreich-like ataxia and retinitis pigmentosa subsequent to the onset of ataxia. Neurological symptoms included ataxia, dysarthria, hyporeflexia, and decreased proprioceptive and vibratory sensations. TTP deficiency in humans specifically affects the central axons of dorsal root ganglion cells and the retina, with minor involvement of the peripheral sensory nerve, optic nerve, and pyramidal tract [131, 132]. Previously we have observed that the neuroprotective properties of αT3 are more potent than that of αTOC [114, 115]. In the present study, we observe that orally supplemented αT3 may be transported to tissues even in the absence of TTP. Taken together, these findings warrant clinical studies testing the efficacy of oral αT3 supplementation in humans suffering from inherited mutations in the gene encoding TTP and also in those that are TTP-sufficient but suffer from or are at a high risk of neurodegenerative diseases.
2.1.3.9 Unequivocal distribution and metabolism of αTOC and αT3

Vitamin E enters the circulation from the intestine in chylomicrons. The conversion of chylomicrons to remnant particles results in the distribution of newly absorbed vitamin E to all of the circulating lipoproteins and ultimately to tissues. This enrichment of lipoproteins with vitamin E is a key mechanism by which vitamin E is delivered to tissues [124]. In the liver, newly absorbed dietary lipids are incorporated into nascent very low density lipoproteins. The liver is responsible for the control and release of αTOC into blood plasma. In the absence of TTP, αTOC is not secreted back into the plasma. Excess vitamin E is not accumulated in the liver, but is excreted, mostly in bile [124]. Results of the current study show that αT3 levels in the liver of rats and of TTP-deficient mice were much lower than the levels of this vitamin E isoform in most peripheral tissues studied. Such observation argues against a central role of the liver in delivering oral αT3 to peripheral tissues. TTP has the ability to bind to both αTOC as well as αT3. The affinity to bind αTOC is several-fold higher than that for αT3 [120]. Thus under conditions of coexistence, αTOC is expected to clearly outcompete αT3 for binding. Although our studies with the TTP-deficient mice indicate the existence of TTP-independent mechanisms for the tissue delivery of oral αT3, observations in the rat indicate that the mechanisms for transporting αTOC and αT3 seem to compete such that transport of αTOC is favored. Thus, co-supplementation of αTOC and αT3 is likely to compromise tissue delivery of αT3.
Tocopherol and tocotrienol are metabolized by side-chain degradation initiated by cytochrome P450 (CYP)-catalyzed N-hydroxylation followed by β-oxidation. CYP3A4 and CYP4F2 are involved in the degradation of tocopherol. Both tocopherol and tocotrienol in particular induce the expression of CYP3A4 and CYP3A5 by activating the pregnane x receptor (PXR), a nuclear receptor regulating a variety of drug-metabolizing enzymes [133]. Quantitatively, tocotrienols are degraded to a larger extent than their counterparts with saturated side chains. The pronounced quantitative differences in the metabolism between individual tocopherol as well as between tocotrienol and tocopherol in vitro suggest a corresponding lack of equivalence in vivo [134].

2.1.4 Conclusions

The efficiency of αTOC as a chain-breaking antioxidant, combined with its prevalence in the human body led biologists to almost completely discount the “minor” vitamin E molecules as topics for basic and clinical research. Recent discoveries have led to a serious reconsideration of this conventional wisdom [135]. All eight tocols in the vitamin E family share close structural similarity and hence possess comparable antioxidant efficacy. Yet, current studies of the biological functions of vitamin E indicate that members in the vitamin E family possess unique biological functions often not shared by other family members [136-139]. They have been implicated with the prevention of various neurodegenerative disorders, cancers and the possible reduction of
infarct associated with heart attack and stroke due to antioxidant dependent and independent mechanisms alike. Numerous beneficial functions of tocotrienol alone have been reported during the last two decades [126, 138, 140].

To date, the vast majority of research and literature on vitamin E has been associated with αTOC, the most bioavailable of all the vitamin E isoforms found in human tissue [124]. In contrast, it has been reported that only 1% of the entire literature on vitamin E addresses tocotrienols [103]. As such, αTOC has garnished the most attention by both consumers and researchers, which has led it to be the best characterized of all the vitamin E isoforms in terms of antioxidant function, specific tissue bioavailability and transportation. As a consequence of the focused nature of vitamin E research towards αTOC, and the assumption that other vitamin E homologues have little or no bioactivity in the human body, all recommended daily allowances (RDAs) released by the Food and Nutrition Board for vitamin E intake are based solely on the consumption of αTOC [103]. The oversight of the possible usefulness of vitamin E isoforms other than alpha-tocopherol has led to the unfortunate misconception and association that alpha-tocopherol and vitamin E are synonymous. The current work represents the first evidence documenting the tissue distribution of a lesser characterized vitamin E isomer, αT3, following long-term supplementation. It is clear that orally taken αT3 may be successfully delivered to several vital organs. This transport efficiency seems
to be down-regulated under conditions of αTOC co-supplementation. Studies with TTP-deficient mice revealed that αT3 may be transported to tissues by TTP-independent mechanisms. These findings warrant a more careful consideration of the under-reported vitamin E isomers and establish the relevance for the subsequent chapter detailing the neuroprotective properties of αT3 as related to stroke.
2.1.5 FIGURES

Figure 2.1 | 8 Naturally occurring vitamin E isoforms. Within the two families of tocopherol and tocotrienol, 4 different R-groups for the chromanol ring exist: α, β, γ and δ. Tocopherols and tocotrienols only differ in the degree of saturation found in the hydrocarbon tail, with tocotrienols containing three unsaturations.
Figure 2.2 | Schematic representation of the study design aimed at examining the long-term effects of oral vitamin E supplementation in rats. Female rats were maintained on vitamin E-deficient diet (TD 88163, Harlan) and divided into the following four groups supplemented (5 days/week) with: (i) αT3 (5mg/kg body weight) (ii) αTOC (5mg/kg body weight), (iii) αT3 + αTOC (2.5 + 2.5 mg/kg body weight), and (iv) placebo vitamin E-stripped corn oil (volume matched). These rats were identified as first-generation (G) breeders, ie., G=1. Offspring from all groups were nursed from their mother until 4 weeks of age. On the fifth week of age, the offspring were weaned and supplemented with their respective isoform of vitamin E for 1 week. This was followed by tissue harvest from vitamin E-supplemented rats in G = 2, 3 and 5. The placebo group females, fed with E-deficient corn oil alone, lost fertility and did not reproduce, G=3. The mean duration taken to generate each generation is indicated in weeks against each G row. Sample size: For G=2, αTOC n=3M and 4F, αT3 + αTOC n=4M and 3F, and αT3 n=3M and 3F. For G=3, αTOC n=4M and 3F, αT3 + αTOC n=4M and 0F (no females were born), αT3 n=3M and 3F and 3F. For G=5, αTOC n=4M and 6F, αT3 + αTOC n=4M and 4F, αT3 n=4M and 4F. M, male; F, female.
Figure 2.3 | α-Tocotrienol and α-tocopherol levels in the skin of rats. Animals were maintained on vitamin E-deficient diet and supplemented with either αT3, αTOC, or co-supplemented with αT3 + αTOC as indicated in the figure. Open and closed bars represent αTOC data from male and female rats, respectively. Hatched and cross-hatched bars represent αT3 data from male and female rats, respectively. Data represent mean ± SD. *P* < 0.05 is designated by letters a-e: a, higher in corresponding gender-matched group in the same generation; b, lower than in corresponding gender-matched group in the same generation; c, higher than in corresponding gender-matched and supplementation-matched group in G2; d, higher than in corresponding gender-matched and supplementation-matched group in G3; e, higher in females compared to corresponding males in the same generation and supplementation group. G, generation.
Figure 2.4 | α-Tocotrienol and α-tocopherol levels in abdominal adipose and gonads of rats. Animals were maintained on vitamin E-deficient diet and supplemented with αT3, αTOC, or co-supplemented with αT3 + αTOC as indicated in the figure. Data from G5 are shown. Open and closed bars represent αTOC data from male and female rats, respectively. Hatched and cross-hatched bars represent αT3 data from male and female rats, respectively. Data represent mean ± SD. $P < 0.05$ is designated by letters a-c: a, higher than in corresponding gender matched in the same supplementation group; b, higher in females compared to corresponding males in the same generation and supplementation group; c, lower compared to αTOC levels in the corresponding gender-matched co-supplemented group. G, generation.
Figure 2.5 | α-Tocotrienol and α-tocopherol levels in the heart of rats. Animals were maintained on vitamin E-deficient diet and supplemented with αT3, αTOC, or co-supplemented with αT3 + αTOC as indicated in the figure. Open and closed bars represent αTOC data from male and female rats, respectively. Hatched and cross-hatched bars represent αT3 data from male and female rats, respectively. Data represent mean ± SD. *P < 0.05* is designated by letters a-e: a, higher than in corresponding gender-matched αTOC supplemented group in the same generation; b, lower than αTOC levels in the corresponding gender-matched αTOC supplemented group in the same generation; c, lower than corresponding gender-matched αTOC levels in the same tissue in co-supplemented rats; d, higher than corresponding supplementation-matched females in G2. e, higher in females compared to corresponding males in the same generation and supplementation group. G, generation.
Figure 2.6 | α-Tocotrienol and α-tocopherol levels in the lungs of rats. Animals were maintained on vitamin E-deficient diet and supplemented with αT3, αTOC, or co-supplemented with αT3 + αTOC as indicated in the figure. Open and closed bars represent αTOC data from male and female rats, respectively. Hatched and cross-hatched bars represent αT3 data from male and female rats, respectively. Data represent mean ± SD. *P* < 0.05 is designated by letters a-e: a, higher than in corresponding gender-matched αTOC supplemented group in the same generation; b, lower than αTOC levels in the corresponding gender-matched αTOC supplemented group in the same generation; c, lower than in corresponding gender-matched αTOC levels in the same tissue of co-supplemented rats; d, higher than in corresponding gender-matched co-supplemented rats in G2. e, higher in females compared to corresponding males in the same generation and supplementation group. G, generation.
Figure 2.7 | α-Tocotrienol and α-tocopherol levels in the vastus lateralis skeletal muscle of rats. Animals were maintained on vitamin E-deficient diet and supplemented with αT3, αTOC, or co-supplemented with αT3 + αTOC as indicated in the figure. Open and closed bars represent αTOC data from male and female rats, respectively. Hatched and cross-hatched bars represent αT3 data from male and female rats, respectively. Data represent mean ± SD. P < 0.05 is designated by letters a-d: a, higher than in corresponding gender-matched αTOC supplemented group in the same generation; b, lower than in corresponding gender-matched αTOC levels in the same tissue of co-supplemented rats; c, higher than in corresponding gender-matched αT3 supplemented rats in G2; d, higher in females compared to corresponding males in the same generation and supplementation group. G, generation.
Figure 2.8 | α-Tocotrienol and α-tocopherol levels in the brain and spinal cord of rats. Animals were maintained on vitamin E-deficient diet and supplemented with αT3, αTOC, or co-supplemented with αT3 + αTOC as indicated in the figure. Open and closed bars represent αTOC data from male and female rats, respectively. Hatched and cross-hatched bars represent αT3 data from male and female rats, respectively. Data represent mean ± SD. $P < 0.05$ is designated by letters a-d: a, higher than in corresponding gender-matched αTOC supplemented group in the same generation; b, lower than αTOC levels in the corresponding gender-matched αTOC supplemented group in the same generation; c, lower than in corresponding gender-matched αTOC levels in the same tissue of co-supplemented rats; d, higher in females compared to corresponding males in the same generation and supplementation group. G, generation.
Figure 2.9 | α-Tocotrienol and α-tocopherol levels in the blood of rats. Animals were maintained on vitamin E-deficient diet and supplemented with αT3, αTOC, or co-supplemented with αT3 + αTOC as indicated in the figure. Open and closed bars represent αTOC data from male and female rats, respectively. Hatched and cross-hatched bars represent αT3 data from male and female rats, respectively. Data represent mean ± SD. *P < 0.05* is designated by letters a-d: a, higher than in corresponding gender-matched αTOC supplemented group; b, lower than in corresponding gender-matched αTOC levels in the tocopherol supplemented group in the same generation; c, lower than in corresponding gender-matched αTOC levels in the same tissue of co-supplemented rats; d, higher in females compared to corresponding males in the same generation and supplementation group. G, generation.
Figure 2.10 | α-Tocotrienol and α-tocopherol levels in the liver of rats. Animals were maintained on vitamin E-deficient diet and supplemented with αT3, αTOC, or co-supplemented with αT3 + αTOC as indicated in the figure. Open and closed bars represent αTOC data from male and female rats, respectively. Hatched and cross-hatched bars represent αT3 data from male and female rats, respectively. Data represent mean ± SD. P < 0.05 is designated by letters a-d: a, higher than in corresponding gender-matched αTOC supplemented group; b, lower than αTOC levels in the corresponding gender-matched αTOC levels in the tocopherol supplemented group; c, lower than corresponding gender-matched αTOC levels in the same tissue of co-supplemented rats; d, higher in females compared to corresponding males in the same generation and supplementation group. G, generation.
Figure 2.11 | α-Tocotrienol and α-tocopherol levels in tocotrienol supplemented TTP-deficient mice. Top left: An adult (7 wk old) TTP-deficient (-/-) mouse born from homozygous parents maintained on tocotrienol supplementation compared to a wild-type (+/+ ) mouse of the same background. Mice were maintained on long-term tocotrienol supplementation as described in section 2.1.2.1. Open and closed bars represent αTOC data from male and female mice, respectively. Hatched and cross-hatched bars represent αT3 data from male and female mice respectively. Data represent mean ± SD. *, P < 0.05, represents significantly higher (lower, in the case of liver) than αTOC level in the corresponding gender-matched tissue.
2.2.1 Unique neuroprotective properties of the natural vitamin E α-tocotrienol

2.2.1.1 Background

On average, every 40 seconds, someone has a stroke in the United States making it the 3rd leading cause of death [23]. Stroke is a broad term that applies to a variety of related pathologies involving cerebrovascular disruption of blood flow. Prior to a discourse of how the natural vitamin E isoform αT3 is neuroprotective in stroke-related pathology, it is important to classify the different conditions of stroke in terms of their prevalence. As it relates to our in vivo research, our animal models specifically address ischemic stroke, which according to the American Heart Association accounts for the vast majority (87%) of all clinically presented stroke cases [23]. Intracerebral and sub-arachnoid hemorrhages constitute the remaining 13% of strokes (approximately 8% and 5% respectively) and differ greatly in both their etiology and pathology.

Briefly, intracerebral hemorrhage occurs when a vulnerable blood vessel within the brain bursts, allowing blood to leak inside the brain. The sudden increase in intracranial pressure can cause damage to the neurons around the rupture site. If the amount of blood increases rapidly, the sudden buildup in pressure can lead to unconsciousness or death. Intracerebral hemorrhage usually occurs in selective parts of the brain, including the basal ganglia, cerebellum, brainstem, or cortex. The most common cause of
intracerebral hemorrhage is high blood pressure (hypertension) which is affected by dietary sodium content. Less common causes of intracerebral hemorrhage include trauma, infections, tumors, blood clotting deficiencies, and abnormalities in blood vessels. Subarachnoid hemorrhage occurs when a blood vessel just outside the brain ruptures and the area between the arachnoid membrane and the pia mater rapidly fills with blood. A patient with subarachnoid hemorrhage may have a sudden, intense headache (referred to as a thunderclap headache), neck pain, and nausea or vomiting. It may arise spontaneously or due to trauma with the sudden buildup of pressure outside the brain causing rapid loss of consciousness or death. Subarachnoid hemorrhage is most often caused by cerebral aneurysms - small areas of rounded or irregular swellings in the arteries. Where the swelling is most severe, the blood vessel wall becomes weak and prone to rupture. While hemorrhagic stroke is not addressed in the present work, it should be noted that the principle mechanisms of αT3 neuroprotection discussed in the context of ischemic stroke in this dissertation may be applicable to hemorrhagic stroke as well. Future investigation is warranted.

2.2.1.2 Stroke-related risk factors

Ischemic stroke can be influenced by a myriad of disease and known risk factors. The most common etiology is due to the narrowing of the arteries in the neck or head. This is most often caused by atherosclerosis which is
directly influenced by dietary factors over time. High fat diets leading to elevated LDL cholesterol and triglyceride levels are significant risk factors for atherogenesis. If the arteries become too narrow, blood cells may collect and form blood clots. These blood clots can block the artery where they are formed (thrombosis), or can dislodge and become trapped in smaller or more distant arteries of the brain (embolism). A thrombotic stroke occurs when diseased or damaged cerebral arteries become blocked by the formation of a blood clot within the brain. Clinically referred to as cerebral thrombosis or cerebral infarction, this type of event is responsible for almost 50% of all strokes [23]. Cerebral thrombosis can also be divided into an additional two categories that correlate to the location of the blockage within the brain: large-vessel thrombosis and small-vessel thrombosis. Large-vessel thrombosis is the term used when the blockage is in one of the brain's larger blood-supplying arteries such as the carotid or middle cerebral artery, while small-vessel thrombosis involves one (or more) of the brain's smaller, yet deeper penetrating arteries (Φ:0.2 - 15mm). This latter type of stroke is also called a lacunar stroke. An embolic stroke is also caused by a clot within an artery, but in this case the clot (or emboli) was formed somewhere other than in the brain itself. Often from the heart, these emboli will travel the bloodstream until they become lodged and cannot travel any further. This naturally restricts the flow of blood to the brain and results in almost immediate physical and neurological deficits. Again, a high fat diet is a critical risk factor for coronary heart disease which may lead to embolic stroke. While these are the most common causes of
ischemic stroke, there are many other possible causes including traumatic injury to the blood vessels of the neck, or disorders of blood clotting.

The rodent model of temporal middle cerebral occlusion employed by our laboratory mimics the effects of an acute ischemic attack that could be caused by a thrombotic or embolic event. In either natural occurrence of stroke, diet itself is a significant contributor to the etiology of the ischemic episode. The dietary dynamics that contribute to risk factors under the umbrella definitions of metabolic syndrome and cerebrovascular disease are well known [141]. That dietary elements may also reduce the risk of stroke incidence and severity is less popularized.

2.2.1.3 Dietary factors affecting stroke

DIETARY FATS

The nutritional benefits of α-linolenic acid (ALA), an essential omega-3 polyunsaturated fatty acid, include a lower risk of cardiovascular disease and lower incidence of stroke [142]. In a rodent model of temporal middle cerebral artery occlusion, a 500 nmol/kg bolus of ALA injected into the jugular vein 2 hours post-reperfusion was found to significantly reduce the volume of infarct as compared to control animals [143]. Whether a diet enriched with ALA would also have the same effect remains to be seen. I hypothesize, however, that it would be difficult to significantly enrich the brain’s lipid profile with ALA via diet
as the body is selective in how body tissues and their subsequent lipid profiles are organized [144].

High cholesterol diets have not only been implicated in a higher incidence of stroke due to atherosclerosis, but recently have also been implicated in inducing a larger volume of infarct in rodents following transient occlusion of the middle cerebral artery [145]. Specifically, rodents kept for 6 wk on a hyper-cholesterolemia diet (36% fat compared to normal chow’s 5% fat) had 50% larger infarct volumes as compared to controls following stroke. This was explained by a diet-induced effect on the cerebral microvasculature - causing it to undergo a pro-inflammatory and pro-thrombogenic phenotype.

PROTEIN

There is very little published in scientific literature to suggest how a high protein, low carbohydrate, ketogenic diet will affect stroke. While ketogenic diets have reportedly had great success in the prevention of epileptic seizures, little is understood about the molecular mechanism behind such protection or whether that could translate to neuroprotective properties in stroke. As an aside, ketogenic diets are generally also higher in fat content which may induce a pro-atherogenic response, which as previously described induces a higher incidence of stroke and larger infarcts.
CARBOHYDRATES

Diabetes is a major risk factor for development of ischemic cerebrovascular disease. Patients with diabetes are at least two times more likely to have a stroke than non-diabetic subjects and they are more likely to suffer increased morbidity and mortality after stroke [146]. The hallmark pathology of diabetes is sustained hyperglycemia. In both humans and animals, neuronal damage initiated by stroke-induced ischemia is increased by concomitant hyperglycemia [147, 148]. Hyperglycemia has also been associated with increased oxidative stress suggesting that this may contribute to more severe dysfunction following cerebral ischemia. One explanation for increased volume of infarct due to high blood sugar levels is that hyperglycemia can result in superoxide formation by both enzymatic and spontaneous oxidation of glucose [149]. Superoxide is a reactive anion that is both directly toxic to neurons and an initiator of free-radical generation. Therefore, a carbohydrate heavy diet that induces hyperglycemia prior to stroke will have an acute affect in increasing infarct volume in a rodent model of ischemic stroke. Furthermore, diabetic rats that induce a hyperglycemic response from standard chow (60% carbohydrate) also experience larger volumes of infarct compared to non-diabetic controls [150].
MINERALS

Hypertension, or high blood pressure, is a leading risk factor for ischemic stroke and has been linked to the induction of larger volumes of infarct in rodent models of stroke-induced ischemia [151]. High sodium consumption has been positively correlated with an increase in blood pressure and subsequent reductions in dietary intake of sodium have been reported to significantly reduce blood pressure, the risk of hypertension and therefore the incidence and severity of stroke.

Another mineral which modulates infarct size following ischemic stroke is magnesium. Magnesium has dual modes of action, both direct and indirect, in reducing the volume of infarct following stroke. First, an indirect role for dietary magnesium has been reported in the reduction of blood pressure associated with hypertension [142]. Additionally, a direct role for magnesium in the reduction of infarct volume following stroke-induced ischemia has been described by its role in the prevention of excitotoxicity by blocking NMDA receptors [152]. The NMDA receptor is an ionotropic receptor for glutamate. Following stroke, glutamate excitotoxicity has been implicated as a key mediator of stroke-induced neurodegeneration.
DIETARY INTAKE

A very broad means by which diet may directly influence infarct size in ischemic stroke is by caloric restriction or intermittent fasting. Ischemic stroke studies in rats have shown that intermittent fasting induces a mild stress to neurons that under circumstances of extreme duress, such as stroke, make them more resilient to oxidative, metabolic and excitotoxic insults caused by stroke-induced ischemia [153]. Similarly, fluid balance may directly affect infarct size in ischemic stroke as well. In particular, some scientists have hypothesized that hypernatremia, due to a cellular loss of water or increased salt loading may improve infarct outcomes in ischemic stroke by promoting an anti-edema action following reperfusion [154]. It is my contrary belief, as supported by an article published in Stroke [155], that inducing hypernatremia will only exacerbate infarct volume, as hypernatremia may also cause hypertension which has been implicated in the induction of larger infarcts in rat models of ischemic stroke [151].

MICRONUTRIENTS

A wide array of compounds classified as micronutrients may prevent induction of infarct volume following stroke-induced ischemia. For example, the B vitamins, such as B6 (pyridoxine), B9 (folate), and B12 (cobalamin), may reduce stroke risk and infarct volume via effects on plasma homocysteine and
antioxidant defense [156, 157]. Dietary antioxidants, such as vitamin E (alpha-tocopherol) and vitamin C may also reduce the volume of infarct following stroke-induced ischemia by inhibiting free-radical mediated damage [158]. Recently, our laboratory has published evidence that the vitamin E isoform, αT3, has neuroprotective antioxidant independent mechanisms not attributed to the other vitamin E isoforms [114]. The mechanism of action is attributable to inhibition of two key enzymatic mediators of stroke-induced neurodegeneration: c-Src kinase and 12-lipoxygenase (12-Lox).

2.2.1.4 Ischemic stroke and glutamate excitotoxicity

The etymology of the word ischemia is derived from the Greek words iskhein which means “keep back” and haima meaning “blood”. Ischemic stroke occurs when an artery to the brain is blocked. The brain depends on continuous blood flow to deliver oxygen and nutrients to the brain, and take away carbon dioxide and cellular waste. If an artery is blocked, neurons cease to be able to maintain homeostasis and subsequent trafficking of essential lipids, proteins, nutrients and waste. The terminal result is cell death, mediated by a cascade of extracellular and intracellular factors; the latter of which include two specific targets of αT3 neuroprotection, the aforementioned c-Src kinase and 12-Lox.
The body of research in this chapter stems from our laboratory’s striking finding that in cultured neurons, nM concentrations of αT3, but not αTOC, blocked cell death in a model of glutamate toxicity [114, 115, 118]. Glutamate toxicity and subsequent reactive oxygen species proliferation are major contributors of cell death within the nervous system and are directly involved in the pathogenesis of stroke [114, 142]. Specifically, an ischemia-induced deficiency of cellular ATP provokes a profound loss of ionic gradients and membrane depolarization in neurons. The degradation of ionic gradients is associated with a large, sustained rise (100 – 300 μM) in the extracellular concentration of the neurotransmitter glutamate [159]. The release of glutamate initiates a positive feedback loop, with the activation of glutamate receptors further decreasing ionic gradients and consuming ATP, both of which promote further release of glutamate. This cascade of events culminates in a rapid rise of reactive oxygen species (ROS) which depletes cellular stores of glutathione (GSH) and initiates a cell death cascade involving the metabolism of arachidonic acid via 12-Lox [114]. Neurons in the brain are enriched with arachidonic acid (AA). An estimate of fatty acid composition (wt%) in rat brain suggest that roughly 13% of total brain lipids are AA [160]. The massive release of AA from the lipid bilayer has been implicated in brain ischemic insult [161]. Following release, this free fatty acid is known to be metabolized, primarily by 12-lipoxygenase in the brain [162]. Figure 2.12 provides a schema of the glutamate excitotoxicity pathway.
Micro-injection experiments confirmed that the αT3-specific targets of neuroprotection were relegated to the cytoplasm in cultured neurons [102]. HT4 neurons injected with $10^{-19}$ mol (sub-attomole) αT3 in the cytoplasm were protected from glutamate-induced degeneration whereas nuclear injected neurons were not spared (Fig. 2.13). No benefit was observed in neurons micro-injected, cytoplasmic or nuclear, with αTOC. The mechanism of αT3 neuroprotection in glutamate-induced neuronal cell death was found to be mediated by the aforementioned cytosolic targets: c-Src kinase (Src) and 12-Lox (Fig. 2.14) [114]. We previously reported that glutamate-induced Src activation represents a major checkpoint in oxytosis of HT4 neuronal cells [114]. Furthermore, it has been reported that Src deficiency or inhibition of Src activity in mice is neuroprotective following stroke [163]. Subsequently, we observed that 12-Lox serves as a potential substrate for phosphorylation by Src and that attenuation of 12-Lox activity by αT3 or similarly by a known inhibitor (BL15), protects neurons subjected to excitotoxic glutamate challenge in vitro [102, 114]. This chapter focuses on the 12-Lox mechanism of cell death, and subsequent neuroprotection by αT3.

2.2.1.5 Mechanism of 12-Lipoxygenase action

The first Lox activity was described over 34 years ago via the transformation of arachidonic acid (AA) to 12-S-hydroxyeicosatetraenoic acid (12-S-HETE) in human platelets [164]. Since that time, three distinct isoforms
of 12-Lox have been identified and named on the basis of the cells in which they were first discovered; platelet, leukocyte and epidermis. While each isoform carries out the same general function, namely hydrogen abstraction from a lipophilic molecule, each has a unique gene structure, amino acid sequence and tissue distribution. Tissue distribution of the isoforms and relative homology has been best characterized in mice [165]. From murine models, leukocyte 12-Lox reportedly has the most widespread distribution in a variety of tissue types, including the nervous system, while platelet and epidermis 12-Lox are specifically expressed in platelets and skin respectively. Epidermal 12-Lox is reported to share 60% of its amino acid identity with platelet and leukocyte 12-Lox. Similarly, murine platelet 12-Lox is reported to be 58% identical to the leukocyte isozyme.

Despite variances in amino acid sequences and tissue distribution, the active site of each isozyme is highly conserved. The structure of 12-Lox at the active site is composed of an N-terminal beta-barrel domain and a C-terminal domain containing a hydrophobic substrate-binding site. A non-heme iron atom is coordinated by three histidine residues and the carboxy-terminal isoleucine. The oxidation of ferrous iron (Fe$^{2+}$) to ferric iron (Fe$^{3+}$) activates the enzyme which is then capable of excising a hydrogen from arachidonic acid at carbon #10 (Fig. 2.15). This leads to the formation of a radical metabolite of arachidonic acid (due to loss of a proton) that rapidly reorganizes its double bonds to take on a slightly more stable conformation. Next, insertion of
molecular oxygen generates a hydroperoxide radical which is reduced to the hydroperoxide anion by the simultaneous oxidation of iron to the ferric state. A proton is then accepted to form the hydroperoxide. The newly formed 12-S-hydroperoxyeicosatetraenoic acid (12-S-HPETE) is then released from the active site of 12-Lox whereby it may be reduced to form 12-S-HETE. 12-S-HPETE alone has been reported to cause cell death [166]. Yet to be published work by our laboratory has also demonstrated that 12-S-HPETE (5 μM) can induce cell death in cultured HT4 neurons treated with BSO to deplete GSH levels (Figure 2.16).

The overall objectives of the experimentation described in this chapter were to examine the in vivo significance of oral αT3 supplementation and 12-Lox in stroke-induced neurodegeneration and to identify possible mechanisms of the 12-Lox mediated metabolites 12-S-HPETE and 12-S-HETE in cell death. To study the in vivo significance of 12-Lox and oral αT3 supplementation as related to stroke, we employed two different models of rodent middle cerebral artery (MCA) occlusion. In 12-Lox knockout mice, we used an intraluminal monofilament model of transient MCA occlusion whereby a small nylon filament was surgically directed via the internal carotid artery to block the origin of the MCA for 1h before it was removed to simulate reperfusion [102]. To study the stroke-related neuroprotection orally supplemented αT3, we used spontaneously hypertensive rats (SHR) which are reported to have more reproducible stroke-induced infarct volumes [167].
To further improve upon the consistency of infarct size in the αT3 supplemented rats, we employed a model of permanent MCA occlusion [102].

While both methods of rodent MCA occlusion are present in the literature as acceptable in vivo models of stroke, they are not without their limitations. The intraluminal monofilament method of MCA occlusion is known to produce stroke-related infarcts of variable size due to the blinded nature of the procedure. Proper MCA occlusion using this approach can only be confirmed via laser doppler flowmetry which assesses relative blood flow in the stroke hemisphere. Meanwhile, the permanent MCA occlusion model in rats is non-reversible, thereby precluding study of reperfusion biology. This procedure also necessitates an invasive approach by drilling a small hole in the rat skull to properly visualize and occlude the MCA. Although these methods represent the best available models of stroke in small animals today, their respective limitations have contributed to the failure of stroke therapeutic clinical trials. For this reason, we developed a large animal, pre-clinical model of stroke in which we are currently testing oral αT3 supplementation prior to a clinical study. Development of this unique model is the focus of chapter 3 in this dissertation.

A large body of evidence indicates that mitochondrial dysfunction plays a critical role in the pathophysiologic mechanisms of acute neurodegeneration [168, 169]. Indeed, metabolic impairment, mitochondrial Ca$^{2+}$ accumulation and increased oxidative stress all contribute to neurodegeneration following
acute stroke [170, 171]. Causes of mitochondrial dysfunction include disruption of the electron transport chain (ETC) and induction of the permeability transition pore (PTP). Impaired ETC activity further exacerbates the cellular energy crisis during ischemic insult [169]. The PTP is a transmembrane protein channel that upon forming dissipates mitochondrial hydrogen ion gradients and induces mitochondrial swelling [172]. To elucidate potential mechanisms of 12-Lox mediated AA metabolite cell death, we isolated live mitochondria from rat brain cortex and incubated them in the presence of 12-S-HPETE and 12-S-HETE. Using single and dual-beam spectrophotometry we studied the effects of the eicosanoids on mitochondrial inner membrane potential and susceptibility to the permeability transition pore. Their effects on mitochondrial respiration were analyzed using a Clark-type electrode system.

2.2.2 Materials and methods

2.2.2.1 Mouse model of acute ischemic stroke

12-Lox knockout (B6.129S2-Alox15tm1Fun) and the corresponding background C57BL6/J mice were obtained from Jackson Laboratory, MI. Transient focal cerebral ischemia was induced in 8 to 10 week old male mice by middle cerebral artery (MCA) occlusion. The mice were anesthetized with 1% to 1.5% halothane in oxygen-enriched air delivered through a facemask.
Occlusion of the right middle cerebral artery was achieved by using the intraluminal filament insertion technique previously described [173, 174]. Briefly, a 6-0 nylon monofilament was inserted into the internal carotid artery, via the external carotid artery. Then the filament tip (≈1.0mm length and 0.25mm width) was positioned for occlusion at a distance of 6mm beyond the internal carotid artery-pterigopalatine artery bifurcation. We observed that this approach results in a 60% to 70% drop in cerebral blood flow as measured by laser Doppler (DRT4, Moor Instruments). Once the filament was secured, the incision was sutured and the animal was allowed to emerge from the anesthesia in its home cage. After 60min of occlusion, the animal was briefly re-anesthetized with halothane in oxygen-enriched air, and reperfusion was initiated via withdrawal of the filament. This surgical protocol typically results in a core infarct limited to the parietal cerebral cortex and caudate putamen of the right hemisphere. Throughout the surgery, rectal temperature was maintained at 37±0.5°C through the use of a homeothermic blanket system. Mice were given a 0.5 ml subcutaneous injection of lactated Ringer solution at the conclusion of the surgical procedure, and returned to their home cage for 72 hours [174].

2.2.2.2 Determination of infarct volume

Brains were rapidly removed, placed in a -80°C freezer for 2min, and then sectioned into five 2-mm-thick coronal sections. Sections were incubated
for 15min in 2,3,5-triphenyltetrazolium (TTC), with rotation every 2min to allow uniform tissue staining. TTC is a viability stain frequently used to define infarct regions in rodents [175]. In normal tissue, dehydrogenase activity reduces TTC to formazan, which stains red. In infarct affected tissue, however, dehydrogenase activity is impaired, leaving the region unstained. The TTC solution was maintained at 37°C throughout the staining process. Following staining, the sections were fixed in 10% buffered formalin solution. The brain slices were photographed using Inquiry software (Loats Associates, Inc). The images were used to determine infarct size as a percentage of the contralateral hemisphere after correcting for edema, as previously described [173, 174]. The infarct extended from caudate putamen into surrounding cortex, and was visible in 4 of 5 slices of the brain from control wild-type mice.

2.2.2.3 Spontaneously hypertensive rat stroke studies I and II

Spontaneously hypertensive rats (SHR) were obtained from Harlan (Indianapolis, IN). These inbred albino rats have been derived from a nucleus colony obtained from the National Institutes of Health, Bethesda, Maryland.

STUDY 1. SHR (n=32; male; 4 wk old, Harlan, Indianapolis, IN) were randomly divided into 2 groups: control and supplemented groups. All rats were maintained on vitamin E-deficient laboratory chow (TD88163; Harlan) from 4 to 12 wk of age until stroke was performed. Because of the isoprenoid
side-chain, tocotrienol is sensitive to oxidative modification. Supplementation of rodent chow with tocotrienol has resulted in poor delivery of tocotrienol to the brain [113]. We have observed that oral gavaging results in significant delivery of tocotrienol to the brain [118]. Thus, that route of administration was chosen for this study. The control group was orally gavaged with vitamin E-stripped corn oil in a volume matching the mean volume of the supplement in the test group. The supplement, Tocomin 50%, contained 50% tocotrienol and was provided by Carotech Sdn Bhd (Malaysia). Stock supplement solution (0.3 g Tocomin per ml) was made in vitamin E-stripped corn oil. The test group was orally gavaged with the supplement oil at a high dosage of 1 g Tocomin per kg body weight. The supplementation was done daily (5d/wk) for 8 wk. Stroke was performed at 12 wk of age 20 to 24 h after the last supplementation.

**STUDY 2.** SHR (n=42; male; 4 wk old, Harlan) were randomly divided into 2 groups: control and supplemented. All rats were maintained on vitamin E-deficient chow (TD88163; Harlan) from 4 to 17 weeks of age until stroke was performed. The control group was orally gavaged with vitamin E-stripped corn oil with volume matching the mean volume of the supplement in the test group. Stock solution of tocotrienol supplement (0.06 g tocotrienol/ml) was made in vitamin E-stripped corn oil. The test group was orally gavaged with the supplement oil at a moderate dosage of 50 mg tocotrienol per kg bw. Incorporation of orally supplemented vitamin E to the brain is a slow process.
Longer supplementation period improves bioavailability of vitamin E to the brain [118, 176]. Compared to the protocol of study I where supplementation was performed for 8 wk, the duration of supplementation in study II was increased to 13 wk (5d /wk). Stroke was performed at 17 wk of age 20 to 24 h after the last supplementation. For both studies, rats were maintained under standard conditions at 22±2° C with 12:12 hours dark:light cycles. All animal protocols were approved by the Institutional Laboratory Animal Care and Use Committee (ILACUC) of the Ohio State University, Columbus, Ohio.

2.2.2.4 Spontaneously hypertensive rat stroke

Male SHR weighing 250 to 350g were fasted for 24 hours before surgery. Because pre-ischemic hyperglycemia may increase infarct size, [177] animals were fasted in an attempt to control for glucose levels. SHR were chosen because infarction has been reportedly produced in this species with little variability in infarct size [167]. Halothane (1.5% to 2.0%), mixed with oxygen and nitrogen, was delivered through a nose cone using a flow regulator. The tail artery was cannulated with a polyethylene catheter to monitor blood pressure and to obtain blood samples for assessing physiological variables. Permanent focal neocortical ischemia was produced by tandem right common carotid artery (CCA) and MCA occlusion as described [167]. Core body temperature was maintained at 37° C throughout the procedure with a heat lamp connected to a rectal thermistor. PaO₂ was
maintained above 80mmHg during the surgical procedure and mean arterial pressure was maintained above 90 mmHg, the lower limit of auto-regulation in SHR, by adjusting the halothane concentration. Immediately after CCA/MCA occlusion, all wounds were sutured closed and the animals were allowed to recover from anesthesia. The Institutional Laboratory Animal Care and Use Committee approved all procedures. Surgeries for each experiment were completed over a 2-wk period on rats delivered from a single shipment for each of the two studies.

Arterial blood pressure was monitored throughout the surgical procedure and then checked 2 to 4 h after surgery when animals were recovered from the anesthesia. Arterial blood PaO$_2$, PaCO$_2$, pH, glucose, and hematocrit were measured just after tail artery cannulation and repeated just before MCA occlusion. Rectal temperatures were recorded at 2 to 4 h after CCA/MCA occlusion. Hematocrit was measured again during decapitation. When the CCA/MCA occlusion surgery was done under the controlled conditions as described above, none of the monitored physiological variables were predictive of infarct size. Despite this fact, to avoid variations in infarct size potentially attributable to physiologic alterations, we had decided that rats with post CCA/MCA occlusion PaO$_2$<70 mmHg, mean arterial blood pressure<100 mmHg, rectal temperature>39°C or a >5% drop in hematocrit would be excluded from the study. Specifically, regarding the temperature exclusion criteria Morikawa et al reported no differences in infarct size in rats.
maintained at 36°C for 2 hours after MCA occlusion versus those kept at 39°C [178]. Furthermore, no association between infarct size and temperature was seen for rats in the 36°C and 39°C groups [178]. For harvest of brains, animals were anesthetized with halothane and decapitated 24 h after CCA/MCA occlusion in all experiments. Because infarct volume provides an objective numeric value, it was chosen as the primary outcome measure. Functional scoring systems were not used because of the subjective nature of assessments and the arbitrary numeric value assignment [179]. Infarct volume was measured 24 h after MCA occlusion because infarct margins are maximally delineated by this time [178, 179].

2.2.2.5 12-Lipoxygenase phosphorylation in brain tissue

Rat brain tissues (100 to 150 mg) from study II were used to detect 12-Lox phosphorylation. For extraction, brain tissues were pulverized in liquid nitrogen and then homogenized in lysis buffer (20mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 1 mmol/L Na₂EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na₃VO₄, 1 μg/ml leupeptin and 1 mmol/L PMSF) using a teflon homogenizer. After homogenization, tissue lysate was centrifuged at 15,000 rpm at 4°C for 20min. After the first spin, supernatant was collected and centrifuged again at 15,000 rpm at 4°C for an additional 20 min. The clear supernatant was collected and protein concentration determined using BCA protein reagent.
Immobilized phospho-tyrosine mouse mAb (P-Tyr-100, Cell Signaling Technology Inc) was used to immunoprecipitate tyrosine-phosphorylated proteins (200 μg protein per sample). The immunoprecipitated proteins were separated on a 10% SDS-polyacrylamide gel electrophoresis, and probed with anti-12-Lox polyclonal antiserum (Cayman Chemical Inc).

2.2.2.6 Histological analysis of spontaneously hypertensive rat brain

Brains were rapidly removed from the cranium. A small portion of the non-infarcted occipital lobe was removed for vitamin E analysis and the remainder of the brain was placed in neutral buffered 10% formalin for a minimum of 3 to 4 wk. For study II, 6 rats per group were used for histological examinations. Brains were dehydrated and embedded in paraffin. Coronal sections, 10 μm thick, were cut at 500 μm intervals. The sections were stained with hematoxylin and eosin (H&E).

STUDY 1. Each brain section was magnified using a photographic lens and the infarct area traced onto paper. Each drawing was then retraced onto a digitizing tablet interfaced to a computer (Matrix v2.0), which computed infarct areas for each segment.

STUDY 2. A digital photograph of each brain section was taken using a camera attached to microscope. Snappy version 1.0 was used to capture photographs of brain sections. Infarct area from digital photographs was
measured using WoundMatrix software version 2.0 as described previously [180]. Total infarct volume was calculated by summing the infarcted areas of sequential sections and by multiplying the sum with the thickness of sections. Image analysis for the experiment was done by a technician blinded to the study group. Intra-observer variability using this method on two separate occasions was excellent (product moment coefficient of correlation r=0.98, n=12). All results were analyzed for statistical significance using a two-tailed Student t test.

2.2.2.7 Immunohistochemistry

Sections of infarct site and contralateral control site of the post-stroke brain were collected in OCT or formalin for different immunohistochemical analyses.

Fluoro-jade staining. Fluoro-Jade is an anionic fluorochrome capable of selectively staining degenerating neurons in brain slices. The histochemical application of Fluoro-Jade results in a simple, sensitive and reliable method for staining degenerating neurons and their processes. Compared to conventional methodologies, Fluoro-Jade is a sensitive and more definitive marker of neuronal degeneration than H&E or Nissl type stains, while also being comparably sensitive yet considerably simpler and more reliable than
suppressed silver techniques. To determine the neuronal degeneration, frozen brain sections (10µm) were stained using Fluro-Jade procedure [181].

**PP60src and phospho-Src staining.** Formalin-fixed brain tissue were embedded in paraffin and sectioned. The sections (4 µm) were deparaffinized and stained with mouse monoclonal antibody to Src (Upstate Cell Signaling Solutions) and rabbit polyclonal antiserum antiphospho-Src (Upstate Cell Signaling Solutions).

2.2.2.8 Vitamin E extraction and analysis

Vitamin E extraction and analysis of rat brains were performed as described in methods section 2.1.2.2 using an HPLC-coulometric electrode array detector (Coularray Detector, model 5600 with 12 channels; ESA Inc). This system enables the simultaneous detection of tocotrienols and tocopherols in the same run as described previously by our laboratory [118, 119].
2.2.2.9 Rat cortical mitochondria isolation

Mitochondria were isolated from cortical brain tissue of Sprague Dawley rats as previously described [182]. Briefly, rats were decapitated and the brains were quickly removed and rinsed in PBS. Cortices were surgically dissected out and homogenized in isolation buffer (215 mM mannitol, 75 mM sucrose, 0.1%BSA, 20 mM HEPES, 1 mM EGTA, pH adjusted to 7.2 with KOH). Following homogenization, mitochondria were selectively isolated using Percoll gradient centrifugation to separate them from cellular fractions. Mitochondria were pelleted and concentration was determined via protein assay.

2.2.2.10 Mitochondrial state-3 respiration in presence of 12-S-HPETE

Prior to experimentation, pelleted mitochondria from rat brain cortex were re-suspended in isolation buffer at a concentration of 0.5 mg/ml. Next, they were incubated for 5 min in the presence of either 12-S-HPETE (BIOMOL, Plymouth, PA. 0.5\(\mu\)mols/mg mitochondrial protein and 1.5\(\mu\)mols/mg mitochondrial protein) or a matching volume of ethanol (vehicle control). Following incubation period, state 3 respiration was determined by measuring the consumption of oxygen in solution using an Apollo 4000 Free-Radical Analyzer (World Precision Instruments, New Haven, Conn) after induction by 4
mM succinate at 1 min and 160 μM ADP at 7 min. Respiration was calculated as the rate of oxygen consumption per mg protein over time.

2.2.11 Mitochondrial inner-membrane potential and PTP induction in response to AA metabolites and αT3

Cortical mitochondria inner membrane potential (ΔΨ) and permeability transition pore (PTP) opening were measured using an Aminco DW-2000 dual beam spectrophotometer. ΔΨ was determined by measuring wavelength at 511-533 nm in the presence of 10 μM safranin [183] following mitochondria incubation with 12-S-HPETE (1μM), 12-S-HETE (1μM), AA (1μM), or matching volume of ETOH (vehicle control). Induction of PTP was determined by measuring the absorbance due to mitochondrial swelling at 540nm following 5 minutes of treatment with 12-S-HPETE (1 μM), AA (1 μM) or αT3 (1 μM) + 12-S-HPETE (1 μM) and 10 min of incubation in the presence of Ca2+ (100 μM).

2.2.2.11 Visualization of mitochondrial inner membrane potential in presence of 12-S-HPETE

PBS (control) or 12-S-HPETE (0.3 μM) were directly delivered to HT4 neurons in the cytosol via a micromanipulator Femtojet B 5247 and Injectman NI 2 (Eppendorf™) microinjection system coupled to a specialized phase-
contrast Zeiss™ microscope. PBS and 12-S-HPETE were co-injected with Dextran Alex Fluor 488 at 3 mg/ml for visualization. Cells were allowed to incubate for 2 hours after which 50nM TMRM was added to the cultures for an additional 30 min in order to visualize mitochondrial membrane potential (ΔΨ).

2.2.2.12 Statistics

Bar graphs represent mean±SD. Difference between 2 means was tested by Student t test. A value of $P<0.05$ was interpreted as a significant difference. Comparisons among multiple groups were made by ANOVA. $P<0.05$ was considered statistically significant.

2.2.3 Results and discussion

2.2.3.1 12-Lox-deficient mice are resistant to stroke mediated damage

Pursuant to our previously reported finding that 12-Lox deficient neurons are resistant to glutamate-induced death and that 12-Lox represents a key target for αT3 action [114], we observed that 12-Lox-deficient mice were resistant to stroke injury (Fig. 2.17). Significantly, 12-Lox-deficient mice exhibited more than 80% reduction in infarct volume as compared to wild-type controls, demonstrating the significance of 12-Lox in mediating stroke-induced
damage (Fig. 2.17). The compelling neuroprotective effects of αT3 in relation to 12-Lox in vitro led us to question the significance of this nutrient in vivo.

2.2.3.2 α-Tocotrienol prevents stroke damage in spontaneously hypertensive rats, inhibits 12-lipoxygenase phosphorylation

In SHR stroke study 1, experimental stroke outcome data revealed that the Tocomin-fed (50% αT3) rats tended ($P=0.057$, Fig. 2.19A) to have reduced brain injury after stroke as compared to the control group. While not statistically significant, this trend was the impetus for proceeding with a second stroke study in which potentially confounding variables were limited in study design. The study was repeated with a modified experimental design, including a more pure form of the αT3 supplement, longer supplementation period (5 additional weeks of supplementation), and a larger sample size. The purified αT3 supplementation increased brain αT3 levels without significantly changing brain αTOC (Figure 2.18C, D). The brain is known to retain its vitamin E levels effectively under conditions of dietary vitamin E deficiency [184]. Consistently, we did not observe any significant drop in the αTOC level of the brain of the control rats maintained on vitamin E deficiency (Figure 2.18C). Thus, other antioxidant systems such as the GSH system in the brain are not likely to be affected under the conditions of our study. In SHR stroke study 2, αT3-supplemented rats were significantly protected against stroke-induced brain injury ($P<0.05$, Figure 2.19B). Analysis of the brain tissue
collected from the stroke site as well as from the contralateral non-stroke site of the brain demonstrated that although phospho-12-Lox was not detected in the non-stroke site, 12-Lox was clearly tyrosine-phosphorylated in the tissue from the stroke site. Such stroke-associated phosphorylation of 12-Lox was lower in the brain of rats supplemented with αT3 (Fig. 2.19C). These findings corroborated nicely with results of αT3 prevention of 12-Lox phosphorylation in cultured HT4 neurons subjected to glutamate excitotoxicity (Fig. 2.14).

2.2.3.6 Hypothetical mechanisms of αT3 specific inhibition of 12-Lox

The structural difference between αT3 and αTOC is limited to the 3 unsaturations present in the phytyl tail of αT3. This small yet defining difference must be a critical link to the differences observed in the neuroprotective capabilities of each isoform. This leads me to hypothesize that one possible mechanism by which αT3 could inhibit 12-Lox activity is through steric hindrance, or non-competitive inhibition. The hydrophobic phytol tail of the vitamin E homologs allows for penetration into biological membranes and interaction with other lipophilic molecules[185, 186]. The unique nature of the unsaturations present in the tail of αT3 will induce a curved structural conformation as compared to a more ordered and linear molecular conformation in αTOC[114]. This unique structural conformation may permit binding of αT3 at an allosteric site such that it changes the conformational state of the active site on 12-Lox. In this instance, when AA is released from
the lipid bilayer, steric hindrance forces would prevent it from binding with high affinity to 12-Lox.

A similar hypothesis is that αT3 itself may compete for the active site of 12-Lox serving as a competitive inhibitor, thereby preventing AA from being metabolized. The various 12-Lox isozymes have substrate specificity for lipophilic molecules other than AA. For example, leukocyte 12-Lox has high affinity for linolenic acid (AA precursor) and even larger molecules such as phospholipids, cholesterol esters and low density lipoproteins [187, 188]. Epidermis lipoxygenase has high affinity for metabolizing linoleic acid methyl esters [189]. This suggests that other lipophilic compounds, such as αT3, may compete for the 12-Lox active site with AA. This hypothesis was directly tested by our laboratory in a small study using computer modeling of leukocyte 12-Lox to determine potential docking sites of αT3 [114]. The computer modeling took into account ten possible docking positions of αT3 and the binding energies associated with each. It found that while αT3 didn’t bind directly to the active site, it may directly interact with 12-Lox at the opening of a solvent cavity adjacent to the active site of AA metabolism, thereby inducing steric hindrance and preventing AA from binding and being metabolized.

A final hypothesis is that αT3 may prevent 12-Lox from localizing at the plasma membrane following activation. It has been previously reported that 12-Lox is sequestered in the cytosol until activation and subsequent translocation to the lipid membrane by thrombin stimulation [165].
Incorporation of αT3 into the lipid bilayer may alter the membrane fluidity and environment such that 12-Lox is not able to localize and exert an effect on arachidonic acid released from the lipid bilayer. While this would not explain enzyme inhibition of 12-Lox in the traditional sense, it represents one hypothetical explanation for the neuroprotective properties of αT3 in the prevention of AA metabolism.

2.2.3.3 Histochemical analyses of αT3 supplementation effects in stroke brain

The histochemical application of Fluoro-Jade results in a simple, sensitive, and reliable method for staining degenerating neurons and their processes [181]. More prominent Fluoro-Jade staining in brain sections from control rats compared with sections from supplemented rats (Fig. 2.20) was consistent with stroke-induced brain injury results shown in Figure 2.19. Previously, we have reported that activation of c-Src represents a key mechanism that contributes to neurodegeneration [115]. Furthermore, we reported that activated c-Src kinase can phosphorylate 12-Lox [102]. Tyrosine residue Y416 represents a major auto-phosphorylation site in c-Src activation loop that is also responsible for c-Src activation [190]. This data presents first in vivo evidence indicating that stroke is associated with c-Src activation at the injury site (Fig 2.20 - A3 vs. B3). Stroke-associated c-Src activation was partly suppressed in αT3 supplemented rats (Fig. 2.20 - C3).
2.2.3.4 12-Lipoxygenase mediated metabolite, 12-S-HPETE, induces mitochondrial dysfunction, αT3 prevents mt permeability transition pore opening

Activated 12-Lox metabolizes arachidonic acid released from the lipid membrane bilayer into the eicosanoid 12-S-HPETE (Fig 2.15). We have found suggestive evidence that 12-S-HPETE alone is neurotoxic when injected into the cytosol of GSH depleted neurons (unpublished – Fig. 2.16). A potential mechanism of action for 12-S-HPETE induced cell death has yet to be reported in the literature. Mitochondrial dysfunction is known to occur and contribute to neuronal cell death [168, 169, 191]. Challenged mitochondria lose active respiratory function, defined as rate of oxygen consumption following addition of state 3 respiratory substrate ADP in isolated mitochondria. Figure 2.21 demonstrates that 12-S-HPETE significantly inhibits the actively respiring state of functional mitochondria isolated from the cortex of rat brain in a dose dependent manner.

The mitochondrial inner membrane potential (mtΔΨ) is responsible for maintaining the proton motive force that drives respiration [192, 193]. Isolated mitochondria from rat cortex that were incubated with 12-S-HPETE (1 μM) had compromised mtΔΨ (Fig. 2.22) as compared to vehicle or arachidonic acid controls (Fig. 2.23). This outcome was visualized in HT4 neurons that received cytosolic microinjection of 12-S-HPETE and the mtΔΨ reporter
tetramethylrhodamine methyl ester (TMRM) (Fig. 2.24). Permeability transition pore (PTP) opening represents another component of mitochondrial dysfunction. The protein channel complex effectively negates the partitioning of protons across the inner membrane of mitochondria, thereby reducing mtΔΨ and arresting state 3 respiration. 12-S-HPETE (1 μM) exacerbated Ca²⁺ induction of the PTP (Fig. 2.25) as compared to arachidonic acid (1μM) control. This effect was ameliorated by co-incubation with αT3 (1 μm) (Fig. 2.25). Figure 2.26 summarizes the observed effects of 12-S-HPETE on mitochondrial function and the protective effect of αT3 in inhibition of PTP formation.

2.2.3.5 Limitations of mitochondrial study design, potential solutions

This work presents only a preliminary study on the effects of αT3 in preventing 12-S-HPETE mediated mitochondrial dysfunction. Studies to assess the effects of αT3 co-incubation with 12-S-HPETE to prevent respiratory dysfunction and loss of mtΔΨ were inconclusive (data not shown) and demonstrated a limitation of the experimental design in the post-hoc addition of αT3 to isolated mitochondria. As a lipid soluble vitamin, the neuroprotective properties of αT3 may necessitate incorporation into the lipid-bilayer of cells and organelles including mitochondria. A better experimental design therefore, would be to isolate cortical mitochondria from animals on a αT3 dietary supplementation regimen. Note that brain tissue-specific delivery
of tocotrienol isoforms was successfully demonstrated in section 2.1. In this way, αT3 may partition into lipids naturally and provide a more physiologically relevant assessment of mitochondrial protection in the context of 12-S-HPETE perturbation.

2.2.4 Conclusions

In support of a central role of 12-Lox in glutamate-induced neurodegeneration, we have previously reported that inhibitors of 12-Lox prevent death of neuronal cells caused in response to glutamate or GSH-lowering agents [114]. Our case for 12-Lox as a critical mediator of glutamate-induced neurodegeneration was strengthened by the finding that compared with neurons from corresponding wild-type mice, cortical neurons from 12-Lox-deficient mice are resistant to glutamate-induced death [114]. Our current findings demonstrate that 12-Lox deficiency robustly protects against stroke injury. This work also provides first the first characterization of a direct mechanism of cell death for the 12-Lox mediated metabolites of AA. 12-S-HPETE was shown to play a critical role in mitochondrial dysfunction by inhibiting the rate of state 3 respiration, decreasing mitochondrial inner membrane potential and exacerbating PTP opening. This builds a compelling case to look at 12-Lox as a therapeutic target for the management of stroke-related injury in the brain.
In glutamate-challenged neurons, αT3 effectively modulates both 12-Lox as well as c-Src activity to favor survival [114, 115]. In a rodent stroke model, αT3 reduced the volume of the stroke-induced lesion and inhibited 12-Lox phosphorylation. This body of research demonstrates that oral αT3 supplementation may be protective in preventing stroke-induced damage in vivo. In isolated mitochondria, αT3 prevented 12-Lox mediated AA metabolite 12-S-HPETE from inducing the PTP. All of these outcomes support a neuroprotective role for αT3 in preventing stroke-related neurodegeneration at various checkpoints of cell signaling.

While the results are encouraging, much work remains prior to taking αT3 to clinical stroke trials in humans. First, a better understanding of the αT3 specific mechanism of neuroprotection is needed. The minute structural differences between αT3 and αTOC significantly contribute to the substantial differences observed in bioavailability and isoform specific function in pathological conditions such as stroke. While hypothetical mechanisms of neuroprotection have been provided, further characterizations of the unique structural and biological properties of αT3 are warranted. Second, the effects of αT3 neuroprotection need to be studied in terms of functional outcomes, not only biological. Sensorimotor and behavioral studies in animals following acute ischemic stroke have been reported [194, 195]. Future in vivo studies should address the functional significance of αT3 neuroprotection following acute ischemic stroke, preferably in a large animal stroke model that more
accurately depicts the human phenomenon. To that end, while the results in this section provide relevance for αT3 neuroprotection as related to small animal in vivo stroke models, the models themselves have received criticism for not accurately modeling the complexities of the human stroke condition. This is the basis for the next chapter in which we describe a novel pre-clinical model of acute ischemic stroke in canines.
Figure 2.12 | Glutamate excitotoxicity pathway. High concentrations of extracellular glutamate interfere with the uptake of cystine into the cell, leading to decreased intracellular levels of cystine and its reduction product cysteine, with resultant decrease in glutathione (GSH) synthesis. This in turn leads to a proliferation of reactive oxygen species (ROS) that activate c-Src kinase (src). Src is capable of phosphorylating tyrosine residue 527 of 12-Lipoxygenase (12-Lox) which activates and induces translocation of the enzyme to the membrane phospholipid bilayer. Free arachidonic acid (AA) which has been released from membrane phospholipids by phospholipases (ie – phospholipase A2) is substrate for activated 12-Lox mediated metabolism into 12-S-hydroperoxyeicosatetraenoic acids (12-S-HPETE) and 12-S-hydroxyeicosatetraenoic acids (12-S-HETE). The 12-Lox mediated metabolites of AA are known to signal for cell death, however, it has yet to be elucidated if they play a direct role in neurodegeneration.
Figure 2.13 | Cytosolic αT3, but not αTOC, protects neurons from glutamate induced death. HT4 (A–I) were injected with αT3 (10^{-19} mol) into the cytoplasm (A; 90% survival count in 6 experiments) or nucleus (D). (B) Survival of the neuron injected with αT3, whereas the other neurons died and disappeared from the monolayer. Tocotrienol was coinjected with the fluorescent QDot (seen in red). The culture plate containing the surviving cell was repopulated with fresh, healthy HT4 cells to monitor the fate the surviving cell (arrow marked in C) over a period of 18 hours. Calcein AM was used to stain live cells (C). Control cells injected with QDot alone (not shown) or with αTOC (F and G, cytosolic; H and I, nuclear) did not survive (0% survival count
in 5 experiments) against glutamate induced challenge. Cytosolic injection of αT3 protected primary immature cortical neurons (J and K) against glutamate challenge as well. Panels A, D, F, H, J represent images at 0 hour of glutamate challenge. Panels B, E, G, I, K represent images at 18 hours of 10 mmol/L glutamate challenge. Representative illustrations of 5 experiments are shown. Objectively, nuclear injection of αT3 failed to protect in 100% case. TCP (10^{-19} mol) failed to protect in 100% of cases. Cytosolic injection of αT3 protected cells in 90% of all cases. Arrows in the following frames point towards the same cell (A-C, D-E, F-G, H-I, J-K).
Figure 2.14 | c-Src and 12-lipoxygenase in glutamate-induced neuronal death. HT4 cells (A and B) were either treated or not with αT3, BL15, herbimycin, or geldanamycin (as indicated) for 5 min and challenged with buthionine sulfoximine (0.15 mmol/L; BSO, A) or BSO and arachidonic acid (0.05 mmol/L, B) for 24 h. BL 15 is an inhibitor of 12-lipoxygenase. Both herbimycin and geldanamycin inhibit c-Src kinase activity. (A) αT3, 12-Lox inhibitor as well as c-Src inhibitors protected against BSO-induced glutathione depletion dependent loss of cell viability. †Higher than BSO nontreated. *Lower than BSO-treated. (B) αT3, 12-Lox inhibitor, and c-Src kinase inhibitors protected against BSO and arachidonic acid-induced loss of cell viability. †Higher than BSO (A) and nonchallenged (B) groups. *Lower than BSO and arachidonic acid-challenged group. *P<0.001. Glutamate- (10 mmol/L) induced 12-Lox phosphorylation was inhibited by αT3 (250 nmol/L) in HT4 cells (C) as well as in immature cortical neurons (F). Herbimycin also inhibited inducible 12-Lox phosphorylation (E). Cells were either treated or not with αT3 for 5 min and challenged with glutamate (10 mmol/L) or BSO (0.15 mmol/L) and arachidonic acid (0.05 mmol/L) for 15 min (D) or 30 min as indicated. In these experiments, 15 min before challenging, cells were treated with Na3VO4 (0.15 mmol/L) to inhibit tyrosine phosphatases. C, control (nontreated); TCT, α-tocotrienol; H, herbimycin; G, glutamate; AA, arachidonic acid. (G and H) Glutamate-induced phosphorylation was inhibited (G) in cells expressing dominant negative c-Src (K296R/Y528F) and more prominent in kinase-active (Y529F) c-Src overexpressing cells than in wild-type (pUSE) or kinase-dead c-Src (K297R) overexpressing cells (H). Cells were activated with 10 mmol/L glutamate for 15 min.
Figure 2.15 | 12-lipoxygenase mediated metabolism of arachidonic acid.

The oxidation of ferrous iron (Fe$^{2+}$) to ferric iron (Fe$^{3+}$) activates the enzyme which is capable of excising a hydrogen from arachidonic acid at carbon #10. This leads to the formation of a radical metabolite of arachidonic acid (AA) that rapidly reorganizes its double bonds to take on a slightly more stable conformation. Next, insertion of molecular oxygen generates a hydroperoxide radical which is reduced to the hydroperoxide anion by the simultaneous oxidation of iron to the ferric state. A proton is then accepted to form the hydroperoxide. The newly formed 12-S-HPETE is then released from the active site of 12LO whereby it may be reduced to form 12-S-HETE. NOTE: unaffected portions of AA metabolites were truncated in some steps for lack of space.
Figure 2.16 | Arachidonic acid (AA) metabolite, 12-S-HPETE, induces cell death in GSH depleted HT4 neurons. HT4 neurons were treated with BSO (50μM) for 12h to deplete cellular GSH levels. Cytosolic micro-injection (500 femtoliter) of 12-S-HPETE (0.3μM) was neurotoxic under GSH depleted conditions after 24h (A. 0h; B. 24h). Coinjection with red Qdot was used as a tracker. (C) When added (5μM, 24h) to culture media outside the cell, 12-S-HPETE (HP), but not 12-S-HETE (HE), was cytotoxic when co-treated with BSO (50μM) to deplete cellular GSH. αT3 (1μM, 5min before BSO tx) protected. Data (mean ± SD, n=4) shown as %LDH leakage. *P<0.05 significantly different compared to untreated or BSO treated control (con). †P<0.05 significantly lower compared to corresponding 12-S-HPETE treated cells without αT3. (D) GSH levels in cells following treatment with BSO (50μM) for 24h. Mean ± SD (n=4); *P<0.05 compared to untreated cells.
Figure 2.17 | 12-Lipoxygenase-deficient mice are resistant to transient focal cerebral ischemia. Transient focal cerebral ischemia was induced in the C57BL6/J (control) and 12-lipoxygenase knockout (12-Lox-/−) mice by middle cerebral artery occlusion. Coronal sections of brain (72 hours after stroke) were stained with 2,3,5-triphenyltetrazolium (TTC). Fixed sections were photographed using Inquiry software (Loats Associates, Inc.). The images were used to determine infarct size as a percentage of the contralateral hemisphere after correcting for edema. The infarct extended from caudate putamen into surrounding cortex, and was visible in 4 of 5 slices of the brain from control wild-type mice. Data are mean±SD. *P<0.02.
Figure 2.18 | α-tocotrienol (αT3) and α-tocopherol (αTOC) levels in the brain of spontaneously hypertensive rats. Spontaneously hypertensive rats (SHR) were supplemented with Tocomin (50% αTCT) in study #1 and purified αT3 in study #2 as described in methods section 2.2.2.3. Following respective study supplementation periods and stroke, a small portion of the non-infarcted occipital lobe was removed for vitamin E analysis. Figures (A) and (C) represent the effect of oral supplementation on brain αTOC. (B) and (D) represent the effect of oral supplementation on brain αT3. *P<0.01 significantly different compared with corresponding control group.
Figure 2.19 | Oral αT3 supplementation protects against stroke-induced injury and 12-Lox phosphorylation in SHR brain. (A) Study #1 infarct volume in response to stroke. P-value of 0.057 nearly misses statistical cut-off (P<0.05) for study. (B) Study #2 infarct volume in response to stroke. (C) αT3 supplementation lowered 12-lipoxygenase phosphorylation in the stroke-site brain tissue of SHR. The brain tissue sample was harvested 24 h after stroke from a predictable area of infarct core in the MCA supplied territory. This includes the primary motor cortex and the primary somatosensory cortex.
Figure 2.20 | Histological analyses of post-stroke infarct zone cortical sections. Fluoro-Jade (A1, B1, C1), Src (A2, B2, C2) and phosphor-Src (A3, B3, C3) staining. In the contralateral hemisphere of the brain in control rats (A1), there was negligible Fluoro-Jade positive staining. The stroke affected hemisphere of the brain from αT3 supplemented rats (C1) contained fewer Fluoro-Jade positive cells compared with the stroke affected hemisphere of control rats (B1). In the non-stroke affected section of the brain, Src was present (A2) but not in phosphorylated form (A3). Stroke did not influence Src expression, but clearly induced Src phosphorylation and activation. Such stroke-induced Src activation was less in the brain sections of αT3-supplemented rats as compared to controls (C3 vs. B3). For all sections shown, brain was harvested 24 h after stroke. Bar=100μm.
Figure 2.21 | Inhibition of mitochondrial ATP synthesis (state 3 respiration) by 12-S-HPETE. Isolated mitochondria from the cortex of rat brain (0.5 mg/ml) were incubated for 5 minutes with either ethanol vehicle (A), 0.5 μmoles/mg mitochondrial protein 12-S-HPETE (B) or 1.5 μmoles/mg mitochondrial protein 12-S-HPETE (C). State 3 respiration was determined by measuring the consumption of oxygen in solution using a Clark-type electrode following induction by 4 mM succinate (*) at 1 minute and 160μM ADP (†) at 7 minutes. Each trace is representative of one of 3 different runs per group. 12-S-HPETE (HP -1.5 μmoles/mg mitochondrial protein) significantly inhibited state 3 respiration (†, rate of oxygen consumption after addition of ADP) as compared to ETOH control. (D) Mean rate of oxygen consumption ± SD; ETOH control (n=3) vs. HP (n=3). *P<0.05.
Figure 2.22 | 12-S-HPETE and 12-S-HETE compromise mitochondria inner membrane potential. Brain mitochondria were isolated from 10 wk old female Sprague Dawley rats (n=4). Inner membrane (IM) potential (ΔΨ) of mitochondria incubated with either ETOH (vehicle), 12-S-HPETE or 12-S-HETE was determined using dual wavelength spectroscopy (533-511nm) as the difference in absorbance following the addition of succinate (1mM) less the uncoupler CCCP (5 uM). Chart shows a representative graph of absorbance vs. time for ETOH (black), 12-S-HPETE (blue) and 12-S-HETE (red) treated mitochondria. Numbers on chart represent the addition of: 1) treatment (1 uM 12-S-HETE or 12-S-HPETE or equal volume of ETOH vehicle), 2) rotenone (5 uM), 3) succinate (1 mM), 4) CaCl$_2$ (100 uM), 5) EGTA (1 mM), 6) CCCP (5 uM). After addition of succinate (4), 12-S-HPETE and 12-S-HETE incubated mitochondria demonstrate loss of membrane potential as compared to ETOH control.
Figure 2.23 | Loss of mitochondrial membrane potential following treatment with 12-S-HPETE and 12-S-HETE. Decrease in mitochondrial inner membrane potential, calculated as mean percent difference in absorbance between succinate (1 mM) and CCCP (5 um) values in control (ETOH vehicle only) versus treatment with arachidonic acid (AA, 1 uM), 12-S-HPETE (HP, 1 uM) and 12-S-HETE (HE, 1 uM). Data are mean ± SD calculated from 3 separate runs for each treatment. *P<0.05 HE or HP treatment vs ETOH control.
**Figure 2.24 | 12-S-HPETE induced loss of mitochondrial membrane potential in neurons.** Cultured HT4 neurons were treated with 10 mM BSO 12 hours prior to cytoplasmic injection with either PBS (A and B) or 12-S-HPETE (C and D, 0.3 µM). Microinjection was coupled with Dextran Alex Fluor 488 for visualization. Following microinjection, cells were permitted to incubate for 2 hours after which 20 µM tetramethylrhodamine methyl ester (TMRM) was added to visualize mitochondrial membrane potential. Cytosolic microinjection of 12-S-HPETE (D) reduces TMRM signal intensity, representative of loss of mitochondrial membrane potential, as compared to control (B).
**Figure 2.25 | α-Tocotrienol protects mitochondria from 12-S-HPETE exacerbated permeability transition pore opening.** Mitochondrial swelling and opacity caused by Ca\(^{2+}\) induction of permeability transition pore opening was measured spectrophotometrically at 540 nm. Prior to the addition of Ca\(^{2+}\), mitochondria were incubated with arachidonic acid (AA, 1 µM), 12-S-HPETE (1 µM), or α-tocotrienol (1 µM) + 12-S-HPETE (1 µM) for 5 min. Representative trend lines are shown (n=3).
Figure 2.26 | Summary diagram of 12-S-HPETE effects on mitochondrial dysfunction. 12-S-HPETE (some evidence of 12-S-HETE) effectively inhibited state 3 respiration and inner membrane potential in isolated brain mitochondria and cultured HT4 neurons. 12-S-HPETE also induced the permeability transition pore (PTP), which leads to mitochondrial swelling and dysfunction. α-Tocotrienol inhibited PTP induction by 12-S-HPETE – suggestive of another potential mechanism of αT3 neuroprotection in stroke-related pathology.
CHAPTER 3

NOVEL PRE-CLINICAL MODEL OF ACUTE ISCHEMIC STROKE

3.1 INTRODUCTION

Stroke is currently the third leading cause of death in the U.S. with 780,000 Americans afflicted by a new or recurring stroke each year; accounting for nearly 1 in every 16 deaths [23]. In spite of the high incidence and mortality of such a devastating disease stroke remains a very difficult pathology to accurately model. While a variety of potential therapeutics have shown promise in cell culture and small animal models of stroke, the vast majority of clinical trials to test the efficacy of such modalities have failed [196, 197]. As of 2000, 75 different therapeutic strategies have been tested in acute stroke clinical trials, while only 2 have been widely accepted to be beneficial (tPA on the basis of the NINDS trials and aspirin on the basis of the combined results of IST and CAST) [198]. Further emblematic of the difficulty in accurately modeling acute ischemic stroke is the 2008 request for applications
from the National Institutes of Health and Canadian Stroke Network for a Stroke Preclinical Trials Consortia in recognition of “the translational barriers that exist today in stroke research” [199].

To address the apparent gap between laboratory and clinical research, we have developed a novel pre-clinical model of acute focal ischemia in canines using an interventional radiology approach. The advantages of using a canine model are numerous. First, the size and anatomical feature set of the canine brain more closely mimics human brain as compared to small animal stroke models. Canines have a highly evolved gyrencephalic neocortex with a white to gray matter ratio that more closely approximates humans [200, 201]. Second, their neurovascular architecture accommodates an array of endovascular devices and interventional radiology techniques permitting a minimally invasive approach to the surgery while providing real-time visualization of occlusion. For example, endovascular MCA occlusion is made possible by deploying a soft platinum matrix coil clinically used to treat intracranial aneurisms [202]. This approach necessitates only two small femoral artery punctures to navigate the neurovasculature and deploy the device in the M1 segment of the MCA under guided fluoroscopy. Finally, the proposed method of MCA occlusion in canines offers a highly reproducible and relatively inexpensive alternative to non-human primate models of acute focal ischemia. While anatomically closer to humans than canines, the use of
non-human primates pose additional ethical, veterinary and housing considerations that obligate larger fiscal and personnel requirements [203, 204].

Taking these factors into account along with our experimental results, the proposed pre-clinical model of transient MCA occlusion in canines spans the translational gap that exists between laboratory and clinical stroke research. This model serves as an ideal platform to further elucidate the neuroprotective properties of the natural vitamin E αT3 prior to clinical study.

3.2 Materials and methods

3.2.1 Endovascular Canine MCA Occlusion

All experimentation was approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University. One day prior to percutaneous intervention, mongrel canines (n=5) with a body weight of 20-30 kg received a 300 mg loading dose of clopidogrel. On the day of surgery, the animals were sedated with telazol (6 mg/kg bw IM, vol<3cc.) and anesthetized (1.5-2.0% isoflurane). Continuous cardiac rhythm, respiration rate, end-tidal CO2, and oxygen saturation were monitored for control of physiologic parameters. Canine body temperature was maintained around the normal range of 38-39.2° C using a convective warming system (Gaymar Thermacare, Orchard Park, NY).
Bilateral common femoral artery access was obtained using 5 French sheaths (Arrow, Erding, Germany). Under fluoroscopic guidance (GE Medical OEC 9800 Plus Cardiac, GE Healthsystems, Piscataway, NJ) a five French guide catheter (Boston Scientific, Natick, MA) was advanced into the vertebral artery (VA) and was attached to a pressurized drip. The animal was administered 2000 units of heparin as a bolus. A 4 French catheter (Boston Scientific) was then placed in the right vertebral artery to provide access into the basilar artery system and allow for periodic infusion of spasmolytic agents as needed (papaverine 0.3 mg/ml delivered at 1ml aliquots). Arteriography allowed for documentation of vasodilatation in the intended territory. Microcatheter techniques were then used to access the MCA via the circle of Willis (Fig. 3.1). Through the vertebral artery (VA) guide catheter a SL-10 microcatheter (Boston Scientific) with a microwire was advanced into the MCA. Once the microcatheter was in place, an embolic coil (3x20 Ultrasoft Matrix2 Platinum Coil, Boston Scientific) was delivered into either MCA to include the entire M1 segment. The 4F catheter and the 5F catheter were then used to perform digital subtraction angiograms (DSAs) of the internal carotid arteries and the vertebrobasilar circulation in order to confirm complete occlusion of the MCA via fluoroscopic contrast injection without evidence for either circle of Willis collaterals or any pial collateral formation that may have developed during the occlusion. The coil was repositioned if necessary in order to achieve complete occlusion. Angiographic evidence for incomplete occlusion or pial collateral formation reconstituting the occluded territory were
considered exclusion criteria for MRI analysis. Once the coil was positioned in place, the microcatheter was drawn back into the third spinal arterial ramus. ICA and VA DSAs were repeated every 15 minutes to confirm continued occlusion. Occlusion of the MCA lasted for 1 h. At the end of the transient occlusion, the microcatheter was advanced back into the MCA and the coil was captured and retrieved. VA and ICA DSAs were repeated to confirm reperfusion of the occluded territory. The catheters were then removed. A blood draw for activated clotting time was then obtained. Depending on the result of the activation time, a weight based calculated dose of protamine was delivered to the canine to reverse the effects of heparinization. The sheaths were then removed and pressure was applied at the arteriotomy sites for hemostasis. The canine was then brought out of anesthesia, extubated and the arteriotomy sites were periodically checked for hematoma. Post-operative veterinary care was provided to the canines for 24 h prior to MRI.

3.2.2 3T Magnetic Resonance Imaging

Evaluation of the infarct lesion was accomplished using 3T MRI (Philips Healthcare, Andover, MA) 24 h after MCA reperfusion. The animal was sedated with telazol (6 mg/kg bw IM, vol<3 cc) and anesthetized (1.5-2.0% isoflurane) throughout the MRI scans (approximately 1 h). While under anesthesia in the magnet, the canine’s heart rate, respiratory rate and body temperature were monitored. All MR imaging was performed under the
guidance and supervision of a trained technician at the Wright Center of Innovation for Biomedical Imaging (Columbus, OH). The image processing software ImageJ (NIH, Bethesda, MD) was used for infarct volume calculation from coronal T2-weighted MR images (3mm slice thickness). Raw MR images were converted to standard DICOM (Digital Imaging and Communications in Medicine) format and transferred to an image processing workstation. After appropriate software contrast enhancement of the images, manual planimetry was performed to delineate infarct region, ipsilateral hemisphere and contralateral hemisphere. We employed this technique to quantitate stroke injury as a fraction of contralateral hemisphere and total brain volume. Correction for edema induced midline shift in hemispherical volume was incorporated into infarct volume calculations as previously described[205].

3.2.3 Histology

Canines were euthanized (euthasol, IV, 1ml/4.6kg) immediately following MR imaging. Next, necropsy was performed to isolate the brain. Continuous 3mm coronal slices were collected throughout the ipsilateral and contralateral hemispheres using a canine brain matrix. Sections were rinsed in PBS, embedded in OCT compound (Sakura Finetek, Torrance, CA) and frozen at -80°C. OCT embedded slices were subsequently cut in 10μm thick sections on a Leica CM 3050 S cryostat (Leica Microsystems, Wetzlar, Germany) and mounted onto slides for histological determinations.
Hematoxylin and eosin staining of frozen canine brain tissue was performed to contrast gross stroke pathology in infarct affected and contralateral control tissue.

To determine neuronal degeneration, frozen brain sections (10μm) were stained using Fluoro-Jade procedure[181]. Fluoro-Jade is an anionic fluorochrome capable of selectively staining degenerating neurons in brain slices. Compared to conventional methodologies, Fluoro-Jade is a sensitive and more definitive marker of neuronal degeneration than Nissl type stains, while also being comparably sensitive yet considerably simpler and more reliable than suppressed silver techniques. Tissue sections were analyzed by fluorescence microscopy (Axiovert 200M) and images were captured using Axiovert v4.6 software (Zeiss, Germany).

3.3 Results and discussion

3.3.1 Vertebrobasilar approach to MCA occlusion

Intuitively, endovascular access to the MCA is via the internal carotid artery (ICA) which contributes to the deficiency of published MCA occlusion models that purposefully explore alternative routes [206]. We observed that the ICA to MCA approach was not feasible in canines because of the tortuosity of the canine ICA (Fig 3.1). Our experiments identified that the basilar artery (BA) to MCA approach was effective to provide the endovascular access
needed for our purposes. Originally, navigation of the canine ICA was attempted with an array of small diameter microwire (.010” - .014”) and microcatheter (1.3F) systems. However, none were capable of advancing to the MCA origin without significant stretching, distortion and risk of perforation of the ICA’s tortuous loops. Further exploration of the canine cerebrovascular architecture (Fig. 3.2) revealed the BA approach to be adequately large and straight to accommodate the FASdasher 14 microwire and SL-10 microcatheter. Femoral artery (FA) catheterization allowed access to the vertebral arteries (VA) that branch off of the left and right subclavian arteries near the aortic arch. The VA run along the outside of the spinal cord and eventually split between vertebrae C2 and C3 into the right and left spinal ramus arteries which unite to form the anterior spinal artery (ASA). The microwire was advanced from either VA into the anterior spinal artery (ASA) via the spinal ramus artery (SRA). The ASA continues intracranially to become the BA. Next, the microwire was directed from the BA around the Circle of Willis into either the left or right MCA depending on which side appeared more favorable under c-arm fluoroscopy (Fig. 3.3A). The smaller diameter of the PCOM in relation to the BA requires constant monitoring of contrast filling under fluoroscopy to assure the PCOM is not unintentionally occluded by the microwire or microcatheter (Fig. 3.3B). The effects of PCOM occlusion were documented in a separate canine not included in the infarct volume calculations of the present study (Fig. 3.4). Occlusion of the PCOM in this particular canine induced an infarction of the diencephalon. As the stroke
evolved, the canine had difficulty in regulating body temperature presumed to be due to hypothalamic failure. To reduce the risk of inadvertent PCOM occlusion, the matrix coil was quickly deployed into the MCA upon successful tracking of the microcatheter across the PCOM (Fig. 3.3C). After placement of the matrix coil in the M1 segment of the MCA, the microcatheter was then retreated to the origin of the SRA. Successful MCA occlusion was verified by ICA injection of contrast agent under fluoroscopy. During the 1 h MCA occlusion period, both ICAs were routinely subjected to arteriograms in order to confirm occlusion of the ipsilateral MCA and opacification of the contralateral hemisphere (Fig. 3.3D). After 1 h of occlusion, the matrix coil was retrieved and patency of the MCA territory confirmed reperfusion (Fig. 3.3E). Slower than normal filling of the reperfused MCA was documented and determined to be a consequence of the rapid onset of edema and vasospasm. A post-reperfusion BA arteriogram was performed in order to confirm opacification of the PCOMs, BA and both MCAs (Fig 3.3F). Physiological parameters recorded throughout the surgical procedure remained consistent before, during and after occlusion (Table 3.1).
Table 3.1 | Physiological parameters pre -, during -, and post-occlusion.

3.3.2 **Angiographic visualization of MCA occlusion**

A noteworthy strength of this pre-clinical stroke model is the real-time visualization of MCA occlusion under c-arm fluoroscopy. In order to verify occlusion, we introduced guide catheters into both femoral arteries so that one catheter could facilitate delivery of the matrix coil to occlude the MCA while the second could be positioned in either ICA for arteriograms of the MCA territories (Fig. 3.5) or in the VA to confirm patency of the vertebrobasilar system and PCOM around the microcatheter. Furthermore, the ischemic territory affected by MCA occlusion can be visualized under c-arm fluoroscopy by observing the phenomenon of contrast diffusion into arterioles and capillary beds -termed “blushing” (Fig. 3.6). Only a few seconds after the initial bolus injection of contrast into the right ICA, absence of blushing in the region circled
in Fig. 3.6C defines the ischemic territory at risk for developing infarct. The ability to visualize the placement of the matrix coil as well as the affected ischemic territory allows for repositioning of the coil as needed in order to reproduce a consistent ischemic territory across multiple animals.

3.3.3 Infarct volume assessment

Twenty-four hours after MCA reperfusion, high resolution T2-weighted images of coronal brain slices from 3T MRI revealed infarct associated with the region of the cerebral cortex supplied by the MCA (Fig. 3.7). To quantitate the infarct volume, manual planimetry was performed to delineate the infarct region, ipsilateral and contralateral hemispheres (Fig. 3.8a). Only slices from the anterior aspect of the brain including the olfactory lobe to the posterior aspect of the cerebrum were used. Hemispheric volumes were determined from T2-weighted images by use of the following neuroanatomic landmarks: falx cerebri, pineal gland, fissura longitudinalis, infundibulum, sylvian aqueduct, and third ventricle. Lesion areas were then summed and multiplied by the slice gap thickness to obtain an infarct volume uncorrected for edema. A substantial midline shift, evidenced by the displacement of the third ventricle, is emblematic of the supratentorial lesion. As a result, the relative hemispherical size is distorted which in turn leads to a misrepresentation of true hemispherical infarct volume. Due to the strong presence of edema induced swelling in canine brain at 24 h post MCA reperfusion, we used a
previously reported method for edema corrected lesion volume calculation [205]. This calculation is based on 3 assumptions: 1) compression of the contralateral hemisphere is comparable to compression of the entire healthy brain tissue, whereas the lesion is not compressed. 2) The contralateral hemisphere is compressed to the same extent as the affected hemisphere is extended. Total brain volume does not change; and 3) volume extension occurs only within the lesion, not in the unaffected tissue. Taking these factors into account, the mean percent hemispherical infarct volume corrected for edema in the four canines as reviewed by two independent observers was 30.9±2.1 and 31.2±4.3, respectively (Fig. 3.8). Three dimensional reconstructions of axial MRI slices using Osirix software provided a clear visual representation of infarct size in relation to hemispherical volume (Fig. 3.9).

3.3.4 Stroke-induced pathology

Hematoxylin and eosin staining of the stroke induced infarct tissue of the neocortex at 24h time point revealed condensed, pyknotic nuclei as compared to contralateral control tissue (Fig. 3.10A, B, D, E). Positive Fluoro-Jade immunofluorescence staining was used to indicate neuronal degeneration in infarct affected tissue as compared to contralateral controls (Fig. 3.10C, F).
3.3.5 Comparison to intraluminal thread model of MCA occlusion

When the intraluminal suture model of MCA occlusion in rats was first reported by Koizumi et al in 1986 and later modified by Longa et al in 1989, it introduced a novel non-invasive small animal model of acute focal ischemia [207, 208]. To this day the model continues to be the most frequently used method to test potential therapeutic stroke agents in vivo [209]. Several variations of techniques and approaches to induce MCA occlusion in rodents have been published since the model’s inception, however, none have been as widely accepted [210-212]. Despite the popularity of the intraluminal suture model, there remain significant limitations to the approach which have been extensively documented in the literature and contribute to the high degree of variability in lesion volume and location across and within studies [196, 209, 213-217]. First, real-time placement of the occluder in the MCA cannot be visualized. Consequently, overshooting or undershooting the MCA origin is a common occurrence that can contribute to variability in lesion volume outcomes. While laser doppler flowmetry (LDF) improves the reliability of occluder placement, it only provides information on the relative decrease in blood flow at a single point; thereby making it difficult to determine if the territory is fully or only partially occluded. A significant advantage of the canine guided fluoroscopy approach is the appreciation of M1 segment MCA occlusion in real-time using angiograms. This allows for visualization of the entire ischemic territory and confirms partial or full occlusion of the MCA.
Further contributing to the variability of stroke lesion volume in the intraluminal thread model is the potential for premature reperfusion of the MCA territory which has been documented to occur in 25% of experimental animals [209]. While premature reperfusion was not encountered in our study, repeated monitoring of the MCA territory via c-arm fluoroscopy allows for adjustment of the matrix coil as needed to ensure complete occlusion of the MCA throughout the ischemic event.

Finally, it has been reported that after placement of the intraluminal suture tip in the proximal MCA, the remaining suture in the ICA is occlusive to the anterior choroid artery and the hypothalamic artery; thereby producing unintentional subcortical lesions [218]. In our pre-clinical model we have documented that occlusion is specific to the MCA supplied territory and by retreating the microcatheter to the SRA origin we can prevent unintentional occlusion of arteries, such as the PCOM, which remain patent under fluoroscopy.

The high degree of variability in lesion volume associated with the intraluminal suture model is not limited to experimentation in small animals such as rodents. In translating the intraluminal suture model to non-human primates, Freret et al reported a 58% standard deviation around the mean infarct volume from marmosets (n=4) subjected to transient MCA occlusion [219]. In contrast, the standard deviation around the mean (n=4) in canines using the current matrix coil approach was found to be less than 15% for both
observers. Similarly, other endovascular models employing the ICA route to MCA occlusion in non-human primates have reported large standard deviations in infarct volume across animals [220, 221]. The appreciably tighter standard deviation in stroke induced lesion volumes using our model suggests that fewer experimental animals would be required to achieve statistical significance in studying potential therapeutic targets.

3.3.6 Comparison to craniectomy model of MCA occlusion

As it relates to other large animal models of MCA occlusion, our proposed method of endovascular occlusion differs in that it offers a minimally invasive approach (Fig. 3.12). Several variations of transorbital MCA occlusion via vessel clamp in non-human primates have been reported [222-226]. While variants of this approach have touted highly reproducible infarct volumes, they necessitate an invasive approach including removal of the orbital globe, transection of the optic nerve and opthalmic artery, and craniectomy of the posteromedial orbit [224]. To date, the effects of the orbital wound and trauma induced inflammatory response have not been resolved separately from the stroke induced pathology. The close proximity of the trauma to the stroke site, including removal of the skull and dura covering the anterior circle of Willis, warrants further investigation.
Concurrent with the development of our endovascular approach, we separately attempted a craniectomy in the canine whereby a small cranial window (2 cm x 2 cm) was created in the parietal bone and the dura excised to expose the MCA for vessel clip occlusion. However, this approach was quickly abandoned after 24 h MR imaging of the head revealed significant inflammation adjacent to the craniectomy site and edema induced herniation of the cortex through the cranial window (Fig. 3.11). Advances in the field of interventional neuroradiology have outdated such an invasive approach as trauma associated with the endovascular model is reduced to two small puncture sites in the anatomically distant femoral arteries.

3.3.7 Model limitations, potential pitfalls and solutions

Advantages aside, the endovascular model as described is not without its own unique set of challenges and potential pitfalls. First, there was a steep learning curve to navigating the canine intracranial vasculature. Several animals were required early on in model development to familiarize the surgeon with the tactile skill set and tools necessary to achieve successful endovascular navigation. Second, the canine vertebrobasilar system is prone to microcatheter and microwire arresting vasospasm. Significant precaution must be taken to avoid prolonged exposure of the microcatheter to the PCOM and BA. Microwire movement must also be limited to the purposeful advancement of the wire to the MCA origin as quickly as possible to avoid
spasm. In the case of spasm occurring, waiting for it to resolve for a short interval (5-10min) or delivering a small dose of spasmolytic agent generally overcame the problem. Finally, in some canines MCA cannulation was simply not possible because of variations in their cerebrovascular anatomy. In this instance, and when appropriate, catheters and wires were removed and the canines were recovered to be transferred out of the study.

3.4 Conclusions

Successful translation of stroke therapeutics research from the laboratory to the clinic has failed to meet expectations. Contributing to the discrepancy in laboratory and clinical results are the limitations of small animal models to produce consistent infarct volumes across and within studies, and the vast anatomical and functional differences between small and large mammalian brains. To date, large animal models of stroke in non-human primates have employed an invasive transorbital approach which has the potential to introduce trauma associated complications in addition to the cost prohibitive nature and ethical considerations associated with the use of non-human primate subjects. The endovascular canine model of transient MCA occlusion serves as an ideal pre-clinical stroke model benefiting from guided fluoroscopic occlusion of the MCA, and high inter-animal reproducibility in a cost effective large animal model.
To say that clinical trials are expensive is an understatement. National spending on clinical trials in the United States was nearly $24 billion in 2005 [227]. Proceeding to clinical trials prior to evaluating the target therapeutic in a robust pre-clinical setting is a risky endeavor. As it relates to the clinical translation of our α-tocotrienol stroke research, the canine pre-clinical stroke model provides the best bridge between laboratory and clinical stroke study. Experiments employing the model are in progress with canines receiving α-tocotrienol supplements or placebo controls for a period of 10 wk. It is our hope that this model will serve as an effective tool to improve upon the success rate of translating potential stroke therapeutics, such as α-tocotrienol, to the clinical setting.
Figure 3.1 | Vertebrobasilar approach to MCA occlusion in canines. The majority of intraluminal middle cerebral artery (MCA) occlusion models in animals approach the MCA via the internal carotid. In canines, this approach is not feasible due to the tortuosity of the internal carotid artery (ICA) that prevents navigation with microwires as small as .010” in diameter. (A) Stretching and perforation of the ICA were encountered when trying to advance a microcatheter into the ICA. (B) The alternative to this approach was to advance a microwire into the basilar artery (BA), along the posterior communicating artery (PCOM), and into the MCA. Subsequently a microcatheter was positioned into the MCA and a 3x20 Ultrasoft Matrix2 Coil (Boston Scientific, Natick, MA) was deployed for 1 hour to induce MCA territory infarction. Following 1h of occlusion, the coil was retrieved and the MCA territory reperfused as confirmed under fluoroscopy. 1=ICA, 2=MCA, 3=ACA, 4=PCOM, 5=BA.
Figure 3.2 | Non-invasive fluoroscopic guidance of canine cerebrovascular system. In order to assess the canine cerebrovascular system and to guide the approach as described in Fig 3.1, real-time angiographies were performed using Omnipaque™ Iohexol (GE Healthcare, UK) contrast under c-arm fluoroscopy. The still frame is representative of a left vertebral artery (LVA) contrast injection. In all angiograms, left and right sides are opposite due to the supine orientation of the canine during surgery. From bottom of frame to the top: RVA = right vertebral artery, LVA = left vertebral artery, C3 = vertebrae C3, LSRA = left spinal ramus artery, RSRA = right spinal ramus artery, C2 = vertebrae C2, ASA = anterior spinal artery, C1 = vertebrae C1, BA = basilar artery, LSCA = left superior cerebellar artery, RSCA = right superior cerebellar artery, LPCA = left posterior cerebral artery, RPCA = right posterior cerebral artery, LPCOM = left posterior communicating artery, RPCOM = right posterior communicating artery, LMCA = left middle cerebral artery, RMCA = right middle cerebral artery, ACA = anterior cerebral artery.
Figure 3.3 | Fluoroscopic guidance of right middle cerebral artery occlusion. Under guided c-arm fluoroscopy, (A) the microwire was advanced from the BA, along the PCOM to the origin of the RMCA. (B) RICA contrast injection confirmed placement of the wire in the RMCA. (C) The microcatheter was tracked along the microwire into the RMCA. From the microcatheter the matrix coil was deployed into the RMCA occluding the M1 segment. (D) LICA contrast injection confirmed that the LMCA was still intact, along with the ACA, BA, and RPCA while the RMCA remains occluded. (E) Following 1h occlusion, the matrix coil was retrieved and RICA contrast injection confirmed that the RMCA had reperfused. (F) BA contrast injection revealed that the Circle of Willis was intact. While slower than the LMCA, the RMCA still perfused. Slower post-reperfusion filling of the ischemic territory was interpreted to be a consequence of the early-onset of edema.
Figure 3.4 | 3T MRI evidence of posterior communicating and basilar artery affected stroke. Prolonged placement of the microcatheter along the PCOM (A) and BA (B) en route to the MCA may produce unintended occlusive events in their respective territories (arrows). To avoid PCOM and BA occlusion, the microcatheter must be advanced quickly for coil deployment and then rapidly retreated to the spinal ramus arterial origin.
Figure 3.5 | Enhanced contrast angiography of left middle cerebral artery occlusion. LICA and RICA contrast injections were enhanced with a colored blood flow look-up table and overlaid to demonstrate the effectiveness of blocking the LMCA with a matrix coil. Note the lack of perfusion beyond the matrix coil which is situated at the origin of the LMCA as compared to the normal perfusion of the RMCA territory.
Figure 3.6 | Angiographic assessment of the ischemic territory at risk of stroke-induced infarction. (A) A matrix coil has been placed in the RMCA as described in materials and methods. (B) Injection of the RICA with contrast agent under c-arm fluoroscopy (color enhanced) demonstrates the coil occluded segment of the RMCA as compared to the LMCA. (C) Two seconds later, the contrast agent has perfused into small capillary vessels. While the unaffected LMCA territory blushes normally, the ischemic RMCA supplied territory is not patent and remains black; defining the stroke area at risk.
Figure 3.7 | Coronal slices of MCA territory stroke-induced brain lesion. T2-weighted MRI (TR=3000 ms, TE=100 ms, Field of View=145 mm, Slice Thickness=3.0 mm, Echo Train Length=15, Acquisition Matrix=256x256, Number of Averages=2) was performed 24 h after reperfusion of the MCA using a Phillips 3T system. Coronal brain sections demonstrate cortical edema (arrow), indicative of MCA territory infarcted lesion, are ordered from the anterior aspect (top left) to the posterior (bottom right) of the cortex.
Figure 3.8 | Stroke-induced infarct volume as determined by MRI.
Mongrel canines (n=4) were subjected to 1 h MCA occlusion via the vertebrobasilar system. Following 24 h reperfusion, coronal images were analyzed to determine the volume of the stroke induced lesion. Two observers calculated infarct volume independently. (A) Mean percent hemispherical lesion volumes (n=4): Observer 1 = 30.9±2.1, Observer 2 = 31.2±4.3; (B) Bland-Altman observer comparison of ratio (observer 1/observer 2) vs. average, bias = 1.0003±0.066.
Figure 3.9 | Volumetric reconstruction of stroke-induced infarct lesion. T2-weighted MRI coronal slices (acquisition details in Fig. 3.6) were volume rendered using Osirix software (32-bit, version 3.0.2). The color contrast from blue to orange to white represents increasing signal intensity. The eyes (high intensity signal) have been intentionally kept in the field of view for orientation purposes (A and B). Stroke-induced infarct appears orange throughout a large portion of the right hemisphere. (A) Oblique view. (B) Anterior/posterior view. Coronal sectioning through the brain reveals MCA territory infarct and ventricular compression in the right hemisphere (C and D in oblique view).
Figure 3.10 | Histological analysis of post-stroke infarct and control tissue. Following stroke, reperfusion and 24 h MRI, the canines (n=4) were euthanized and brain tissue was cryosectioned in 10μm coronal sections for histological analyses. H&E staining of non-infarcted contralateral hemisphere tissue (A) and infarcted stroke tissue (D) at 10x magnification. H&E staining of non-infarcted contralateral hemisphere tissue (B) and infarcted stroke tissue (E) at 20x magnification. Nucleus (blue, DAPI) and degenerative neuron staining (green, FluoroJade) of non-infarcted contralateral hemisphere tissue (C) and infarcted stroke tissue (F) at 20x magnification.
Figure 3.11 | Exploration of alternative MCA occlusion models: a canine craniectomy trial. Previously reported alternatives to endovascular MCA occlusion in large animals include a craniectomy approach. Following the creation of a 2x2cm cranial window in the canine (n=1) skull, the left MCA was occluded for one hour with a microvascular clip. 3T MRI was performed 24 h after reperfusion. (A) Coronal MR images reveal inflammatory response due to the invasive nature of the procedure in creating the cranial window (star) and (B) herniation of the cortex out of the cranial window due to swelling and edema (arrow). To date, models employing the craniectomy approach have not resolved trauma induced injury separately from stroke pathology.
3.12 | Minimally invasive endovascular approach to middle cerebral artery occlusion. A considerable strength to the proposed model of canine ischemic stroke is the minimally invasive approach. Two small femoral artery punctures are used to navigate microwires, microcatheters, and occlusive coils under guided c-arm (inset photo) fluoroscopy. The surgeon can access all branches of the circle of Willis and visualize in real-time the occlusion event using angiographic contrast injections under c-arm fluoroscopy.
CHAPTER 4

SUMMARY AND GENERAL DISCUSSION

5.1 Summary

This work describes the therapeutic potential for nutrient intervention of stroke-related pathologies. The research spans both basic and applied science to expound upon the mechanistic and functional benefits of nutrient-based stroke interventions. This dissertation began with three primary objectives:

- To characterize a basis for nutritional intervention of acute ischemic stroke risk factors by using niacin-bound chromium in the prevention of metabolic syndrome.

- To determine the in vivo relevance of the natural vitamin E, α-tocotrienol, in neuroprotection following acute ischemic stroke.

- To develop a pre-clinical model of acute ischemic stroke in canines to bridge the translational gap that exists between laboratory and clinical
stroke research; and to improve upon existing stroke models for testing α-tocotrienol efficacy prior to clinical study.

Chapter 1 – The first chapter introduced the concept of nutritional intervention and disease. A synopsis describing the history of nutrition-related illness and the discovery of essential dietary components, vitamins, that prevented deficiency diseases was presented. This led into a discourse on modern nutrition-associated diseases, such as stroke, which are in contrast largely contributed to by dietary excess rather than deficiency. Metabolic syndrome, a disorder that affects roughly a quarter of the U.S. population, was presented as a conglomeration of risk factors associated with ischemic stroke, which include obesity, insulin resistance, and dyslipidemia. A mouse model of metabolic syndrome was described in which the efficacy of a nutrient based therapeutic, niacin-bound chromium (NBC), was tested. Supplementation with NBC was found to modestly improve glucose clearance and significantly benefit the lipid profile of metabolic syndrome mice as compared to placebo controls. Employing genome-wide microarray expression analysis, a myogenic response was observed in the adipose transcriptome of NBC supplemented mice. The experimental evidence supported the conclusion that the nutritional intervention of NBC in a mouse model of metabolic syndrome demonstrated therapeutic potential.
Chapter 2 – The second chapter focused on a direct nutritional intervention of acute ischemic stroke via α-tocotrienol (αT3). Previous work by our laboratory reported that αT3 possessed neuroprotective properties not shared by other family members in cell culture[114, 115]. Whether αT3 was capable of tissue delivery in relevant concentrations to confer neuroprotection remained to be seen. Sub-chapter 2.1 described a multi-generational supplementation study in which αT3 and the well characterized isoform α-tocopherol (αTOC) were gavaged independently or together in rats maintained on E-deficient chow. Results demonstrated that αT3 was indeed capable of being delivered to brain tissues in physiologically relevant concentrations (in range of 5-10μmol/g). Co-supplementation of αT3 with αTOC compromised the efficiency of αT3 delivery, further supporting the previously published finding that tocopherol transfer protein (TTP) has a higher affinity for αTOC[120]. Surprisingly, however, in mice with a genetic mutation that inhibited TTP transcription, orally gavaged αT3 was still capable of delivery to organs, including brain, at physiologically relevant concentrations. It was concluded that TTP-independent mechanisms of αT3 tissue distribution existed and a reconsideration of the biological relevance of αT3 and the other vitamin E family members was warranted.

Having established the tissue distribution of αT3 in rodents, sub-chapter 2.2 sought to translate the previously reported neuroprotective observations from cell culture studies to in vivo models of rodent stroke. In spontaneously
hypertensive rats orally supplemented with αT3, we found that stroke-induced infarct volume was significantly reduced as compared to controls. 12-Lipoxygenase (12-Lox) is a reported mediator of stroke-induced neurodegeneration[114, 161]. The active form of phosphorylated 12-Lox was limited in stroke affected tissue of αT3 supplemented rats as compared to placebo controls. Further supporting the role of 12-Lox as a key mediator of stroke-induced cell death, 12-Lox deficient mice had significantly smaller infarct volume as compared to wild-type matched controls. 12-Lox metabolizes arachidonic acid from the lipid bilayer into the eicosanoid 12-S-HPETE. We demonstrated first-evidence that 12-S-HPETE caused dysfunction in mitochondria isolated from the cortex of rats by inhibiting state 3 respiration, reducing inner membrane potential, and exacerbating permeability transition pore opening (PTP). 12-S-HPETE induction of PTP was inhibited in mitochondria by co-treating them with αT3.

Chapter 3 – Potential stroke therapeutics have a long history of failure in clinical trials[196, 197]. While the results of nutritional intervention with αT3 in rodent stroke models from chapter 2 showed promise, prior to clinical study, a large animal pre-clinical assessment of efficacy was warranted. In chapter 3 we introduced a novel pre-clinical model of acute ischemic stroke in canines. We innovated on neuroradiological techniques to produce a stroke model that, while minimally invasive, permitted real-time visualization of cerebrovascular
occlusion. The reported approach generated highly reproducible infarct volumes as compared to more invasive and expensive non-human primate models of acute ischemic stroke [219, 222, 223].

5.2 General discussion and closing thoughts

Conceptually, the idea of a nutritional intervention of disease is not new. Nutritional and dietary based remedies for disease have a storied history of use dating back as far as Egyptian times [8]. However, it was only until the late 19th century that a scientific basis for nutritional intervention of disease occurred in the context of deficiency diseases and the discovery of vitamins[13, 19, 20, 228, 229]. Nutrition-based therapeutics have since been identified and classified both as prophylactic and on-demand in terms of their therapeutic potential [10]. Today, scientific research and development for disease therapeutics heavily favors novel drug development. The average cost of developing a new drug in 2006 was estimated to exceed $800 million [230]. The exorbitant cost of new drug development plays a significant role in the price that is passed down to the consumer. A refocus of research efforts toward elucidating the therapeutic mechanism of natural remedies is justified. This dissertation documents that prophylactic supplementation of a natural chromium supplement can decrease plasma LDL, and improve the total cholesterol to HDL ratio in mice similarly to pharmaceutical derived atorvastatins, such as Lipitor [231]. In 2006, Lipitor sales exceeded $12 billion
dollars, making it the best selling drug in the world [232]. That a natural dietary supplement can generate similar outcomes to a commercialized pharmaceutical agent is profound.

As described in chapter 3, of the 75 tested neuroprotective agents for therapeutic treatment of stroke, only 2 have been approved by the FDA [198]. The first, aspirin, is a simple over-the-counter blood thinner. The second, tissue plasminogen activator (tPA) is an endogenous serine protease clot buster. The mechanism of action for both of these compounds is mechanical – that is they are administered to improve blood flow to the affected sooner, not to treat the physiological consequence of the stroke. As the underpinnings of stroke pathology are mechanical by nature, so are a number of new surgical devices aimed at treating the problem. A medical device company, CoAxia, recently received $11.5 million dollars for continued development of a balloon-catheter device that transiently occludes the descending aorta in order to redirect blood volume to the cerebrovascular system in hopes of reducing the severity of the stroke [233]. While an interesting concept, the device still cannot treat the underlying phenomena responsible for neuronal cell death following ischemic event. This dissertation describes a naturally occurring vitamin E isoform, α-tocotrienol, with considerable potential in the prophylactic prevention of stroke-induced neurodegeneration. As a vitamin, toxicity study prior to clinical study is largely unnecessary. Humans have been consuming α-tocotrienol and cooking with its natural source, palm oil, for thousands of
years. The safe dose of tocotrienols for human consumption has been estimated at 200-1000 mg/day [234]. Should clinical study of α-tocotrienol as related to stroke prove beneficial, an immediately available, low-cost, natural, dietary supplement would be available to serve the millions of people afflicted by stroke every year. Regardless of when or if a clinical trial for α-tocotrienol occurs, as this is a natural vitamin with a long history of use, the potential benefits for at-risk stroke should be thoughtfully considered. Furthermore, the work in this thesis focuses on one neurodegenerative disease – acute ischemic stroke. It remains to be seen if αT3 possesses neuroprotective properties in other neurodegenerative disorders such as Alzheimer’s or Parkinson’s disease. Further investigation is warranted.

Interest in nutrition based therapeutics, or nutraceutics, is on the rise. In the U.S. alone, nutraceutics are estimated to be a 170 billion dollar per year industry [235]. The fact that the nutraceutical industry is not governed with the same stringency as pharmaceuticals is both a blessing and a curse. On one hand, the more lenient regulations of the FDA’s Dietary Supplement Health and Education Act (DSHEA) make it easier to bring nutraceutic products to market, ultimately keeping the cost of production and retail price lower. Conversely, without the same level of scrutiny as pharmaceutics, there is little incentive to generate quality peer-reviewed scientific research for nutraceutics. To that end, the DSHEA permits nutraceutics to claim only health, not medical, benefits. The distinction between the two is murky at best and often times
misleading to consumers. An example of a permissible nutraceutical claim by DSHEA standards is “helps to maintain cholesterol levels that are already within the normal range”; whereas an impermissible claim is that a nutraceutic “lowers cholesterol” [235]. The lobbying power of pharmaceutical companies is well documented [236-238]. In this election year, the pharmaceutical industry has already donated nearly four hundred thousand dollars to Democratic and Republican campaigns [239]. However, as nutritional intervention of disease continues to gain popularity as an alternative to traditional western medical practice of pharmaceutical-based therapeutics, more research and increased peer-review accountability of nutrient targets will continue to grow the nutraceutical field and market.
LIST OF REFERENCES


69. Sun, Y., et al., *The biomimetic [Cr3O(O2CCH2CH3)6(H2O)3]+ decreases plasma cholesterol and triglycerides in rats: towards*


